Growth patterns of monosodium urate monohydrate (gouty and urinary) crystals in gel: An *in vitro* study

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Abstract: Monosodium urate monohydrate crystals are deposited in peripheral joints causing gout and elicit an intense localized inflammatory attack whereas in collecting ducts and medullary interstitium as a urinary calculi which causes dysuria, nausea and hematuria. The purpose of present study is to observe possible growth patterns of monosodium urate monohydrate crystals. The crystals were grown in test tubes by single diffusion gel technique and were observed at 7th, 14th, 21st, 28th and 40th day. Needle and spherulite type crystals were observed at 14th day, while arboresque, crystal sheaves, densely branched, dumbbell, mushroom type spherulites, plumose and hexagonal prismatic crystals were observed for the first time. After 40th day complete spherulites were observed with their aggregates. The crystals were characterized by Scanning Electron Microscopy, Energy-Dispersive X-ray and Fourier Transform Infra Red spectroscopies. The pattern of MSUM will be helpful to determine the role of different natural products in the modulation, inhibition or promotion of these crystals by affecting the shape, size, transparency, approximate number and total mass of growing crystals. In case of inhibition or modulation it will be helpful for evaluation the prophylactic management whereas the promotion of the crystal will give an idea about the risk factors of gout and kidney stones.

Keywords: Crystal morphology, characterization, urate.

INTRODUCTION

Urate crystals are found in different forms as uric acid anhydrous, monohydrate and dihydrate, monosodium urate mono hydrate and ammonium acid urate. Mono sodium urate monohydrates (MSUM) are deposited in peripheral joints and periarticular soft tissues causing gout whereas in collecting ducts and medullary interstitium as a urinary calculi. In addition to hyperurecemia some localized factors such as temperature, pH and mechanical stress also play an important role in crystal formation (Sutor and Scheidt, 1968; Choi *et al.*, 2005; Martillo *et al.*, 2014). The pathogenesis of mono sodium urate monohydrate urolithiasis involves urine pH persistently less than 5.5, plasma urate supersaturation beyond 6.8 mg/dL or 405 µmol/L, hyperuricosuria, low urinary volume, dietary intake of high purine and also genetic factors. Epidemiological studies revealed that males are three times more affected than females. Elderly people of more than 60 years have two times more tendency than younger. Symptoms of disease include dysuria, nausea and hematuria (Ngo and Assimos, 2007; Martillo *et al.*, 2014). The MSUM renal calculi also promote calcium oxalate monohydrate crystallization (Grover *et al.*, 2003).

Crystal growth in the gel is a simple, easy and inexpensive *in vitro* technique which provides crystals of different morphologies and sizes along with practical observation of crystal growth stages (Henisch, 1968). Gel serves as an ideal medium for studying crystal deposition diseases such as the formation of atherosclerotic plaque, gall stones, gouty crystals and urinary stones (Patel and Rao, 1982). The gel medium is chemically inert, prevents the turbulence and provides a framework of separated nucleation sites to grow single crystal. The viscous nature, temperature and pH of the gel provide a resemblance with human physiological conditions. However, the size, quality and quantity of growing crystal during the experiment can't be predicted (Robert and Lefaucheux, 1988; Sperka, 1988). Earlier, MSUM crystals have been grown in tetramethoxysilane and sodium meta silicate gel (Kalkura *et al.*, 1995). The present *in vitro* study is an attempt to observe and report possible growth patterns of MSUM which will be helpful to determine the role of different natural products in the modulation, inhibition or promotion of these crystals.

MATERIALS AND METHODS

Chemicals

Acetic acid (glacial) 100 % anhydrous, sodium hydroxide, sodium silicate solution (Merck, Germany), Uric acid crystalline and uric acid sodium salt (Sigma-Aldrich Chemie, Switzerland).

Instruments

Glass test tubes of 25 mm diameter and 150 mm length; IR Prestige-21 FTIR Spectrophotometer Shimadzu; JSM-6380A Scanning Electron Microscope and EDS EX-54175 JMU, JEOL Japan; Nikon Eclipse E 400 binocular microscope, Japan.

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Crystal growth

The single diffusion gel technique was used to grow mono sodium urate monohydrate crystals (Parekh *et al.*, 2009; Choubey, 2011). The gel medium was prepared by mixing sodium meta silicate solution of 1.06 specific gravity, 0.2M NaOH and 2N acetic acid solution to obtain pH 5.18-5.21. After gel formation, 20mL of 0.07M uric acid solution was gently poured along the wall of the test tube drop wise without disturbing the gel and the test tubes were capped. The experiment was carried out at 25±2°C. Test tubes were observed at 7^{th}, 14^{th}, 21^{st}, 28^{th} and 40^{th} day.

Gel removal and collection of dried crystals

The gel inside test tube was detached and transferred into petri dish of diameter 6″ containing water. The harvested crystals were carefully recovered from the gel and dried using absorbent paper, then washed three times with double distilled water and again dried with absorbent paper. 40x photograph of each or every crystal was taken.

Characterization of harvested crystals

The crystals were characterized by SEM, EDS and FTIR. Crystals were coated with gold up to 300 Å using Quick Auto Coater and analyzed directly for SEM imaging. Then EDS measurements were taken and data were recorded for 40 days harvested crystals and standard samples by using the EDS detector. The FTIR spectra of 40 days grown crystals and standard compound were recorded in potassium bromide phase with 4000-400 cm^{-1} spectral range.

STATISTICAL ANALYSIS

Results are expressed as mean ± standard error of mean and were analyzed by unpaired student *t*- test.

RESULTS

Arboresque, crystal sheaves, densely branched, dumbbell, mushroom type spherulites, needles, plumose and hexagonal prismatic crystals were observed (figs. 1 & 2). Weight of harvested crystals, their morphology and density (crystal crowd) are shown in table 1. The SEM image of MSUM spherulite is shown in fig. 3. Seven days, harvested crystals were incompletely formed spherulites. Crystals of the 40 days harvesting period were good quality spherical crystals with cylindrical thread like fibers sticking out. Data from each EDS spectrum shows the mass percentage of detecting elements in testing and standard samples, using ZAF method standardless quantitative analysis. EDS spectra of harvested crystals matched with standard (fig. 4). The FTIR spectra of 40 days harvested crystals and standard compound are shown (fig. 5) with vibration patterns (table 2).

DISCUSSION

Spherulites (sphere like crystals) and needles shape crystals have been observed, while previous studies reported the formation of needles, densely branched, bow shaped and aggregated spherulites (Kalkura *et al.*, 1995). Arboresque (hairy sphere like crystals which gives tree like appearance), densely branched, dumbbell, mushroom type spherulites (umbrella or domed cap like spherical crystal) and crystal sheaves (crystals in the form of bunch of needles) were also observed as MSUM growth patterns for the first time. Plumose (feather like aggregate of small crystals) and hexagonal prismatic (prism like with hexagonal base) crystals which are rarely formed during spherulitic growth (Rosseeva *et al.*, 2009) were also observed. In gouty arthritis, MSUM crystals are found as needles and ball shape in synovial fluid whereas aggregated bow shaped in the articular cartilage (Kalkura *et al.*, 1995). Current FTIR analysis compared with the spectra of previously reported and commercially available compound and confirms harvested crystals as of MSUM.

Needle shape is the basic morphology of the spherulite polymorphs. Radial arrays of crystalline needles originate from a common initial nucleating point and form spherulite. Needles often have some curvature (branching angle). These needles repeated tip splitting in preferred directions of curvature, with the needle tip acting as a template. In addition to radial growth, the thickness of needles also increases. Peripheral needles are thin as compared to needles located at the center (Heijna *et al.*, 2007). In MSUM crystals, urate anions are hydrogen-bonded together to form a purine ring. Sodium ions coordinate to the urate purine ring with water molecules and form crest of sheets. These sheets generate longitudinal axis of the crystal by stacking themselves. The nuclei of urate crystals become stable after gaining critical size and the crystals grow more rapidly along longitudinal axis forming cylindrical thread like fibers. These fibers branch to form space filling pattern followed by spherulite formation. Sometimes, at the growth front of initially grown fibers, secondary fibers nucleate to form crystal sheaves which expand on each side of primary nucleation to develop two eyes (non crystalline region) and shaped into spherical. By increasing supersaturation, eyes become small till the complete spherulite formation (Gránásy *et al.*, 2005; (Martillo *et al.*, 2014). As a combined effect of length and width growth, needle shape crystals become changed into a cylindrical thread like fibers and when the process approaches to equilibrium, needles cease to grow and show as spherulite with thick fibers sticking out (Heijna *et al.*, 2007). Mono sodium urate monohydrate [$NaC_5H_3N_4O_3.H_2O$] crystals, express triclinic symmetry with space group P1, a = 1.0888(5) nm, b=0.9534(3) nm, c = 0.3567(1) nm, α = 95.06˚, β = 99.47˚, γ = 97.17˚ and Z = 2 (Mandel and Mandel, 1976; Kalkura *et al.*, 1995).

Table 1: Growth patterns of MSUM crystals

Growth period (days)	Weight of harvested crystals (mg)	Crystal morphology with order of crystal density
07	12.54±0.01*	Needles ℘ incomplete spherulite.
14	33.30±0.05*	Needles, complete spherulite and their aggregates ℘ crystal sheaves and their aggregates ℘ densely branched spherulites and their aggregates ℘ mushroom shape spherulite ℘ spherulite with arboresque growth ℘ dumbbell shape arboresque spherulite, plumose and hexagonal prismatic crystal.
21	47.00±0.00*	Complete spherulite and their aggregates ℘ dumbbell shape spherulites ℘ mushroom shape spherulites.
28	59.50±0.25	Complete spherulite and their aggregates ℘ in complete spherulites.
40	61.00±0.02*	Complete spherulite and their aggregates.
All values represent mean± SEM of n=5; *P<0.05 showing significant values using unpaired student's *t*-test.		

Table 2: FT-IR wave numbers and vibrations assignment of MSUM crystals

Wave numbers (cm^{-1}) of MSUM=$NaC_5H_3N_4O_3.H_2O$			Bonds / vibrations
Standard (commercially available)	Test (grown)	Reported values (Parekh *et al.*, 2009)	
3599.17	3599.17	3598.00	O-H stretching(weakly H bonded OH vibrations) of water
3059.10	3055.24	--------	
2179.56	--------	--------	
2058.05	2058.05	--------	
----------	1924.96	--------	--------
1737.86	1737.86	1737.70	C=O
--------	1668.43	---------	H-O-H symmetric bending vibrations
1529.55	1527.62	1500.50,1531.40	C=C
1431.18	1429.25	1430.1	--------
1384.89	1382.96	1384.80	C-N
1354.03	1350.17	1351.00	
1259.52	1255.66	1259.50	
1199.72	----------	--------	
1134.14	1130.29	--------	
1002.98	1001.06	--------	
885.33	885.33	842.0, 886.00	N-H rocking
798.53	794.67	800.40	
--------	721.38	722.30, 741.60,766.70	
659.66	626.87	661.50	--------
597.93	594.08	400-600	Oxygen-metallic bond
534.28	530.42	--------	
--------	489.92	--------	

Pathologic crystallization causes atherosclerotic plaque, gall stones, gout and urinary stones. The process of crystal growth is very complex. Therefore the study to find out crystallization promoting or inhibiting factors is important (Natarajan *et al.*, 1997; Kalkura and Natarajan, 2010). The direct observation of crystallization is not possible by *in vivo* models and the mechanism remains unexplained. *In vitro* models not only provide the direct observation of crystal growth, but also devising the meaning of unwanted crystal promotion, modulation or inhibition. Growth of pathologic crystals in gel along with plant extracts and juices gives important information about crystallization promotion, modulation or inhibition by comparing the changes. These changes include shape, size, transparency, approximate number and total mass of crystals (Natarajan *et al.*, 1997). In case of inhibition, it

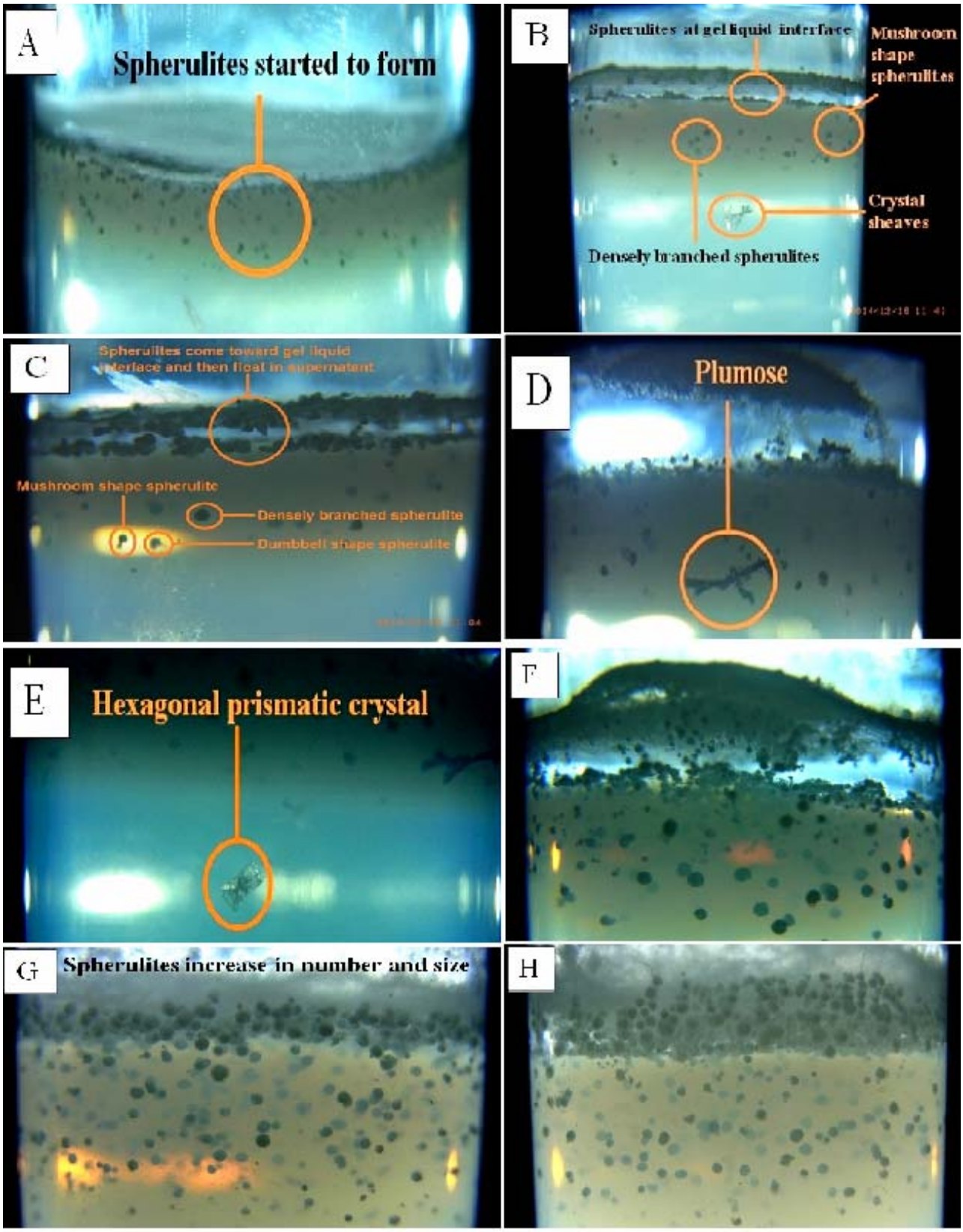

Fig. 1 : MSUM crystals grown in gel medium after (A) 7 days, (B-E) 14 days, (F) 21 days, (G) 28 days and (H) 40 days.

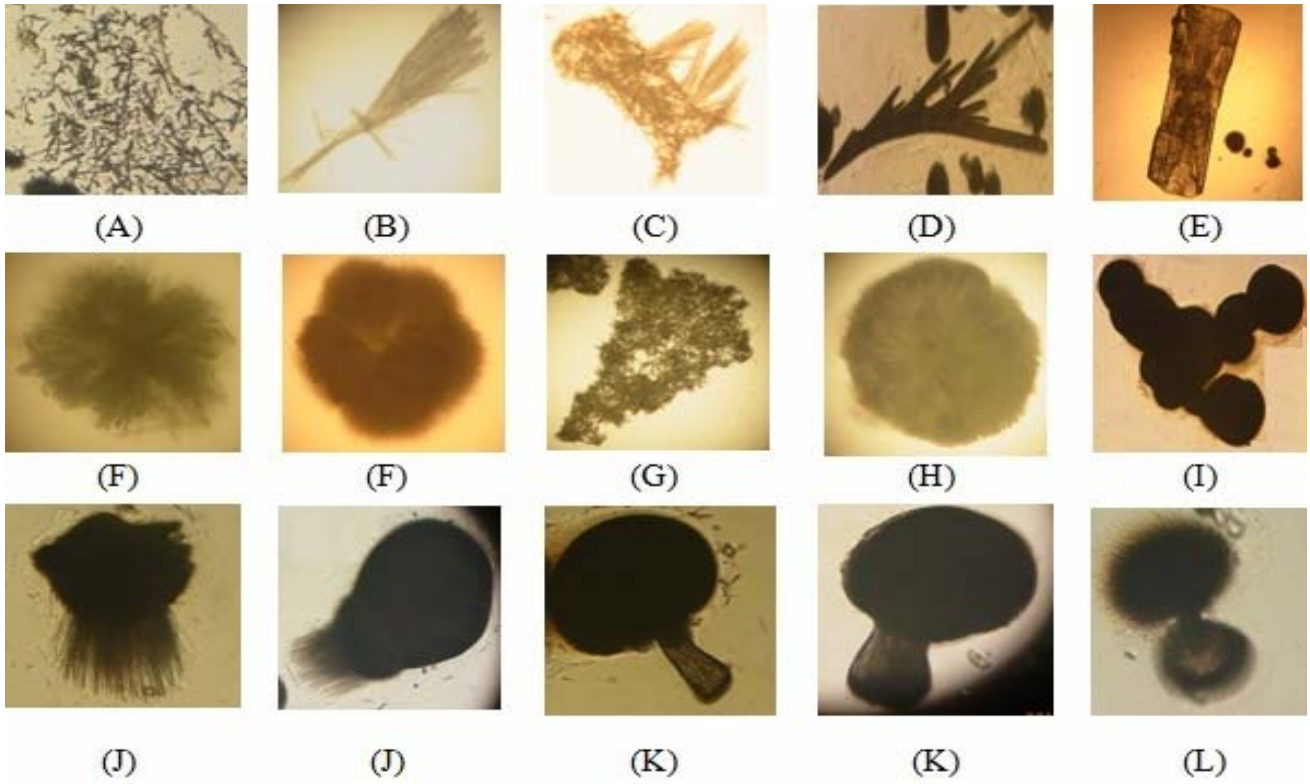

Fig. 2: Microphotographs (40x) of 14 days grown MSUM showing variety of habits. (A) needles, (B, C) crystal sheaves and their aggregates, (D) plumose, (E) hexagonal prismatic crystal (rare case), (F , G) densely branched spherulites and their aggregates, (H , I) complete spherulite and their aggregates, (J) spherulite with arboresque growth, (K) mushroom shape spherulite and (L) dumbbell shape arboresque spherulite.

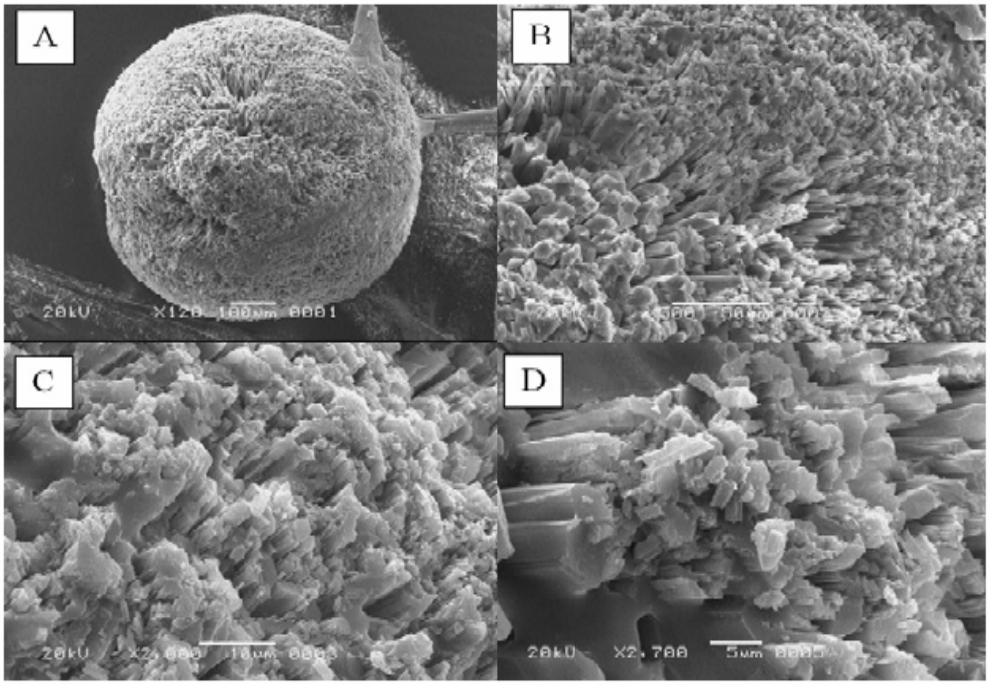

Fig. 3: SEM images displaying completely grown MSUM spherulite at different µm. (A) 100, (B) 50 , (C) 10 and (D) 5.

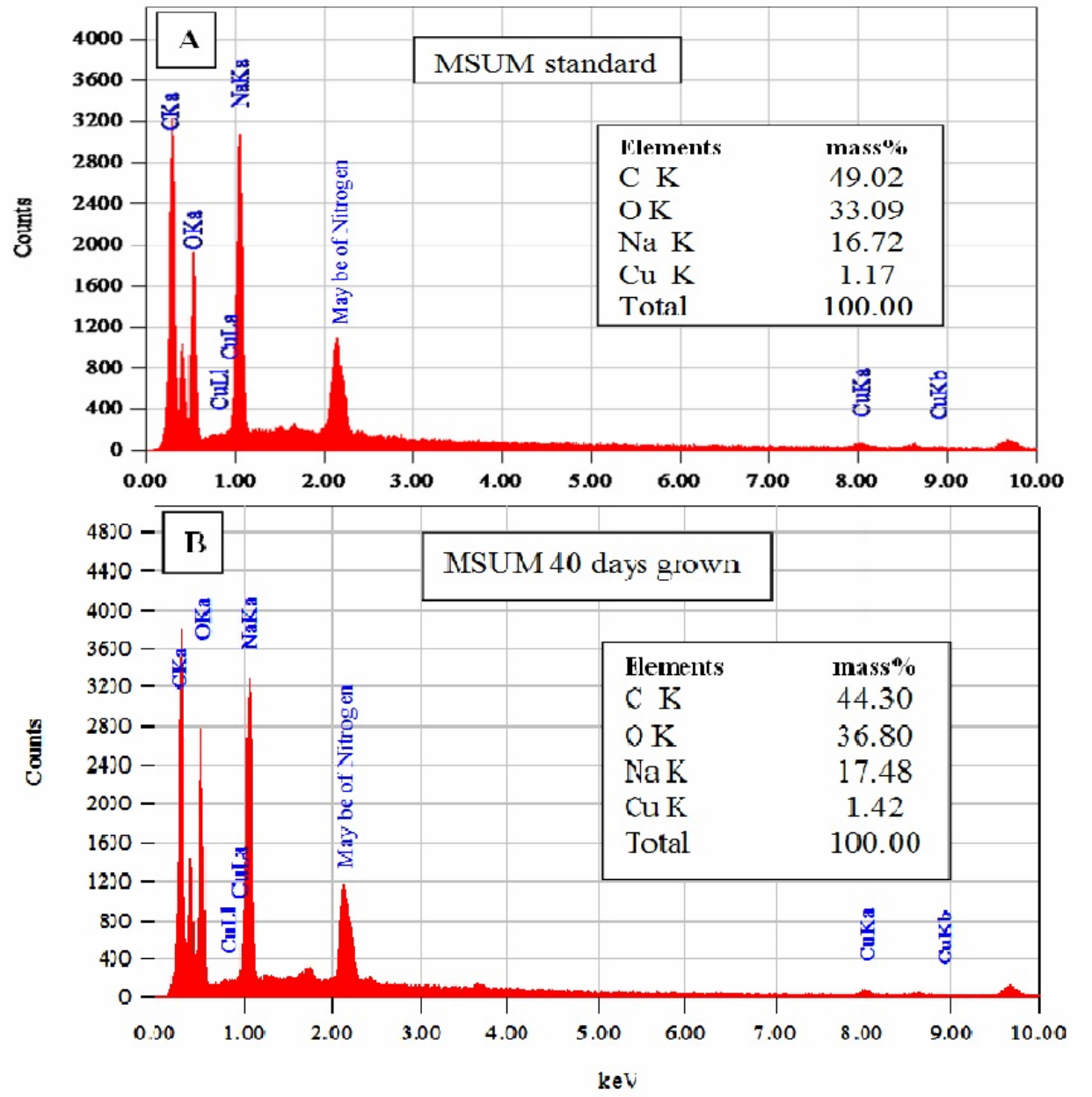

Fig. 4: EDS analysis of MSUM crystals. (A) standard, (B) 40 days grown.

assures prophylactic management by evaluation of nucleation, growth and aggregation of growing crystals. Therefore, this *in vitro* technique provides a multidisciplinary approach in characterizing the grown crystals and help in formulating a strategy for the prevention or dissolution of gouty and urinary crystals. In case of promotion, i.e. increase in size and number of crystals will give an idea about risk factors of these diseases (Ahmed *et al.*, 2016). The technique may be utilized for new means to control crystal growth. As tested substance could be applied during and after the growth of crystals showed the effect of testing substance (natural products), when crystals are starting to form and after their complete formation respectively (Joseph *et al.*, 2005). The present study only shares the MSUM formation and did not discuss about growth kinetics. Atomic force microscopy and Scanning confocal interference microscopy are experimental techniques use to measure growth rates of crystals along different axes (Mcpherson *et al.*, 2000; (Grohe *et al.*, 2006; (Van Driessche *et al.*, 2008). Therefore, these techniques are recommended to understand the crystal growth kinetics.

CONCLUSION

MSUM crystal deposition is well established in the etiology of acute gouty arthritis and urolithiasis. Current study provides different phases of MSUM crystallization

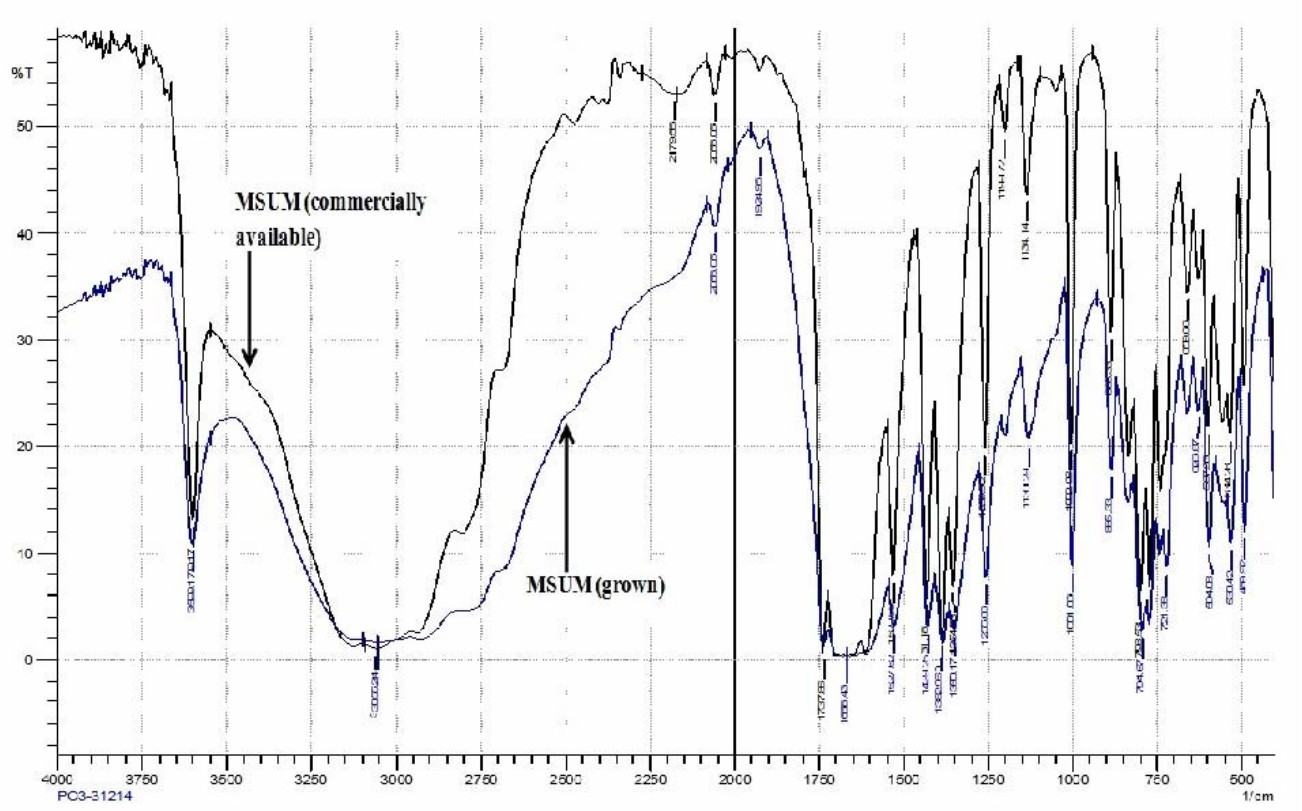

Fig. 5: FTIR spectra of completely grown MSUM crystals compared with commercially available compound.

for the first time. It may also be used as a model to evaluate prophylactic management against gouty and renal stones through *in vitro* assessment of nucleation, growth and aggregation inhibition and modulation of developing or developed crystals by using different natural products.

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