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ORIGINAL PAPER

Growth inhibition of Struvite crystals in the presence of juice of *Citrus medica* Linn.

C. K. Chauhan · M. J. Joshi

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Abstract Struvite, one of the components of urinary stone grows rapidly forming "staghorn-calculi", is a painful urological disorder. It is necessary to study the growth-inhibition of Struvite crystals. This in vitro study has been carried out in the presence of the juice of *Citrus medica* Linn. by using single diffusion gel growth technique. Sodium metasilicate solution of specific gravity 1.05 and an aqueous solution of ammonium dihydrogen phosphate of 0.5 M concentration were mixed so that the pH value 7.0 could be set. After the gelation, supernatant solutions comprising of pure 1.0 M Magnesium acetate (control solution) as well as mixed with the different concentrations of the juice were gently poured on the set gels. From the study of growth-inhibition behavior of Struvite crystals, it was found that *Citrus medica* Linn. inhibits the growth of the crystals. This study may be used for formulating the strategy for prevention or dissolution of Struvite.

Keywords Struvite · Urinary stone · Gel growth · *Citrus medica* Linn. · Inhibition

C. K. Chauhan (✉) · M. J. Joshi
Crystal Growth Laboratory, Department of Physics,
Saurashtra University, Rajkot 360 005, Gujarat, India
e-mail: ckc33@rediffmail.com

M. J. Joshi
e-mail: mshilp24@rediffmail.com

Present Address:
C. K. Chauhan
Physics Department, H. & H. B. Kotak Institute of Science,
Dr Yagnik Road, Rajkot 360 001, Gujarat, India

Introduction

The formation of an urinary calculi is a serious, debilitating problem in all societies throughout the world. It is estimated that approximately 12% of the population will suffer from the disease at some stage in their lives [1]. A large number of people are suffering from urinary stone problem all over the globe. Not only the humans but animals and birds also suffer from the urinary stone problem. The occurrence in some areas is so alarming that they are known as 'Stone Belts'. The area of high incidence of urinary calculi include British islands, Scandinavian countries, Central Europe, Northern Australia, Northern India, Pakistan, Mediterranean countries [2]. The financial costs of the disease are staggering; in the United States, for instance, the health bill for treatment of kidney stones runs to billions of dollars annually [3]. More recent studies suggest that there has been a gradual increase in the annual incidence and a decrease in the age of onset of the disease perhaps the result of change in lifestyle and diet [4].

Majority of the calculi are composed of calcium salts, oxalates and phosphates. Among the phosphates, magnesium phosphates, namely, Ammonium Magnesium Phosphate Hexahydrate (AMPH)—$\{(NH_4)MgPO_4{\cdot}6(H_2O)\}$ commonly known as Struvite and Magnesium Hydrogen Phosphate Trihydrate—$\{MgHPO_4{\cdot}3(H_2O)\}$ 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]. Priestley and Dunn [16]

reported that 41% of the patients have 5-year survival rate with untreated unilateral Struvite stones.

The stone formation requires supersaturated urine. Super-saturation also depends on urinary pH, ionic strength, solute concentration and complexations [2]. Three conditions must coexist for the formation of Struvite calculi; (1) alkaline urine, (2) the presence of urea or ammonia in the urine and (3) higher concentration of minerals in the urine. As it is known, Struvite forms as a consequence of a urinary tract infection by urease producing micro organisms, this urease splits urea and produces ammonia. Further hydrolysis of the ammonia takes place, which produces NH^{4+} ions and increases urine pH and gives neutral or alkaline urine. An elevated urinary pH reduces the solubility of magnesium ammonium phosphate and favors precipitation of Struvite crystals. Higher intake of phosphate (from Proteins) and magnesium based food and lower intake of water gives rise to the PO_4^{3-} and Mg^{2+} ions in the supersaturated urine, which leads to the conditions of formation of Struvite [17].

Urine of a healthy person is under-saturated with regard to Struvite, but because of the conditions provoked by urease-producing microorganisms and the urine complex composition, the precipitation of Struvite can occur. Under such conditions Struvite often precipitates together with apatites and the sediment can easily be attached to the particles of organic matter formed as a consequence of the infection. This mechanism favors the crystal deposition and aggregation, so that Struvite stones grow rather quickly. Struvite stones may grow rapidly over a period of weeks to months and, if not adequately treated, can develop into a Staghorn or branched calculus that involves the entire renal pelvis and calyces. Patients with infected Staghorn calculi who receive no treatment have about a 50% chance of losing the kidney [18, 19].

Therefore, it is very much necessary to study the growth-inhibition of Struvite crystals. In the present investigation, Struvite crystals were grown by single diffusion gel growth technique and the growth inhibition study of the Struvite crystals in the presence of the different concentration of the juice of *Citrus medica* Linn. was carried out.

In the gel growth technique, growth occurs due to reaction between two solutions in a gel medium or achieving super-saturation by diffusion in gel medium. Slow and controlled diffusion of reactants in gels can mimic the condition in a body [20, 21]. Bio-crystallization usually occurs in the slow and steady process in the soft tissues, cavities or vessels. Single diffusion gel growth technique provides the simplified in vitro model of the highly complex growth of urinary calculi in vivo. Growth of crystals with different morphologies is commonly found in bio-crystallization. In the gel growth technique, by changing the growth conditions, crystals with different morphologies and sizes can be obtained. The main advantage is that the crystals can be observed practically in all stages of their growth. The gel growth technique was described in details by Henisch [22], Henisch et al. [23] as well as Patel and Rao [24]. Urinary stones grow in a gel like medium; therefore, they have radially striated growth [25]. The crystal growth by gel method provides simulation of synovial cartilage and other biological fluids [26]. Gel growth (in vitro) of a few urinary stone constituents and the inhibitory role played by some extracts or juices of natural products in crystal growth were studied earlier [27]. This technique has been successfully used to study the growth inhibition of calcium oxalate crystals [28] and calcium hydrogen phosphate dehydrate (CHPD), i.e., Brushite crystals [20] using herbal extracts of *Tribulus terrestris* Linn. and *Bergenia Ligulata* Linn. Growth inhibition studies of Struvite in the presence of some of the herbal extracts of *Boerhaavia diffusa* Linn. [29], *Rotula aquatica Lour* [30] and *Commiphora wightii* [17] were successfully carried out by the present researchers.

In the traditional Indian system of medicine, i.e., Ayurveda, many herbal medicines have been recommended for the treatment of urinary stone problem and some of them have been experimentally evaluated [20, 21, 31]. The Importance of various *citrus* fruits has been described in the Ayurvedic treatises. *Citrus* is grown in tropical and subtropical regions of the world and occupies a wide range of latitude over which it is being cultivated. The north-eastern Indian states are rich treasure of various citrus species and their varieties. In the present growth inhibition study researcher used one of the citrus fruit—*Citrus medica* Linn., commonly known as Baranimbu, Bijaura or Bijoru in Hindi, Citron in English, Cidro in Spanish, Zitronatzitrone in German, Fo shou in Chinese and as Bushukan in Japanese. The fruit is as shown in Fig. 1. The general descriptions,

Fig. 1 Fruits of *Citrus medica* Linn.

propagation, native legends and names, characteristics, constituents, various medicinal uses were discussed in detail elsewhere [32–39]. Its importance is also noted in the Linnean Herbarium situated at the Department of Phanerogamic Botany at the Swedish Museum of Natural History [40] which is one of the largest herbaria in the world.

Struvite type kidney stones thrive in basic conditions of urine and hence the treatment should be the acidification of the urine. It is of prime importance to carry out the search for suitable Struvite inhibitor, which has probably no many side effects. As one of the main chemical constituents in the juice of *Citrus medica* Linn. is citric acid, it has been decided to check its inhibitive effect on Struvite crystals. Therefore, the growth inhibition study of Struvite crystals has been carried out by the present researchers using natural fruit juice of *Citrus medica* Linn. under in vitro conditions to identify the potency of inhibition, which can be further studied in vivo.

Experimental technique

The single diffusion gel growth technique was used to study the growth and inhibition behavior of Struvite crystals in the presence of different concentration of the juice of *Citrus medica* Linn. The double distilled water and AR grade chemicals were used to grow the Struvite crystals. Sodium metasilicate (SMS)—{Na_2 Si O_3, $9H_2O$} solution of specific gravity 1.05 was used to prepare the gel. An aqueous solution of ammonium dihydrogen phosphate (ADP)—{NH_4 H_2 PO_4, $2H_2O$} of 0.5 M concentration was mixed with the SMS solution in appropriate amount so that the pH value 7.0 could be set for the mixture. The gel solution of 20 ml was transferred into test tubes of 140 mm length and 25 mm diameter. All test tubes and other glassware were autoclaved at 120°C for 15 min. Here, the silica gel was chosen so that it remains stable and does not react with the reacting solutions or with the product crystal formed. After gelation took place, 20 ml supernatant solutions of pure 1.0 M Magnesium acetate—{C_4 H_6 Mg O_4, $4H_2O$} prepared with different concentration of the juice of *Citrus medica* Linn. were gently poured on the set gels in test tubes. For each test tubes, 20 ml supernatant solutions of 1.0 M Magnesium acetate were prepared by taking different volumes of the juice of *Citrus medica* Linn. and distilled water. Composition and the pH of the supernatant solutions are as shown in Table 1. Figure 2 shows the schematic diagram of the single diffusion gel growth technique.

After pouring supernatant solution, the test tubes were capped with airtight stopples. The pouring of solutions on the set gels in various test tubes was done in the aseptic medium in laminar flow hood to avoid microbial contaminations. The experiment was conducted at the room temperature. The following reaction is expected to occur in the gel between the two reactants.

$$NH_4H_2PO_4 \cdot 2H_2O + (CH_3COO)_2Mg \cdot 4H_2O \rightarrow NH_4MgPO_4 \cdot 6H_2O + 2CH_3COOH \cdots \quad (1)$$

The apparent lengths of growing/dissolving Struvite crystals in each of the test tubes were measured by using a traveling microscope of least capacity 0.001 cm at regular time interval. The apparent lengths of growing/dissolving Struvite crystals at different depth from the gel–liquid interface in each of the test tubes were measured. The depths of dissolution from the gel–liquid interface were measured at regular time interval for each of the test tubes. The statistical analysis of the single factor ANOVA was also carried out. The days for the complete dissolution in each of the test tubes were also noted. The whole experiment was conducted twice.

Table 1 The composition and the pH of the Supernatant Solutions

Number of the supernatant solution	Composition of the supernatant solution			pH of the supernatant solution
	Volume of juice of *Citrus medica* Linn. (ml)	Volume of distilled water (ml)	Magnesium acetate (g)	
S·S.-1	00	20	4.288	7.80
S·S.-2	02	18	4.288	5.87
S·S.-3	04	16	4.288	5.40
S·S.-4	06	14	4.288	5.01
S·S.-5	08	12	4.288	4.87
S·S.-6	10	10	4.288	4.75
S·S.-7	12	08	4.288	4.67
S·S.-8	14	06	4.288	4.57
S·S.-9	16	04	4.288	4.50
S·S.-10	18	02	4.288	4.43
S·S.-11	20	00	4.288	4.35

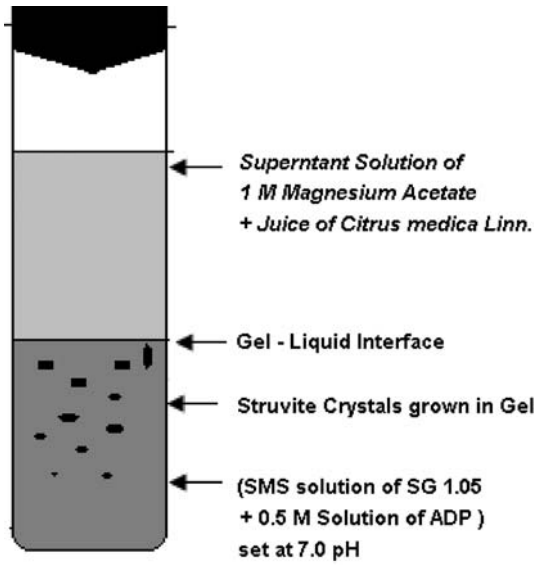

Fig. 2 Schematic diagram of single diffusion gel growth technique

Results and discussion

Struvite crystals with different morphologies like dendritic type, prismatic type, rectangular platelet type, needle type were grown in the gel [41]. Figure 3 shows the photograph of the grown Struvite crystals in the gel medium. It was observed that as the concentration of the juice of *Citrus medica* Linn. was increased in the supernatant solution, the number of grown Struvite crystals in the silica hydro gel medium decreased and also average size of the Struvite crystals decreased.

After pouring of the supernatant solution, dendritic type crystals were grown in the gel at the gel–liquid interface. The growth rates of Struvite crystals, at the end of 2nd and 4th day, growing in the gel at the gel–liquid interface for different concentration of supernatant solutions are presented in Table 2. It can be noticed from Table 2 that the growth rate of crystals and hence the size of the crystals decreases as the concentration of *Citrus medica* Linn. increases. It was observed that the length of crystals growing in the gel at gel–liquid interface increased up to first 4 days in the cases of the supernatant solutions no. 1 or *S·S.-1*(i.e., without any inhibitor) and *S·S.-2*; and then they started dissolving. The length was increased up to first 3 days in the case of *S·S.-3* and the dimension remained unchanged up to the end of 4th day; and then started dissolving. In the cases of the *S·S.-4, S·S.-5* and *S·S.-6*, length of the crystals growing in gel at gel–liquid interface increased just up to first 2 days; and then they started to dissolve gradually. It was observed that Struvite crystals, grown in the gel at the gel–liquid interface, dissolved completely within 23 days in case of *S·S.-3*; within 18 days in the case of *S·S-4*; and within 12 and 10 days in case of *S·S.-5* and *S·S.-6*, respectively. It was also noted that in the case of *S·S.-7* and for other higher concentrations, i.e., for *S·S.-8* to *S·S.-11*, Struvite crystals can not grow at the gel–liquid interface; it can be clearly noticed in the Fig. 3. This study is important as it is conducted under the growth conditions. The simple dissolution is tested by placing the already grown crystal in an appropriate solution. The usual aim is to achieve the inhibition and dissolution of growing calculi in a body, where the required nutrients for the growth are being continuously supplied. Altogether, the same thing is mimicked in this in vitro experiment.

Table 2 Growth rates of the Struvite crystals growing in the gel at gel–liquid interface for the different concentrations at the end of 2nd and 4th day

Number of supernatant solution	Growth rate (cm/days)	
	At the end of Day 2	At the end of Day 4
S·S.-1	0.608	0.309
S·S.-2	0.300	0.192
S·S.-3	0.250	0.125
S·S.-4	0.240	Dissolution starts
S·S.-5	0.225	Dissolution starts
S·S.-6	0.240	Dissolution starts

At the gel–liquid interface the concentration gradients of the nutrients are the maximum and hence it is important to study the effect of juice of *Citrus medica* Linn. on the growing crystals. The growth and dissolution of Struvite at the gel–liquid interface is shown by the plots of average length versus time period in Fig. 4. It was found that the crystals grown at the gel-liquid interface dissolved up to a certain extent in control solution *S·S.-1*, i.e., in the absence of the juice. It may be due to the formation of acetic acid as shown in chemical reaction equation (1). But it was observed that the dissolution of the crystals grown at the gel–liquid interface was faster for different concentrations of *Citrus medica* Linn., i.e., for the *S·S.-2* to *S·S.-6*. It was noticed that Struvite crystals can not grow at the gel–liquid interface in the cases of *S·S.-7* to *S·S.-11*, due to the effect of higher concentration of the juice of *Citrus medica* Linn. in the supernatant solutions. The dissolution rates of grown crystals in the gel at gel–liquid interface for the different concentration of *Citrus medica* Linn. are given in Table 3, which suggests that the dissolution rate increases with the increasing concentration of *Citrus medica* Linn. in the supernatant solution. This further suggests that at the interface the already observed dissolution due to the formation of acetic acid is enhanced by the presence of juice.

Growth and dissolution of grown Struvite crystals at different depth in gel from the gel–liquid interface for *S·S.-1*, i.e., in the absence of the inhibitor, is shown by the plots of average length versus time period in Fig. 5. It was observed that the length of growing crystals in the gel at gel–liquid

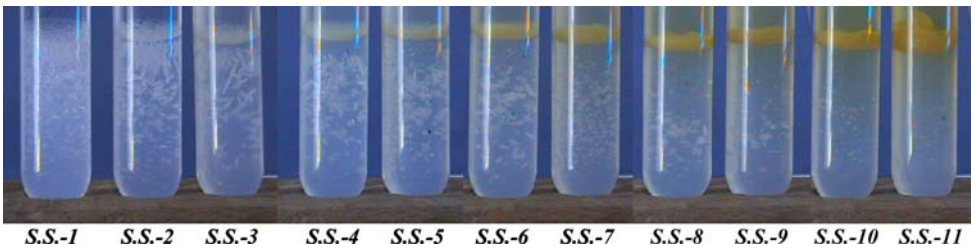

Fig. 3 Photograph of the Struvite crystals grown in gel medium in test tubes with different concentration of the juice of *Citrus medica* Linn.

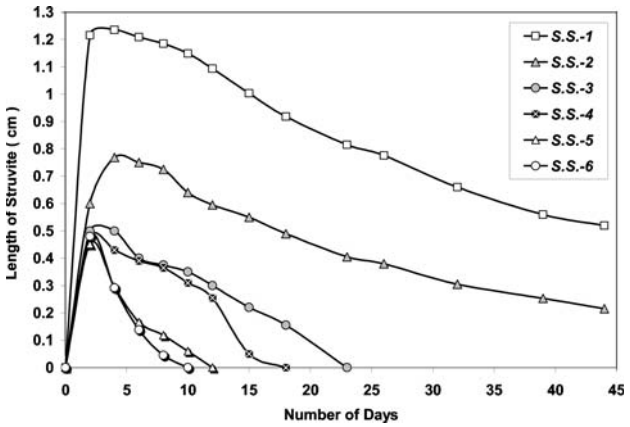

Fig. 4 Growth and dissolution of Struvite at the gel–liquid interface

Table 3 Dissolution rates of the Struvite crystals in the gel at gel–liquid interface for the different concentrations

Number of the supernatant solution	Dissolution rate (cm/days)
S·S.-2	1.38×10^{-2}
S·S.-3	2.63×10^{-2}
S·S.-4	3.00×10^{-2}
S·S.-5	4.51×10^{-2}
S·S.-6	6.00×10^{-2}

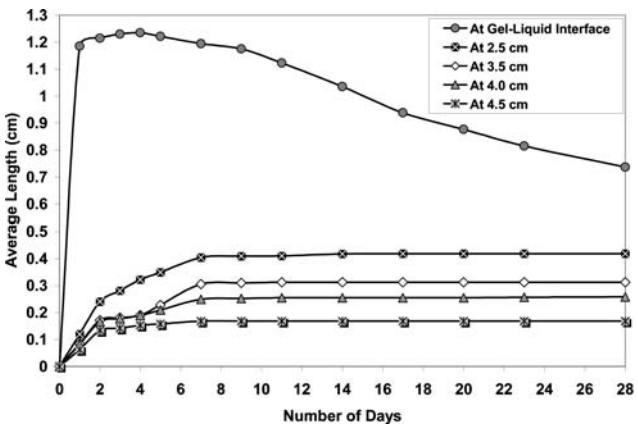

Fig. 5 Growth of Struvite at different depth from the gel–liquid interface in the absence of inhibitor

interface increased up to first 4 days in this case and then they started dissolving, due to the formation of acetic acid. It was noticed that the length of the crystals at different depth from the gel–liquid interface increased up to first 7 days, and then it remained constant up to about 45 days. The formation of acetic acid is the maximum at the gel-liquid interface due to high concentration gradients and faster reactions taking place. But as one goes towards the bottom of the test tubes the diffusion of reactants is comparatively less and the amount of acetic acid produced is also less, which does not dissolve the growing crystals and hence a steady growth of crystals is observed. As the depth of the gel column increases from the gel–liquid interface, the sizes of the grown crystals were found to be gradually smaller.

Figure 6 shows the histograms depicting the maximum length of the grown Struvite crystals in the gel media for different concentrations of *Citrus medica* Linn. It was observed that the maximum dimensions of the grown crystals in the gel media decreased with the increasing concentration of *Citrus medica* Linn. in the supernatant solution.

At different depths from the gel–liquid interface the grown crystals were measured and the phenomenon of the on set of dissolution was noted down. The depth of dissolution is defined as the depth from the gel–liquid interface up to which either the grown crystals were dissolved completely or no crystal can grow at all. Figure 7 shows the plots of depth of dissolution versus time in days. It is found that the depth of dissolution increases with time. In pure control solution *S·S.-1,* it was almost parallel to the *x*-axis and for solutions of higher concentrations of the juice it is pushed deeper and deeper in to the gel. It is also noted that the depth of dissolution is increased slowly for the lower concentration, while it is increased rapidly for the higher concentration, e.g., the depth of dissolution of 5.00 cm is achieved in just 15 days for *S·S.-10* and *S·S.-11*; while it is achieved in 39 days for *S·S.-4*. Single factor ANOVA, i.e., Analysis of variance, was also carried out; which shows that the difference in the depth of dissolution was highly significant at 0.001 level. Figure 8 shows the histograms of depth of dissolution after 10 and 15 days for the different concentrations, it is noticed that the depth of dissolution increases rapidly with increase in the concentration of *Citrus medica* Linn. juice.

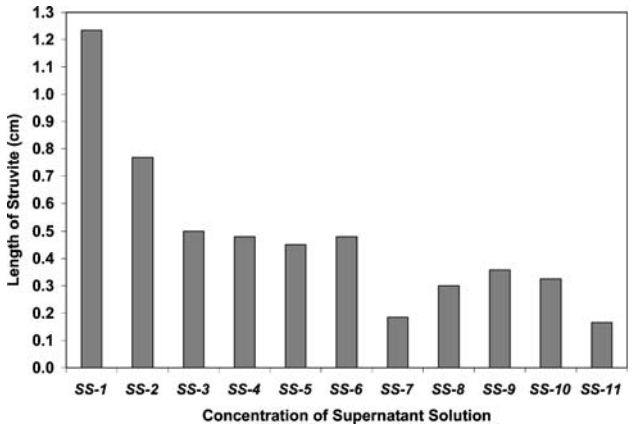

Fig. 6 Maximum length of Struvite grown in the gel media in test tubes with different concentration

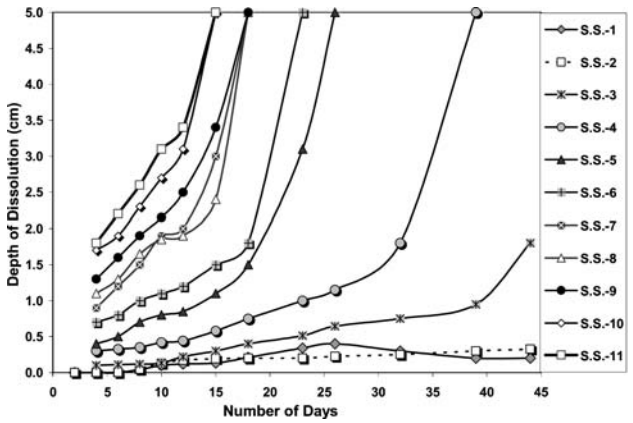

Fig. 7 Depth of dissolution versus number of days

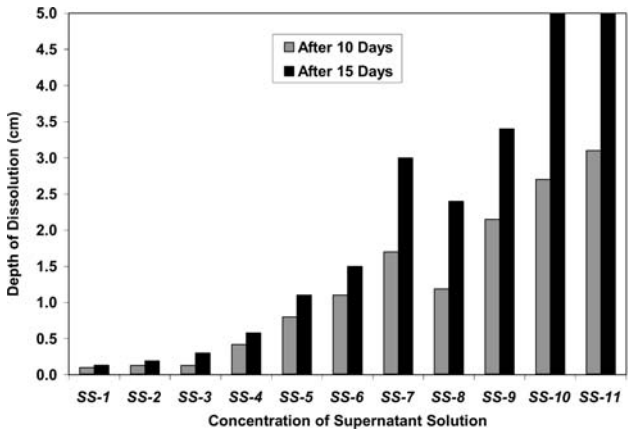

Fig. 8 Depth of dissolution after 10 and 15 days for the different concentration

The growth and dissolution of Struvite crystals grown at the different depths in the gel from the gel–liquid interface for *S·S.-2* to *S·S.-11* have been studied. Plots of average apparent length of growing crystals versus time in days for different depths in the gel from the gel–liquid interface are shown in Fig. 9a–j. As the depth of the gel column increases from the gel–liquid interface, the average length of the grown crystals decreases gradually. It was also observed that as the concentration of the juice of *Citrus medica* Linn. was increased in the supernatant solution, average size of the grown Struvite crystals decreased. It can be seen that the period of dissolution, i.e., the time taken for the complete dissolution of the crystals, is less for the crystals grown near the gel–liquid interface, whereas the period of dissolution is more for the crystals grown at the different higher depth from the gel–liquid interface. At the gel–liquid interface the dissolution is faster due to the presence of sufficient amount of the juice of *Citrus medica* Linn. and also acetic acid as an added advantage. However, amount of the juice of *Citrus medica* Linn. and acetic acid decreases down towards the bottom of the gel column, which may be responsible for the delayed dissolution. For the cases of *S·S.-7* to *S·S.-11, concentration* of the juice of *Citrus medica* Linn. was such that no crystal can grow at the gel-liquid interface. It is clear from the Fig. 9f–j that Struvite crystals grow only at the higher depth and it can not grow at the gel–liquid interface in the cases of *S·S.-7* to *S·S.-11*. It can be noticed from the Fig. 9j for *S·S.-11* that the dissolution period is same for two different depths and the average length of the crystals is also nearly the same, which indicates that the higher amount of *Citrus medica* Linn. juice brings almost sufficient concentrations in the gel column to dissolve crystals nearly uniformly. Altogether, a histogram of Fig. 10 shows the numbers of days required for the complete dissolution of grown crystals in the gel media for different supernatant solutions. It is seen that, as the concentration of the juice of Citrus *medica* Linn. in the supernatant solution increases, the number of days required for the complete dissolution decreases. For example, one can notice from the histogram that it took 70 days for the complete dissolution for *S·S.-2* and just 15 days for *S·S.-11*.

Several attempts have been made to check the growth inhibition of different urinary type crystals by employing the gel growth technique. Joseph and Joshi [21] reported the inhibitory effect of tartaric acid and tamarind on CHPD crystal. Earlier an in-vitro growth inhibition study on one of the urinary type Brushite crystals in the presence of citric acid and lemon juice along with the artificial reference urine and natural urine was reported by Joshi and Joshi [31], which also showed strong inhibition of growth of CHPD crystal. Also, the crystal growth and dissolution of brushite crystals is studied by taking different concentrations of citric acid by Parekh and Joshi [42]. Recently, a modified gel growth technique has been proposed by Parekh and Joshi for the micro crystal growth and in situ

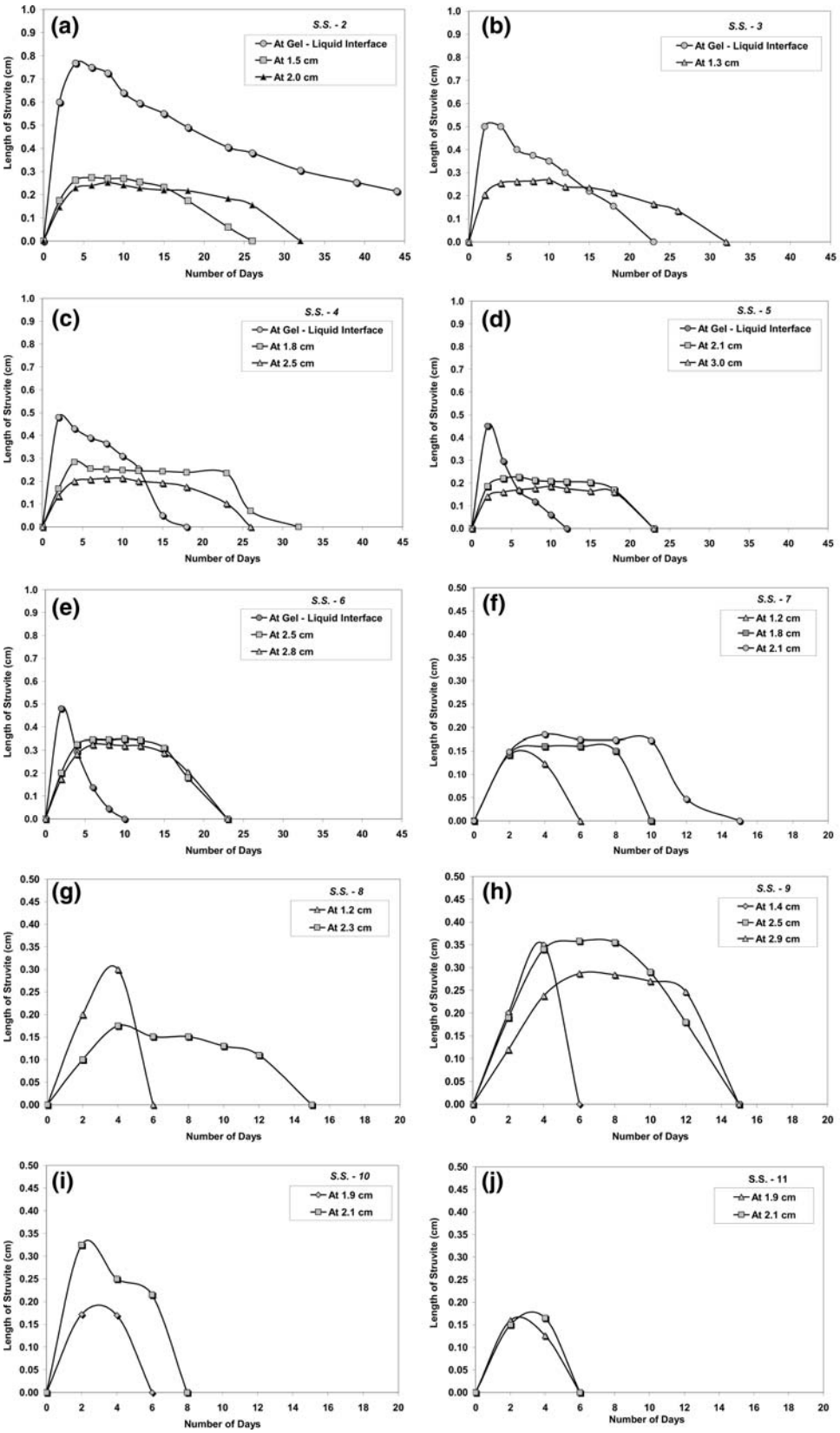

Fig. 9 a–j Growth and dissolution of Struvite at different depth from the gel–liquid interface for *S·S.-2* to *S·S.-11*

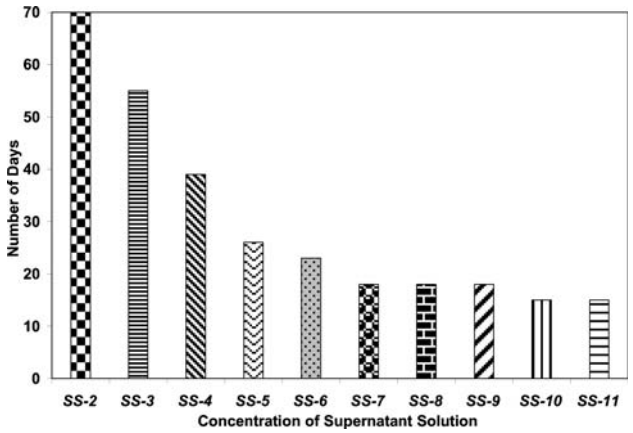

Fig. 10 Number of days required for complete dissolution for the different concentration

observations, which has been successfully tested for Brushite micro-crystal growth inhibition in the presence of citric acid [43].

A treatment with alkali, usually in the form of magnesium potassium citrate or potassium citrate, is very common to increase urinary citrate and reduce the rates of stone formations in the patients of hypocitraturic calcium nephrolithiasis [44–46]. A critical review on preventive treatment of nephrolithiasis with alkali citrate is written by Mattle and Hess [47]. Inasmuch as the most of the earlier studies on citrate inhibition have been mainly concentrated on calcium oxalate monohydrate and Brushite crystals, the present investigation has been carried out to prove the citrate inhibition in Struvite crystals also. Holly Nash [48] reported that the risk of the formation of Struvite in cats can be minimized by slightly acidifying the urine pH by dietary regulations. For human being Acetohydroxamic acid (AHA) is the most widely used irreversible inhibitor of bacterial urease. AHA has a high renal clearance, can penetrate the bacterial cell wall, and acts synergistically with several antibiotics. Although in-vivo studies have demonstrated that AHA inhibition of bacterial urease decreases urinary alkalinity and ammonia levels even in the presence of infection, 20% of patients experience associated adverse effects. These include phlebitis, deep venous thrombosis, and hemolytic anemia. In addition, the use of AHA in patients with impaired renal function (serum creatinine level > 2.5 mg/dL) limits its effectiveness and increases its toxicity [15].

The juice of the fruits of *Citrus medica* Linn. mainly contains Citric acid, Ascorbic acid, Hespiridin, Campesterol, Stigmasterol, Sitosterol and Cholesterol [49]. Therefore, it is believed that the large amount of citric acid is playing an important role in the growth inhibition of Struvite crystals. The acidic nature of the pH of the supernatant solution indicates that the juice is of acidic nature. It can be concluded that *Citrus medica* Linn. is found to be a potent inhibitor for Struvite crystal growth in vitro. This in vitro study may be helpful to carry out further in vivo studies.

Conclusions

As the concentration of the juice of *Citrus medica* Linn. in the supernatant solution was increased, the number of grown Struvite crystals in the silica hydro gel media was decreased. With the increasing concentration of the juice of *Citrus medica* Linn., the dimensions, i.e., the average apparent length of the crystals, were reduced due to inhibitive effect produced by the juice. Growth rate of Struvite crystals was decreased as the concentration of the juice of *Citrus medica* Linn. increased. It was noticed that the dissolution rate increases with the increasing concentration of the juice of *Citrus medica* Linn. It is found that the depth of dissolution was increased slowly for the lower concentration, while it increased rapidly for the higher concentration of the juice of *Citrus medica* Linn. From the single factor ANOVA, it is found that the difference in the depth of dissolution was highly significant at 0.001 level. As the concentration of the juice in the supernatant solution increased, the total number of days required for the complete dissolution decreased. Although the stone formation process occurring in the human body is quite complex, and takes place in a dynamic environment; from the present study one can suggest that the juice of *Citrus medica* Linn. inhibits the growth of Struvite crystals in vitro. This study may be used for formulating the strategy for prevention or cure.

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