Effect of *Blumea balsamifera* Extract on the Morphology and Nucleation of Calcium Oxalate Crystals in Artificial Urine

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***Abstract*—Blumea balsamifera or "sambong" is a known natural cure for kidney stone formation; however, there are limited scientific studies which quantify and explain how the extract inhibits kidney stone formation. In this study the effect of B. balsamifera extract on the morphology and nucleation of calcium oxalate crystals in an artificial urine environment was studied using microscopic and turbidimetric analyses. Results showed a reduction in crystal size from 38.21 ± 5.65% to 76.47 ± 5.45%. However, no significant difference was found in crystal sizes as extract dosage varies. Crystal morphology showed a shift from Calcium Oxalate Monohydrate (COM) to Calcium Oxalate Dihydrate (COD). Turbidity assay showed a decrease in turbidity slope as the extract inhibited nucleation. The nucleation mechanism was also modified by the extract. The dependence of nucleation rate on supersaturation was lost in the presence of the extract. These results suggest that the medicinal properties of B. balsamifera is attributed to the shift in crystal shape and size, and an inhibition of nucleation.**

***Keywords*—*Blumea balsamifera* extract, crystallization, nucleation, calcium oxalate stone, kidney stones**

I. Introduction

Nephrolithiasis or kidney stone formation is caused by crystallization of solids from supersaturated urine. The crystals grow and aggregate into larger crystals, which upon retention, are harmful and can impair kidney functions. Kidney stones are composed of various minerals such as struvite, uric acid, cysteine and calcium oxalate. Among these, the most common type of kidney stones are calcium oxalate stones found in 76% of cases with more than 60% of cases in the form of Calcium Oxalate Monohydrate (COM) [1]. COM is a picket fence-shaped crystal while Calcium Oxalate Dihdyrate (COD) is octahedral in shape. COD is considered a less pathogenic hydrate of calcium oxalate as it is generally smaller, easier to eliminate through urine and aggregates to a lesser extent relative to COM [2].

Several medications and treatment methods currently exist to treat kidney stones. Among these are medications from *B. balsamifera*, locally known in the Philippines as *sambong*. Clinical studies showed a significant reduction on in vivo stone dissolution using urine from *B. balsamifera* consuming individuals, but the mechanism was not explained [3]. Recent studies showed that *B. balsamifera* shifts crystal morphology from COM to COD [4] and increase crystallization rate [5] but its effect on nucleation was not studied.

In this study, the effect of *B. balsamifera* on the nucleation of calcium oxalate crystals will be explored and quantified. This will be able to provide additional scientific grounds, on mechanism behind the medicinal property of *B. balsamifera*.

II. Materials and Methods

A. Extract Preparation

B. balsamifera leaves were cut dried at 40 °C for six days and crushed into a powder form. Extraction was conducted using a Soxhlet apparatus with ethanol as the extracting solvent [6]. The extraction is performed at 80 °C with constant stirring for 6 hrs. A total of 7 g of crushed *B. balsamifera* leaves and 700 mL of 99.5% ethanol were used for every extraction. After extraction, ethanol was boiled-off in a water bath at 80 °C to obtain a crude extract.

B. Artificial Urine Preparation

Artificial urine was prepared using the formulation by Burns and Finlayson (1980) as recommended by a comparative study on various formulations of artificial urine for in vitro studies (Table 1) [7].

Table 1. Artificial urine formulation by Burns and Finlayson

Compound (mmol/L)		Compound (mmol/L)	
Sodium Chloride	105.5	Potassium Chloride	63.7
Sodium Phosphate	32.3	Calcium Chloride	3.5
Tri-Sodium Citrate	3.21	Sodium Oxalate	0.32
Magnesium Sulfate	3.85	Ammonium Hydroxide	17.9
Sodium Sulfate	16.95	Ammonium Chloride	0.0028

C. Morphology Study

Artificial urine and extract-containing samples were crystallized for 3 days at room temperature. Each sample was placed under a light microscope using a hemocytometer to measure crystal sizes. Pictures were taken from each sample at magnifications of 100× and 400× under the light microscope. The pictures were then analyzed using ImageJ 1.50i [8].

D. Nucleation Study

A turbidity assay was developed by Hess *et al.* In this method, the increase in turbidity measured at 620 nm corresponds to an increase in detectable particles or stable nuclei [9]. Turbidimetry was performed at various levels of supersaturation ratios (17.5, 20.0, 22.5, 25.0) and extract dosages (0.0 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 2.0 mg/mL, 5.0 mg/mL) using Perkin Elmer Lambda 850 UV-Vis spectrophotometer. Three replicates were made for every combination. Absorbance in the samples were measured using. For every 1-mL cuvette replicate, volumes of artificial urine, extract, calcium chloride solution, and sodium oxalate solution were added sequentially. Water replaces the extract in the control replicates (those with extract dosage of 0.0

mg/mL).

E. Phytochemical Assay

The phytochemical assay was performed by the Standards and Testing Division of the Department of Science and Technology - Industrial Technology Development Institute from 50 g of cut *B. balsamifera* leaves.

III. Results and Discussion

A. Morphology Study

Fig. 1 shows the arithmetic and area mean Feret diameters of calcium oxalate crystals at different supersaturation ratios.

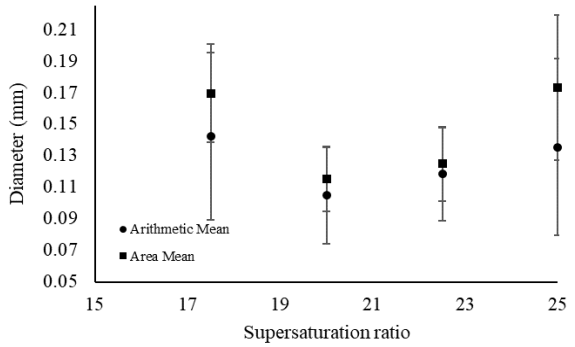

Fig. 1. The mean Feret diameter of calcium oxalate crystals at different supersaturation ratios have a statistically insignificant difference with P-values of 0.1882, 0.3118, 0.3960 and 0.1880 at supersaturation ratios of 17.5, 20 22.5 and 25, respectively.

The area mean Feret diameters are consistently bigger than the arithmetic mean. This is due to the nature of calculation. The area mean diameter allocates a weight that is dependent on the projected area of the particles. Regardless of the type of mean used, crystal diameter decreased from a super saturation ratio of 17.5 to 20 and increased from 20 to 25. However, the difference between the area mean and arithmetic mean Feret diameters is statistically insignificant.

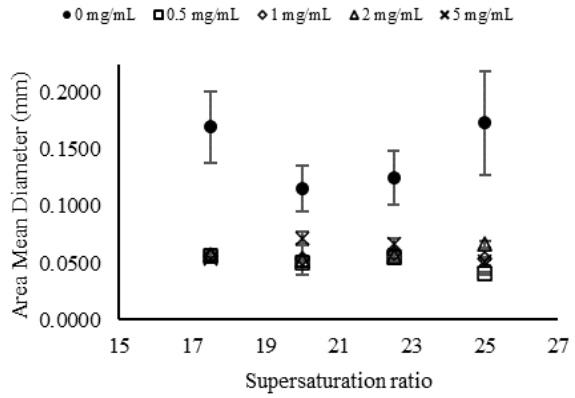

Fig. 2. The area mean Feret diameter significantly decreased in the presence of the extract ($P = 7.203 \times 10^{-6}$).

Fig. 2 shows that the extract reduced the size of the crystals across all supersaturation ratios. Two-factor ANOVA showed that supersaturation ratio had no significant effect on the Area mean diameter ($P = 0.7288$) while the effect of the extract is significant ($P = 7.203 \times 10^{-6}$). Further statistical analysis omitting the control 0 mg/mL of extract showed that the concentration is not a significant factor ($P = 0.3219$). Thus, only the presence of the extract even at the lowest concentration of 0.5 mg/mL reduced the size of calcium oxalate crystals.

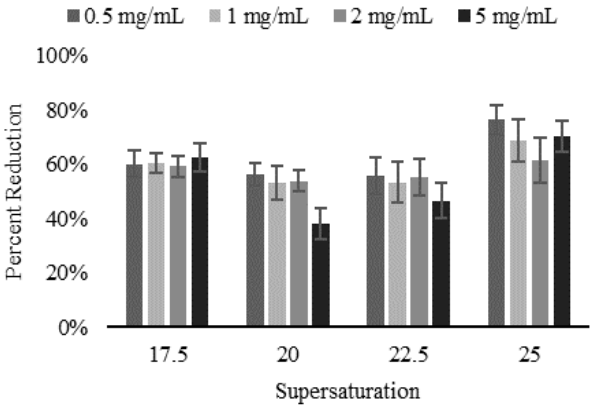

Fig. 3. The crystal size decreased by 60% on average and is not dependent on extract concentration across all supersaturations.

Fig. 3 shows that the extract significantly reduced the size of the crystals ranging from 38.21 ± 5.65% to 76.47 ± 5.45%. Percent reduction in crystal size does not generally correlate with extract concentration except at a supersaturation ratio of 20 where percent reduction linearly decreases with the extract concentration (RSQ = 0.9402). However, this is an exception as poor coefficient of determination values were observed at other supersaturation ratios.

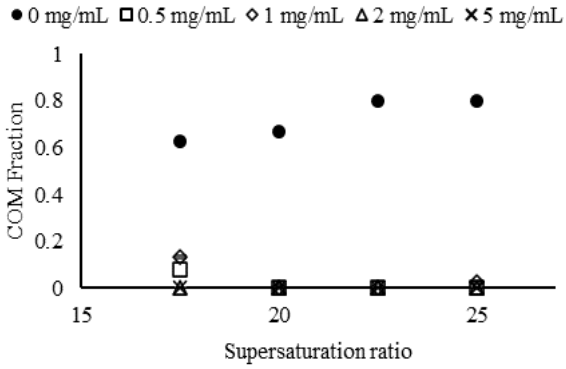

Fig. 4. Presence of the extract converted most if not all the crystals from COM to COD.

Fig. 4 shows that across all supersaturation ratios, COM fraction is reduced from 60–80% to 0%. The reduction in size is due to a shift in crystal morphology from COM to COD. COD is the less pathogenic hydrate of calcium oxalate crystals. It is unstable at high supersaturation ratios and is eliminated prior to reaching a pathological size. Formation of COD was reported for other organic crystal growth modifiers such as polyacrylate, Poly-l-aspartate polyglutamate [10], Osteopontin [11], and other plant extracts such as Khella plant [12] and previous studies on *B. balsamifera* [4].

B. Nucleation Study

A typical plot of adsorption versus time shown in figure 5 has a short period of time where absorbance reading is small followed by an increase in absorbance consistent with the observation of Hess *et al.* [9].

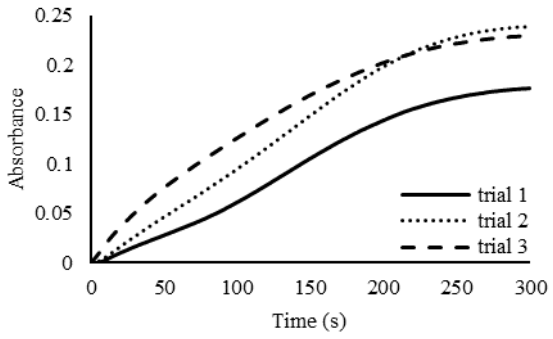

Fig. 5. A typical plot of experimentally observed absorbance across time shows a monotonically increasing absorbance until a maximum was reached.

The turbidity slope was determined from the linear region in the absorbance plot. Fig. 6 shows that the turbidity slope increases with supersaturation ratio. Regression results in a strong linear correlation between the turbidity slope and supersaturation ratio (RSQ = 0.9906). This is consistent with classical nucleation theories where nucleation rate is proportional to supersaturation [13].

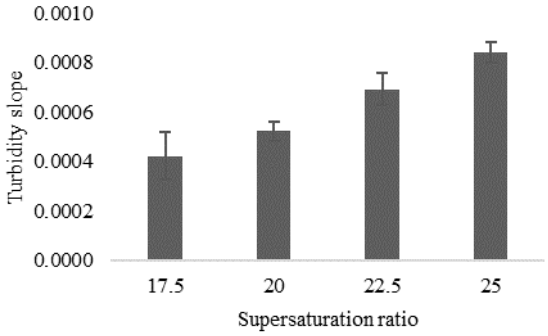

Fig. 6. The average turbidity slope increases with the supersaturation ratio.

Fig. 7 shows that as the concentration of *B. balsamifera* extract increases, the linear correlation between the turbidity slope and supersaturation ratio weakens. The coefficient of determination decreased from 0.99 to 0.8928, 0.5884, 0.3814 and 0.0995 at 0.5, 1, 2, and 5 mg/mL of extract respectively.

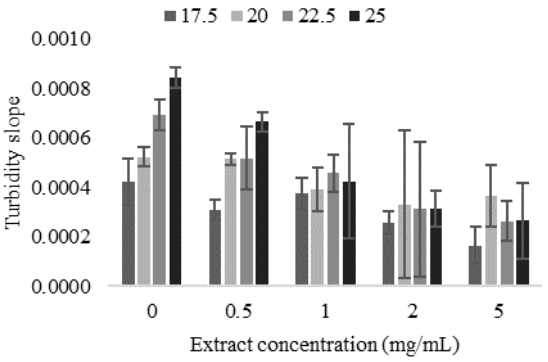

Fig. 7. Increasing the supersaturation ratio increases the turbidity slope but the presence of increasing extract concentration changes this trend and the linear dependence of turbidity slope on supersaturation ratio.

The turbidity slope generally decreases with increase in extract concentration as shown in Fig. 8. Single factor ANOVA shows that the change in turbidity slope is not significant at 0.5 and 1 mg/mL of extract with a P-value of 0.3546 and 0.0696, respectively. However, the reduction is significant at 2 and 5 mg/mL of extract with a P-value of 0.0149 and 0.0127, respectively. A similar decrease in turbidity slope was reported from the extract of *Ammi visnaga* (Khella). Further analysis showed that *Ammi visnaga* extract increased the change in Gibbs free energy for the crystallization process making nucleation thermodynamically less favorable [12].

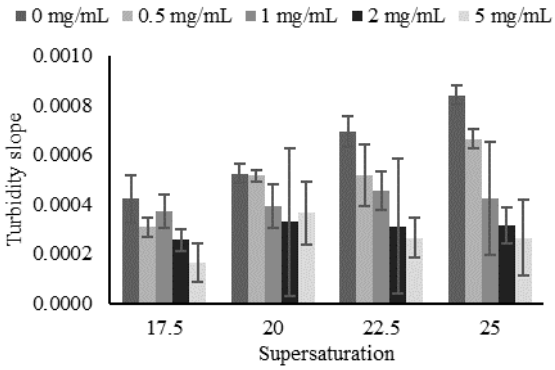

Fig. 8. Turbidity slope generally decreases with increasing extract concentration at all supersaturation ratios.

Percent inhibition is computed from the change in magnitude of the turbidity slope with and without the extract. Fig. 9 shows that percent inhibition varies from 1.58% to 68.52%, depending on the extract concentration and supersaturation ratio. In general, higher percent inhibition is obtained at higher supersaturation and extract concentration. However, percent inhibition does not show a linear dose dependence on concentration particularly at high extract concentrations. At an extract concentration below 2 mg/mL, percent inhibition still exhibited poor linear correlation with extract concentration (RSQ = 0.6438, 0.9030, 0.9440, 0.9081 at supersaturation ratios 17.5, 20, 22.5 and 25).

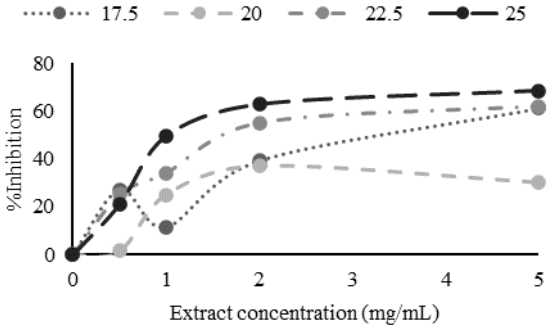

Fig. 9. Percent inhibition is not linearly correlated with extract concentration. Lines connect data points and are not from a model.

C. Extract Composition

Table 2. Phytochemical assay results

Compound	Relative amount
Sterols	+++
Triterpenes	−
Flavonoids	+
Alkaloids	++
Saponins	++
Glycosides	+
Tannins	−

Table 2 summarizes the phytochemical analysis of *B. balsamifera.* Results show an abundance of sterols, followed by alkaloids and saponins. Flavonoids were detected in the

extract, while tannins and triterpenes are absent.

Saha & Verna attributed the inhibitory effects of *Bergenia ciliata* extract on polyphenolics, such as saponins, alkaloids, flavonoids and terpenoids [14]. In a patent, the inhibitory effect of catechins and the compounds present in green tea extract were attributed to chelation of calcium ions and solubilization of crystals [15].

IV. Conclusion

Blumea balsamifera extract has a significant effect on the morphology and nucleation of calcium oxalate crystals at varying supersaturation ratios. The extract reduced the crystal size by 38.21±5.65% to 76.47±5.45% across supersaturation ratios of 17.5 to 25 due to a COM to COD shift in crystal morphology. The reduction in size is not significantly affected by supersaturation ratio nor intensity of extract concentration. *B. balsamifera* extract also significantly changed the nucleation behavior of calcium oxalate as the linear dependence of the turbidity slope with supersaturation ratio is lost and overall inhibited by the extract regardless of the concentration.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

All authors contributed equally in this study. CMontealegre conceptualized the topic, analyzed data and wrote the final paper. JTNolasco, JVBautista and RTQuintero conducted the experiment, analyzed the data, and wrote parts of the paper. All authors approved of the final version.

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