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Inhibition of calcium oxalate crystals growth by *Macrotyloma uniflorum* (Lam.) Verdc, *Phaseolus lunatus* Linn, and *Phaseolus vulgaris* Linn: An *in vitro* study

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Abstract
Purpose: The purpose of the study is to explore all possible morphological features of calcium oxalate monohydrate and calcium oxalate dihydrate crystals and their habits in case of inhibition.
Methods: The study was carried out on glass slide to observe the growth and inhibition of calcium oxalate monohydrate (COM) crystals by using infusions (5-20%) of *Macrotyloma uniflorum* (Lam.) Verdc, *Phaseolus lunatus* Linn. and *Phaseolus vulgaris* Linn. The reagents of double diffusion gel technique was used for this purpose.
Results: Calcium oxalate crystals are divided into three types, calcium oxalate monohydrate, calcium oxalate dihydrate and calcium oxalate trihydrate. These types are further divided into sub types on the basis of their morphology. In case of calcium oxalate monohydrate (COM), these crystals are donut, dumbbell, needles, platy, prismatic, rosette, round edges and X-shaped. Whereas, calcium oxalate dihydrate (COD) are reported as the elongated large rods and tetragonal bipyramidal forms. In the present study dendritic or arborescent (tree like platy crystals) were observed for the first time as the part of a COM growth. Long chain loose agglomerates and compact aggregated crystals are the common pattern of calcium oxalate crystals. All tested infusions caused growth inhibition of calcium oxalate crystals. Smaller zones of nucleation and defected shape of the grown crystals; declare as different patterns of growth inhibition.
Conclusion: This study gives an extensive information about morphology, aggregation and growth inhibition of calcium oxalate crystals.

Keywords: Calcium oxalate, crystallization, *Macrotyloma uniflorum* (Lam.) Verdc, *Phaseolus lunatus* Linn, *Phaseolus vulgaris* Linn, microscopic study, urolithiasis

Introduction
The formation of kidney stone (urolithiasis) is a cascade of crystal nucleation, growth, aggregation and retention of crystal within the renal tubules [1]. Small crystals usually adhere to the urothelial surface and then increase into comparatively larger particle. Calcium oxalate crystals are 50% of urinary calculi, found in three forms [2]. The thermodynamically stable monohydrated form or whewellite (COM; $CaC_2O_4.H_2O$), the metastable dihydrate or weddellite (COD; $CaC_2O_4.2H_2O$) and trihydrate or caoxite (COT; $CaC_2O_4.3H_2O$). COM, the major component of kidney stones often accompanied by small amounts of COD. Whereas COT results from bacterial indisposition of the renal tract [3]. Medicinal plants have been employed to treat kidney stones during ages. Herbal medicines are of high demand because of the wide range of medicinal properties with higher safety margin and low cost [2]. *Macrotyloma uniflorum* (Lam.) Verdc., *Phaseolus lunatus* Linn., and *Phaseolus vulgaris* Linn., belong to family Papilionaceae. All the three legumes are commonly available in Pakistan. *M. uniflorum* is also found in Africa, Australia, Bhutan, India, Indonesia, Myanmar, Nepal, Philippine and Sri-Lanka. The seeds are light or deep reddish brown in color with orbicular-reniform in shape. *P. lunatus* is native to tropical America and now widely cultivated throughout the tropics. The seeds are brown, red, purple, and black in color with sub-rhombic reniform in shape. *P. vulgaris* is also native to tropical America with dark red seed of reniform shape. Widely cultivated in tropical and temperate regions of the world [4-7]. The seeds of *M. uniflorum* [8], *P. lunatus* [9] and *P. vulgaris* [10] are reported to possess antiurolithiatic activity.

The present study was carried out on glass slide by using reagents of double diffusion gel technique[11] to observe the growth habits of calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD) and its inhibition by infusions (5-20%) of *Macrotyloma uniflorum* (Lam.) Verdc., *Phaseolus lunatus* Linn., and *Phaseolus vulgaris* Linn.

Experimental

Apparatus and Instruments

Nikon Eclipse E 400 binocular microscope, Japan; Ricoh CX4 Digital Camera, Japan; Microscope slides 25.4x76.2 (1"x3") Universal Health Care Products, China; Whatman filter paper # 02, Whatman International Ltd., England.

Chemicals and Reagents used

Acetic acid (glacial) 100% anhydrous, calcium chloride dihydrate, magnesium acetate tetra hydrate, orthophosphoric acid 85%, oxalic acid dihydrate, sodium silicate solution (Merck, Germany).

Plant material, identification and sample preparation

The seeds of *Macrotyloma uniflorum* (Lam.) Verdc., *Phaseolus lunatus* Linn., and *Phaseolus vulgaris* Linn., were purchased and identified by a taxonomist, in the Department of Botany, University of Karachi. The voucher specimen number of *Macrotyloma uniflorum* (Lam.) Verdc., (G.H.No.86483), *Phaseolus lunatus* Linn., (G.H.No.86451) and *Phaseolus vulgaris* Linn., (G.H.No.86536) were deposited in the Herbarium of University of Karachi. The dried seeds were separately grinded to powder and then passed through 600 µm sieves and kept in an amber bottle at room temperature before commencing the experiment.

Preparation of infusion

The powdered seed (20g) of each plant was separately soaked in 100 ml of de-ionized water for 24 h for 20% infusion, then filtered thrice to get clear filtrate. The dark brown (*Macrotyloma uniflorum*), light brown (*Phaseolus lunatus*) and milky white(*Phaseolus vulgaris*) infusions were obtained. From these 20% infusions, dilutions of 5,10 and 15% infusion were prepared. Freshly prepared clear infusions were used for experiment.

Method of crystal growth and inhibition

The different stages of the growth of calcium oxalate crystals were studied under compound microscope. Crystals were grown on glass slide marked left and right at 26±2 °C. A drop of gel media (pH 5.02-5.17) was put at the mid of the glass slide. Gel media allowed to convert into good quality gel. Gel formation occurs in 5 min. Single drop of 1 M oxalic acid was dropped to the left and 1 M calcium chloride and magnesium acetate (1:1) solution was dropped to right side of properly formed gel on the glass slide. The glass slide was observed under microscope till it was completely dried. In case of crystal inhibition studies, a drop of tested infusion was also added at right side just after the addition of calcium chloride and magnesium acetate solution.

Results

Morphological features and growth patterns of calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) were observed. COM showed donut, dumbbell, needles, platy, prismatic, rosette, round edges, x-shaped and COD have been seen in the elongated large rods and tetragonal bipyramidal forms. Dendritic or arborescent (tree like platy crystals) were observed for the first time (**Photograph 1; Figure-2**). Long chain loose agglomerates and a short, compact aggregate are the common pattern of calcium oxalate crystals. This pattern is so much important in the sense that after crystal-crystal collision, short and then long chain agglomerates are formed. These agglomerates convert into short compact aggregates and then change into small kidney stone. This explains why calcium oxalate stones are found as a major proportion of kidney stones. COM crystals were starting to grow from needles to play and then to prismatic shape and other different behavior as crystals with rounded edges, donut, platy and rosette before reaching towards equilibrium state. Numerous agglomerates and aggregates were common in almost all observations showing the maximum contribution of calcium oxalate in urolithiasis. Crystals grow in size and as a consequence of crystal-crystal collision loose small agglomerates are formed which are composed of only little crystals. Agglomerates gradually increase in size and become large, as more and more crystals take part in crystal-crystal collision. Then void spaces of agglomerate fill to change into densely packed opaque spheres to form aggregates. Agglomerates also possess composites of platy crystals arranged in the form of flower petals forming a flower like structure, rosettes. Dendritic or arborescent crystals are tree like platy crystals, with well-defined dendritic side branches in one or more direction from the central point. Dendritic crystals were observed for the first time as a growth phase of COM **(Photograph-1)**.

The infusions of *M. uniflorum*, *P. lunatus* and *P. vulgaris* showed defected/ deformed crystals. The presence of normal and defected/ deformed crystals provide a comparison to the degree of crystal growth/crystal inhibition. The absence of long chain agglomerates in each treatment (except 15% MU) showed a good positive relation with antiurolithiatic effect. The following results were obtained from the COM inhibitory activity of *M. uniflorum*, *P. lunatus* and *P. vulgaris* infusions (table-1 and photograph-2).

- **5% MU, PL and PV:** The slide of PL contains less crystals as compare to MU and PV. X-shape and rosettes were common in PL and MU whereas, donut and platy crystals were observed in MU and PL respectively. PV just shows normal rosettes.
- **10% MU, PL and PV:** In this case the number of crystals increased, suggested that less effective inhibition than 5% infusions. The rosettes were common in MU and PL. Whereas in PV, tetragonal bipyramidal and elongated large rod shape crystals of COD were observed.
- **15% MU, PL and PV:** Agglomerates of defected crystals were observed in MU. Donuts were observed in PL. The less number of crystals were observed in 15 % PL as compared to 10% infusion. The PV showed tetragonal bipyramidal crystals of COD and their defected forms.
- **20% MU, PL and PV:** The rosettes were observed in PV. Whereas, defected crystals were prominent in MU and PL.

Discussion

The formation and inhibition of calcium oxalate crystals are of great importance for antiurolithiatic medicine. Crystals are generally grown from microns to several centimeters. Stacks of microscopic COM crystals appear as growth layers in the

form of the hill which is known as hillock growth. These stones attached to tips renal papilla and when detached, hinder urine flow or even urine obstruction in the ureter due to their large enough size. COM crystals are large cationic particulates presenting more calcium ions than COD at their surface. These ions have a stronger affinity for anionic molecules in renal epithelial cell membranes and therefore make strong adhesion contacts with renal epithelial cells, form stable aggregates instead of excretion and causes retention of mineral in renal collecting ducts for urolithiasis [12].

The adhesive strength of the COM crystal is of following order (100) > (121) > (010) faces [13]. These crystals exhibit larger areas of (100) face having the largest adhesion strength to form aggregates and strong attachments to resist their detachment during urine flow. Single COM crystal form bundles of crystals by stacking on top of each other and thus numerous bundles combine to form kidney stones. COM has a large number of (100) faces as a point of attachment with other crystals together with fastest growth faces (121), (021), and (010). The stability of crystal aggregates totally depends on intermolecular adhesion forces(like van der Waals, hydrogen, ionic and rarely covalent bonds) between the crystal faces and specific functional groups on urinary constituents and affinity for a surface[12]. Calcium oxalate growth inhibitors such as citrate, chondroitin sulfate, inulin, osteopont in serum albumin, Tamm-Horsfall protein and transferrin contain a high percentage of anionic groups (polyanions) [14] with many acidic amino acid residues take part in phosphorylation and glycosylation, and thus bind to Calcium oxalate surface [15]. These carboxylic acid rich moieties (e.g. glutamic acid and aspartic acid) adsorbed to flat, positive charge COM (100) faces. In other words, these inhibitors masking the binding sites of (100) faces towards renal epithelial cells. Excess negative charge on the adsorbed crystal(s) create charge repulsion towards negatively charged renal epithelial cells resulting the inhibition of attachment. This adsorption reduces the adhesive strength of these (100) faces and frustrate the attachment of other crystals. This phenomena reduce the rate of crystal growth by inhibiting classical mechanisms of layer-by-layer hillock growth. This phenomenal results face size reduction during crystal growth. This growth retardation of COM (100) faces yielding a tabular crystal habit. This Inhibitor-crystal interaction with crystal surface via a range of intermolecular forces [12, 14, 16]. Normal human urine likely contains factors that can modulate calcium oxalate crystallization into COD. Urinary inhibitors of crystal growth can cause preferential crystallization of COD, rather than COM. COD crystals are found in the healthy people and urine of stone formers and are routinely excreted during urination [17]. COD exhibits negligible area of (100) face for adhesion contacts. It contains dominant (101) faces of weak adhesion strength in the bipyramidal habit. So, less stable, aggregates and attachments reducing their tendency to form stones. Thus, COD play an important role against stone disease [12]. COD has weak adhesion contacts with epithelial cells thus, more readily excreted. So, it is proposed that *in vivo* COD formation protects against urolithiasis [12, 17].

The seeds of *M. uniflorum* [8], *P. lunatus* [9] and *P. vulgaris* [10] are reported to possess antiurolithiatic activity to a greater extent because of its potassium and magnesium contents as well as of phytic acid content which may be helpful in protecting the formation of stones. Anthocyanins, flavonoids and phenolic acids are reported as antioxidants from the seeds of *M. uniflorum, P. lunatus* and *P. vulgaris*. It is evident from various reports that regular intake of potassium or magnesium above the recommended concentration, suppresses the formation of stones because potassium promotes urinary citrate excretion and together with magnesium it further inhibits crystal formation[18]. Magnesium is also an inhibitor of urinary stone. It can compete with calcium for oxalate and thus form complexes with oxalate with the formation of magnesium oxalate, which is more soluble than calcium oxalate [19]. It is further reported that magnesium ion has the property to destabilize calcium oxalate ion pairs and thus reduces the size of the aggregates. The magnesium ion's inhibitory effect remains stable in an acidic environment and synergistic with citrate [20]. Phytic acid plays an important role to inhibit the calcium oxalate crystallization [21]. The antilithiatic role of phytic acid is contributed by its ability to bind with calcium to reduce its bioavailability and its antioxidant action [22]. Cytotoxic substances with oxidative capacity and hyperoxaluria induces renal tubular cell injury by the production of free radicals due to lipid peroxidation in proximal tubules [23] with antioxidant defensive system depletion and the failure of the calcium pump. Calcium and oxalate accumulate and then precipitate in the presence of membrane fragments to form stones [22]. Renal epithelial cell injuries in renal papilla invites whewhellite to form attached renal calculi and development of whewellite papillary calculi. Antioxidant activity play an important role to avoid calculi formation [24] and by protecting membrane injury prevents calcium oxalate retention [22].

Inhibition of urinary crystals showed in terms of their number and morphology. The dark regions in the experiment showed the crystals crowd (more nucleation, growth and aggregation). Spaces between crystals show a less degree of agglomeration/aggregation. In most cases, the presence of normal and defected crystals at the same slide gives morphological changes to compare the degree of inhibition. To clarify the crystal images segmented images were used. Long chain loose and compact aggregated crystals are the common pattern of calcium oxalate crystals. In the present study aggregated crystals were less observed or present as defected form. Although, after careful study we just obtained an idea about the degree of inhibition in terms of the number of crystals (crystal crowd/cloud). Further studies are required to assure crystal inhibition.

Conclusion

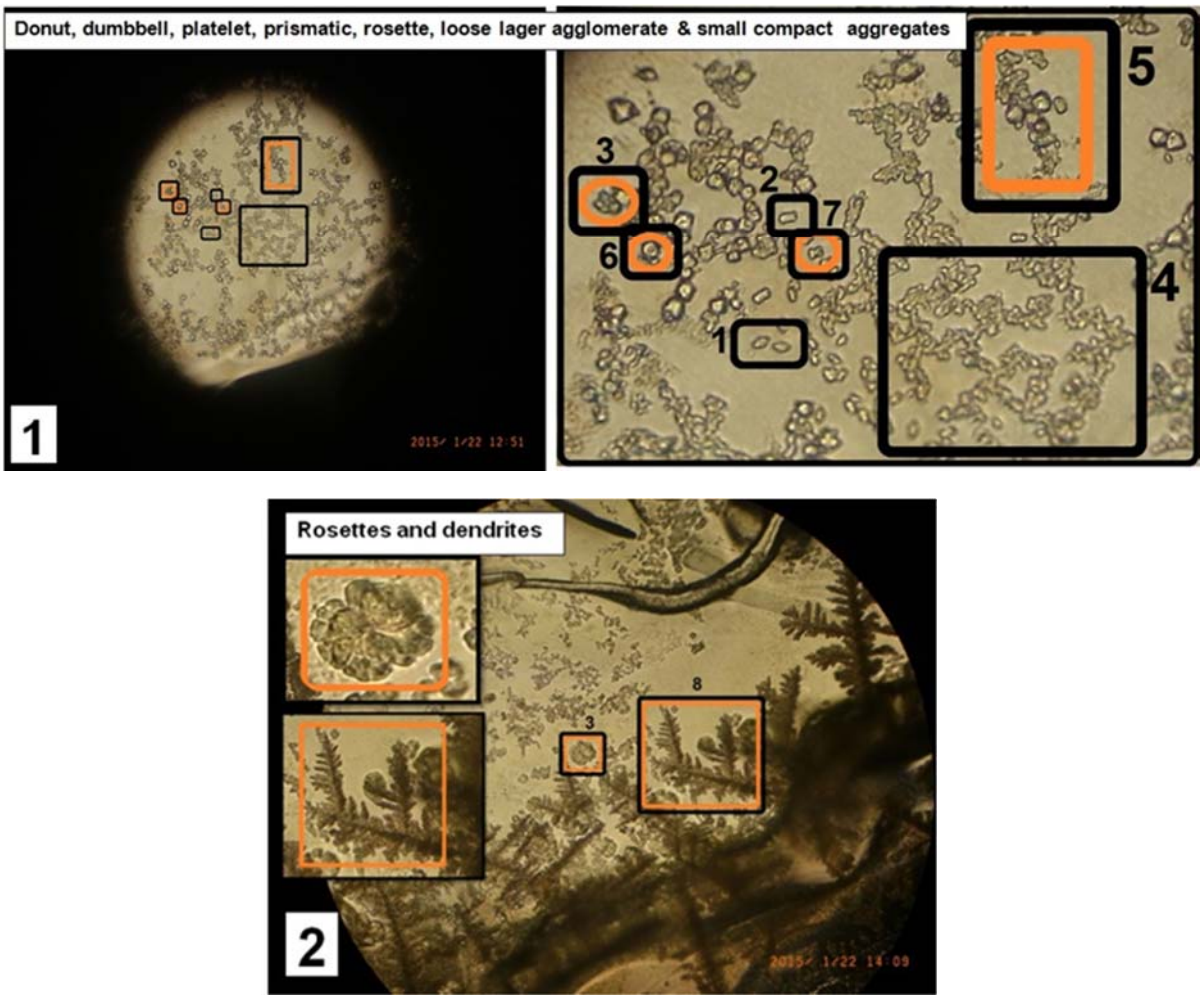
The present microscopic study of calcium oxalate crystal growth and its inhibition by traditionally reported *M. uniflorum*, *P. lunatus* and *P. vulgaris* is uniquely simple and provide rapid analysis of antiurolithiatic activity. The theme of the study has not been published elsewhere in any journal or other citable form. In the present study dendritic were observed for the first time as the part of a COM growth. The study gives a complete picture of all COM growth phases and their qualitative inhibition on a glass slide for the first time. It was a preliminary study and doesn't has any quantitative and statistical analysis. Now, the authors are looking forward to focus different other scientifically based authentic aspects of the same study and to justify the obtained results in future.

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

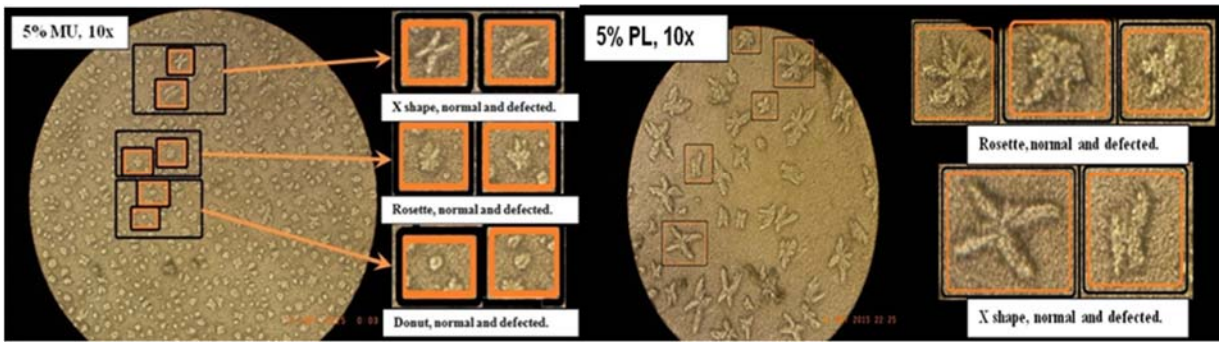
Table 1: Types of calcium oxalate crystals observed under microscope by using 5, 10, 15 and 20 % infusions of *Macrotyloma uniflorum, Phaseolus lunatus* and *Phaseolus vulgaris*

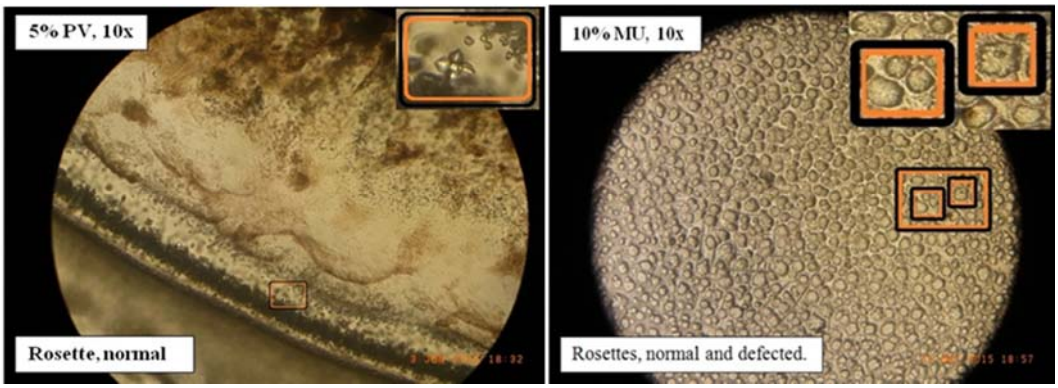
Crystal Shape	Treatments of infusions (percentage)
COM	
Donuts	MU(5) ; PL(5,15)
Dumbbell	PV(20)
Platy	MU and PL(5)
Rosettes	MU, PL and PV(5,10)
X-shape	MU and PL(5)
COD	
Elongated large rods	PV(10)
Tetragonal bipyramidal	PV(10,15)

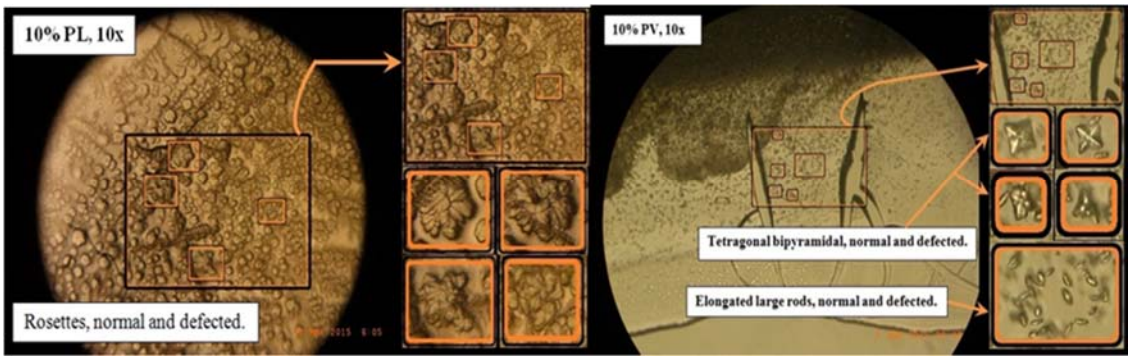
Keys: MU= *Macrotyloma uniflorum*; PL= *Phaseolus lunatus*; PV= *Phaseolus vulgaris*; COM=Calcium oxalate monohydrate; COD=Calcium oxalate dihydrate.

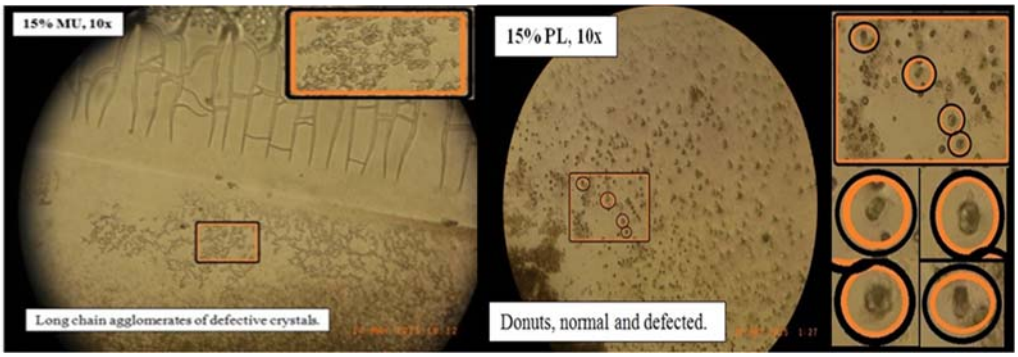


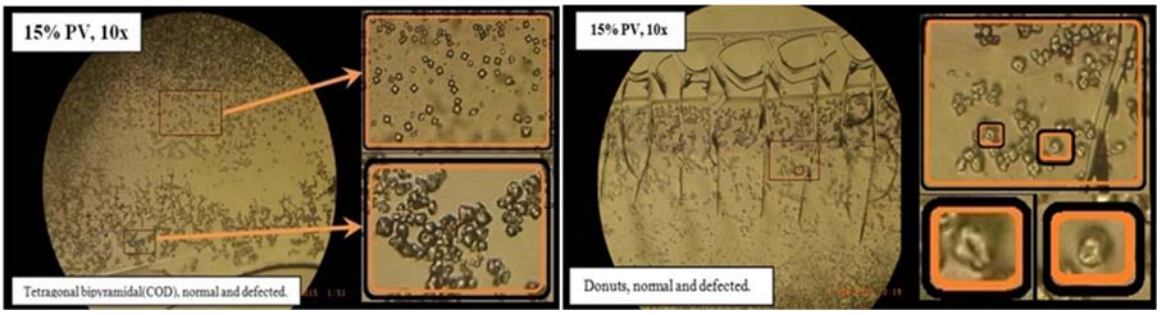
Photograph 1: COM crystal formation under compound microscope (at 10x magnification) prismatic(1), platy or platelet shaped(2) [16]; rosettes(3), loose larger agglomerate(4) small compact aggregates(5); donut (6), dumbbell (7) [25].

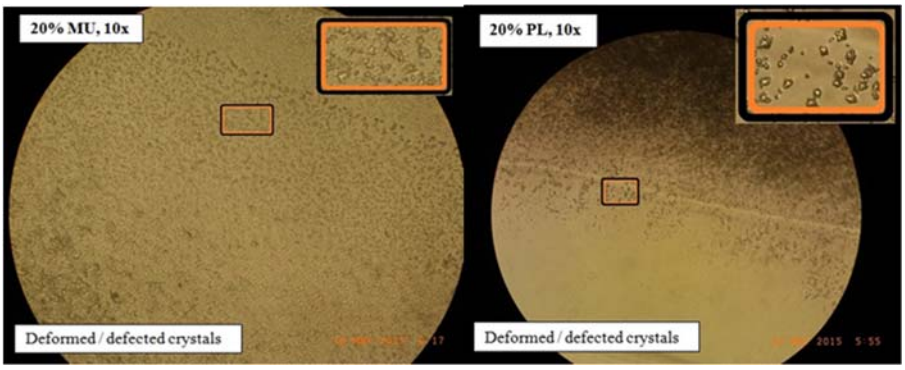


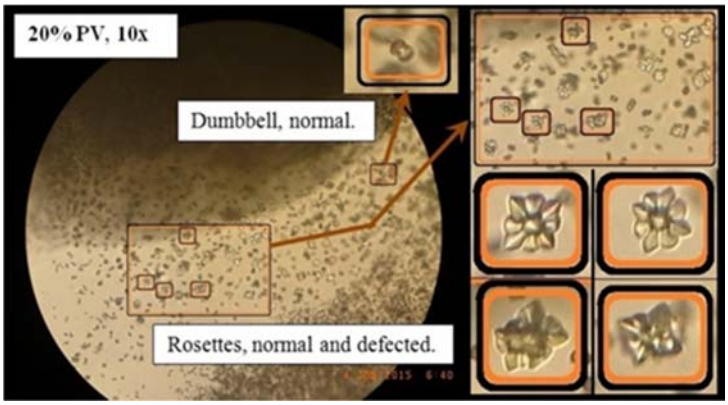

5% PV, 10x
Rosette, normal
10% MU, 10x
Rosettes, normal and defected.


10% PL, 10x
Rosettes, normal and defected.
10% PV, 10x
Tetragonal bipyramidal, normal and defected.
Elongated large rods, normal and defected.


15% MU, 10x
Long chain agglomerates of defective crystals.
15% PL, 10x
Donuts, normal and defected.


15% PV, 10x
Tetragonal bipyramidal(COD), normal and defected.
15% PV, 10x
Donuts, normal and defected.


20% MU, 10x
Deformed / defected crystals
20% PL, 10x
Deformed / defected crystals



Photograph 2: The inhibitory effects on calcium oxalate crystals by using *Macrotyloma uniflorum*, *Phaseolus lunatus* and *Phaseolus vulgaris* infusions.

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