Morphological Inhibition of Calcium Oxalate Monohydrate by Roselle (*Hibiscus sabdariffa* L.)

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Abstract

Calcium oxalate (CaOx) can be crystallized in several forms and morphologies. In this research, we investigated the crystallization of CaOx by the precipitation of calcium chloride and sodium oxalate in the absence and presence of roselle (*Hibiscus sabdariffa* L.). The effects of roselle concentration and $[Ca^{2+}]/[C_2O_4^{2-}]$ concentration on CaOx crystal forms and morphologies were studied. The crystals were characterized by scanning electron microscopy (SEM), and the quantities of Ca^{2+} in aqueous solution were detected by atomic absorption spectrometry (AAS). The results show that roselle concentration and $[Ca^{2+}]/[C_2O_4^{2-}]$ concentration affected the crystal morphologies that mainly were hexagonal, octahedral and dendritic. Higher roselle concentration increased the formation of calcium oxalate dihydrate (COD) crystals as the octahedral shape. Roselle may act as a good inhibitor for kidney stones since it induces the COD crystals that are easily excreted in urine. This research may ensure the application of roselle as herbal medicine to prevent kidney stones.

Keywords: Crystallization; Kidney stone; Calcium oxalate; Roselle; *Hibiscus sabdariffa* L.; Scanning electron microscopy

1. Introduction

Roselle (*Hibiscus sabdariffa* L.), an annual shrub, belongs to the Malvaceae family and occurs widely throughout tropical countries including Thailand, where it is known as Krachiap Daeng [1,2]. Various components of the plant (flower, leaves, calyx and corolla) are prepared as a drink and for herbal medicinal purposes [3]. Its red persistent calyx is the major component having a sour taste and is generally used as a beverage and food colorant [1]. It is traditionally used as an antibacterial, antifungal, hypocholesterol-emic, diuretic, uricosuric, mild laxative and antihypertensive substance [4-7]. It is also claimed as a Thai folk medicine for kidney stones [4].

Kidney stones are commonly composed of calcium oxalate (CaOx), especially calcium oxalate monohydrate (COM) micro-crystals [8-10]. COM is the most thermodynamically stable form at room temperature in nature [9,11]. When food containing high oxalic acid, such as spiny pigweed (*Amaranthus lividus* L.) and cocoa (*Theobroma cacao* L.) are consumed, they cause the formation of CaOx crystals in urine. Generally, the produced crystal is COM. Humans normally have biological control mechanisms to prevent COM crystallization in urine by inducing inhibitors that decrease nucleation, growth, and aggregation of COM crystals [8,12]. In particular, inhibitors in urine will transform COM to calcium oxalate dihydrate (COD) [10,13].

In the past decade, interactions between stone crystals and organic matrixes, such as citrate, osteopontin, aspartate, glutamate, poly-(styrene-alt-maleic acid) (PSMA) and glycosaminoglycans have been investigated [8,9,11,14]. Only recently has the application of herbs been applied for control of COM crystal development in vitro, especially in Thailand. Interaction mechanisms between organic molecular additives and inorganic crystals in vitro, during the formation of stones remain poorly understood [11]. The applicability of herbs such as roselle (*Hibiscus sabdariffa* L.) in inhibiting kidney stones remains unanswered. In the present study, we investigated the crystallization of CaOx crystals from aqueous solution in the absence and presence of roselle. The effects of roselle concentration and $[Ca^{2+}]/[C_2O_4^{2-}]$ concentration on CaOx crystal formation, and compositions were studied. In addition, we used atomic absorption spectrometry (AAS) for detecting the Ca^{2+} in aqueous solution, and scanning electron microscopy (SEM) to describe the morphologies and surfaces of CaOx crystals as well as their compositions with energy dispersive X-ray spectroscopy (EDS).

2. Materials and methods

2.1. $CaCl_2$ and $Na_2C_2O_4$ solutions

All preparations and experiments were conducted at room temperature (25 ^oC), and all chemicals were analytical grade. $CaCl_22H_2O$ (ASP Ajax Finechem) and $Na_2C_2O_4$ (ASP Ajax Finechem) were from the same stock solutions of 1 M in distilled (dI) water. CaOx crystals were produced by using the required concentrations of $CaCl_2$ (1, 4 mM) and $Na_2C_2O_4$ (1, 4 mM) diluted from the stock solutions with dI water. With regard to the previous study [15], the most appropriate $[Ca^{2+}]/[C_2O_4^{2-}]$ concentration is 1:1 because surface characteristics could not distinctively classify the differences, when compared with 2:1 and 3:1 using SEM; the lowest Ca^{2+} concentration was chosen.

2.2 Roselle-extracted solutions

Dried roselle calyxes were ground into coarse powder and then extracted by ethanol (MERCK). After 24 h equilibration, they were filtered and evaporated as roselle-extracted matrix. The matrix was diluted with dI water to 0.25, 5, 10 and 50 g/L, which were used as the crystal modifier of roselle-extracted solution.

2.3 Crystallization of CaOx crystals in absence and presence of roselle

In a typical experiment, $CaCl_2$ (1 mM, 20 mL) was added to $Na_2C_2O_4$ (1 mM, 20 mL) in five well-beakers. Subsequently, the roselle modifier was added to the solutions, 0.25 to 50 g/L along with vigorous stirring for 1 minute. Then the mixtures were covered with a glass plate for 24 h until solutions crystallized. As a reference, the CaOx was prepared in the absence of roselle. In the same tract, the final concentration of $CaCl_2$ (4 mM, 20 mL) added to $Na_2C_2O_4$ (4 mM, 20 mL) was treated to the same conditions.

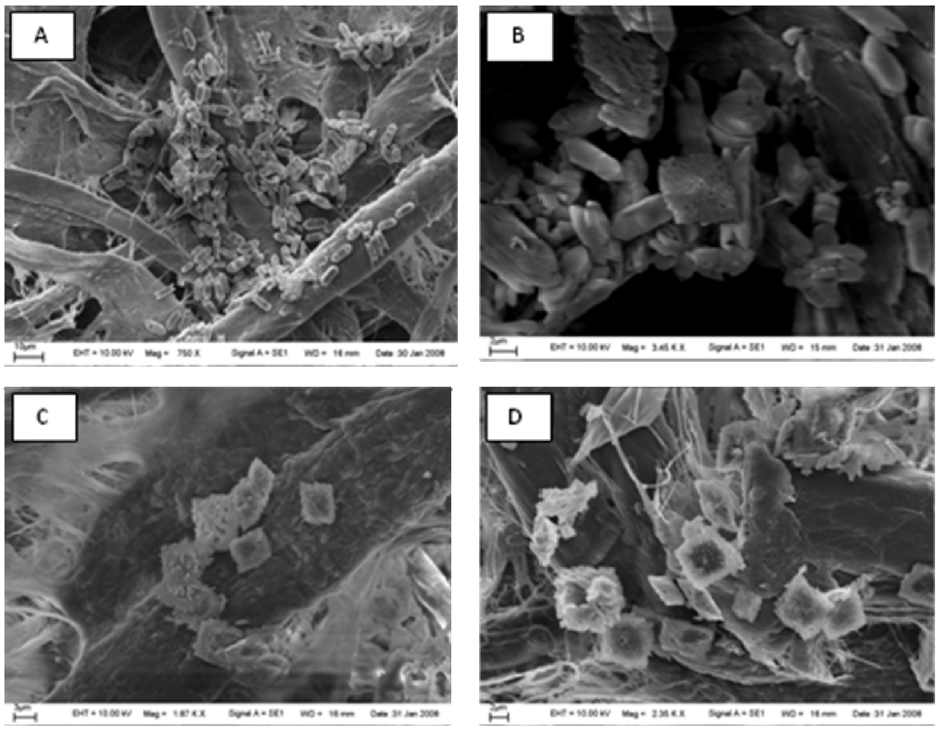
2.4 Detection and characterization

Quantities of Ca^{2+} in solutions were determined after filtration of the mixtures by AAS (model AA-6501F, SHIMADZU). Dried CaOx crystals were characterized for their morphologies by SEM (model 1450 VP, LEO) with an accelerating voltage of 20 kV. Chemical elements of crystals were also characterized by using EDS as the detector in SEM.

3. Results and discussion

3.1 Effect of roselle concentration

The effect of varying amounts of roselle on the morphologies and the sizes of CaOx particles at 25 ^{o}C under the standard analysis conditions are exhibited in Fig. 1. Fig. 1A indicates that all CaOx particles were hexagonal plate-like in shape of COM [8-10,13,16,17]. When roselle increased in concentration, the amount of COM particles decreased, and those of COD gradually increased. Most CaOx particles had the octahedral shape of COD [13,16,17] (Fig. 1E). This is because roselle contains mainly anthocyanin, cyanidin, delphinidin [18], ascorbic acid [19,20], citric acid [21], succinic acid, and oxalic acid [20]. Hence, these functional groups of organic compound pigment and acid could transform the structure of COM to COD. In conclusion, the higher concentration of roselle inhibits the formation of COM, and promotes the formation of COD.



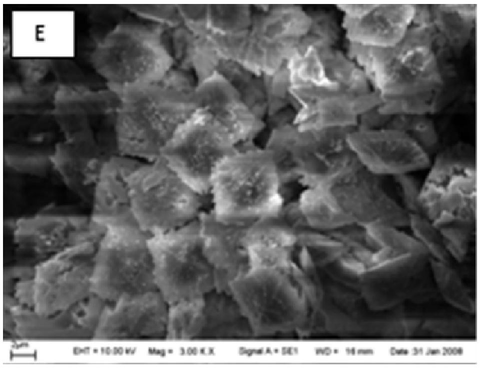


Figure 1 SEM micrographs of CaOx particles received from the absence and presence of roselle at 25 °C. [Ca^{2+}]: 1 mM and [$C_2O_4^{2-}$]: 1 mM, [roselle]: (A) 0, (B) 2.5, (C) 5, (D) 10 and (E) 50 g/L, the scale bars = 10 µm (A), 3 µm (C), 2 µm (B, D, E).

After $CaCl_2$ reacted with $Na_2C_2O_4$, the crystallization of CaOx occurred so that the stable COM was the dominant phase. In the absence of roselle (Fig. 1A), Ca^{2+} concentration in the aqueous solution was very low. When concentrations of roselle were added to the solutions, Ca^{2+} concentrations gradually decreased (Fig. 2). Due to the reaction between roselle and Ca^{2+} ions in the aqueous solutions, COD then could be formed and increased with the roselle concentrations.

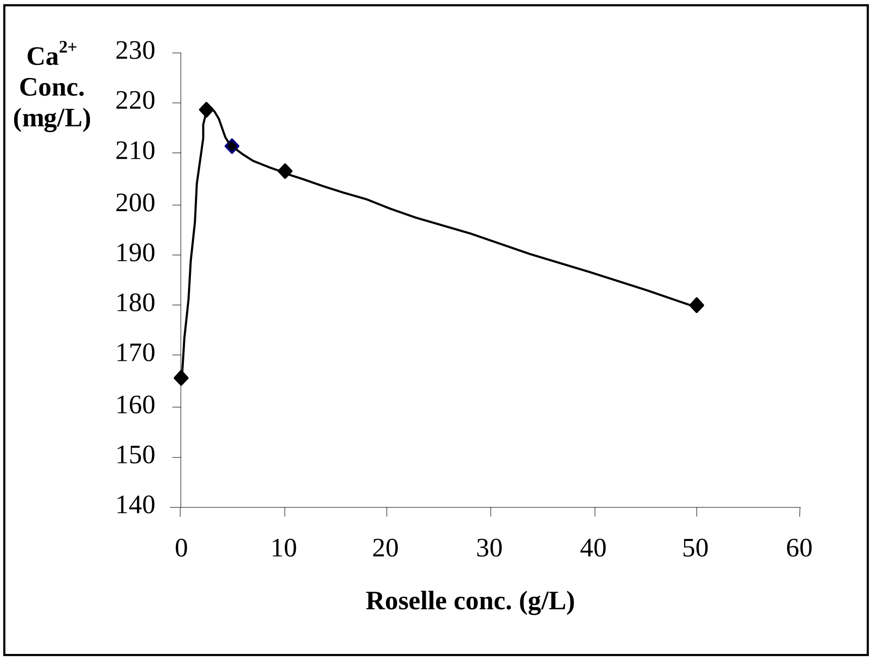


Figure 2 Ca^{2+} concentration in relation with roselle concentration from the reaction between $CaCl_2$ (1 mM, 20 mL) and $Na_2C_2O_4$ (1 mM, 20 mL) at 25 °C.

Surface structures of COM and COD differ in their affinities for cell membranes [11]. COM has a higher affinity for renal tubule cells [22] and for cell membranes as compared with COD [23]. Hence, a preferential adsorption to cell membranes of COM crystals induces kidney stones [11]. In contrast, COD prevents kidney stones because it is easily excreted in urine [22,24]. It is suggested that roselle act as a good inhibitor for kidney stones once they induce the formation of COD. This is consistent with its practice in Thailand of using roselle as a diuretic to excrete kidney stones [4]. The presence of functional groups of organic compound pigment and acid in roselle may inhibit the formation of COM by reacting with calcium instead of oxalate.

The compositions of crystals from $CaCl_2$ and $Na_2C_2O_4$ were characterized with EDS and found to consist of carbon (C), oxygen (O), calcium (Ca) and gold (Au). Au was found because the crystals had to be sealed with Au before detecting by SEM. Hence, the resulting compositions were confirmed, that the crystals were CaOx.

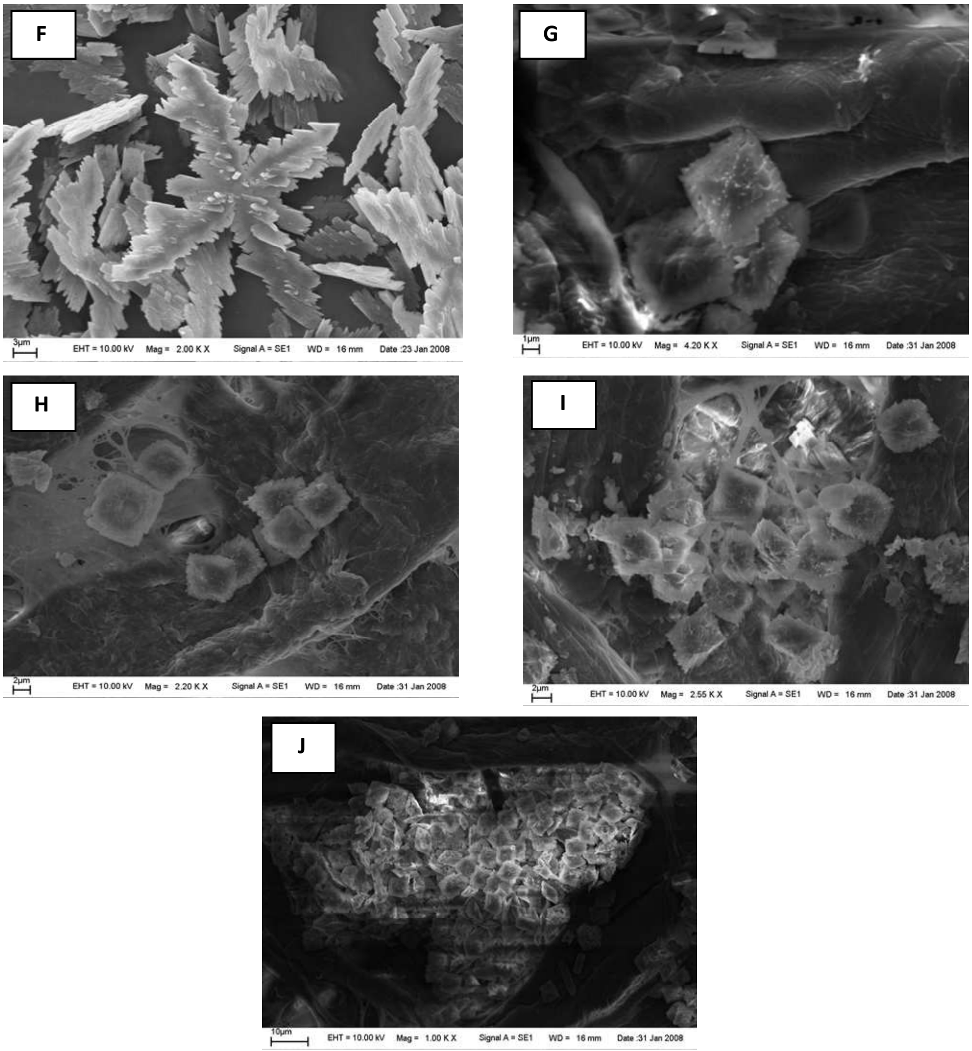


Figure 3 SEM micrographs of CaOx particles received from the absence and presence of roselle at 25 ^oC. [Ca^{2+}]: 4 mM and [$C_2O_4^{2-}$]: 4 mM, [roselle]: (F) 0, (G) 2.5, (H) 5, (I) 10 and (J) 50 g/L, the scale bars = 10 µm (J), 3 µm (F), 2 µm (H, I), 1 µm (G).

3.2 Effect of [Ca^{2+}]/[$C_2O_4^{2-}$] concentration

Varying [Ca^{2+}]/[$C_2O_4^{2-}$] concentration would change the CaOx crystals and the CaOx-modifier crystals reaction, leading to morphological variation of CaOx crystals [11]. At higher [Ca^{2+}]/[$C_2O_4^{2-}$] concentration of 4:4, Fig. 3F shows the COM particles to be dendritic in shape [10,25], compared with a hexagonal shape when the concentration is 1:1 (Fig. 1A). The amount of COD particles of octahedral shape gradually increased with roselle concentration, instead of COM particles, the same as the concentration of 1:1. The compositions of crystals in the concentration of 4:4 were characterized also by EDS. The chemical elements were the same as the concentration of 1:1.

4. Conclusion

Our findings clearly indicated that the roselle concentration, applied as a herbal inhibitor, and [Ca^{2+}]/[$C_2O_4^{2-}$] concentration affected morphologies of CaOx crystals. Main crystal morphologies found were hexagonal, octahedral and dendritic. Higher roselle concentration increased the formation of COD crystals with an octahedral shape. Roselle may act as a good inhibitor to control CaOx crystals, and is applied as herbal medicine to prevent kidney stones, since it induces COD crystals to form that are easily excreted in urine.

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6. References

[1] Hirunpanich V., Utaipat A., Morales N. P., Bunyapraphatsara N., Sato H., Herunsale A. and Suthisisang C., Hypocholesterolemic and Antioxidant Effects of Aqueous Extracts from the Dried Calyx of *Hibiscus sabdariffa* L. in Hypercholesterolemic Rats, J. Ethnopharm, Vol. 103, pp. 252-260, 2006.

[2] Prasongwatana V., Woottisin S., Sriboonlue P. and Kukongviriyapan V., Uricosuric Effect of Rosell (*Hibiscus sabdariffa*) in Normal and Renal-Stone Former Subjects J. Ethnopharm Vol. 117, pp. 491-495, 2008.

[3] Herrera-Arellano A., Flores-Romero S., Chavez-Soto M. A. and Tortoriello J., Effectiveness and Tolerability of a Standardized Extract from *Hibiscus Sabdariffa* in Patients with Mild to Moderate Hypertension: a Controlled and Randomized Clinical Trial, Phytomed., Vol. 11, pp. 375-382, 2004.

[4] Farnworth N. R. and Bunyapraphatsara N., Thai Medicinal Plants, Prachachon Press, Bangkok, 1992.

[5] Onyenekwe P. C., Ajani E. O., Ameh D. A. and Gamaniel K. S., Antihypertensive Effect of Roselle (*Hibiscus sabdariffa*) Calyx Infusion in Spontaneously Hypertensive Rats and a Comparison of Its Toxicity with that in Wistar Rats, Cell Biochem. Func., Vol. 17, pp. 199-206, 1999.

[6] Ajay M., Chai H. J., Mustafa A. M., Gilani A. H. and Mustafa M. R., Mechanisms of the Anti-hypertensive Effect of *Hibiscus sabdariffa* Calyces, J. Ethnopharm, Vol. 109, pp. 388-393, 2007.

[7] Wright C. I., Van-Buren L., Kroner C. I., and Koning M. M. G., Herbal Medicines as Diuretics: A Review of the Scientific Evidence, J. Ethnopharm, Vol. 114, pp. 1-13, 2007.

[8] Qiu S. R., Wierzbicki A., Orme C. A., Cody A. M., Hoyer J. R., Nanocollas G. H., Zepeda S. and De Yoreo J. J., Molecular Modulation of Calcium Oxalate Crystallization by Osteopontin and Citrate, Proc. Natl. Acad. Sci. USA, Vol. 101, pp. 1811-1815, 2003.

[9] Sheng X., Jung T., Wesson, J. A. and Ward M. D., Adhesion at Calcium Oxalate Crystal Surfaces and the Effect of Urinary Constituents, Proc. Natl. Acad. Sci. USA, Vol. 102, pp. 267-272, 2004.

[10] Thongboonkerd V., Samangoen T. and Chutipongtanate S., Factors Determining Types and Morphologies of Calcium Oxalate Crystals: Molar Concentrations, Buffering, pH, Stirring and Temperature, Clin. Chim. Acta, Vol. 367, pp. 120-131, 2005.

[11] Yu J., Tang H., Cheng B. and Zhao X., Morphological Control of Calcium Oxalate Particles in the Presence of Poly-(Styrene-Alt-Maleic Acid), J. Solid State Chem., Vol. 177, pp. 3368-3374, 2004.

[12] Oehlschlager S., Fuessel S., Meye A., Herrmann J., Froehner M., Albrecht S. and Wirth M. P., Role of Cellular Oxalate in Oxalate Clearance of Patients with Calcium Oxalate Monohydrate Stone Formation and Normal Controls, Urol., Vol. 73, pp. 480-483, 2009.

[13] Jung T., Kim W. S. and Choi C. K., Biomineralization of Calcium Oxalate for Controlling Crystal Structure and Morphology, Mater. Sci. Eng.: C, Vol. 24, pp. 31-33, 2004.

[14] Shirane Y., Kurokawa Y., Miyashita S., Komatsu H. and Kagawa S., Study of Inhibition Mechanisms of Glycosaminoglycans on Calcium Oxalate Monohydrate Crystals by Atomic Force Microscopy, Urol. Res., Vol. 27, pp. 426-431, 1999.

[15] Pachana K., The Application of Nanotechnology to Study Surface Mechanism of Dissolution and Formation of Kidney Stone (Calcium Oxalate) with the Inhibitory and/or Accelerator Substances, Burapha University Press, Chonburi, 2008.

[16] Walton R. C., Kavanagh J. P., Heywood B. R. and Rao P. N., Calcium Oxalates Grown in Human Urine under Different Batch Conditions, J. Cryst. Growth, Vol. 284, pp. 517-529, 2005.

[17] Yu H., Sheikholeslami R. and Doherty W. O. S., Calcium Oxalate Crystallization in Silica and Sugar Solutions-Characterization of Crystal Phases and Habits, Powder Tech., Vol. 160, pp. 2-6, 2005.

[18] Hong V. and Wrostlad O., Use of HPLC Separation/ Photodiode Array Detection for Characterisation of Anthocyanin, J. Agri. Food Chem., Vol. 38, pp. 708-715, 1990.

[19] Wong P. K., Yusof S., Ghazali H. M. and Man Y. B. C., Physico-Chemical Characteristics of Roselle (*Hibiscus sabdariffa* L.), Emer. Gr. Pub. Ltd., Vol. 32, pp. 68-73, 2002.

[20] Fasoyiro S. B., Ashaye O. A., Adeola A. and Samuel F. O., Chemical and Sorability of Fruit-Favored (*Hibiscus sabdariffa*) Drinks, Wrl. J. Agri. Sci., Vol. 1, pp. 165-168, 2005.

[21] Carvajal-Zarrabal O., Hayward-Jones P. M., Orta-Flores Z., Nolasco-Hipolito C., Barradas-Dermitz D. M., Aguilar-Uscanga M. G. and Pedroza-Hernandez M. F., Effect of *Hibiscus Sabdariffa* L. Dried Calyx Ethanol Extract on Fat Absorption-Excretion, and Body Weight Implication in Rats,

J. Biomed. Biotech., Vol. 2009, p. 5, 2009.

[22] Wesson J. A., Worcester E. M., Wiessner J. H., Mandel N. S., and Kleinman J. G., Control of Calcium Oxalate Crystal Structure and Cell Adherence by Urinary Macromolecules, Kidney Int., Vol. 54, pp. 952-957, 1998.

[23] Mandel N., Crystal-Membrane Interaction, J. Am. Soc. Nephrol., Vol. 5, pp. S37-S45, 1994.

[24] Wang L., Zhang W., Qiu S. R., Zachowicz W. J., Guan X., Tang R., Hoyer J. R., Yorero J. J. D. and Nancollas G. H., Inhibition of Calcium Oxalate Monohydrate Crystallization by Combination of Citrate and Osteopontin J. Cryst. Growth, Vol. 291, pp. 160-165, 2006.

[25] Petrova E. V., Gvozdev N. V., and Rashkovich L. N., Growth and Dissolution of Calcium Oxalate Monohydrate (COM) Crystals, J. Optoelec. Adv. Mater., Vol. 6, pp. 261-268, 2004.