Arab Journal of Urology

ISSN: (Print) 2090-598X (Online) Journal homepage: https://www.tandfonline.com/loi/taju20

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To cite this article: Sarmistha Saha & Ramtej J. Verma (2013) Inhibition of calcium oxalate crystallisation *in vitro* by an extract of *Bergenia ciliata*, Arab Journal of Urology, 11:2, 187-192, DOI: 10.1016/j.aju.2013.04.001

To link to this article: https://doi.org/10.1016/j.aju.2013.04.001

Arab Journal of Urology (2013) **11**, 187–192

Arab Journal of Urology
(Official Journal of the Arab Association of Urology)

www.sciencedirect.com

UROSCIENCE

ORIGINAL ARTICLE

Inhibition of calcium oxalate crystallisation *in vitro* by an extract of *Bergenia ciliata*

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Received 8 February 2013, Received in revised form 5 April 2013, Accepted 7 April 2013
Available online 9 May 2013

KEYWORDS

Calcium oxalate;
Bergenia ciliata;
Anti-crystallisation;
Nucleation;
Aggregation

ABBREVIATIONS

CaOx, calcium oxalate;
COM, CaOx monohydrate;
COD, CaOx dihydrate;
IC_{50}, concentration for 50% inhibition;

Abstract ***Objective:*** To evaluate the effectiveness of an extract obtained from the rhizomes of *Bergenia ciliata* (Saxifragaceae) on the inhibition of calcium oxalate (CaOx) crystallisation *in vitro*.

Materials and methods: A hydro-alcoholic extract (30:70, v/v) of rhizomes of *B. ciliata* was prepared at different concentrations (1–10 mg/mL). The crystallisation of CaOx monohydrate (COM) was induced in a synthetic urine system. The nucleation and aggregation of COM crystals were measured using spectrophotometric methods. The rates of nucleation and aggregation were evaluated by comparing the slope of the turbidity of a control system with that of one exposed to the extract. The results were compared with a parallel study conducted with a marketed polyherbal combination, Cystone, under identical concentrations. Crystals generated in the urine were also analysed by light microscopy. Statistical differences and percentage inhibitions were calculated and assessed.

Results: The extract of *B. ciliata* was significantly more effective in inhibiting the nucleation and aggregation of COM crystals in a dose-dependent manner

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Peer review under responsibility of Arab Association of Urology.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.aju.2013.04.001

OD, optical density

than was Cystone. Moreover, the extract induced more CaOx dihydrate crystals, with a significant reduction in the number and size of COM crystals.

Conclusion: An extract of the traditional herb *B. ciliata* has an excellent inhibitory activity on crystalluria and therefore might be beneficial in dissolving urinary stones. However, further study in animal models of urolithiasis is needed to evaluate its potential anti-urolithiatic activity.

Introduction

The incidence of urinary calculi is increasing worldwide and calcium oxalate (CaOx, CaC_2O_4) is the predominant component of most stones, followed by struvite, cystine, uric acid and other compounds [1]. The overall probability of forming stones differs in various parts of the world, and is estimated at 1–5% in Asia, 5–9% in Europe, and 13% in North America. The recurrence rate of renal stones is ≈ 75% in a 20-year span [2]. CaOx stones are found as two different types, the monohydrate (COM) or Whewellite, and the dihydrate (COD) or Weddellite. COM, the thermodynamically most stable form, more common in clinical stones than COD, has a greater affinity for renal tubular cells, and is thus responsible for the formation of stones in the kidney [3].

The crystallisation of the CaOx begins with increased urinary supersaturation, with the subsequent formation of the solid crystalline particles within the urinary tract. This is followed by nucleation, by which stone-forming salts in supersaturated urinary solution coalesce into clusters, that then increase in size by the addition of new constituents [4]. These crystals then grow and aggregate with other crystals in solution, and are ultimately retained and accumulated in the kidney [5]. Renal injury promotes crystal retention and the development of a stone nidus on the renal papillary surface, and further supports crystal nucleation at lower supersaturation levels [6]. Therefore, levels of urinary supersaturation correlate with the type of stone formed, and reducing supersaturation is effective in preventing stone recurrence [7]. Therefore, if this progression of crystallisation can be prevented, then lithiasis could also be prevented.

It is well known that glycosaminoglycans [8], citrate, magnesium and orthophosphate [9] are strong inhibitors of CaOx crystallisation, but these macromolecules are not expected to increase in the urine because of their high molecular weight, and the clinical use of these inhibitors has been limited. Many studies showed the effect of various additives on the *in vitro* inhibition of CaOx crystallisation, such as metallic ions and their complexes [10], sodium dodecyl sulphate [11], α-ketoglutaric acid (a normal physiological constituent of urine) [12], maleic acid copolymers [13] and a protein from human kidney [14].

The modern techniques available in the management of urinary calculi depend on the size and location of calculi, the degree of obstruction, kidney function and associated functions [15]. Open surgical procedures for treating urinary stones are currently not used and were replaced by modern techniques, including extracorporeal lithotripsy or ureteroscopy. Despite the availability of these minimally invasive techniques, with dietary modifications the recurrence rate is still expected to be nearly 50% [16]. Therefore, alternative or complementary medicines with minimal side-effects might be useful. Traditional herbal medicines provide many opportunities for the development of potential therapeutic drugs, in the form of either extracts alone, in combination with other herbs, or in the form of phytochemical compounds isolated from them.

There are several reports related to anti-crystallisation compounds extracted from medicinal plants, and *Bergenia ciliata* (Saxifragaceae) is among those that have been advocated as traditional medicines for kidney stones. Several plant-derived drugs have been used in India and elsewhere which claim to dissolve urinary calculi *in vitro* [17]. An extract from the herb *Herniaria hirsuta L.,* a plant that traditionally is used in Morocco for treating lithiasis, promoted the nucleation of CaOx crystals, increasing their number but decreasing their size [18]. Another medicinal herb, *Tribulus terrestris* extract, induced a concentration-dependent inhibition of nucleation and the growth of CaOx crystals [19].

B. ciliata, commonly known as Paashaanbhed in the Indian Systems of Medicine, is used as a tonic for treating fevers, pulmonary infections, and hypoglycaemia, and has anti-inflammatory, antioxidant and antifungal properties [20]. The major chemical constituents reported from *B. ciliata* are gallic acid, bergenin (+)-afzelechin, 11-O-galloyl bergenin, paashaanolactone, β-sitosterol and β-Sitosterol-d-glucoside [21,22].

In a recent study [23], a phenolic compound isolated from the leaves of *B. ciliata* was effective in dissolving CaOx and calcium phosphate urinary stones. In a previous study [24], the administration of a 70% methanolic extract from the rhizomes of *B. ciliata* had a significant protective effect on the histopathological changes in an animal model of hyperoxaluria induced by ethylene glycol.

Cystone is a marketed composite herbal formulation specifically developed for managing urolithiasis or renal calculi. This formulation has been approved by regula-

tory authorities in India as an Ayurvedic formulation, and has been available in clinical practice for the past 60 years for treating urinary calculi [25].

Therefore, in the present study, we investigated the effectiveness of an extract of *B. ciliata* on CaOx crystallisation *in vitro* and compared it with a control and the marketed polyherbal drug Cystone.

Materials and methods

The plant material was collected in September 2011 and was taxonomically identified and authenticated as rhizomes of *B. ciliata* by Dr. Yogesh T. Jasrai, Department of Botany, Gujarat University, India. The vouched specimens were deposited in the herbarium for future reference.

A hydro-methanolic extract of the rhizomes was prepared to extract both the polar and non-polar active components present in the rhizomes. The plant material was thoroughly cleaned, dried under shade, coarsely powdered, and the extract prepared. Briefly, 5 g of powder was soaked with 100 mL of methanol:water (70:30, v/v) at room temperature and then filtered twice through Whatman filter paper No. 1. After evaporating the solvent, the crude extract was dried under vacuum and stored in an air-tight container at 4 °C. The dried extract was dissolved in ultrafiltered water and used for further study. In a pilot study, we found that the inhibition of nucleation and aggregation by the extract was not statistically significant at 100, 200, 400 and 500 µg/mL. However, the extract showed significant inhibition at >800 µg/mL, and thus we used concentrations up to 10 mg/mL in the present study. The hydro-alcoholic extract and Cystone were dissolved in distilled water to give concentrations of 1, 2, 5, 7.5 and 10 mg/mL for both the nucleation and aggregation assays.

The synthetic urine was prepared according to the method of Burns and Finlayson [26], prepared freshly each day and the pH adjusted to 6.0.

Nucleation assay

The inhibitory activity of the extracts on the nucleation of CaOx crystals was determined by a spectrophotometric assay [27]. Crystallisation was initiated by adding calcium chloride (4 mmol/L) and sodium oxalate (50 mmol/L) solutions to artificial urine, both prepared in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 and 37 °C. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus became optically detectable) in the presence of the extract and that of the control with no extract. The absorbance (optical density, OD) was recorded at 620 nm, and the percentage inhibition calculated as (OD (experimental)/OD (control))/100.

Aggregation assay

The rate of aggregation of the CaOx crystals was determined by the method of Hess et al. [28] with slight modifications. The COM crystals were prepared by mixing both the solutions of calcium chloride and sodium oxalate at 50 mmol/L. Both solutions were then equilibrated in a bath for 1 h at 60 °C. The solutions were then cooled to 37 °C and then evaporated. The COM crystals were then dissolved with Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 to a final concentration of 1 mg/mL. The absorbance at 620 nm was recorded at 30, 60, 90, 180 and 360 min. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract with that obtained in the control. The percentage inhibition was calculated as $(1-S_i/S_c)/100$, where S_i is the slope of the plot in the presence of inhibitor (extract) and S_c the slope of the control plot (with no inhibitor).

The results were expressed as the mean (SEM), and analysed statistically using linear regression, one-way anova followed by the Tukey's multiple comparison test, with $P < 0.05$ taken to indicate significance. The 50% inhibitory concentrations (IC_{50}) were calculated by probit analysis and assessed using the chi-squared test.

Results

The *in vitro* inhibitory effect of extracts of *B. ciliata* on various phases of CaOx crystallisation was determined by the time course of turbidity measured in synthetic urine at extract concentrations of 1, 2, 5, 7.5 and 10 mg/mL. The light micrographs at 6 h in the control system showed the formation of both types of CaOx crystals, as bi-concave, oval and dumb-bell shaped COM and octahedral COD, with significant aggregations (Fig. 1). The arrows in Fig. 1 show both types of crystals, but *B. ciliata* extract at the highest concentration (10 mg/mL) inhibited crystal formation, with no COM crystals and a few COD crystals, as shown by the arrows (Fig. 2). Moreover, there were fewer COD crystals with

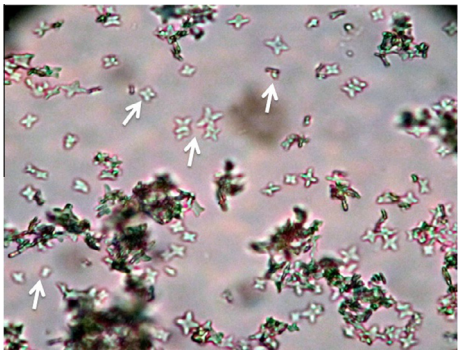

Figure 1 CaOx crystallisation induced in the control (light microscopy × 100).

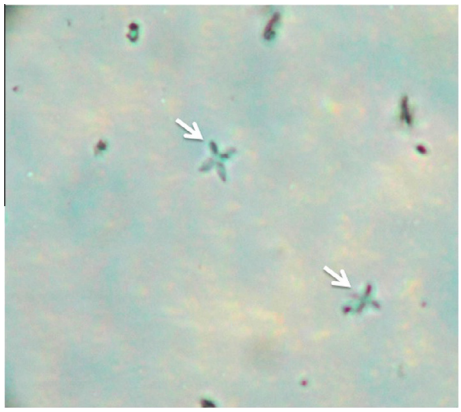

Figure 2 *B. ciliata* (10 mg/mL) inhibited the crystallisation of CaOx (light microscopy × 100).

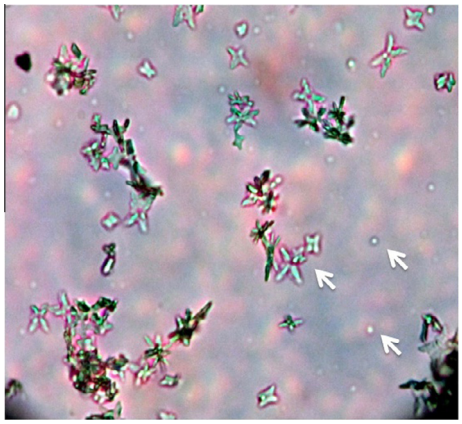

Figure 3 Inhibition of CaOx crystallisation by Cystone (10 mg/mL) (light microscopy × 100).

the extract. Cystone was less effective than the extract, with numerous COM and COD crystals when used at the same concentrations (Fig. 3), with the arrows showing a moderate number of COD crystals and a few COM crystals. Thus, the highest concentration of the *B. ciliate* extract was associated with only COD crystals.

The extract of *B. ciliata* at various concentrations also had a significant inhibitory effect on nucleation and aggregation, compared with Cystone. In the nucleation assay the number of crystals formed was estimated as the turbidity of the solution. The absorbance of the control was subtracted from that obtained with the extract. There was a steep decrease in the absorbance with increasing concentration of the extract (Fig. 4). The percentage inhibition of the extract was 32–92%, whereas with cystone it was 17–62% (Fig. 5A and B, Table 1). The dose-dependent increase in percentage inhibition of nucleation by the extract had a coefficient of regression (r^2) of 0.965. The IC_{50} of the plant extract was 2.3 mg/mL, compared with 8.1 mg/mL for Cystone.

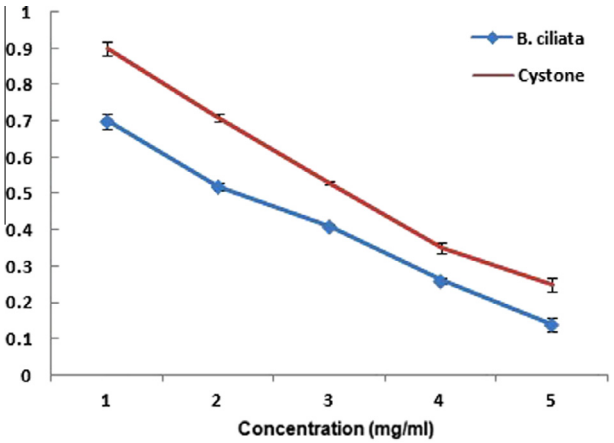

Figure 4 The plot of turbidity as influenced by *B. ciliata* extract and Cystone at various concentrations.

Similarly, in the aggregation assay, *B. ciliata* extract showed a significant dose-dependent inhibition of the aggregation, with percentage inhibitions of 58–97% ($P < 0.001$, Fig. 6A). The IC_{50} of the plant extract was 0.9 mg/mL. However, Cystone also showed inhibitory activity on crystal aggregation, but was less potent than the plant extract at the same concentration range (Fig. 6B), with a percentage inhibition of 35–71% and an IC_{50} of 2.92 mg/mL, and in a concentration-dependent manner. The r^2 was > 0.9 ($P < 0.01$) for all plots (Figs. 5 and 6).

Discussion

We used a classical model of synthetic urine supersaturated with calcium chloride and sodium oxalate to determine the growth and aggregation of CaOx crystals. The normal urine in the human is not a static solution, as new solutes are constantly being added and subtracted from the solution. However, it is difficult to mimic the urinary tract *in vitro*, but the growth of crystals in synthetic urine in a static environment can be useful to some extent for explaining the growth of urinary calculi in humans.

The extract of *B. ciliata* promoted the formation of COD crystals rather than COM crystals. The extract might contain some phytochemicals that inhibit the growth of COM crystals. COD crystals are less tightly bound to the epithelial cell surfaces than COM crystals, and therefore cause less tubular injury [29]. A similar inhibition of COM stones was also reported for *Tamarindus indica* pulp [30]. The *B. ciliate* extract also contains some substances that inhibit the aggregation of COM crystals. Crystal aggregation is the most critical step, as it occurs very rapidly and has a considerable effect on particle size, and aggregated crystals are commonly found in urine and renal stones [31].

B. ciliata has several polyphenolic constituents, e.g., alkaloids, flavonoids, saponins, terpenoids, and tannins

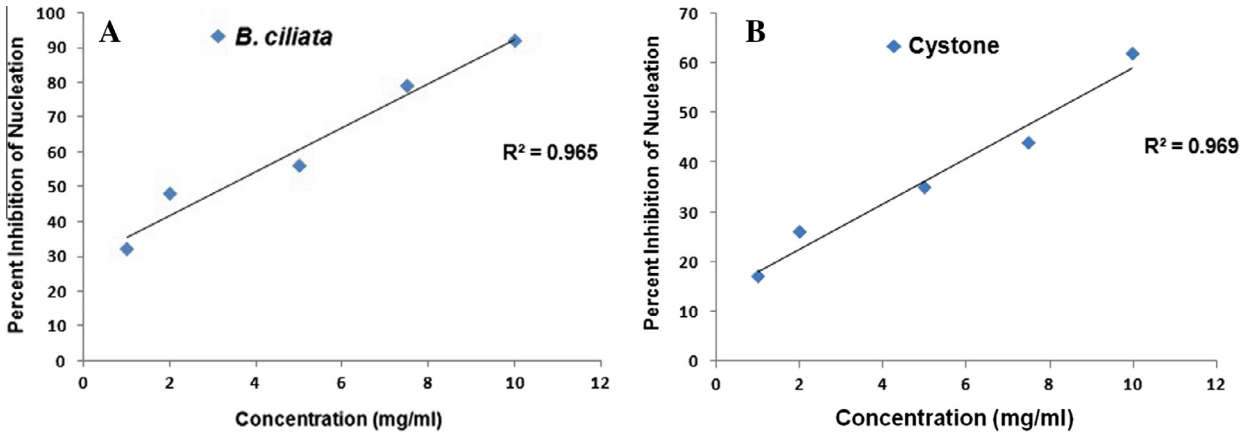

Figure 5 The effect of (A) *B. ciliata* extract, and (B) Cystone, on the nucleation assay. Values are the mean (±SEM) and were significantly different from the control, as well as within the groups, at $P < 0.001$.

Table 1 The effect of *B. ciliata* and Cystone on the nucleation and aggregation of COM crystallisation.

Variable and concentration (mg/mL)	Mean (SEM)% inhibition	
	Cystone	*B. ciliata*
Nucleation		
1	17 (1.1)	32 (1.3)[a,#]
2	26 (1.2)	48 (0.8)[a,#]
5	35 (1.4)	56 (0.5)[a,#]
7.5	44 (1.5)	79 (1.4)[a,#]
10	62 (1.7)	92 (1.2)[a,#]
Aggregation		
1	35 (1.2)	58 (1.1)[a,*]
2	42 (1.7)	62 (1.4)[a,*]
5	52 (1.4)	73 (1.7)[a,*]
7.5	65 (0.9)	85 (1.1)[a,*]
10	77 (1.1)	97 (1.4)[a,*]

Results are expressed as mean ± SEM.
Level of significance [†]$P < 0.05$; [#]$P < 0.01$; [*]$P < 0.001$.
[a] as compared with cystone group between the same dose levels.

[32]. Saponins are known to have anti-crystallisation properties by disaggregating the suspension of mucoproteins, the promoters of crystallisation [33]. A saponin-rich fraction of *Herniaria hirsuta* was also found to be a potent inhibitor of CaOx stone formation in an animal model, and of CaOx crystal formation *in vitro* [34]. However, the contribution of other phytochemicals on the reported activities cannot be excluded.

The present results indicated a lower IC_{50} for the plant extract than for Cystone, and we conclude that the extract from rhizomes of *B. ciliata* was more potent than Cystone, which contradicts a previous study showing that Cystone was more potent than a phytochemical compound isolated from an ethanolic extract of the leaves of *B. ciliata* [23]. This might be because the rhizomes contain more polyphenolic compounds like gallic acid and bergenin than do the leaves [22]. Moreover, a hydro-alcoholic extract contains both polar and non-polar phytochemical compounds, and thus includes major polyphenolic compounds like tannins and saponins. Microscopy showed that the extract reduced the number of crystals and could be used to differentiate COM and COD crystals. This suggests that phytochemicals from the plant exert their action

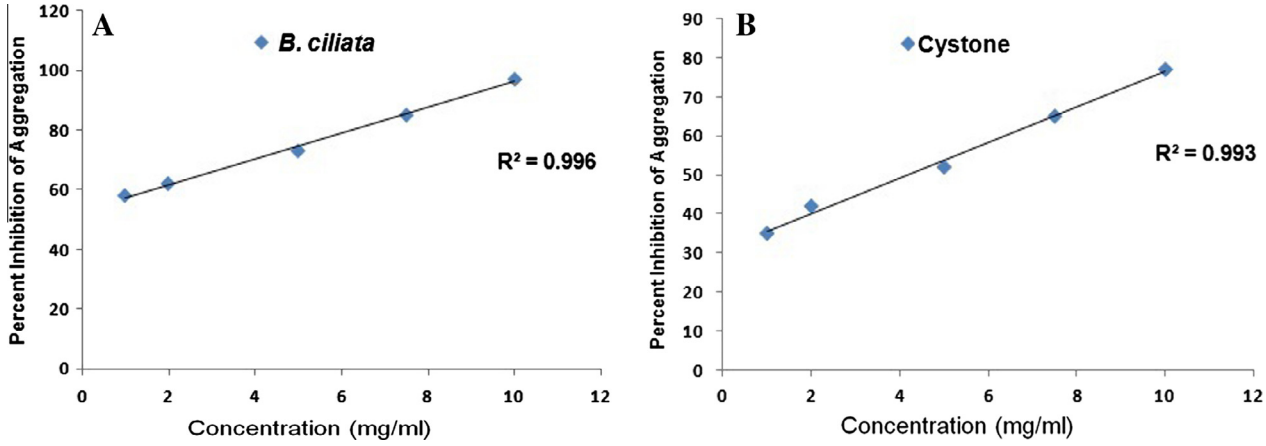

Figure 6 The effect of (A) *B. ciliata* extract, and (B) Cystone, on the aggregation assay. Values are the mean (±SEM) and were significantly different from the control, as well as within the groups, at $P < 0.001$.

directly on the crystals. Moreover, the appearance of more COD crystals is highly beneficial, as COM crystals have a higher affinity for adhering to the renal epithelial cells than do COD crystals [35].

In conclusion, the plant extract of B. ciliata can inhibit the nucleation and aggregation of CaOx crystallisation *in vitro*. However, the mechanism of action of the extract in animal models of lithiasis needs to be investigated. As the observed activity of the plant extract might be due to other phytochemicals present in it, further characterisation and isolation of the major active components from the plant extract are required.

Conflict of interest

None.

Acknowledgements and funding

The financial assistance to Sarmistha Saha from Lady Tata Memorial Trust (LTMT/AD/Q2/2011–2012) in the form of a Senior Research Fellowship, is acknowledged with thanks.

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