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In vitro Growth and Inhibition Studies of Monosodium Urate Monohydrate Crystals by Different Herbal Extracts

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Abstract: Problem statement: A large number of people in the world have been suffering from gout for centuries. Gout is the term given to a metabolic condition in which the signs and symptoms results from the deposition of crystals of Monosodium Urate Monohydrate (MSUM) in various connective tissues. **Approach:** It was quite difficult to grow good quality large MSUM crystals because uric acid was having very low solubility in any known solvents. **Results:** Attempts were made in the present investigation to grow MSUM crystals *in vitro* by single diffusion gel growth technique, which is quite suitable to mimic the growth of MSUM crystals *in vivo* up to a certain extent. Good quality needle type crystals were grown in the gel, which were characterized by FT-IR, Powder X-ray diffraction and Thermo-gravimetry. The characterization study confirmed the formation of MSUM crystals in the experiments. Herbal extract solutions of *Boswellia serrata* Linn., *Aerva lanata juss* ex. *Schult*, *Routula aquatica* Lour. and *Boerhavia diffusa* Linn., were used for the growth inhibition study of MSUM crystals. Aqueous herbal extract solutions were poured as supernatant solutions along with the control solution on the set gels during the crystal growth experiment. The growth observations and the measurements of crystal dimensions using optical microscope suggested that *Routula aquatica* Lour. and *Aerva lanata juss* ex. *Schult* extracts exhibited good crystal growth inhibition results. **Conclusion:** *Routula aquatica* Lour. extract dissolved MSUM crystals after 15 days of pouring the supernatant solution on the gel. This *in vitro* study may be helpful for *in vivo* studies, which may further lead to develop a preclinical formulation for gout treatment.

Key words: Crystals, powder X-ray diffraction, FTIR, thermal stability, herbal extracts

INTRODUCTION

Gout is the term given to a group of metabolic conditions, in which the signs and symptoms results from the deposition of crystals of Monosodium Urate Monohydrate (MSUM) in various connective tissues and joints. It is also an auto inflammatory disorder associated with deposition of MSUM crystals in joints and periarticular tissues. The recent advances, however, suggest that the innate immune system may drive the gouty inflammatory response to MSUM[1,2]. The deposition of these crystals, result from the raised levels of uric acid in blood (hyperuricemia) and various body fluids. Hyperuricemia is not essential requirement of diagnosis of gout, but the risk of gout and its presence increases with the degree and duration of hyperuricemia. The risk factor of gout is also associated with the uric acid and urate compound nephropathy[3]. Nephropathy of uric acid and urate substances is associated with the formation of Monosodium Urate (MSU), ammonium urate and uric acid in urinary tract system. The MSU is rarely observed in renal calculi in spite of human urine often being supersaturated with monosodium urate[4-6]. Only exceptionally pure monosodium urate calculi have been described and these were of vesical origin[7]. Hyperuricosuria,

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simulated by the addition of dissolved sodium urate, promotes the crystallization of Calcium Oxalate. It could also act as an effective promoter of calcium oxalate crystallization according to *in vitro* experiments[8,9].

A very few attempts have been made to grow the uric acid or urate crystals. Irusan *et al*.[10] have reported the growth of ammonium urate crystals using the gel growth technique, whereas Kalkura *et al*.[11] have reported the growth of spherulites of monosodium urate monohydrate and Kalkura *et al*.[12] have studied crystallization of pure uric acid.

From this description of accumulated knowledge about the role of Monosodium Urate Monohydrate (MSUM) crystals in gout or gouty arthritis as well as urolithiasis, it is evident that till today there is no sufficient information available to clarify all aspects related to crystallization behavior under physiological conditions and its different characteristics. It is, therefore, important to grow and characterize MSUM crystals and study the inhibitive effect of various herbal extracts on its growth. The growth and inhibition study provides very useful information regarding the potent herbal extracts or herbal formulations, which can inhibit the growth of crystals *in vitro* and may be useful in prevention or cure of this crystal induce ailments.

MATERIALS AND METHODS

The growth of MSUM crystals is not widely reported. In the present investigation, glass test-tubes of 25 mm diameter and 150 mm length were used as crystal growth apparatus. Sodium metasilicate solution of 1.05 specific gravity and 0.2 M, NaOH solutions were added in equal amounts. This mixture was acidified by 2N, acetic acid in such manner that the pH values within 4.5-5.0 are obtained. This mixture was poured in equal volumes in different test tubes and allowed to set into the gel form. Within 48 h, good quality gels were set. After setting the gel, the 0.07 M uric acid solution was poured gently on that. Good quality needle shaped crystals were grown, which are shown in Fig. 1. The probable reaction for the formation of MSUM is as follows:

$$NaOH + C_5H_4N_4O_3 \rightarrow NaHC_5H_2N_4O_3 \cdot H_2O$$

Different plants were selected for the preparation of extracts, which are *Boswellia serrata Roxb* (gum resin), *Routula aquatica* Lour. (roots) and *Boerhaavia diffusa* Linn. (roots) and *Aerva lanata* Juss ex. *Schult* (root). Hot aqueous extracts are obtained by taking 50 g of plant materials placed in a beaker and then 400 mL of distilled water was added to the plant material in the ratio 1:8, which was heated in a boiling water bath until the kwath reduces in half of the original volume. All extracts were air dried in a rotary vacuum evaporator to a syrupy consistency and then in a steam bath to thick, pasty consistency. All extracts were stored in glass vials kept in air tight plastic boxes at-20°C.

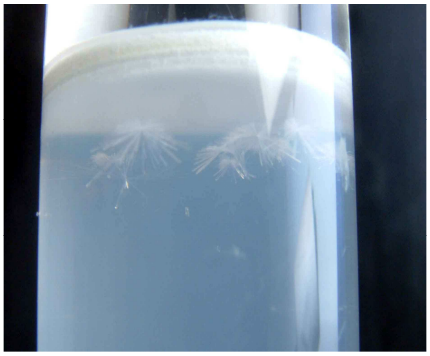

Fig. 1: Crystal growth of MSUM crystal

Figure 1 shows the grown crystals. The crystals have been characterized by different techniques.

FT-IR spectrum was recorded on Shimadzu 8400 set up in 400-4000 cm^{-1} range using powdered samples in KBr medium. Powder XRD was carried out on Philips X'pert using Cu K_α radiation. Thermogram was recorded on Pyrif 1, Perkin Elmer, in atmosphere of air, from room temperature to 900°C at a heating rate of 15°C min^{-1}.

RESULTS

The molecular structure of MSUM is as Shown in Fig. 2. Figure 3 shows FT-IR spectrum of MSUM crystals. Table 1 gives assignments to the peaks.

From the FT-IR spectrum one can confirm the presence of keton group (C = O), carbon-nitrogen bond (C-N), N-H stretching and rocking as well as the presence of water of hydration in the sample.

Figure 4 is the powder XRD pattern and Table 2 gives the data. The samples are highly crystalline in nature.

Figure 5 is the thermo-gram of MSUM, which shows that the compounds is stable up to 100°C and slowly give up water of hydration and on wards 170°C starts losing crystalline water immediately. The associated water molecule is completely removed at 240°C and becomes anhydrous. Subsequently, when the temperature is further increased, the mass loss occurs very sharply between 370-480°C with the release of gases like hydrogen cyanide, carbon dioxide and carbon monoxide. This is followed by a slow process between 480-750°C with loss of nearly 75-80% of original mass.

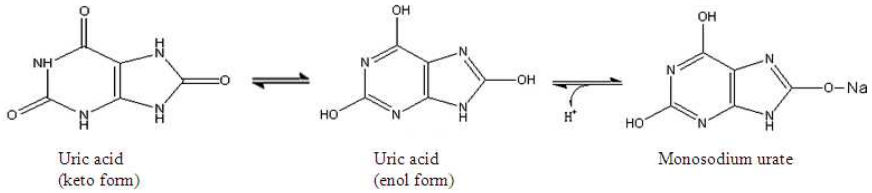

Fig. 2: Chemical structure of MSU

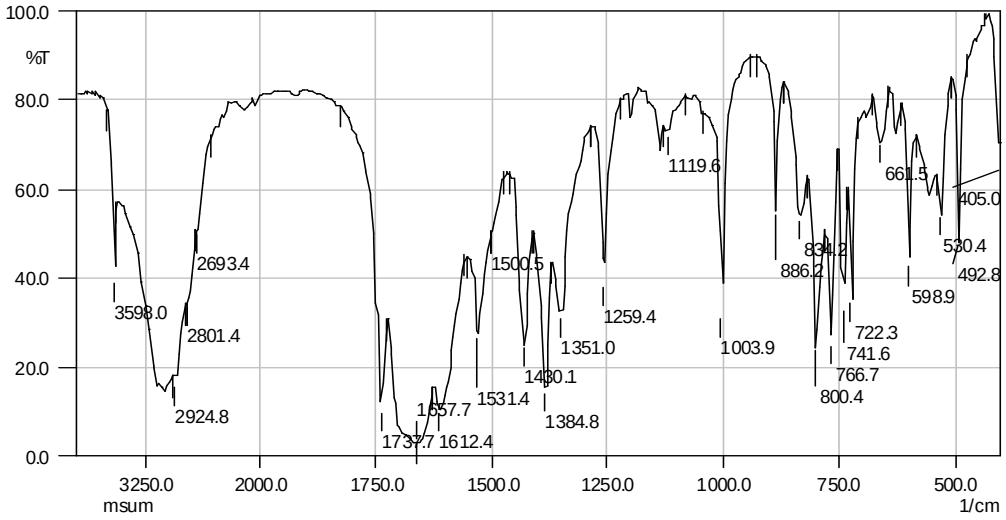

Fig. 3: Shows FT-IR spectrum of MSUM crystals

Table: 1 Assignments of observed absorptions in FT-IR spectrum

Monosodium urate monohydrate $NaHC_5H_2N_4O_3$

Assignments	Observed vibrational frequencies (cm^{-1})
C = O	1737.7
C = C	1531.4, 1500.5
C-N	1259.4, 1351.0, 1384.8
N-H stretching	2924.8
N-H rocking	722.3, 741.6, 766.7, 800.4, 886.0, 842.0
O-H stretching	3598.0
Oxygen-metallic bond	400-600

Table 2: Powder XRD data of MSUM crystals

2θ	d(Å)	Height	FWHM
9.3700	9.43082	73.4	0.5034
11.600	7.62221	86.0	0.4947
17.913	4.94785	93.5	0.5017
18.889	4.69429	185.2	0.5381
25.466	3.49486	95.9	1.2240
26.121	3.40877	78.8	1.3220
28.246	3.15688	476.9	0.5930
29.316	3.04410	67.7	0.4938
30.789	2.90170	75.7	0.8045
33.632	2.66260	193.3	0.5999
35.404	2.53332	69.4	1.6593
36.390	2.46695	94.9	0.7224
39.933	2.25581	57.8	0.6052
40.948	2.20223	70.8	0.8910
44.334	2.04158	86.2	0.6576
53.773	1.70337	63.2	1.6051

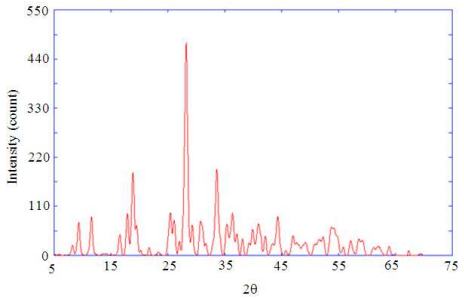

Fig. 4: Powder X-ray diffractogram of the MSUM crystals

Finally, it is converted into Na_2O, which remains stable up to the end of the analysis. One water molecule is found to be associated with the crystals.

However, no work is reported on the *in vitro* growth and inhibition study of MSUM crystals. Figure 6 shows the test tubes having the mixture of uric acid and solution of herbal extracts, which were used for inhibition study. In the case of pure uric acid the average length of grown crystals was 0.675 cm. But for the *B. serrata*, containing supernatant solution, no considerable change was observed. The same effect was observed in the case of *B. diffusa.*

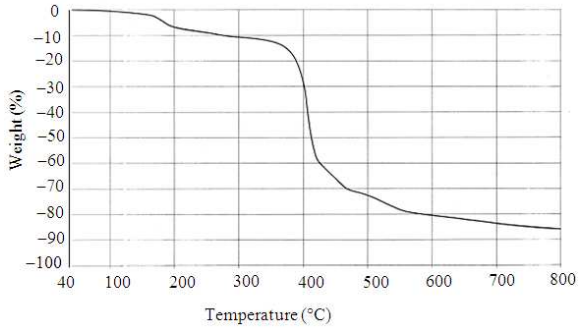

Fig. 5: Thermogram of the MSUM crystals

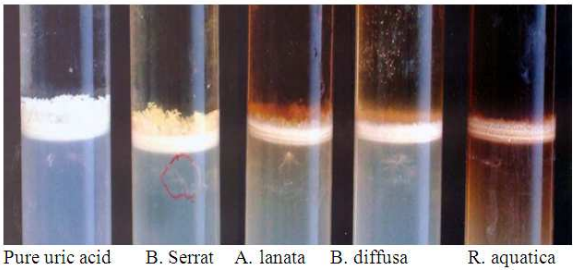

Fig. 6: Comparative study of growth inhibiton study of MSUM crystals

Table 3: Observed crystal length

Solutions	Initial Length of crystals	Length of crystals after 7 days	Remarks
Pure Uric acid	0.675	0.675	No change
B. serrata+ Uric acid	0.664	0.653	Minute change
R. aquatica+ Uric acid	0.880	0.812	After 15 days crystals completely dissolved
B. diffusa+ Uric acid	0.600	0.586	Minute change
A. lanata+ Uric acid	0.865	0.768	Remarkable change

Result of inhibition study of MSUM crystals in terms of observed crystal length after seven days of pouring the herbal extract solutions is shown in Table 3. From the Table 3, one can easily conclude that *R. aquatica* and *A. lanata* give comparatively good inhibition than the others. However, the ethanolic herbal extract solutions almost gave the same results. As uric acid is completely insoluble in ethanol, it was observed that by adding ethanolic extract the re-precipitation occurred in the supernatant solution. But aqueous extract of *B. diffusa* (80 mg mL^{-1}) was found mildly active. These results were found to be encouraging for the *in vivo* studies and the formulation of the drug. This study may be helpful to design the therapies for the prevention and cure of gout.

DISCUSSION

Monosodium urate (MSU, $NaHC_5H_2N_4O_3$) is a white powder and soluble in water to the extent up to 120 mg dL^{-1}[13], for the sake of comparison a much more limited solubility of uric acid in water is reported[14], which is only 6.5 mg dL^{-1}. Allen *et al.*[15] have found that urate solubility in solutions of 140 mEq L^{-1} sodium content is temperature dependent, with a two fold drop in solubility between 37 and 25°C. Moreover, Wilcox *et al.*[6] have carefully reinvestigated the solubility of uric acid and monosodium urate and have found that both are dependent on pH. Uric acid is more soluble with increasing pH, whereas the MSU is less soluble. The solubility of urate in plasma is somewhat greater than the saturation value in aqueous solutions of 0.13 M sodium. Actual determination of solubility of monosodium urate in human plasma (or serum) indicates that the saturation occurs at concentrations about 7 mg dL^{-1}[16-18]. Monosodium urate mainly forms due to the reaction between the Na^+ ion and the urate ion of uric acid. Due to limited solubility of uric acid in any known solvent it is difficult to grow MSUM crystals.

Grases *et al.*[19] have studied the structure of urate renal calculi after precipitation of ammonium and sodium urate from the synthetic urine; on the other hand, Grover *et al.*[20] have studied the *in vitro* growth of calcium oxalate crystals in the presence of pre-incubated seed crystals of uric acid and MSU with undiluted human urine.

FT-IR spectroscopy is an excellent tool to identify various chemical bonds in a compound. Kalkura *et al.*[11] have reported the IR spectra of spherulites of monosodium urate monohydrate. The FTIR spectroscopic study has proved the presence of all functional groups and bonds in MSUM crystals.

Howell *et al.*[21] have reported the powder XRD patterns and d values of uric acid, MSUM and Disodium Urate Dihydrate (DSUD), but unit cell parameters were not reported. The Powder XRD of tophaceous deposits in gout also pin points MSUM as the prime accuse. It is very difficult to obtain the single crystal XRD data on the present MSUM crystals because they get degraded immediately upon removal from the test tube upon exposure of the atmosphere. However, the present powder XRD study suggests that samples are crystalline in nature.

Thermogravimetric Analysis (TGA) is performed to assess the thermal stability of the substance. Earlier, Schneitzler *et al.*[22] have reported TGA of purine derivatives such as aminophylline, theoophylline, caffeine and uric acid. It was found that uric acid was

stable up to 375°C and thereafter the mass loss occurred through rapid process. However, the MSUM crystals having one water molecule associated with it and became anhydrous at 240°C and above 370°C the mass loss became very rapid. The stabilities of uric acid and MSUM are comparable.

The growth and inhibition study of various bio-material crystals, particularly, responsible for ailments related to urinary stones were studied. Joshi *et al*.[23] reported the effect of herbal extracts on the growth of calcium oxalate as well as struvite type urinary crystals by Chauhan *et al*.[24]. Recently, Parekh *et al*.[25] have reported the growth inhibition of hydroxyapatite crystals by herbal extracts. From the Table 3, one can easily notce that *R. aquatica* and *A. lanata* give comparatively good inhibition than the others. However, the ethanolic herbal extract solutions almost gave the same results. As uric acid is completely insoluble in ethanol, it was observed that by adding ethanolic extract the re-precipitation occurred in the supernatant solution. But aqueous extract of *B. diffusa* (80 mg mL^{-1}) was found mildly active. These results were found to be encouraging for the *in vivo* studies and the formulation of the drug. This study may be helpful to design the therapies for the prevention and cure of gout.

CONCLUSION

The growth of MSUM crystals was carried out by using single diffusion gel growth technique, which is well suited to mimic the conditions of the growth of crystals *in vivo*. Very thin, transparent, needle type crystals are observed near the gel-liquid interface. FT-IR spectrum of MSUM crystals confirms the presence of a water molecule, metal-oxygen bond, O-H stretching, N-H rocking and stretching, C = C bond, C = O carbonyl group (keton group) and C-N bond. The powder XRD results confirm the crystalline nature of the sample. MSUM crystals are thermally unstable and finally decompose into Na_2O. One water molecule is associated with the crystal. . In the case of 1% aqueous extract solutions of *B. serrata and B. diffusa* the inhibition was not significant, but in the case of 1% aqueous extract solutions of *R. aquatica* and *A. lanata*, good amount of inhibition was shown. Till today there is no major remedy is available to dissolve the MSUM crystal in synovial fluid, which is responsible for gout. This study may help the prevention or cure of gout.

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