Growth inhibition of struvite crystals by the aqueous root extract of Rotula aquatica

C K Chauhan1, M J Joshi1,* and A D B Vaidya2
1Crystal Growth Laboratory, Department of Physics, Saurashtra University, Rajkot 360 005, Gujarat, India
2Kasturba Health Society, ICMR Advanced Center for Reverse Pharmacology in Traditional Medicine, 17 Khandubhai Desai Road, Vile Parle (W), Mumbai 400 056, India

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Formation of urinary stone is a serious and debilitating problem throughout the world. In the present study, the inhibitory effect of aqueous extract of root of Rotula aquatica was investigated against struvite crystals (one of the components of urinary stone) grown in vitro using single diffusion gel growth technique. For setting the gel, sodium metasilicate solution (specific gravity 1.05) and 0.5 M aqueous solution of ammonium dihydrogen phosphate were mixed, so that the pH of the mixture could be set at 7.0. Equal amounts of supernatant solution of magnesium acetate (1.0 M) prepared with 0.0%, 0.5% and 1% concentrations of the extract were gently poured on the set gels. It was observed that the number, dimension, total mass, total volume, growth rate and depth of growth of struvite crystals decreased with the increasing extract concentration in the supernatant solutions. The enhancement of dissolution rate and fragmentation of struvite crystals suggested potential application of the extract for inhibition of struvite type urinary stone.

Keywords: Bio-crystallization, Gel growth, Kidney stone, Rotula aquatica, Struvite

The formation of urinary or kidney stone is a serious debilitating problem throughout the world. The usual components of urinary stones are crystals such as brushite, struvite, calcium oxalate, hydroxyapatite, etc.1-3 Struvite, chemically known as ammonium magnesium phosphate hexahydrate ((AMPH)-(NH4)MgPO4·6(H2O)) is also called triple phosphate or urase stone. It is a biomineral and occurs as crystallite in urine and accounts for 30% of all kidney stones worldwide.4,7 In humans, struvites are formed as a result of urinary tract infection with ureolithic microorganisms that split urea and cause persistently alkaline urine.5-10 The conditions and the pathophysiology of the formation of struvite have been reported earlier.11

The single diffusion gel growth technique is preferred to grow struvite crystals, as it provides the simplified in vitro model of the highly complex in vivo growth of urinary calculi. The gel growth technique has been described earlier in detail,12-14 and is also used to study the growth inhibition of calcium oxalate,15 and calcium hydrogen phosphate dihydrate crystals16 using herbal extracts. A modified gel growth technique has been reported for the growth inhibition study of calcium hydrogen phosphate dihydrate microcrystal using citric acid.37

In Ayurveda, herbal extracts and certain juices are commonly recommended for urinary stone treatments and some of them have been evaluated for their role as inhibitor of urinary crystals.11,16,18-23 Rotula aquatica (belonging to family boraginaceae and commonly known as pashanbheda), a rheophytic woody plant is distributed in India, Sri Lanka, China, tropical southeastern Asia, Africa, Brazil and Latin America.24,25 The plant is scattered throughout peninsular and eastern India in the sandy and rocky beds of streams and rivers. It is used as an Ayurvedic medicine for treatment of cough, cardiac disorder, blood disorders, fever, ulcers, poisons, dysuria, bladder stone, cancer, piles and venereal diseases.26-35

In Ayurveda, it is a well-known lithontriptic (stone-dissolving) drug.36 In India, it is one of the most extensively used medicinal plants to dissolve urinary calculi.37

The root tuber of R. aquatica is astringent, bitter, diuretic, cooling, laxative and also lithotrpic. A decoction of root is diuretic and used for treating stones in bladder.37 The aqueous extract of the root shows antioxidant and antiurolithic activity. The sterol, rhabdiol, polyphenols (tannins), glycosides and ureide allantoin have been isolated from the roots,38-40 which have been reported in the treatment of stones. Sterol and rhabdiol have been found to be active to induce diuresis and allantoin is responsible for diuretic activity.39 The antiurolithic activity of
the root extract has been attributed to its diuretic activity. The aqueous extract of *R. aquatica* root has shown crystal dissolving activity against monosodium urate monohydrate type urinary calculi\textsuperscript{23}, whereas ethyl acetate extract exhibits significant antilithic activity against struvite and calcium oxalate stones\textsuperscript{41}. The stems have also found use in diuretic decoctions and found effective in preventing cell proliferation of pancreatic cancer cell lines\textsuperscript{38}.

Earlier study has demonstrated stone inhibitory effect of *R. aquatica* root decoction in male Wistar rats\textsuperscript{42}; the decoction has been found to be non-toxic over the 45-day treatment period and reduces calcium and oxalate ion concentration in urine, confirming its stone inhibitory effect. The aqueous extract of roots has also demonstrated the anti-inflammatory potential as well as anti-oxidant activity, which provides a scientific basis for the use of the plant in the management of crystal-induced arthropathy\textsuperscript{43}. Moreover, acute toxicity studies have revealed that root extract is safe at all doses when administered orally to rats up to a dose of 2000 mg kg\textsuperscript{-1}. No mortality has observed during the 14 days of the observation period\textsuperscript{43}.

In the present study, the inhibitory property of the aqueous extract of *R. aquatica* root has been investigated against urinary type struvite crystals grown *in vitro* using the single diffusion gel growth technique.

**Materials and Methods**

**Chemicals**

Sodium metasilicate (SMS) - {Na\textsubscript{2}SiO\textsubscript{3}, 9H\textsubscript{2}O}, ammonium dihydrogen phosphate (ADP) - {NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}, 2H\textsubscript{2}O}, magnesium acetate - {C\textsubscript{6}H\textsubscript{5}MgO\textsubscript{4}, 4H\textsubscript{2}O} and all the reactants used were of analytical grade (Merck, Mumbai, India) and procured from Krishna Scientific Traders, Rajkot.

**Preparation of *Rotula aquatica* root extract**

The roots of *Rotula aquatica* were collected from Savantwadi, Maharashtra in the month of February and authenticated by Dr. M R Almeida, Taxonomist, Mumbai. The roots were cleaned, dried, powdered (50 g) and added to distilled water (400 ml). The mixture was heated in a boiling water bath until it reduced to half of the original volume. The extract was dried in a rotary vacuum evaporator to a syrupy consistency and then in a steam bath to thick, pasty consistency. The extract was reddish-brown in color and pasty in nature and showed the presence of sugar, phenol, tannin and saponin\textsuperscript{44}. The extract was prepared at Dr Vaidya’s Lab at Mumbai and stored in glass vials kept in airtight plastic boxes in refrigerator. The 0.5\% and 1.0\% concentrations of the extract were used to determine inhibitory effect on struvite type urinary calculi.

**Single diffusion gel growth technique**

An aqueous solution of 0.5 M ammonium dihydrogen phosphate (ADP) was mixed with the sodium metasilicate (SMS) solution of specific gravity 1.05 in appropriate amount to set the pH value 7.0 for the mixture, and then transferred into the autoclaved test tubes (length 140 mm and diameter 25 mm) to set into gel form. All test tubes and other glassware were autoclaved at 120°C for 15 min. The silica hydro gel was preferred, as it remained stable and did not react with the solutions or with the crystals formed. After the gelation, 20 ml supernatant solution of pure 1.0 M magnesium acetate (control solution) and 20 ml supernatant solutions of 1.0 M magnesium acetate prepared with 0.5\% and 1.0\% concentrations of the extract of *R. aquatica* were gently poured on the set gels in the respective test tubes. This was done in the aseptic medium in laminar flowhood to avoid microbial contamination. The measured pH value of the supernatant solutions was 8.00, 6.95 and 6.76, respectively. After pouring supernatant solution, the test tubes were capped with airtight stopples. The experiment was conducted at the room temperature. The apparent length of growing/dissolving struvite crystals in each test tube was measured by a traveling microscope of least count 0.001 cm at different depths from the gel-liquid interface at regular time intervals. The total mass and volume of the grown crystals in each test tube were measured after removal of crystals from the respective gels.

**Statistical analysis**

The single factor analysis of variance (ANOVA) was also carried out to check the comparison of values of apparent length of struvite crystal in the control and *R. aquatica* groups. The statistical analysis was carried out using MS excel. From the ANOVA single factor statistical analysis, it was clear that the variation in the average length with time in each case was highly significant at 0.05 level.
Results and Discussion

Growth inhibition studies of struvite crystals

The growth inhibition study is important as the growth of calculi continues to occur with the supply of nutrients from the urine and simultaneously the inhibition process has to be achieved. Similarly, in vitro growth inhibition studies using gel growth technique, nutrients are constantly supplied to the growing crystals from the reactants and the dissolution or inhibition is to be checked for the selected solutions. Growth inhibition reports on struvite crystals are scarce in literature. Earlier, in vitro inhibition of struvite crystal growth in artificial urine by acetohydroxamic acid (AHA) has been reported. The AHA primarily acts as a urease inhibitor, which may disrupt the struvite growth. Recently, we have reported that Boerhaavia diffusa extract, Citrus medica juice and Commiphora wightii extract exhibit strong inhibitory effect on the struvite.

Morphology of grown struvite crystals

The gel-grown struvite crystals exhibit different morphologies, viz. dendritic, prismatic, rectangular platelet and needle type, depending upon the location of growth. At gel-liquid interface, dendritic-type crystals and at higher depth in the gel from gel-liquid interface, prismatic-type crystals were observed. It was found that the crystals grown in the test tubes without the extract were transparent to translucent diaphaneity. On the other hand, some of the crystals grown in the test tubes with the extract showed dark brown colorization which might be due to inclusion of the extract in the crystals.

Growth of struvite crystals

The number of grown struvite crystals and their average apparent length in silica-hydro gel medium decreased with the increasing concentration of the extract in the supernatant solution. The average length of prismatic-type crystals was 0.7 cm in the case of control solution, which was decreased to 0.4 cm and 0.2 cm in the presence of 0.5% and 1% extract, respectively (Fig. 1). The growth rate of dendritic-type struvite crystals growing in the gel at gel-liquid interface after adding different concentrations of supernatant solutions is presented in Table 1. The growth rate decreased with the increase in the concentration of the extract. The length of crystals growing in the gel at gel-liquid interface increased up to first 4 days for control solution, whereas it increased only for first 2 days and 1 day in the case of 0.5% and 1% extract solutions, respectively, followed by dissolution. The maximum apparent length of grown dendritic-type struvite crystals at the gel-liquid interface decreased with increase in concentration of the extract (1.235 cm, 0.85 cm and 0.638 cm in control and 0.5% and 1% extract containing supernatant solutions, respectively). Interestingly, extent of inhibition of growing struvite crystals at gel-liquid interface at the end of 1st day was 37.13% and 46.16% for 0.5% and 1% concentrations of extract, respectively, demonstrating clearly the inhibitory effect of the extract.

Similarly, the maximum depth of growth (i.e., the depth from the gel-liquid interface in gel up to which struvite crystals were growing) decreased with the increase in concentration of the extract. After 2 days of pouring, it was 5.2 cm for control solution, which decreased to 3.0 cm and 2.8 cm for 0.5% and 1% concentrations of the extract, respectively. This suggested that the extract impeded the diffusion

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<th>R. Aquatica</th>
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Fig. 1—Variation in size of grown prismatic-type struvite crystals for different supernatant solutions [(A) Control, i.e., without inhibitor; (B) 0.5% R. aquatica extract; and (C) 1% R. aquatica extract]
process of reactants occurring in the gel column for the nucleation and subsequently the growth of crystals. Thus, reduction in the depth of growth indicated the inhibition by the extract. Moreover, it was found that as the depth increased, the average apparent length of grown struvite crystals decreased. The maximum apparent length of the grown crystals at 3 cm depth from the gel-liquid interface was 0.365 cm for control, which decreased with increase of concentration of the extract (0.194 cm and 0.125 cm for 0.5% and 1% extract solutions, respectively).

Dissolution of struvite crystals
The growth and dissolution of struvite crystals at gel-liquid interface is shown by plots of average length versus time period in Fig. 2. The dendritic-type struvite crystals grown in the gel at gel-liquid interface dissolved completely within 28 days and 33 days for 0.5% and 1% concentrations of the extract, respectively. It was observed that struvite crystals grown at gel-liquid interface also dissolved to some extent even in the absence of extract. It might be due to partial dissolution of the crystals in acetic acid produced as a byproduct of the chemical reaction which formed the compound for crystals growth. But, dissolution was enhanced in the presence of different concentrations of extract. The dissolution rate of grown struvite crystals in the gel at gel-liquid interface was lower for control \((1.8 \times 10^{-2} \text{ cm/days})\), while it was higher for different concentrations of the extracts \((3.3 \times 10^{-2} \text{ cm/days} \text{ and } 2 \times 10^{-2} \text{ cm/days} \text{ for } 0.5\% \text{ and } 1\% \text{ extract solutions, respectively})\). It was observed that percentage of enhanced dissolution rate was higher for 0.5% concentration of the extract than that of 1% \((83.33\% \text{ and } 11.11\%, \text{ respectively})\).

Figure 3a shows that the length of growing crystals in the gel at gel-liquid interface increased up to first 4 days and then started dissolving due to the formation of acetic acid. It was also noticed that length of crystals at different depths from gel-liquid interface increased up to first 7 days, and then remained constant. As the depth of the gel column increased from gel-liquid interface, the average apparent length of the grown crystals gradually became smaller. The length of growing crystals in the gel at gel-liquid interface increased up to first two days and merely for one day for 0.5% and 1% concentrations of the extract, respectively (Fig. 3b and c).

Fig. 2—Growth and dissolution of struvite at gel-liquid interface

Fig. 3—Growth and dissolution of struvite at different depths from the gel-liquid interface [(a) Control solution; (b) 0.5% \(R.\ aquatica\) extract; and (c) 1% \(R.\ aquatica\) extract]
Fragmentation of struvite crystals

The phenomenon of fragmentation or fracture of the grown struvite crystals due to the presence of the extract was quite interesting and deserved further attention. The incorporation of extract not only allowed the crystalline face to dissolve further, but presumably weakened the existing bonds, leading to cracking and further fracture into fragments. The depth of fragmentation of grown crystals (i.e. the depth from the gel-liquid interface up to which the crystals started breaking) near the gel-liquid interface was noticed for 0.5% and 1% concentrations of the extract (Fig. 4).

It was also observed that at higher depths some of the fragmented crystals retained their critical size. As the concentration of the extract was low at higher depths in the gel column than at the gel-liquid interface, it did not allow crystals to dissolve completely after the fragmentation, but retained a steady state, i.e., a balance between the growth and dissolution. The average length of crystals after fragmentation was found even less than 1 mm. After fragmentation, dimensions of crystals were far less than 5 mm, i.e. the maximum dimension of urinary crystal or calculi which can easily pass through the urinary tract. The total mass and volume of struvite crystals also decreased with the increasing concentration of extract (Fig. 5).

Conclusion

The reduction in number, dimensions, total mass, total volume, growth rate, depth of growth, as well as enhanced dissolution rate and fragmentation of grown struvite crystals indicated the potential inhibitory effect of R. aquatica extract.

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