Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2016; 5(2): 91-95
Received: 02-01-2016
Accepted: 03-02-2016

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Inhibition and modulation of calcium oxalate monohydrate crystals by phytic acid: An *in vitro* study

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Abstract
The aim of the study was to explore the inhibition and modulation of calcium oxalate monohydrate crystals into calcium oxalate dihydrate by phytic acid. The study was carried out on glass slides by using phytic acid (1 - 5%) solutions. All tested solutions inhibited the growth and modulated calcium oxalate monohydrate crystals. Donuts, rosettes and X-shape crystals of calcium oxalate monohydrate along with their defected forms were observed. Whereas, the presence of calcium oxalate dihydrate crystals as elongated rods and tetragonal bipyramidal crystals revealed the modulated forms of calcium oxalate monohydrate. Smaller zones of nucleation declare as general patterns of growth inhibition. This study gives valuable information about inhibition and modulation pattern of calcium oxalate crystals. Further studies are required to confirm the results of present study.

Keywords: Calcium oxalate, crystallization, phytic acid, microscopic study, urolithiasis.

Introduction
Phytic acid (myo- inositol hexa kis phosphoric acid) is found as phosphorus in storage form in most mature seeds. It plays a vital role in seed growth and development. It was first discovered in seeds by Hartig as small round particles of potato starch grain size [1]. Then isolated from plant seeds by Pfeffer in 1872 and identified as inositol compound by Winterstein in 1909 [2]. Phytic acid is a strongly negative charged compound (Figure-1) and form complex with multivalent cations, especially of calcium, iron, magnesium and zinc. These complexes are soluble in acidic medium in the stomach and precipitate at neutral pH in the intestine, causing poor absorption of minerals and trace elements and makes phytic acid as an anti nutrient agent. However, balanced nutrition makes it a less significant problem. On the other hand, phytic acid is also reported as anticalcification, anticancer, antioxidative, hypoglycemic and hypocholesterolemic. In the early stage of urolithiasis, the use of phytic acid has been reported. In 1958, Henneman and co-workers used high doses of phytate as sodium salt (8.8 g/day) to treat stone-former patients with idiopathic hypercalciuria. Phytic acid acts as an antiurolithiatic compound by inhibiting calcium oxalate and calcium phosphate crystallization in renal tissues and urine [1]. The present study was carried out on glass slides by using reagents of double diffusion gel technique [3] to observe the calcium oxalate monohydrate (COM) growth inhibition and modulation patterns by phytic acid (1 - 5%) solutions.

Experimental
Apparatus and Instruments
Nikon Eclipse E 400 binocular microscope, Japan ; Ricoh CX4 Digital Camera, Japan; Microscope slides 25.4 x 76.2 (1 " x 3 ") Universal Health Care Products, China; Whatman filter paper # 02, Whatman International Ltd., England.

Chemicals and Reagents
Acetic acid (glacial) 100% anhydrous, calcium chloride dihydrate, magnesium acetate tetra hydrate, orthophosphoric acid 85%, oxalic acid dihydrate, sodium silicate solution (Merck, Germany), phytic acid solution (Fluka- Sigma Aldrich, Switzerland).

Preparation of solution
Stock solution of 5% Phytic acid was prepared by using 10ml of Phytic acid solution (50% W/W in water) in 100ml of distilled water. The reference solutions of 4, 3, 2 and 1% were prepared by serial dilution method using stock solution.

Method of crystal growth and inhibition
Earlier reported method [4] was adopted to perform the study.

Results

The results showed that there is growth inhibition and modulation in COM crystals. Out of 8 different forms of COM crystals (Photograph-1), only donut, rosettes and X-shape crystals with their defected forms were observed. Which strengthen the crystallization inhibition. The most frequently and abundantly observed crystals were donut along with elongated rod shape crystals. It may be said that donuts were modulated into elongated rods and then deformed into irregular entities. The following results were obtained (Table-1; Photograph-2).

- **1% PA:** Majority of crystals were consists of elongated rods with little clusters. Few donuts along with defected aggregates were also observed.
- **2 and 3% PA:** Segregated donuts and their clusters were observed along with their defected forms. More aggregates were observed than 1% PA.
- **4% PA:** Rosettes and X-shape crystals were found in defected form. A few clusters of rod shape and defected crystals were observed. Less number of segregated crystals and their less crowd showed maximum inhibition of crystallization as comparable to 1,2 and 3% PA.
- **5% PA:** Tetragonal bipyramidal crystals and their defected forms were also found.

Discussion

Calcium oxalate crystals are classified into three forms, calcium oxalate monohydrate (COM) or whewellite, calcium oxalate dihydrate (COD) or weddellite and calcium oxalate trihydrate (COT) or caoxite. The COM crystals are the major component of kidney stones. Whereas, COT results from bacterial indisposition of the renal tract [5]. These forms can be further classified on the basis of their morphology. COM crystals are donut, dendritic, dumbbell, needles, platy, prismatic, rosette, round edges and X-shaped. COD are reported as the elongated large rods and tetragonal bipyramidal forms [4] with less calcium ions, negligible area of (100) face for adhesion contacts on the surface of COD make them weakly adhesive with renal epithelial cells. Therefore, COD instead of retention as urolith, routinely excreted during urination, and therefore it may be said that *in vivo* COD formation protects against urolithiasis [6, 7]. Phytic acid plays an important role in calcium oxalate and calcium phosphate crystallization inhibition [1]. The antiurolithiatic role of phytic acid is contributed by its ability to bind with calcium to reduce its bioavailability and its antioxidant action [8]. Antioxidant activity by protecting membrane injury prevents calcium oxalate retention and hence plays an important role to avoid calculi formation. Lipid peroxidation in proximal tubule produces free radicals. These free radicals cause renal tubular cell injury. This invites retention of calcium oxalate monohydrate crystals in membrane fragments to form attached stone. This stone is unable to excrete out during urination and favors urolithiasis [8-10].

The critical additionable oxalate concentration required to induce crystallization is known as metastable limit. It is inversely proportional to oxalate over load (urine super saturation) [11]. Metastable limit significantly lower in recurrent stone formers and shows spontaneous nucleation of calcium oxalate. Whereas, it is significantly higher in healthy subjects or during effective urolithiasis treatment [12]. Phytic acid also inhibiting calcium oxalate crystallization by increasing metastable limit [13].

In the present study, the degree of crystallization inhibition was observed as

4%PA > 5%PA > 3%PA > 2%PA > 1%PA (on the basis of crystal crowd or crystal density) and 5%PA > 4%PA > 3 and 2%PA > 1%PA (on the basis of crystal aggregates) (Photograph-2). It was also observed for the first time that phytic acid solution not only makes COM crystals defected but also modulate COM into COD and make these COD crystals defected. Therefore, it may be proposed that phytic acid acts as an antiurolithiatic compound not only due to its calcium chelation and antioxidant activity but also has a potential to make COM defected, modulate COM into COD and further make COD defected (Figure-2). It is a preliminary study and further studies are required to justify and confirm the obtained results.

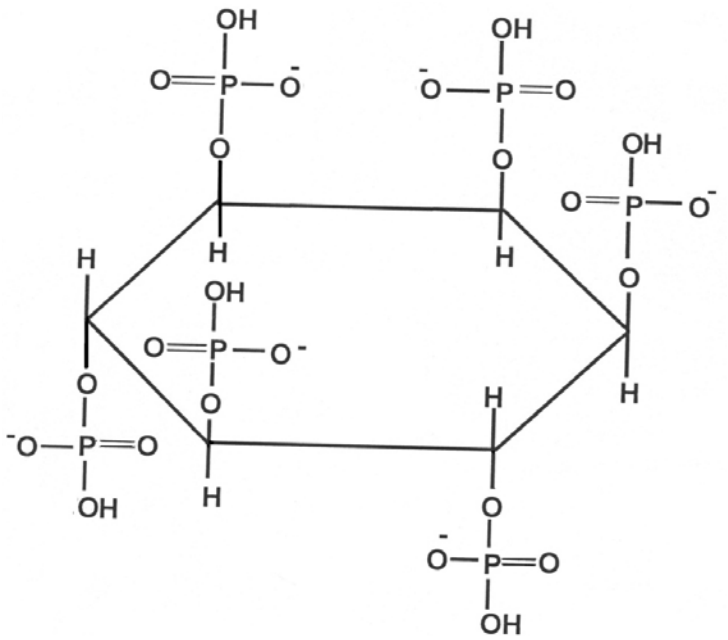


Fig 1: Chemical structure of Phytic acid.

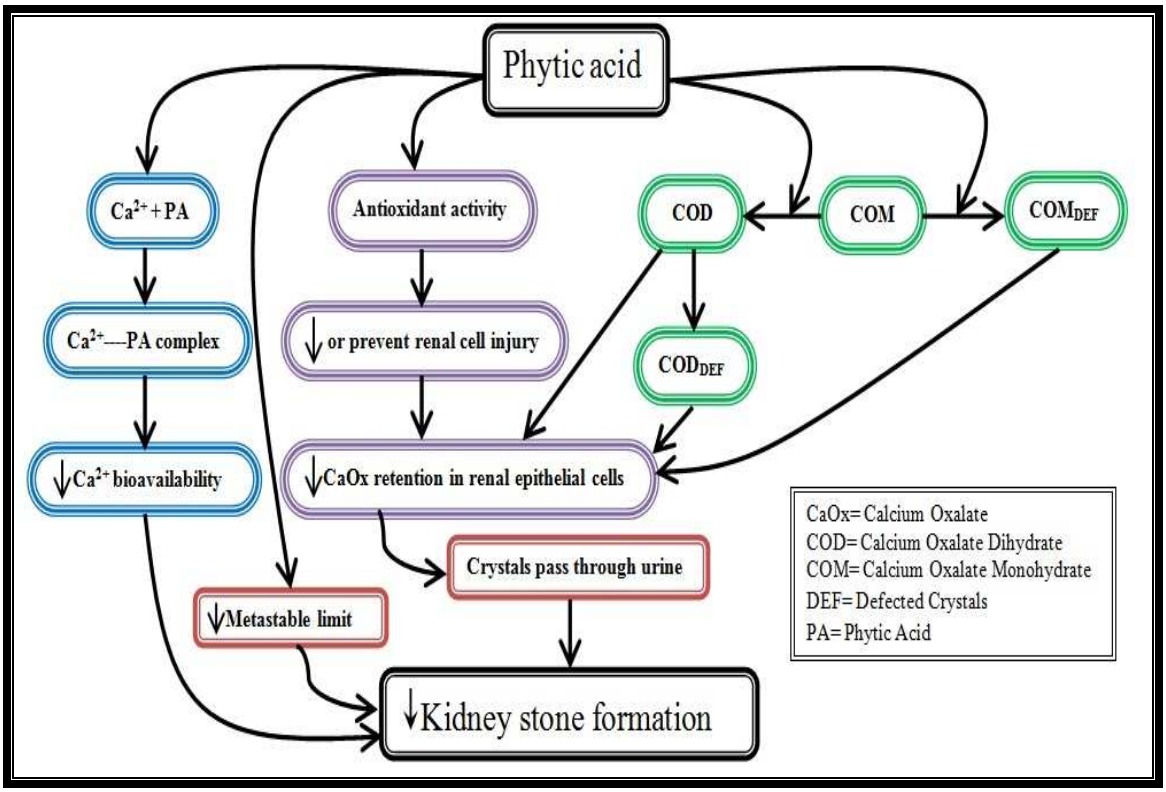
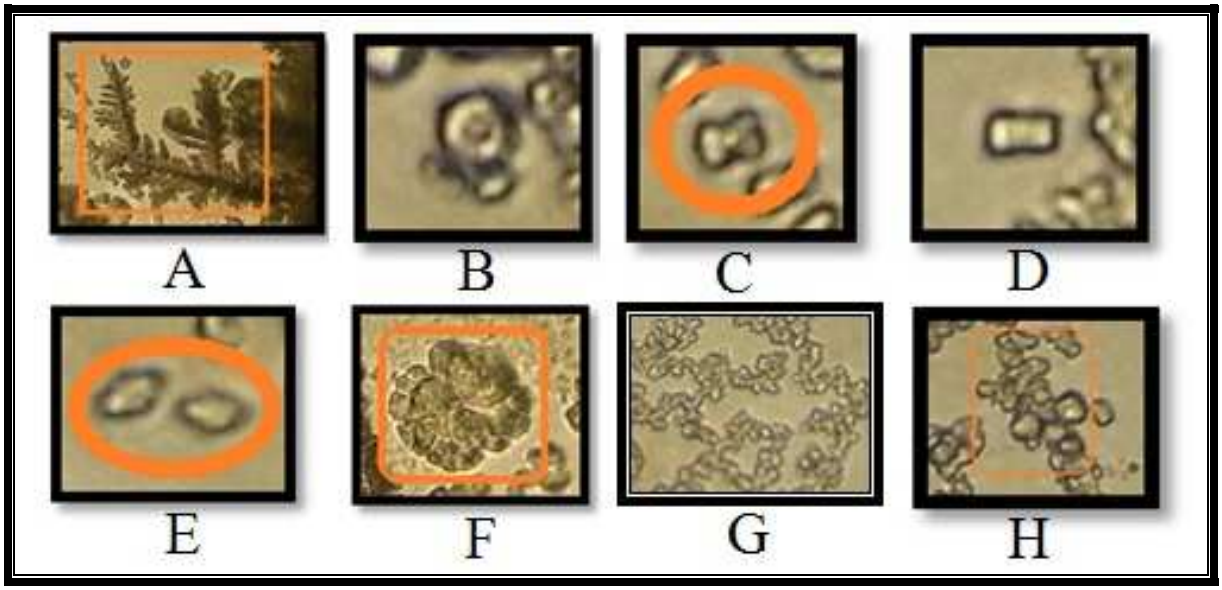


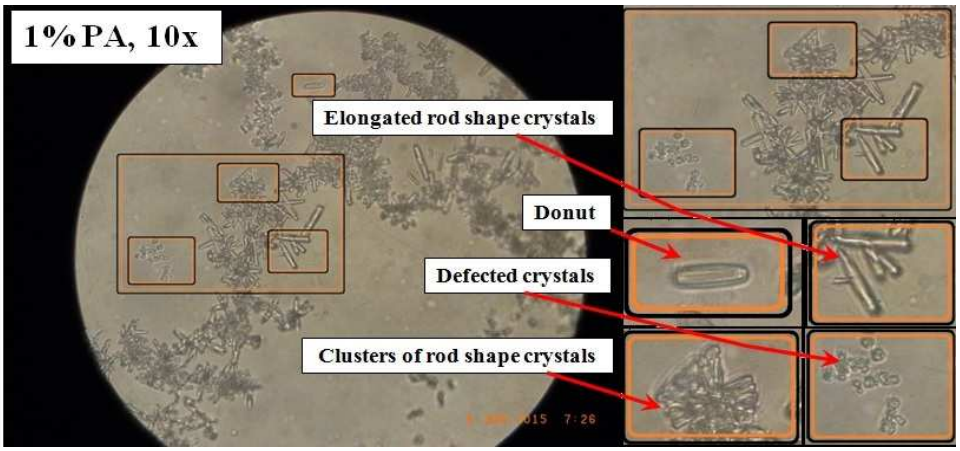
Fig 2: Proposed antiurolithiatic mechanism of Phytic acid.

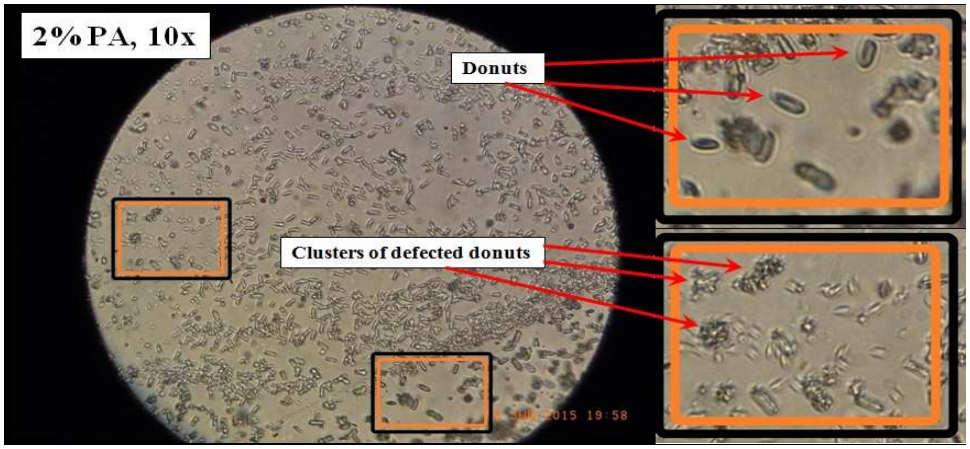
Table 1: Types of calcium oxalate crystals observed under microscope by using 1,2,3,4 and 5% solutions of Phytic acid.

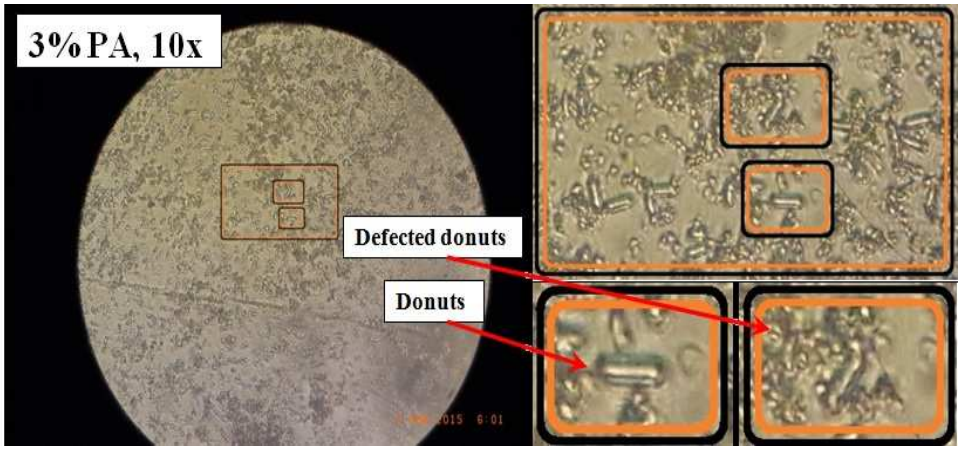
Crystal Shape	Solutions (percentage) treatment
COM	
Donuts	PA(1,2,3)
Rosettes	PA(4)
X-shape	PA(4)
COD	
Elongated large rods	PA(1,4)
Tetragonal bipyramidal	PA(5)
Keys: COM=Calcium oxalate monohydrate; COD=Calcium oxalate dihydrate; PA= Phytic acid	

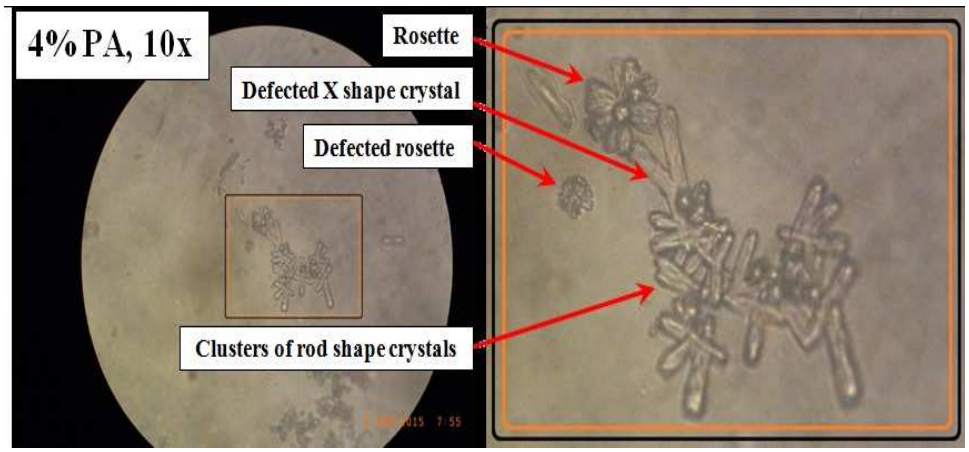


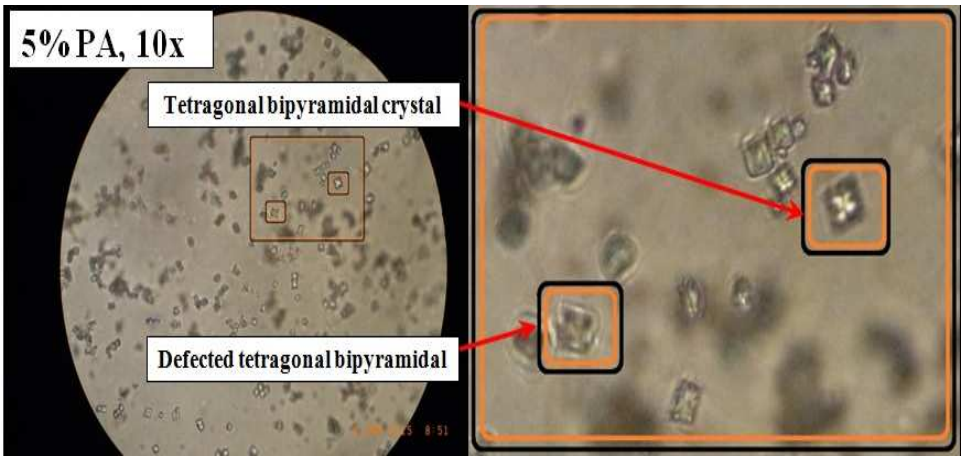
Photograph 1: Differential types and morphologies of COM crystals [4]. (A) Arborescent or Dendritic, (B) Donut, (C) Dumbbell, (D) Platy, (E) Prismatic, (F) Rosette, (G) Loose lager agglomerate, (H) Compact aggregate.


1% PA, 10x
Elongated rod shape crystals
Donut
Defected crystals
Clusters of rod shape crystals


2% PA, 10x
Donuts
Clusters of defected donuts


3% PA, 10x
Defected donuts
Donuts


4% PA, 10x
Rosette
Defected X shape crystal
Defected rosette
Clusters of rod shape crystals



Photograph 2: The inhibitory effects of phytic acid (1 - 5%) solutions on calcium oxalate crystals.

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