Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 1599-1605
Received: 25-05-2019
Accepted: 27-06-2019

Sujitha R
Department of Biotechnology, St. Josephs College, Trichy, Tamil Nadu, India

Dr. A Edward
Department of Biotechnology, St. Josephs College, Trichy, Tamil Nadu, India

Manjula K
Bio Techno Solutions Training and Research Institute, Trichy, Tamil Nadu, India

Correspondence
Sujitha R
Department of Biotechnology, St. Josephs College, Trichy, Tamil Nadu, India

In vitro study of inhibitory effect of *Hibiscus rosasinensis* flower extract on struvite crystals

Sujitha R, Dr. A Edward and Manjula K

Abstract
To investigate the inhibitory effect of aqueous extract of flower of *Hibiscus rosasinensis* on the growth of struvite crystals. Struvite crystals were grown by the single diffusion gel growth technique and the inhibitory effect of aqueous extract of flower of *Hibiscus rosasinensis* on the growth of struvite 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 aqueous extract of flower of *Hibiscus rosasinensis*, the weight of the formed crystals were gradually reduced from 1.44 g to 0.22 g in struvite crystals, respectively. The crystals are harvested from the struvite 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 struvite crystals. Results obtained are indicated that flower of *Hibiscus rosasinensis* have the potential to inhibit the formation of struvite crystals. This study confirms that using aqueous extract of flower of *Hibiscus rosasinensis* can promote the formation of ammonium magnesium phosphate hexahydrate crystals and reduce the nucleation rate of struvite crystals, a major component of triple phosphate urinary stone.

Keywords: Struvite, *Hibiscus rosasinensis* flower, 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 or struvite crystals [2-4]. Among the magnesium phosphates, namely, Ammonium Magnesium Phosphate Hexahydrate (AMPH) commonly known as Struvite and Magnesium Hydrogen Phosphate Trihydrate have also been reported to occur as constituents in renal calculi [5–8] not only in adults but also in children [9, 10]. Struvite calculi, found in 15–20% of urinary calculi [11, 12], are mostly related to urinary tract infections with ureolithic microorganisms in humans and animals [5, 13, 14]. Struvite is also known as triple phosphate stone, infection stone or urase stone. They are found more frequently in women and in persons older than 50 years [15, 16, 17]. Urinary stones are characterized by high recurrence rate therefore requiring a preventive treatment by using the medicinal plants [18, 19].
Hibiscus rosasinensis Linn. (Family Malvaceae) is a plant with flowers widely distributed throughout the world. As a traditional medicine, the fresh juice of the wild flowers is used to treat gonorrhea, the powdered roots are used to treat menorrhagia, and the infusion of the petals is used as a refrigerant drink in fevers. [20] Studies by various researchers have proved that plants are one of the major sources for drug discovery and development. [21] Plants are reported to have antimicrobial, haemolytic, anticancer, anti-inflammatory, antidiabetic, antioxidant properties etc. [22] The chemical constituent shows that the presence of flower : undecanoicacid, tridecanoic acid, tricosanoic acid, tricosan-1-ol, triacontan-1-ol,tartaric acid, stearic acid, pentadencanoic acid, pentacosanoic acid,pentacosan-1-ol, palmitic acid, octanoic acid, octadecadienoic acid, octacosanoic acid, octacosan-1-ol, N-tricosane, N-triacontane, Ntriacontan-1-ol,N-pentacosane, nonanoic acid, nonadecanoic acid, Noctadecane,N-octacosane, N-nonadecane, N-nonacosane, Nhexadecane, N-hexacosane, N-heptadecane, N-heptacosane, Nheneicosane, N-eicosane, N-dotriacontane, N-docosane, myristic acid, montanyl alcohol, margaric acid, lignoceric acid, lauric acid, isotriacontan-1-ol, iso-octacosan-1-ol, hexacosanoic acid, hexacosan-1-ol, heptacosanoic acid, heptacosan-1-ol, heneicosanoic acid,heneicosan-1-ol, docosan-1-ol, decanoic acid, behenic acid, and arachidic acid. [23-27] The anti-arthritic activity of these plants has not been reported yet in any of *in-vitro* models.
In the present investigation, the effects of aqueous extract of flower of *Hibiscus rosasinensis* are used as additives to induce the nucleation and growth of struvite crystals by single diffusion gel growth technique and are reported for the first time. This study incorporated a

multidisciplinary approach in characterizing struvite crystals grown *in vitro* to help formulate prevention or dissolution strategies in controlling urinary stone growth.

Materials and Methods

Materials and instruments

Analytical grade of anhydrous methanol, calcium chloride, magnesium acetate, oxalic acid, sodium metasilicate, ammonium dihydrogen phosphate 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 aqueous extract of flower of *Hibiscus rosasinensis* were collected in the month of march from ponmalaipatti road 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 RS001 dated 20.03.2019.

Preparation of aqueous extracts

The aqueous extract of flower of *Hibiscus rosasinensis* were washed in running water, cut into small pieces and then shade dried for a week at 35-40 ºC, after that it was grinded to a uniform powder of 40 mesh size [6]. The aqueous extract of flower of *Hibiscus rosasinensis* were prepared by soaking 100 g of the dried powder plant materials in 1 L of aqueous by using a 250 ml beaker 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 60 ml (flower) was used to prepare the series (1, 2, 3, 4 and 5 %) of aqueous supernatant concentrations for *in vitro* studies (table 1).

Growth and characterization of Struvite crystals

Glass test tubes were used as a crystallization apparatus and the single diffusion reaction technique was employed [28, 29]. One of the reactants, 0.5 M ammonium dihydrogen phosphate (ADP), was mixed with sodium metasilicate solution the density of 1.04g/cm^3 at pH9.4, so that the pH of the mixture was maintained at 6 and left undisturbed for 2-3 days. After gelation took place, the supernatant solution of 1 M Magnesium acetate was gently poured onto the set gel in various test tubes. After pouring on each supernatant solution, the test tubes were capped with airtight stopples. The experiments were conducted at room temperature (37 °C). The grown Struvite 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 [30].

Table 1: Supernatant solutions added to the set gels for struvite crystals

Supernatant Solutions (SS) (Groups and Treatments)	Compositions
I (Control)	10 ml of 1 M magnesium acetate
II (Distilled water)	5 ml of 1 M magnesium acetate +5 ml of distilled water
III (0.15% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 1% of aqueous extract of flower of *Hibiscus rosasinensis* separately
IV (0.25% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 2% of aqueous extract of flower of *Hibiscus rosasinensis* separately
V(0.50% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 3% of aqueous extract of flower of *Hibiscus rosasinensis* separately
VI(0.75% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 4% of aqueous extract of flower of *Hibiscus rosasinensis* separately
VII(1.00% methanol extract)	5 ml of 1 M magnesium acetate +5 ml of 5% of aqueous extract of flower of *Hibiscus rosasinensis* separately

E] The nomenclature of different additive solution on the growth of struvite crystals

An attempt was made to study the effect of aqueous extract of flower of *Hibiscus rosasinensis* on the growth of struvite 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 Struvite crystals, a series of five different concentrations of 1, 2, 3, 4 and 5% 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 aqueous extract of flower of *Hibiscus rosasinensis* on struvite crystals

The effect of aqueous extract of flower of *Hibiscus rosasinensis* on nucleation and crystallization characteristics of struvite crystals is determined by measuring the weight of the formed crystals. In the gel method, the control using pure Mg $CH_3COO_2{\bullet}4H_2O$ led to the maximum nucleation of crystals growth within 24 h of adding the supernatant solutions Fig. 1 (1a). In the presence of *Hibiscus rosasinensis* flower, nucleation was delayed and reduced masses of the crystals were observed 96 h after adding the supernatant solutions Fig. 1 (1b-g). Morphology of the harvested crystals after addition of aqueous extract of flower of *Hibiscus rosasinensis* as shown in Fig. 2. The largest single struvite crystals having dimensions of 2.5 cm as observed in (Fig. 3a). The sizes of the struvite crystals were reduced from 2.5 cm to 0.6 cm at 1% concentration of extracts was observed in (Fig. 3c-g).With an increase in the concentration of *Hibiscus rosasinensis* flowerfrom 1 to 5% (v/v), the weight of the formed crystals was gradually reduced from 1.44 g to 0.22 g at 1% concentration respectively. The ANOVA statistical analysis was performed and different parameters have been evaluated, and $p<0.05$ has suggested that the correlation is significant as shown in (Table 2).

Recently, growth inhibition studies of Struvite crystals in the presence of some of the herbal extracts [17, 18, 31] were attempted in literature. In the present work, Struvite crystals growth was reduced due to the inhibitory effect of *Hibiscus rosasinensis* flower under *in vitro* conditions. This result

indicates that distilled water did not show any inhibitory activity with regard to crystal growth, whereas the of aqueous extract of flower of *Hibiscus rosasinensis* possessed inhibitory activity due to the presence of bioorganic molecules volatile oil, chiefly sesquiterpene, hydrocarbons, sesquiterpene alcohols, gingerole., starch, tannins flavonoids like galangin [24-27].

Table- 2: ANOVA statistical analysis for harvested struvite crystals

Crystals	Group	Treatents	Mean (gm)±SD
Struvite	A	Control	1.44±0.014
	B	Control+ Distilled water	1.14±0.057
	C	Control+0.15% extracts	$0.69\pm0.014^{a,b}$
	D	Control+0.25% extracts	$0.52\pm0.014^{a,b,c}$
	E	Control+0.50% extracts	$0.45\pm0.014^{a,b,c,d}$
	F	Control+0.75% extracts	$0.33\pm0.014^{a,b,c,d,e}$
	G	Control+1.00% extracts	$0.22\pm0.014^{a,b,c,d,e,f\text{-ns}}$

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 $^{a}p<0.05$, $^{b}p<0.05$, $^{c}p<0.05$, $^{d}p<0.05$, $^{e}p<0.05$.

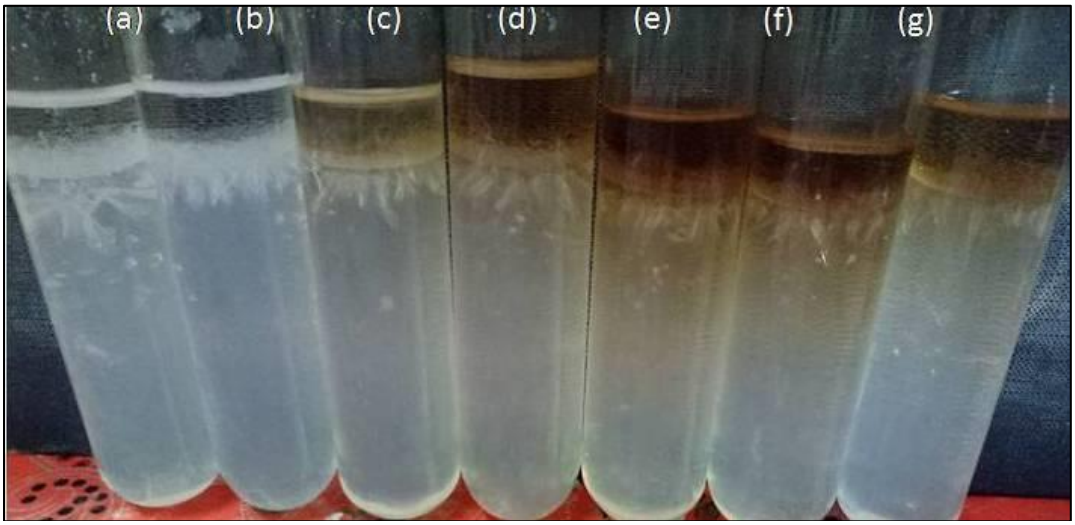

Fig 1: The effect of aqueous extract of flower of *Hibiscus rosasinensis* on struvite crystals in the gel method (a) without any additive (b) with the distilled water (c) with the 1% aqueous extract (d) with the 2% aqueous extract (e) with the 3% aqueous extract (f) with the 4% aqueous extract (g) with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

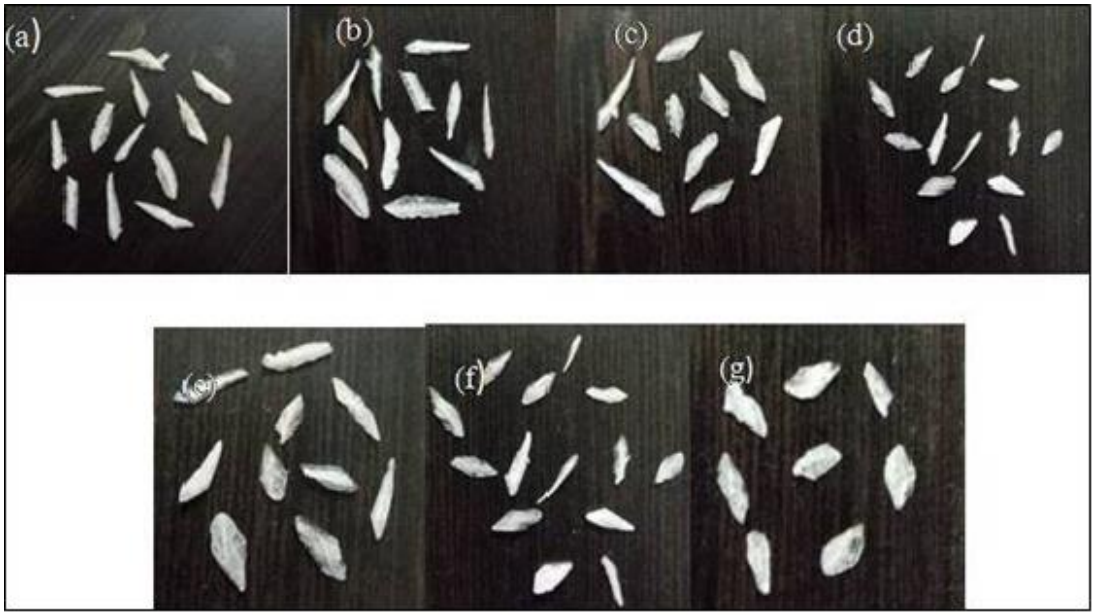

Fig 2: The harvested crystals of struvite obtained from aqueous extract of flower of *Hibiscus rosasinensis* in the gel method (a) without any additive (b) with the distilled water (c) with the 1% aqueous extract (d) with the 2% aqueous extract (e) with the 3% aqueous extract (f) with the 4% aqueous extract (g) with the 5% aqueous extract after 7 days.

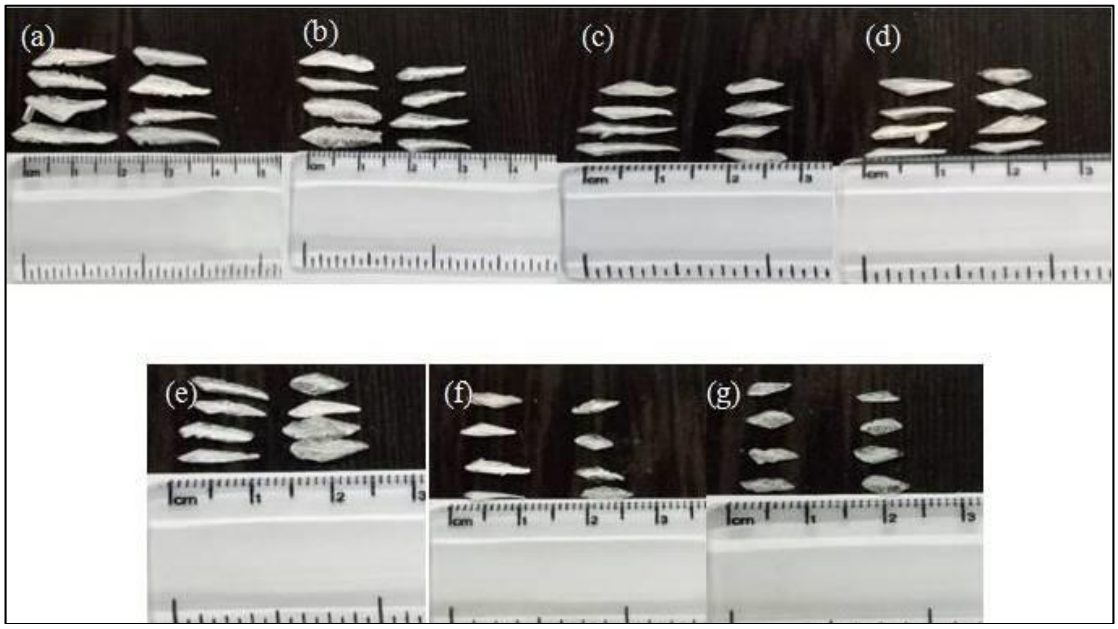

Fig 3: The measurement of struvite obtained from aqueous extract of flower of *Hibiscus rosasinensis* in the gel method (a) without any additive (b) with the distilled water (c) with the 1% aqueous extract (d) with the 2% aqueous extract (e) with the 3% aqueous extract (f) with the 4% aqueous extract (g) with the 5% aqueous extract after 7 days.

Characterization of Struvite crystals

The FTIR spectra of Struvite crystals obtained in the presence and absence of the plant samples are shown in Figure 4.

In Figure 4(a), the band at 2358 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1626 cm^{-1} is due to HOH deformation of water and the peak at 1441 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1007 cm^{-1} is due to V_3 antisymmetric stretching vibration and the peak at 757 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units.

In Figure 4(b), the band at 2358 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1631 cm^{-1} is due to HOH deformation of water and the peak at 1441 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1007 cm^{-1} is due to V_3 antisymmetric stretching vibration and the peak at 757 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units.

In Figure 4(c), the band at 2352 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1636 cm^{-1} is due to HOH deformation of water and the peak at 1441 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1007 cm^{-1} is due to V_3 antisymmetric stretching vibration and the peak at 767 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units.

In Figure 4(d), the band at 2364 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1626 cm^{-1} is due to HOH deformation of water and the peak at 1440 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1004 cm^{-1} is due to V_3 antisymmetric stretching vibration and the peak at 757 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units.

In Figure 4(e), the band at 2362 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1627 cm^{-1} is due to HOH deformation of water and the peak at 1440 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1004 cm^{-1} is due to V_3 antisymmetric stretching vibration and the peak at 757 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units.

In Fig. 4(f), a band at 2364 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1631 cm^{-1} is due to HOH deformation of water and the peak at 1440 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1007 cm^{-1} is due to V_3 antisymmetric stretching vibration and the peak at 757 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units. In the presence of 5% orange juice

Figure 4(g), the band at 2374 cm^{-1} is due to the antisymmetric and symmetric stretching vibration of NH_4 units. The peak at 1600 cm^{-1} is due to HOH deformation of water and the peak at 1438 cm^{-1} is due to the HNH deformation modes of NH_4 units. The band at 1007 cm^{-1} is due to V_3 antisymmetric stretching vibration and the peak at 758 cm^{-1} is due to the water liberational and NH_4 rocking modes. The peak at 568 cm^{-1} is due to the V_4 bending modes of the PO_4 units.

Several researcher have reported crystallization characterization of Struvite crystals using FTIR techniques. The peaks shift from 2358 to 2374 cm^{-1} and from 1441 to 1438 cm^{-1} for HNH deformation modes of NH_4 units previously reported. The shifting further supports that the extract can promote the formation of ammonium magnesium phosphate hexahydrate crystals and reduce the nucleation rate of struvite crystals.

The XRD patterns of struvite crystals obtained in the presence and absence of the methanol extract of *Alpinia purpurata* leaves are shown in (fig. 5). The diffraction peaks obtained were well correlated to the (hkl) indices of struvite phase (JCPDS card number 04-010-2894). The effected *Alpinia purpurata* leaves the nucleation and growth of struvite crystals.

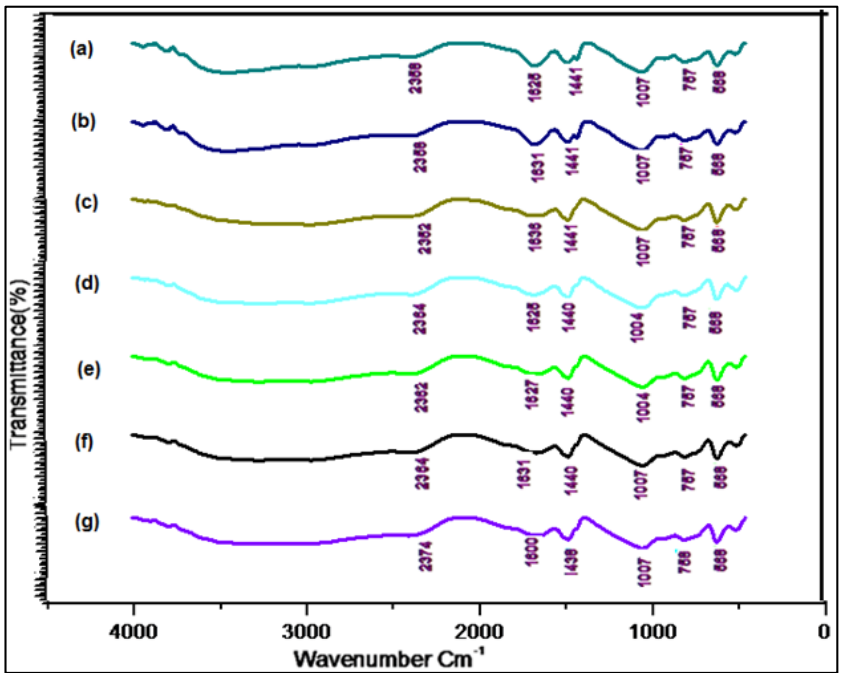

Fig 4: The FTIR spectra of struvite obtained from *Alpinia purpurata* leaves in the gel method (a) without any additive (b) with the distilled water (c) with the 1% aqueous extract (d) with the 2% aqueous extract (e) with the 3% aqueous extract (f) with the 4% aqueous extract (g) with the 5% aqueous extract after 7 days.

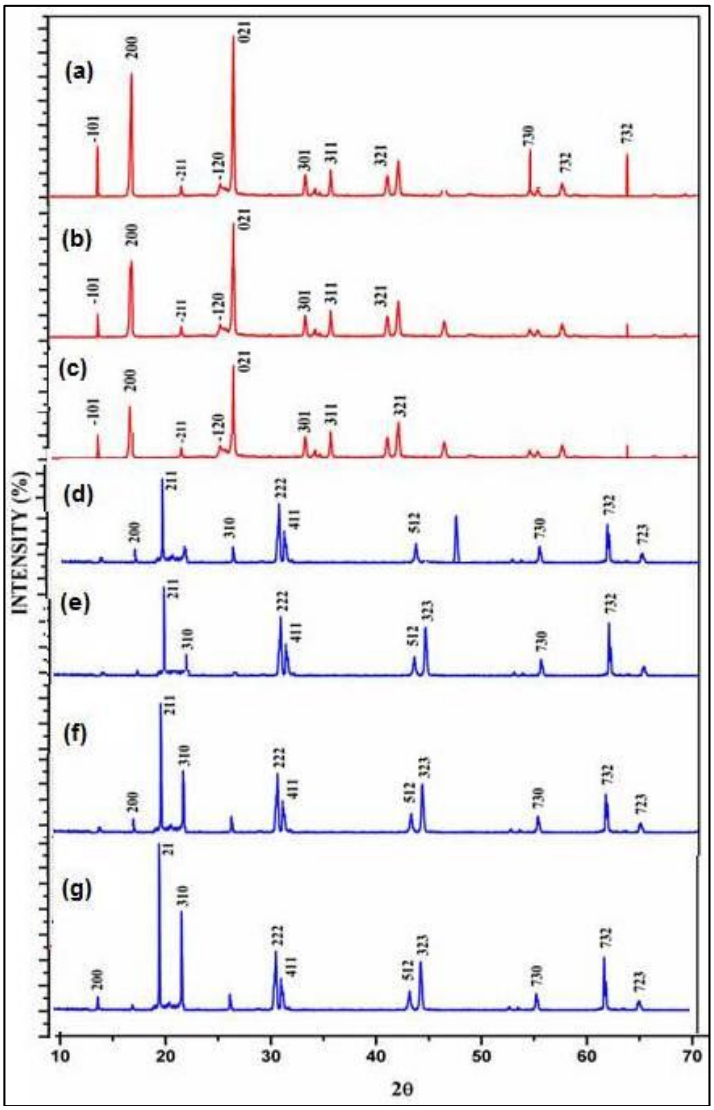

Fig 5: The XRD pattern of struvite obtained from aqueous extract of flower of *Hibiscus rosasinensis* in the gel method (a) without any additive (b) with the distilled water (c) with the 1% aqueous extract (d) with the 2% aqueous extract (e) with the 3% aqueous extract (f) with the 4% aqueous extract (g) with the 5% aqueous extract after 7 days.

Conclusion

Struvite 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 aqueous extract of flower of *Hibiscus rosasinensis* the weight of the formed crystals were gradually reduced from 1.44 g to 0.22 g in struvite 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 struvite crystals showed significant differences ($p<0.05$). This study confirmed that the *Hibiscus rosasinensis* flower extracts can promote the formation of ammonium magnesium phosphate hexahydrate crystals and treat urinary stone by inhibiting the formation of struvite crystals, a major component of triple phosphate urinary stone.

Acknowledgement

S.R acknowledges Dr. S. John Britto, Director, rapinat herbarium, St. Joseph College, Tiruchirapalli, Tamil Nadu for identifying the plants. SR acknowledges Dr. A. Edward,, Assistant Professor, St. Joseph College, Trichy for constant support for this research.

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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