

INHIBITION OF CALCIUM OXALATE CRYSTALLIZATION *IN VITRO* BY EXTRACT OF BANANA CULTIVAR MONTHAN

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ABSTRACT

Objective: To study the effect of banana cultivar Monthan corm extract for its antilithiatic potential under *in vitro* condition.

Methods: Banana cultivar Monthan corm extracts of different solvents with varying polarity were tested for its antilithiatic potential. Kidney stone formation was studied under *in vitro* conditions using three critical assays such as crystal nucleation, aggregation and growth. The effect of extract on the formation and inhibition of crystals were observed spectrophotometrically and the results were presented in this paper.

Results: The results of the *in vitro* assays performed indicate that ethanol extract of Monthan readily prevented crystal nucleation, growth and aggregation.

Conclusion: Monthan corm juice is found to be an effective diuretic, and act as a promoter for inhibitors of crystallization.

Keywords: Lithiasis, Kidney stones, Musa, Banana, Monthan, Calcium oxalate, Nucleation, Crystal growth and aggregation.

INTRODUCTION

Urolithiasis (urinary calculi) is one among the three prevalent disorders in the urinary system. Approximately 80% of these calculi are composed of calcium oxalate and calcium phosphate, followed by cystine, struvite and ammonium acid urate stones. It is a worldwide problem, sparing no geographical, cultural and racial groups. It is a recurrent disease with a relapse rate of 50% [1]. The techniques for removal of calculi such as endoscopic stone removal lithotripsy and extracorporeal shock wave lithotripsy (ESWL), cause traumatic effect of shock waves, persistent residual stone fragments, acute renal disease, possibility of infection, which leads to decrease in renal function [2]. Therefore, antilithiatic drugs from natural sources have assumed greater importance as herbal alternatives which are cost effective with least side effects. The medicinal plants find application in pharmaceuticals, cosmetics, agriculture and food industry. The use of the medicinal herb for curing disease has been documented in history of all civilization. The plant medicine are in great demand in both developing as well as under developed countries for the health care because of their wide biological and medicinal activities, high safety margin and low cost [3, 4].

The market for Ayurvedic medicine is estimated to be expanding at rate of 20% annually in India. The drugs of herbal origin have been used in traditional system of medicines such as Unani and Ayurveda since ancient time. The ayurvedic system of medicine uses about 700 species, Unani 700 species, Siddha 600 species, Amchi 600 species and modern medicine uses around 30 species.

Banana, an herbaceous flowering plants of the genus *Musa*. The generic name is derived from the Arabic word 'mouz'. *Musa* species are grouped according to their ploidy level, that is, proportion of *Musa acuminata* (A) and *Musa balbisiana* (B) in their genome [5, 6]. In the present study, we selected a triploid cultivar Monthan (ABB), most familiar cooking type banana in Southern states of India (Fig. 1). Ancient folk medicines used banana corm juice/ stem juice for all urinary tract problems including kidney stones. In order to ascertain the nature of bioactive components responsible for antilithiatic potential in the banana corm, they were extracted using solvents with different polarity. The study aims at testing the antilithiatic properties of banana corm extracts under *in vitro* conditions.

MATERIALS AND METHOD

Plant sample collection and preparation of extract

The corm of Monthan cultivar was collected from the Orchard of Tamilnadu Agriculture University, Coimbatore. The corms were

cleaned and cut into thin slices and shade dried. The dried corms were powdered and passed through the coarse sieve (0.2mm). The powdered samples were used for extraction. An individual extraction of corm samples were carried out using solvents of increasing polarity such as petroleum ether, benzene, chloroform, ethyl acetate, ethanol, methanol and water. The corm extracts were prepared by hot continuous extraction method using Soxhlet apparatus [7]. The extraction was repeated until the corm samples become colourless. The aqueous extract was prepared by the hot water percolation method [7] addition of 2g of the powdered sample in 100ml of distilled water and kept in a water bath at 60°C for 2 hours. Filtered and centrifuged twice and the supernatant was collected. All the extracts were evaporated in a water bath at 60°C. The residue was stored in an airtight container and refrigerated, which was utilized for the *in vitro* assays.



Fig. 1: Banana cultivar Monthan Corm

EXPERIMENTAL WORK

In vitro assays

Formation of kidney stone involves three critical stages which include nucleation of calcium oxalate crystals, growth and aggregation [8]. These three stages can be analyzed under *in vitro* conditions both in presence and absence of the corm extracts. In order to determine the maximum efficacy of the corm extracts, a varying concentration of all the extracts ranging from 50µg to 1600µg were utilized for these assays.

Nucleation assay

The stone formation begins with the occurrence of nuclei, therefore we chose the classical model for the study of oxalate crystallization

described by Hennequin *et al.* (1993) [9] with some minor modifications. Solutions of calcium chloride and sodium oxalate were prepared separately at a final concentration of 3mM/L and 0.5mM/L respectively in a buffer containing Tris 0.5mM/L and NaCl 0.15mM/L of pH 6.5. Both the solutions were filtered thrice. For the assay, 950 μ l of calcium chloride and varying concentration of corm extracts (final volume of 100 μ l) were pipetted out against a reagent blank (without extract). To this added 950 μ l of sodium oxalate and shook well. The absorbance was measured at 620nm.

Growth assay

Newly formed crystals may combine to form a small hard mass, called calculus. The percentage inhibition of calcium oxalate crystal growth was evaluated in presence and absence of banana corm extracts by the procedure described by Chaudary *et al.* [10], Pak *et al.* [11] and Farooq *et al.* [12]. 4mM calcium chloride and 4mM sodium oxalate of 1ml each were added to a 1.5ml of solution containing sodium chloride (10mM) buffered with Tris (10mM) at pH 7.2. To this 30 μ l of calcium oxalate monohydrate crystal slurry (1.5mg/ml acetate buffer) was added. Consumption of oxalate begins immediately after calcium oxalate monohydrate crystal slurry addition and was monitored for 600 seconds for the disappearance of absorbance at 214 nm, with and without extract. The relative inhibitory activity was calculated as follows:

$$\% \text{ relative inhibitory activity} = ((C-S)/C) \times 100$$

Where 'C' is the rate of reduction of free oxalate without any extract and 'S' is the rate of reduction of free oxalate with Monthan corm extract.

Aggregation assay

The crystals in solution stick together to form large particles called as aggregates. The inhibition in presence of extracts was determined by the method of Hess *et al.* [13]. Calcium oxalate monohydrate crystal seeds were prepared by mixing calcium chloride and sodium oxalate at 50 mM/L. Both the solutions were equilibrated to 60°C in a water bath for 1hour and then cooled to 37°C overnight. The crystals were harvested by centrifugation and then evaporated at 37°C. Calcium oxalate monohydrate crystals were used at a final concentration of 0.8mg/ml buffered with Tris 0.05M / L and NaCl 0.15M / L at pH 6.5. Experiments were conducted at 37°C in the absence of the plant extract after arresting the stirring. The rate of aggregation (IR) was estimated by comparing the slope of the turbidity in the presence of the extract with that obtained in the control.

$$IR = (\text{Turbidity of sample} / \text{Turbidity of control}) \times 100$$

Statistical Analysis

Data were expressed as mean values of three independent experiments (each in triplicate), and statistical evaluation was done

using one-way analysis of variance (ANOVA) followed by Fischers LSD method (Sigma stat version 3.1).

RESULTS AND DISCUSSION

For the treatment of urolithiasis, there exist a large number of plant products as a prophylactic or curative agent in ethanomedicine, but there were very few plants which have been studied extensively. The main drawback in the development of standard drug may be attributed to multi-factorial nature of kidney stones and different chemical composition of renal stones. COM is the primary constituent of the majority of stones formed in urolithiasis. *In vitro* crystal systems are widely used to study processes of crystal nucleation, growth and aggregation [3].

In the present study, the inhibitory potential of the banana cultivar corm extract were tested for the COM crystal formation, which are predominantly present in most of the kidney stones, under *in vitro* conditions. From the graphical representation (Fig. 2), it is evident that the ethanol extract of banana corm cultivars caused dissolution of CaOx crystal nucleation when compared to dissolution by other extracts. CaOx crystals appear hexagonal in shape (control) and the disrupted calculi appear dendritic like and lose the regular hexagonal shape (Fig. 3), revealing that the corm extracts could reduce the size of the crystals formed indicative of antilithiatic activity. The other extracts such as ethylacetate, benzene, chloroform and petroleum ether were found to be less effective in the disruption of calculi. The results were in agreement with Pachana *et al* [14] who reported that an extract of *Tribulus terrestris* promoted the inhibitors of nucleation initiation, by decreasing their size and Pareta *et al* [15] reported that hydroalcoholic extract of *Achyranthes indica* Linn. remarkably inhibits the crystal formation.

In crystal growth assay, various mechanisms have been proposed to explain crystal retention. As a result of crystal growth and agglomeration, particles may be formed that are too large to freely pass through renal tubules. Alternatively, small crystals could be retained by adhering to the surface of the urothelial lining and grow to larger size [16]. Figure 2 and Plate 3 demonstrates the inhibition percentage shown by banana cultivar Monthan on CaOx crystal growth. All the solvent extracts were inhibiting the growth of CaOx, at various concentrations. Ethanol extracts (1600 μ g) shows high inhibitory percent when compared to other solvent extracts (Fig. 4 and 5).

Barros *et al.* [17], showed that the *Phyllanthus niruri* extract did not inhibit CaOx nucleation, but inhibit crystal growth and aggregation. But, in the present study, we could observe the corm extract could prevent the calculi formation at all the three critical stages of stone formation. As the concentration increases, the inhibitory effect also increased.

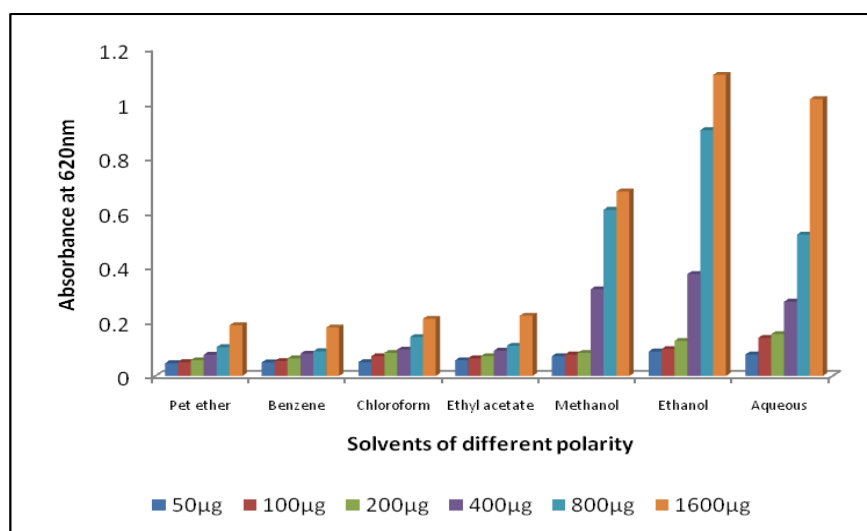
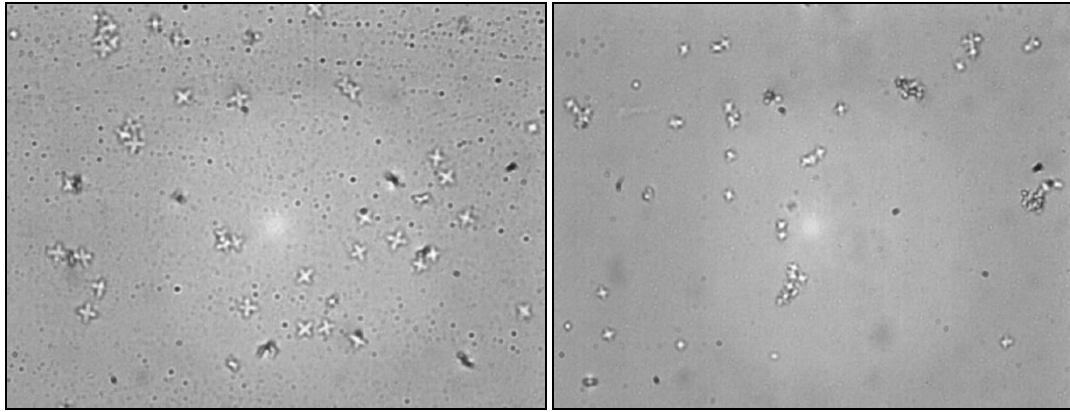


Fig. 2: Effect of banana cultivar Monthan corm extracts on nucleation of CaOx.



a) Without extract

b) With extract

Fig. 3: Effect of banana cultivar Monthan corm extracts on nucleation of CaOx. (Light microscopy, 400x)

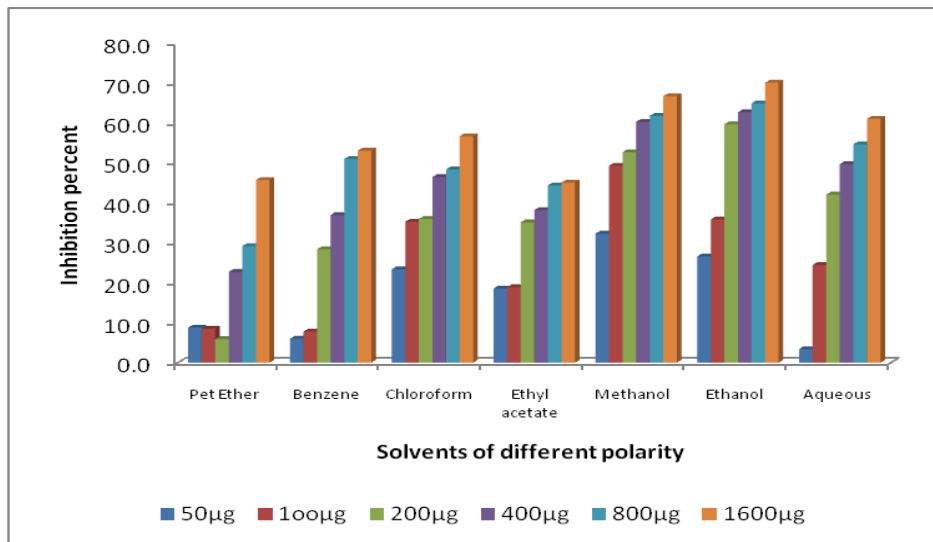
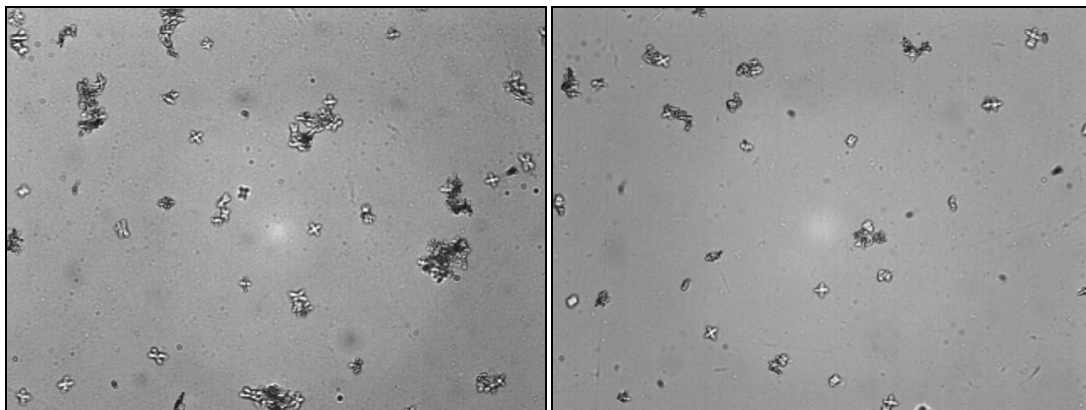


Fig. 4: Effect of banana cultivar Monthan corm extracts on CaOx crystal growth.



a) Without extract

b) With extract

Fig. 5: Effect of banana cultivar Monthan corm extracts on CaOx crystal growth. (Light microscopy, 400x)

Stone crystals bind to one another through a process known as aggregation or agglomeration promoted by strong chemicals and electrical forces. Adhered crystals were held in place and cannot be easily separated and this plays an important role in lithiasis. The inhibitory potential of the different solvents of increasing polarity as given in Figure 6 and 7 revealed that all the extracts exhibited inhibitory factor to a moderate level. Among them ethanol and

methanol extract rendered good prevention against other extracts. The number and morphology of CaOx crystal when observed under light microscope (400X magnification) showed ethanol extract at its higher concentration (1600µg/ml) showed a greater potential towards crystal growth inhibition. Nirmaladevi *et al* [18] reported that crystal aggregation was inhibited by addition of aqueous extract of *Hibiscus rosa-sinensis*.

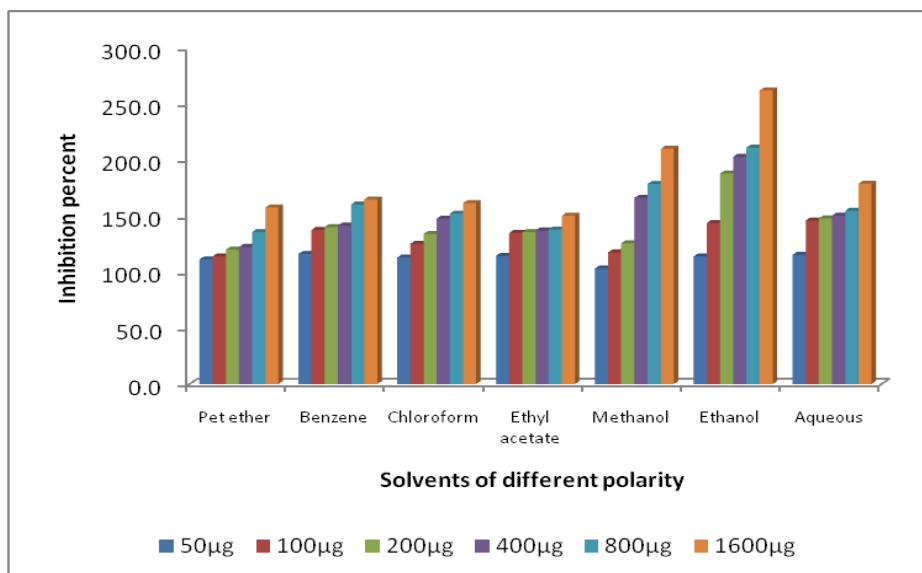


Fig. 6: Effect of banana cultivar Monthan corm extracts on CaOx crystal aggregation.

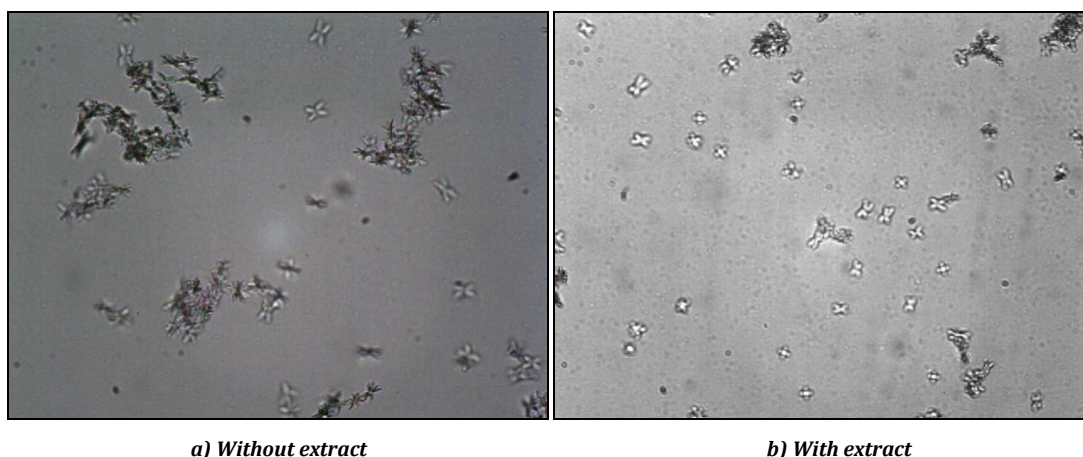


Fig. 7: Effect of banana cultivar Monthan corm extracts on CaOx crystal aggregation. (Light microscopy, 400x)

It is also necessary to compare the effect of aqueous extract with that of the other extracts, since all the parts of banana plant being used in day to day life for various treatments and remedies. In this study, aqueous extract of corm juice was found dissolve crystal nucleation next to ethanol extract, whereas in crystal growth and aggregation it is third best when compared to ethanol and methanol extracts. Further studies need to be conducted in order to understand the regular intake of corm juice in diet may completely eliminate the urinary calculi formation.

CONCLUSION

The results clearly indicate that under *in vitro* conditions, the crystal nucleation, growth and aggregation, was found to express a concentration dependent inhibition. The results when compared among the other non-polar solvents, in all the assays, the ethanol corm extract of banana cultivar Monthan, inhibited crystal nucleation, growth and aggregation better. Critical condition of lithiasis can be treated using polar organic solvent such as ethanol which could render maximum protection thereby preventing injury/damage to kidney by the crystals.

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