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## Urolithiasis in gel: an *in vitro* approach for Whewellite growth patterns to evaluate risk factors and management of urinary stones

**Salman Ahmed, Muhammad Mohtasheemul Hasan and Zafar Alam Mahmood**

**Abstract**

Whewellite crystals are most commonly found in kidneys, ureter and urinary bladder causing urolithiasis. The purpose of the present *in vitro* study was to observe the possible growth patterns and morphology of whewellite crystals. The study was carried out in U- shaped test tubes. Bow shape, donuts and their aggregates, dumbbell, platy, prismatic, rosette, styloid, X-shape and tetragonal bipyramidal crystals were observed. Energy Dispersive X-ray, Fourier Transform Infra-Red spectroscopies and Scanning Electron Microscopy were used to characterize the crystals. This study will be helpful to determine the promotion, modulation and inhibition of the crystals evaluate risk factors and prophylactic management of urinary stones.

**Keywords:** Crystal morphology, characterization, calcium oxalate monohydrate

**1. Introduction**

Urolithiasis is a common clinical problem with high recurrence. Urinary stones are composed of insoluble crystalline compounds in which inorganic phases include oxalate, phosphate and urate salts whereas organic matrix consists of proteins, lipids, polysaccharides and cellular components such as cystine, xanthine, calcium carbonate or hippuric acid [1, 2]. Whewellite (calcium oxalate monohydrate or COM) is the most common in 85% oxalate and phosphate containing calcium stones. The pathogenesis of whewellite formation includes crystal nucleation, growth, aggregation and retention. Cell debris, epithelial cells, red blood cells, urinary casts are the favorite sites for nucleation. The crystal growth starts with several atoms or molecules which form clusters in a supersaturated solution while in aggregation, crystals stick together to form a large particle. The crystal growth is a slow process and urolithiasis requires aggregation and retention followed by accumulation to obstruct the renal passage. The epithelial cell lining of the renal tubules, loop of Henle, Vasa recta, ducts of Bellini and papillary tip provide sites for initial deposition [3]. Glycosaminoglycan is an antiadherent, protective layer covering the inner renal wall and prevents crystal nucleation. The damaged layer favors the attachment of calcium phosphates, glycoprotein aggregates and cellular debris to the walls of renal papilla during early stages of stone formation and induces heterogenous nucleation of crystals. Therefore, small crystals adhered to the urothelial surface and then increase into comparatively larger particle [4]. Whewellite crystals are toxic to renal proximal tubular cells and causes inflammation-mediated cell death. These crystals are participating in both apoptosis and necrosis type of cell death mechanism. These crystals increase free radical generation and lipid peroxidation, which induces cellular injury, collapse mitochondrial functions leading to apoptosis. When these crystals are larger in size induces necrotic cell death by cell membrane rupture. Post apoptotic-necrosis promote crystallization, aggregation, crystal retention, growth, development of stone ultimately loss of renal function [5].

The detailed study of whewellite crystallization is very important to understand the most commonly found urinary stone, which is useful to evaluate risk factors and prophylactic management for urinary stones. Gel method is a simple and inexpensive *in vitro* technique which provides direct access to observe the crystallization process resembled to human physiological conditions [6, 7]. Although, whewellite crystals have been grown in agar and sodium meta silicate gel [8-12]. This study shares the possible growth patterns of whewellite formation are shared in detail to evaluate promotion, modulation and inhibition of these crystals by natural products.

## 2. Materials and Methods

### 2.1 Chemicals

Acetic acid (glacial) 100% anhydrous, calcium chloride dihydrate, magnesium acetate tetra hydrate, oxalic acid dihydrate, sodium silicate solution (Merck, Germany).

### 2.2 Instruments

U - shaped glass tubes of 2.5cm internal diameter, 16cm limb length and 12cm separation between two limbs; JSM-6380A Scanning Electron Microscope and EDS EX-54175 JMU, JEOL Japan; IR Prestige-21 FTIR Spectrophotometer Shimadzu ; Nikon Eclipse E 400 binocular microscope, Japan.

### 2.3 Crystal growth and characterization

The double diffusion gel method was used with some modifications <sup>[10]</sup>. Sodium meta silicate solution of 1.06 specific gravity and 3M acetic acid solution were mixed to prepare gel medium in a U-shaped test tubes. After gel formation, 20ml of 1M oxalic acid solution was gently poured into the left limb, whereas 1M calcium chloride and 1M magnesium acetate solutions in the right limb along the wall of U-tube. The U-tube was capped and observed at 9<sup>th</sup>, 14<sup>th</sup>,

21<sup>st</sup>, 28<sup>th</sup> and 40<sup>th</sup> day. The carefully recovered crystals were washed, dried and characterized by FTIR, SEM and EDS.

### 2.4 Statistical Analysis

Weights of crystals are expressed as mean  $\pm$  standard error of mean and were analyzed by unpaired student *t*- test.

## 3. Results

Morphological features and growth patterns of whewellite were observed. Whewellite crystals were formed as bow shape, donut, dumbbell, platy, prismatic, rosette, styloid and X-shaped, whereas weddellite had been observed as tetragonal bipyramidal form (Figure-1). Weight of harvested crystals, their morphology and density (crystal crowd) are shown in Table-1. The SEM images of crystals are shown in Figure-2. EDS spectrum of testing and standard samples shows the matched mass percentage of detecting elements (Figure-3). The FTIR spectra compared with the spectra of previously reported and commercially available compound and confirm harvested crystals as of whewellite (Table-2; Figure-4).

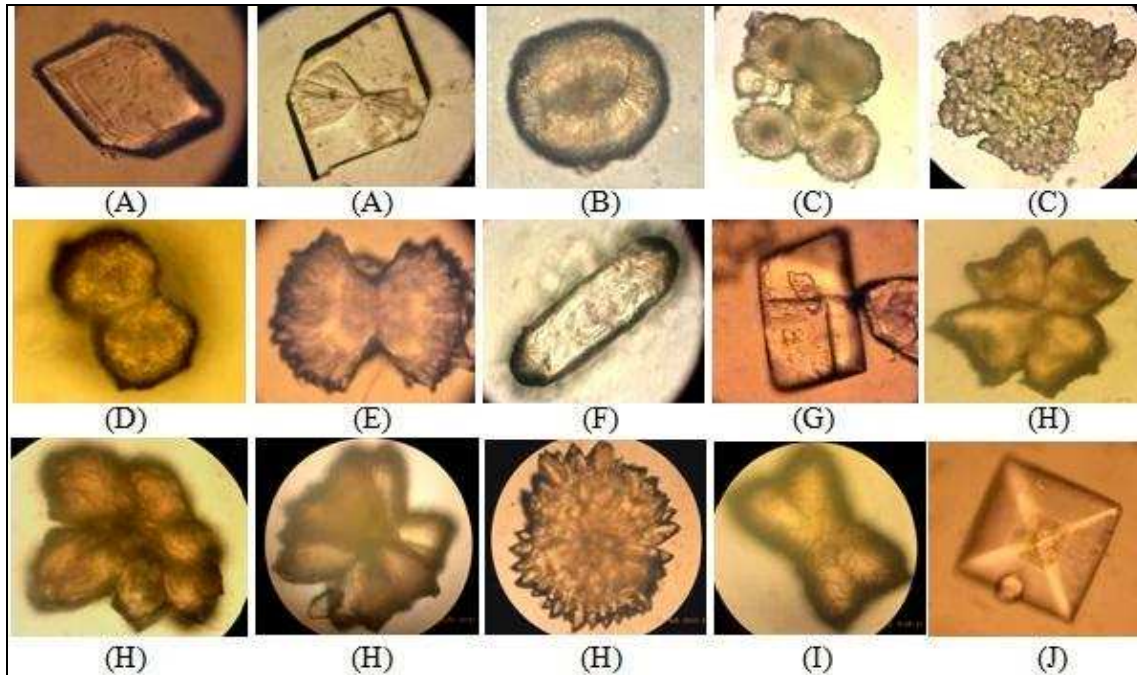
**Table 1:** Growth patterns of whewellite crystals.

Growth period (days)	Weight of harvested crystals (g)	Crystal morphology with order of crystal density
09	0.081 $\pm$ 0.03	Prismatic > dumbbell
14	0.202 $\pm$ 0.00*	Dumbbell > prismatic > aggregated crystals > rosettes
21	0.531 $\pm$ 0.01	Dumbbell > rosettes > aggregated crystals > prismatic > styloid
28	0.705 $\pm$ 0.00*	Dumbbell > rosettes > aggregated crystals > prismatic > donut > styloid and X-shape
40	0.808 $\pm$ 0.01*	Dumbbell > aggregated crystals > tetragonal bipyramidal

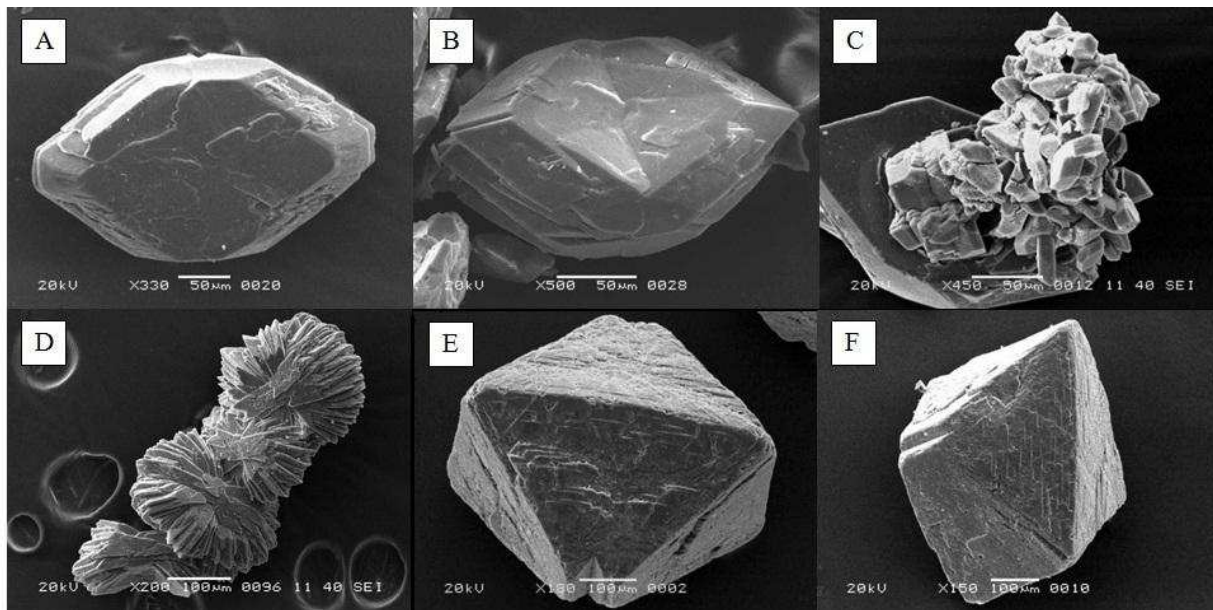
All values represent mean $\pm$  SEM of n=5; \**P*<0.05 showing significant values using unpaired student's *t*-test.

**Table 2:** FT-IR wave numbers and vibrations assignment of whewellite crystals.

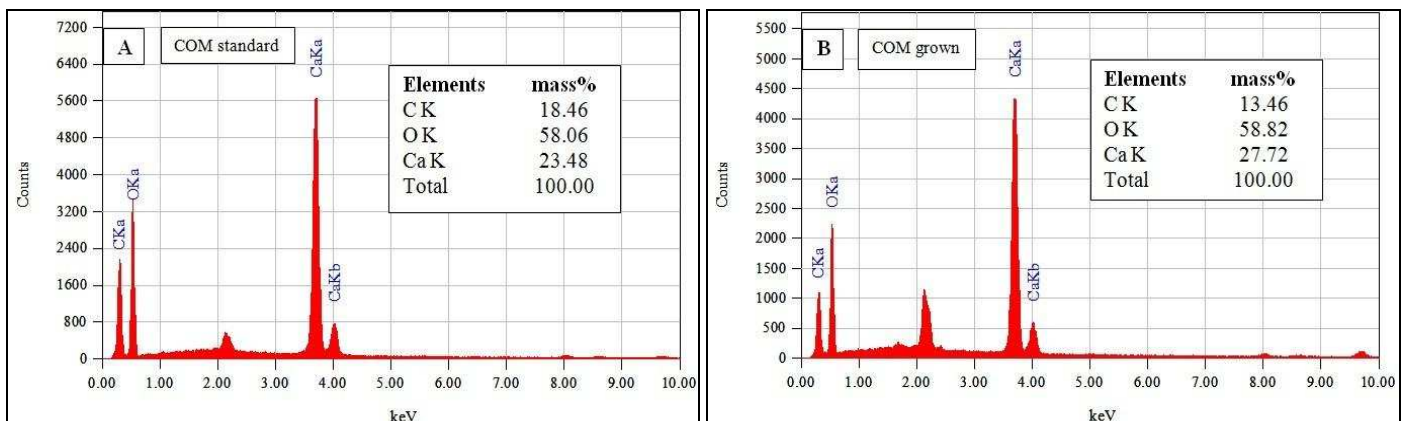
Wave numbers (cm <sup>-1</sup> ) of whewellite crystals			Bonds / vibrations
Standard (commercially available)	Test (grown)	Reported values <sup>[11, 22]</sup>	
3487.30 and 3429.43	3433.29	3500	Antisymmetric OH stretch (strong)
3336.85 and 3259.70	-----	3319	Intermolecular hydrogen bonded OH stretch (strong) indicating water molecule
3059.10	3061.03	3046	Symmetric OH stretch (strong) indicating water molecule
2372.44	-----	-----	-----
1622.13	1622.13	1638	Main antisymmetric carbonyl stretching bands (C = O) indicating carboxylates.
1381.03 and 1321.24	1319.31	1321 and 1323	
-----	1089.78	1030	C-O stretching
950.91	950.91	1000	Symmetric C-H stretch (very weak)
883.40	883.40	884	C-C stretching (presence of two carboxylate anions / existence of the oxalate group)
781.17	781.17	-----	C-H bend
665.44	651.94		
596.00	-----		
516.92	514.99		



**Fig 1:** Microphotographs (40x) of grown whewellite showing variety of habits. (A) prismatic, (B,C) donuts and their aggregates, (D) dumbbell, (E) bow, (F) styloid (G) platy, (H) rosette, (I) X-shape and (J) tetragonal bipyramidal, crystals.



**Fig 2:** SEM images displaying different shapes of whewellite crystals at 50 and 100 μm. (A,B) prismatic, (C) prismatic aggregates, (D) rosette and (E,F) tetragonal bipyramidal.



**Fig 3:** EDS analysis of whewellite crystals (A) standard and (B) 40 days grown.



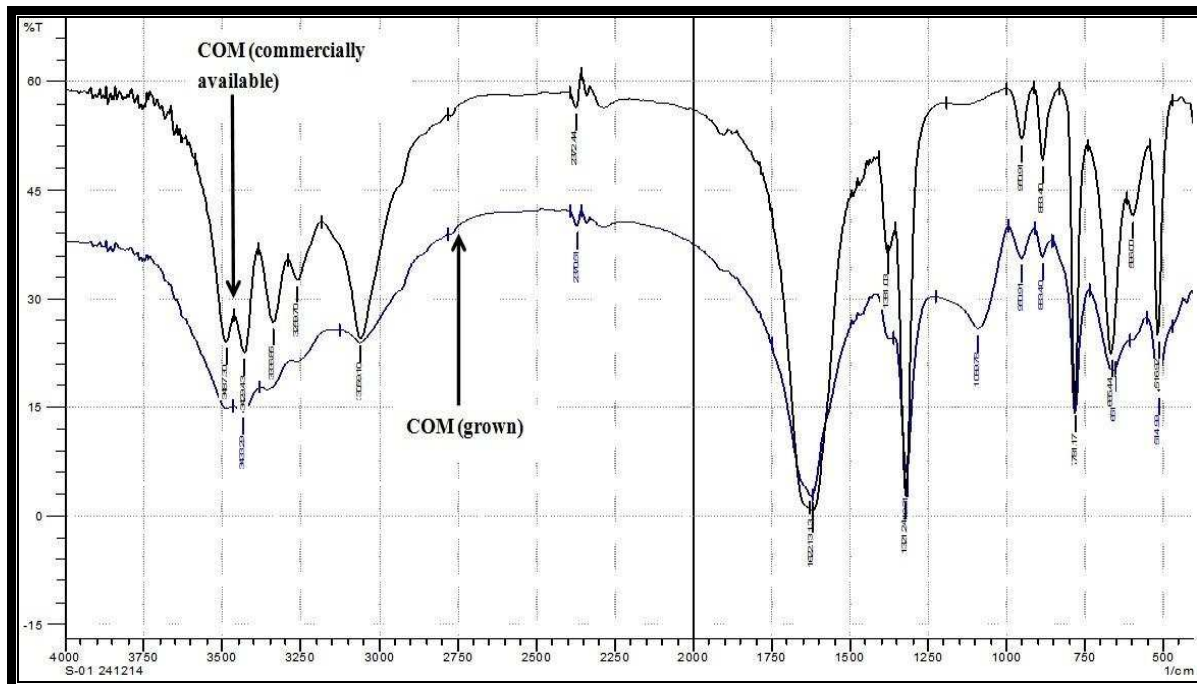


Fig 4: FT-IR spectra of grown whewellite crystals compared with commercially available compound.

#### 4. Discussion

The growth patterns and morphology of whewellite crystals were observed as a donut, platy, prismatic, rosettes, round edges, styloid, loose smaller, larger and compact agglomerate [13-17]; X-shaped crystals and weddellite (calcium oxalate dihydrate) crystals in the tetragonal bipyramidal form [18]. Whewellite crystals make strong adhesion to renal epithelial cells to form stable aggregates instead of excretion. These crystals further cause retention of mineral in renal collecting ducts for urolithiasis [19]. Whewellite has a large number of (100) faces as a point of attachment with other crystals together with fastest growth faces (121), (021), and (010) to form aggregates and strong attachments to resist their detachment during urine flow. Crystals are generally grown from microns to several centimeters. Single microscopic whewellite crystal form bundles of crystals by stacking on top of each other in the form of the hill which is known as hillock growth. These stones attached to tips of renal papilla and when detached cause hindrance in urine flow or even obstruction in the ureter due to their large enough size. Weddellite exhibits negligible area of (100) face for adhesion. In bipyramidal habit, it contains dominant (101) faces of weak adhesion contacts with epithelial cells reducing their tendency to form stones. Thus, weddellite crystals are excreted in urine and do not participate in urolithiasis [19, 20].

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 growth stages [21]. The direct observation of crystallization is not possible by *in vivo* models and the mechanism remains unexplained. When pathologic crystals are growing in gel along with natural products provide a comparison in approximate number, total mass, shape, size and transparency leading to share an important data about promotion, modulation or inhibition of crystals [9]. The studied growth pattern of whewellite will not only confirms traditionally used plants as effective antiurolithiatic agents, but also provides an *in vitro* evaluation of promotion, modulation or inhibition. The promotion of growing crystals gives an idea about the risk factors of urinary stone disease and their inhibition, assures its prophylactic management.

#### 5. Conclusion

The present study provides possible growth patterns and morphology of whewellite crystallization for the first time, which will be useful to evaluate risk factors and prophylactic management of urinary stones.

#### 6. Conflict of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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