

## Determination of nucleation assay for anti-urolithiasis activity from bagasse *Musa acuminata* x *balbisiana* Colla cv. Pisang Awak Legor methanolic extracts using uv-spectrometer and size measurement.

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### ABSTRACT

Determination of inhibition of calcium oxalate (CaOx) crystallisation *in vitro* by nucleation assay is based on the rates of nucleation by comparing the slope of the turbidity of a control system with that of one exposed to the extract, which was found to be inaccurate when the CaOx crystals were formed and precipitated. A need exists for improved methods to determine the inhibition activity in nucleation assay for the study of anti-urolithiasis activity. In this study, the size reduction of CaOx crystals after treated with methanolic extract of *Musa acuminata* x *balbisiana* Colla cv. Pisang Awak Legor bagasse was measured under light microscopy and correlated to the turbidity and percentage of inhibition. Different concentrations of bagasse extract (2, 10, 30, 50 and 100 mg/ml) were investigated and the result shows that high concentration of extract would promote reduction in CaOx's stone size, but it increased the amount of sediment crystals, resulting high turbidity. The stone size was reduced up to 93.76±0.19% in the presence of 100 mg/mL extract while the inhibition percentage was negative (-102.17±0.04%) as the optical density (OD) of the extract was higher than control in the turbidity calculation. The results of this study are expected to provide an understanding on the way of calculating the activity in nucleation assay.



## 1. INTRODUCTION

The remedies have been used for a long time in treating kidney stone disease. Most of the remedies used in traditional medicine systems are taken from plants although they do not have strong systematic clinical evidence yet have a positive effect to the patients except for some composite plants and herbal drugs (Yadav et al. 2011) such as *Herniaria hirsuta* where the extract can decrease the crystal size (Atmani and Khan 2000), *Bergenia ligulata* (Aziz et al. 2005), *Piper nigrum* (Manish et al. 2011), *Dolichos biflorus* (Garimella et al. 2001), *Plantago major* (Verma et al. 2014) etc. Most of the previous findings were done *in vivo* either in animal or human (Yadav et al. 2011).

Banana was a fruit largely consumed all over the world. Different parts of banana plant (*Musa*) including pseudo-stem were known to be used in Ayurveda and other traditional folklore medicine for treatment of various diseases including kidney stone (Kalpana et al. 2013). Studies using banana pseudo-stem has not yet explored extensively in clinical system especially from bagasse from *Musa acuminata x balbisiana* Colla cv. Pokok Awak Legor. This type of *Musa* was in ABB Group.

Nucleation assay is one of the most important steps in stone formation. Generally, the percentage of inhibition stone was calculated using the equation established previously by Hennequin et al. (1993), where the value was obtained from optical density measurement at 620nm using spectrometer. Unfortunately, the method was not suitable when increasing the concentration of extract as the CaOx formation become small particles and increased the turbidity of solution more than control without treatment. It is impossible to mimic all the processes involved in stone formation *in vitro*, thus it is important to choose the most appropriate approach for measuring the inhibition process for *in vitro* study. Hence, this study was conducted to prove that different assessment needs to implement to measure the nucleation assay activity and obtained accurate results.

## 2. MATERIALS AND METHODS

### 2.1 Plant Sample Collection and preparation of extract

Large quantities of the pseudo-stem *Musa acuminata x balbisiana* Colla cv. Pisang Awak Legor have been collected from the village in Padang Serai, Kedah, Malaysia. Each pseudo-stem was cleaned with deionized water and pressed using sugarcane extractor to remove the juice. The bagasse was frozen prior to freeze drying and grinding. 5 g of bagasse was soaked in 300 mL of 80% (v/v) methanol at room temperature for a while and filtered through Whatman filter paper No. 1. The extraction was repeated several times until colorless. The extract was evaporated in rotary evaporator at a temperature not exceeding 40°C prior to freeze dry.

### 2.2 Nucleation Assay

Nucleation assay was done according to Rajeswari et al. (2013) method with some modification. Calcium chloride (5 mmol/L) and sodium oxalate (7.5 mmol/L) solution were prepared in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 and 37°C. A total of 95 µl calcium chloride solutions were added to the different concentration of extract (2, 10, 30, 50, 100 mg/ml) in 96 wells plate. Crystallization was started by adding 95 µl of sodium oxalate. The absorbance (optical density, OD) was recorded at 620 nm after 6 hours of incubation. The size of the CaOx stone was observed and measured using microscope for comparison.

### 2.3 Microscopic observation

The CaOx crystals formed with or without the extracts were observed by using light microscopy (Olympus CX41) equipped with software analysis LS starter and camera Olympus U-CMAD3 (Japan) at 40X magnification.

### 2.4 Statistical Analysis

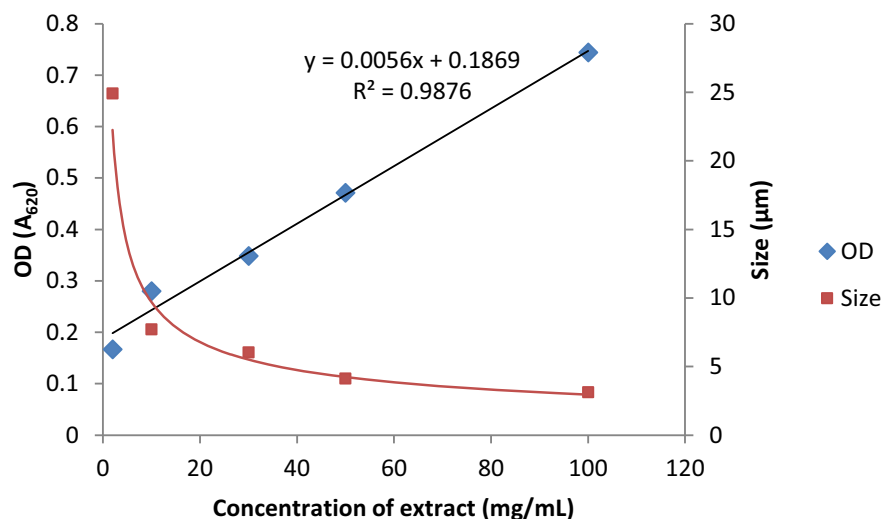
The data obtained was analyzed by two-ways using GraphPad Prism 7. A value of  $p < 0.05$  was considered significant in all cases.

## 3. RESULTS AND DISCUSSION

The methanol was selected as a solvent for extraction based on a previous study by Chilivery et al. (2016) where it showed that methanolic extract from *Argemone mexicana* L. leaves had highest anti-urolithiatic activity compared to petroleum ether, chloroform and water. Moreover, methanol has a polarity index of 5.1 and good in extracting both polar and non-polar substances and easier to concentrate the extract using rotavapor as the boiling point is just 65°C. The relationship between OD and size of CaOx against extract concentration was shown in Table 1. Increase in extract concentrations from 2 mg/mL to 100 mg/mL increased the turbidity, in terms of OD value from 0.167 to 0.744 while the size of CaOx crystal was decreased from 24.9 µm to 3.13 µm. On the other hand, the control (without extract) has higher turbidity and larger CaOx size. The assessment method in anti-urolithiasis activity by measuring the optical density using UV spectrometer in nucleation assay in vitro was established by Hennequin et al. (1993). This method has been followed by most of the studies (Rajeswari et al. 2013; Saha and Verma 2013; Atmani and Khan 2001). The method has been slightly modified and the equation of inhibition percentage used was  $[(Ac - As)/Ac] \times 100$  where, Ac is absorbance without treatment and As is absorbance with treatment. However, our result was contrary to this method, as the OD was not linearly correlated to the size of CaOx (Figure 1). Figure 1 showed that OD increased linearly with the increase in extract concentration, while the size of CaOx was reduced markedly from 2 mg/mL to 10 mg/mL of extract but slowed down afterwards. The result shows that high concentration of extract caused the formation of CaOx's stones into smaller crystals but higher amount, resulting higher turbidity. Hence, the inhibition percentage of nucleation assay using the equation established in by Hennequin et al. (1993) was not suitable in this study. Two-ways ANOVA analyses show the interaction of turbidity and size with concentration of extract is significantly different with  $p$  value  $< 0.0001$ .

**Table 1:** The optical density value and size of CaOx stone at 6 hours of incubation

Sample (mg/mL)	Turbidity (OD)	Size (µm)
Control (0)	0.368±0.04	50.10±0.53
2	0.167±0.03	24.9±1.13
10	0.280±0.01	7.70±0.40
30	0.348±0.02	6.02±0.32
50	0.471±0.03	4.12±0.54
100	0.744±0.04	3.13±0.19



**Figure 1:** Relation between absorbance at 620nm with CaOx size (μm) at difference concentration of extract incubated at 37°C for 6 hours.

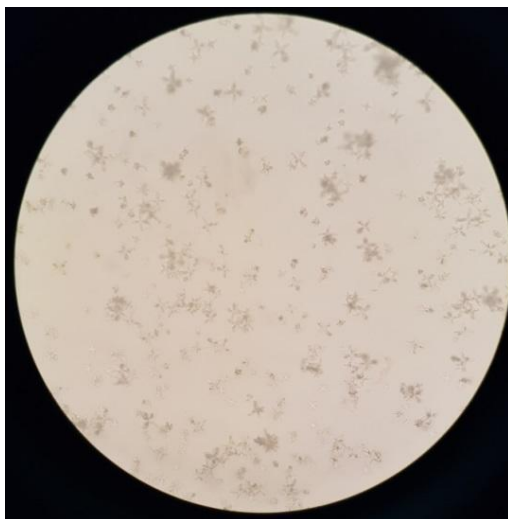
Figure 2 shows the CaOx crystals size under microscopic observation at 40X magnification. The size of stone formation was measured and calculated the inhibition percentage using the equation  $[(S_c - S_s)/S_c] \times 100$  where,  $S_c$  is size of CaOx without treatment and  $S_s$  is size of CaOx with treatment. The size of CaOx stone formation at different concentration of extracts was decreased from  $56.10 \pm 1.84 \mu\text{m}$  to  $2.37 \pm 0.49 \mu\text{m}$  at 0 to 100 mg/mL, respectively. The results were compared to the nucleation assay using OD method as shown in Table 2. The inhibition percentage by size reduction method was  $93.76 \pm 0.19\%$  in the presence of 100 mg/mL extract while the inhibition percentage became negative after treatments with extract concentration exceed 30 mg/mL ( $-27.99\%$  at 50 mg/mL and  $-102.17 \pm 0.04\%$  at 100 mg/mL) in OD method. This is due to the interference of the crystals towards the detection of spectrophotometer which resulted the OD was higher than control in the turbidity calculation.



(A)



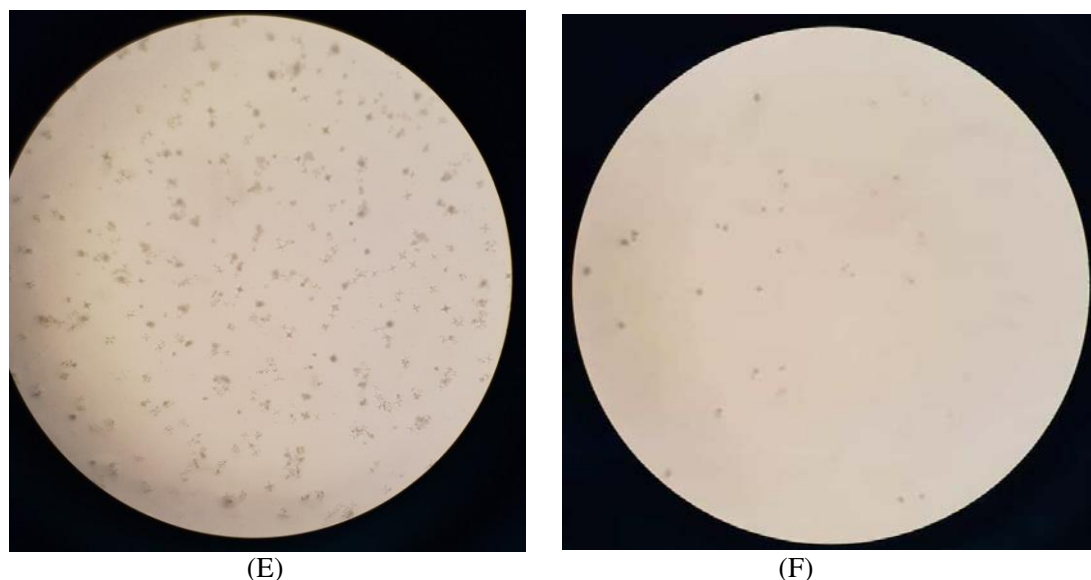
(B)



(C)



(D)



**Figure 2:** Stone formation under light microscopy at 40X magnification in nucleation assay at different concentration of bagasse *Musa acuminata x balbisiana* Colla cv. Pisang Awak Legor methanolic extract incubated at 37°C for 6 hours. (A) 0 mg/mL (B) 2 mg/mL (C) 10 mg/mL (D) 30 mg/mL (E) 50 mg/mL (F) 100 mg/mL

**Table 2:** Percentage of inhibition and size reduction at different concentration of extract

Concentration	Inhibition by OD (%)	Inhibition by size (%)
2	54.62±0.04	50.30±1.13
10	23.91±0.01	84.63±0.4
30	5.43±0.02	87.98±0.3
50	-27.99±0.03	91.78±0.54
100	-102.17±0.04	93.76±0.19

The measurement of the CaOx stone size is proposed in this study as the OD value keeps on increasing with the increase of the extract concentration. However, the size became smaller causes the inability to comply with the commonly used equation in determining the percentage of inhibition. In this case, the increasing of the turbidity were due to the small particles of the CaOx stone after treated with the extracts and resultant the reduction of inhibition percentage. Previous studied by Pachana et al. (2010) showed that the higher concentration of *Tribulus terrestris* extract inhibits the formation of COM (calcium oxalate monohydrate), and promotes the formation of COD (calcium oxalate dehydrate) in the treated group compared to control. This could be one of the reasons that the OD value was increased with the increment of extract concentration, resulted in the negative inhibition percentage. Atmani and Khan (2001) also found that *Herniaria hirsuta* extract promoted the nucleation of CaOx crystals, increasing their number but decreasing their size based on the scanning electron microscopy (SEM) urine analysis which could be another reason the absorbance value increasing when increased the concentration of extract as the crystals

formed were mostly COD. The finding also showed that size reduction gave an advantageous as it can preventing urinary stone formation by inducing the excretion of small particles the kidney and reducing the chance of retention in the urinary tract. Thus, this new approach in assessment of stone inhibition was applied to obtain the accurate result.

Theoretically, stones size less than 5 mm have a high possibility of spontaneous passage through the urinary tract and out of the body on their own with little or no pain which can take up to 40 days or the patient might undergo surgery to remove the stone (Coll et al. 2002). Measurement of Ca<sub>ox</sub> stone size in *in vitro* study will be easier in estimation or screening the potential selected plant extracts as anti-urolithiasis agents since the researcher could determine the accurate size reduction.

## CONCLUSION

In *in vitro* study, determination of nucleation assay would be more suitable by using size determination compared to the absorbance (OD value) due to the interference of the crystals. The accuracy of the activity especially in higher plant extracts concentration could be increased using size determination.

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