

Effect of *Tridax procumbens* extract on calcium oxalate crystallization under *in vitro* conditions

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ABSTRACT

*Kidney stones are from dietary minerals in the urine and there is no suitable drug to use in clinical therapy. Traditionally it is known that *Tridax procumbens* leaf juice possess antilithiatic property. Calcium oxalate (CaOx) crystal nucleation was significantly inhibited by the presence of *Tridax procumbens* extract, when compared to control. The effect of leaf extract on crystal growth assay performed showed a concentration dependent trend. The result of crystal aggregation assay reiterates the fact that the plant extract could readily inhibit crystal formation, which clearly indicates the antilithiatic activity of the plant *Tridax procumbens*.*

Keywords: Lithiasis, kidney stone, male Wistar rats, nucleation, crystal growth, aggregation.

INTRODUCTION

A kidney stone, also known as a renal calculus or nephrolithiasis, is a solid concretion or crystal aggregation formed in the kidneys from dietary minerals in the urine¹. Each year, people make almost 3 million visits to health care providers and more than half a million people go to emergency rooms for kidney stone problems². Various etiological factors have been attributed to stone formation that includes hereditary, dietary, geographical and infective diseases. The recurrence rate is high, being more than 50 percent.

The most common type of kidney stone is calcium containing stones, represent about 80% of all cases, these typically contain calcium oxalate (CaOx) either alone or in combination with calcium phosphate. Calcium oxalate stones are of two forms, calcium oxalate monohydrate (COM) or calcium oxalate dihydrate (COD)³. COM is thermodynamically more stable form, is observed frequently in clinical stones than COD and it has greater affinity for renal tubular cells, thus responsible for the formation of stones in kidney⁴. Human possess mechanisms that biologically controls as well as prevent COM crystallization in urine by inducing inhibitors that decrease nucleation, growth and aggregation of COM crystals⁵.

Herbal drugs have created interest among the people because of its clinically proven effects like immunomodulation, adaptogenic and antimutagenic. The excess intake of synthetic drugs results in higher incidence of adverse drug reactions which has motivated humans to return to nature for safe remedies⁶.

Tridax procumbens is a plant belonging to the Asteraceae family, which have worldwide distribution. Local people known it as “Ghamara” (Hindi), Gaddi Chemanthi (Telugu), Thata poo (Tamil), in English popularly called ‘coat buttons’ and is dispensed for “Bhringraj” by some of the practitioners of Ayurveda. The phytochemical screening of

T. procumbens revealed the presence of alkaloids, saponins, flavonoids (catechins and flavones), fumaric acid, carotenoids and tannins. It is rich in carotenoids, saponins, oleic acid and ions sodium, potassium and calcium. Its flower reported to be rich in Luteolin, glucoluteolin, quercetin and isoquercetin. It is known for its number of pharmacological activities like anti-inflammatory, hepatoprotective activity, wound-healing, antidiabetic activity, immunomodulating property, dysentery, diarrhoea and promotes the growth of hair, and as well as antimicrobial activity against both gram-positive and gram-negative bacteria. Its leaf juice has antiseptic, insecticidal and parasiticidal properties, as a remedy against conjunctivitis. Traditionally, used to check haemorrhage from cuts, bruises and wounds, also as insect repellent too. It is also used as bioadsorbent for chromium (VI)⁷.

The present investigation aimed to better understand the role of *T. procumbens* in urinary stone formation. We evaluated the effect of series of extracts (solvents with different polarity) of this plant (leaves) on CaOx crystallization induced *in vitro*.

MATERIALS AND METHODS

Preparation of *T. procumbens* extracts

The leaves of *Tridax procumbens* were collected fresh from our university campus, cleaned and shade dried. The dried leaves were powdered and passed through the coarse sieve (0.2mm).

About 10g of the powdered sample was taken in a thimble and placed in a Soxhlet apparatus and was extracted by hot percolation method using 200ml of solvents with increasing polarity namely petroleum ether, benzene, chloroform, ethyl acetate, ethanol, methanol and aqueous. The extraction was carried out until the plant materials become colourless. The extract was collected and evaporated in a boiling water bath at 60°C. The residue was kept in a sterile, air tight container and stored in a refrigerator.

The various concentrations of the plant sample tested for their inhibitory potency were 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, 800 µg/mL, 1600 µg/mL and 3200 µg/mL which were prepared at the time of experiment along with a control (no extract).

In vitro Calcium Oxalate Assay

The physicochemical theory of stone formation cogitates urine as a supersaturated solution in which homogenous or heterogeneous nucleation can lead to initiation of crystal formation, which can then aggregates and grow.

Nucleation Assay

The method used was similar to that described by Hennequin *et al*⁸ 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, 950µl of calcium chloride and varying concentration of plant extract were pipetted out against a reagent blank (without extract). To this added 950µl of sodium oxalate and shook well at least for 2-3minutes. The absorbance was measured at 620nm.

Growth assay

The percent inhibition of CaOx crystal growth that was induced *in vitro* was evaluated in the presence of *T. procumbens* extract by the procedure described by Chaudary *et al*.⁹ 4mM calcium chloride and 4mM sodium oxalate of 1ml each were added to a 1.5ml of solution containing NaCl (10mM) buffered with Tris (10mM) at pH 7.2. To this 30µl 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. When plant extract was added into this solution, depletion of free oxalate ions will decrease if the extract inhibits calcium oxalate crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30 seconds incubation with or without the 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 *Tridax procumbens* extract.

Aggregation Assay

The CaOx crystal aggregation inhibition in presence of the extract of *T. procumbens* was determined by the method of Hess *et al.*¹⁰. 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 analyzed by the analysis of variance (one-way ANOVA) to estimate the difference between values of extracts tested.

RESULTS AND DISCUSSION

The various extracts were subjected to nucleation assay to identify the best suited solvent for extraction of *T. procumbens* for treating lithiasis. Among the various solvents tested, the ethylacetate extract was found to be more effective, even with the lowest concentration tested. This was followed by ethanol, methanol, aqueous, chloroform, benzene and petroleum ether extracts.

Inhibition of CaOx crystal nucleation by *Tridax procumbens*

Figure 1. Depicts the effect of different concentration of the ethylacetate extract of *T. procumbens* on the nucleation of calcium oxalate crystals. The percent inhibition with regard to the control (without extract) was found to be in the range of 52-221%. The percentage inhibition was constantly increased with increase in concentration of *T. procumbens*. The inhibitory activity was found to be more prominent at higher concentration of the plant extract. The results were in agreement with Atmani and Khan¹¹, in which the extract of *Hernaria hirsute* was found to possess antilithogenic effect. Plate 1. Exhibits the results of nucleation assay, with and without the extract.

Figure 1. Effect of *Tridax procumbens* ethyl acetate extract on nucleation of CaOx

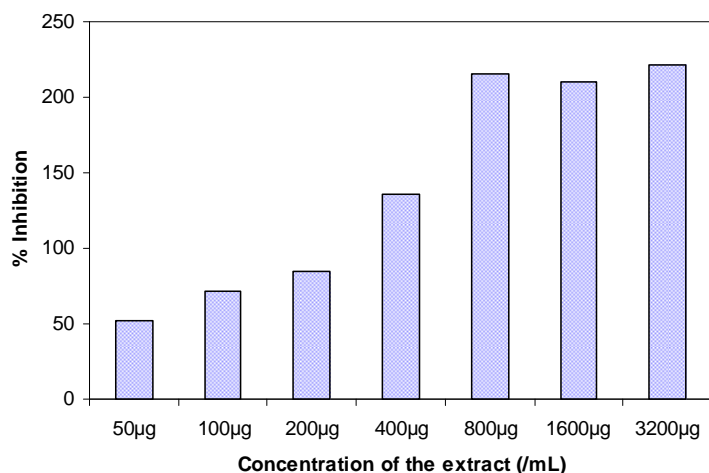
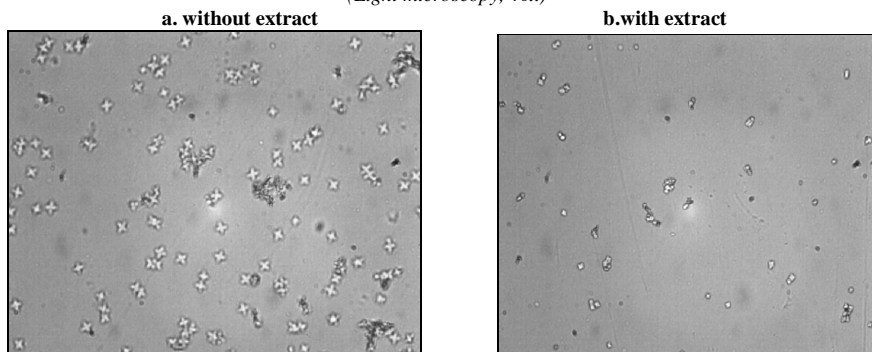


Plate 1. Effect of *Tridax procumbens* on CaOx crystal nucleation
(Light microscopy, 40x)



Inhibition of CaOx crystal growth by *Tridax procumbens*

Figure 2. demonstrates the percentage inhibition shown by *T. procumbens* on the calcium oxalate crystal growth. The ethylacetate extract of *T. procumbens* showed inhibition in a concentration dependent manner. The results of the present study were in agreement with Barros *et al*¹² and Beghalia *et al*¹³ with the extracts of *Phyllanthus niruri* and Algerian medicinal plant respectively. Plate 2. Shows the light microscopic view of CaOx crystal growth in presence and absence of the plant extract.

Figure 2. Effect of *Tridax procumbens* ethyl acetate extract on CaOx crystal growth

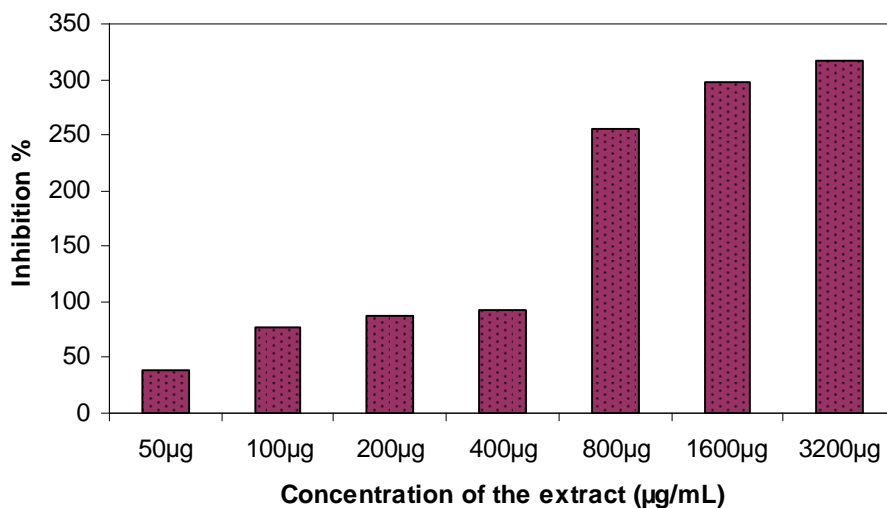
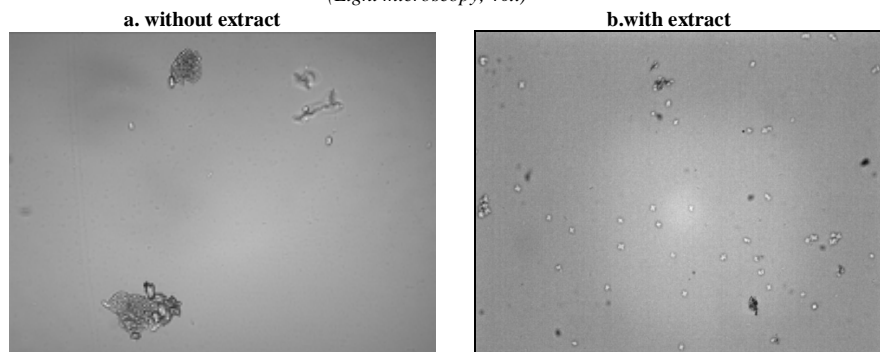


Plate 2. Effect of *Tridax procumbens* on CaOx crystal growth
(Light microscopy, 40x)



Decrease in crystal size and transformed COM to COD crystals, which are less likely to attach with the kidney epithelial cells than COM crystals. Surface structures of COM and COD differ in their affinities for cell membrane. COM has a higher affinity for renal tubule cells and for cell membranes than COD. A preferential adsorption to cell membranes of COM crystals induces kidney stones, whereas COD prevents kidney stones because it is easily excreted in the urine¹⁴.

Inhibition of CaOx crystal aggregation by *Tridax procumbens*

Aggregation is the clumping of small particles in to a large one. The inhibitory potential of the extract was tested with varying concentrations in the aggregated CaOx crystal. The crystal aggregation in presence of plant extract was presented in Figure 3, which showed an increase in inhibition with the increase in concentration of the extract. The inhibition percent was found to be in range of 64-152%. A drastic increase in inhibition was observed in the extract concentration of 800 μ g/mL, 1600 μ g/mL and 3200 μ g/mL (113, 127 and 152% respectively) when compared to 50 μ g/mL, 100 μ g/mL, 200 μ g/mL and 400 μ g/mL (64, 72, 81 and 94% respectively). Barros et al (2003) reported that in vitro studies using the plant extract of *Phyllanthus niruri* reduces CaOx crystal aggregation. Patel et al (2010) revealed that the alcoholic extract of the plant *Pedaliium murex* causes inhibition of crystal formation and aggregation as compared to its aqueous extract.

Figure 3. Effect of *Tridax procumbens* ethyl acetate extract on CaOx aggregation

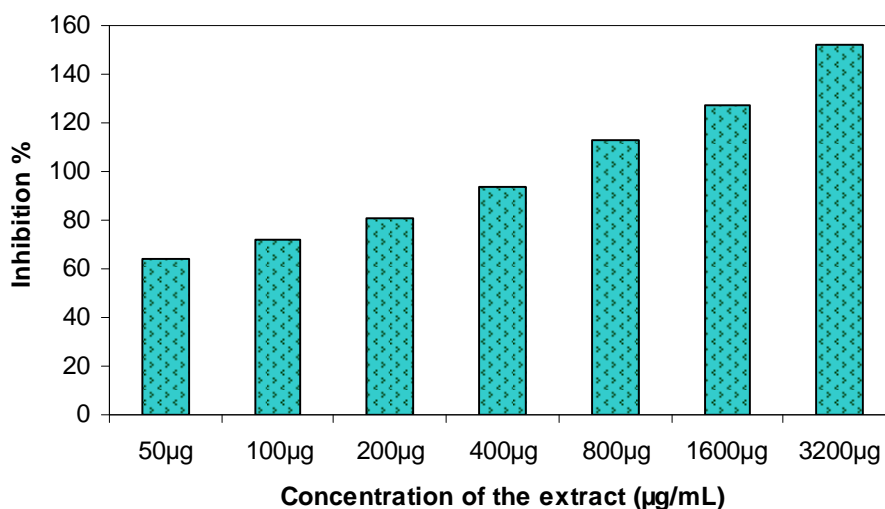
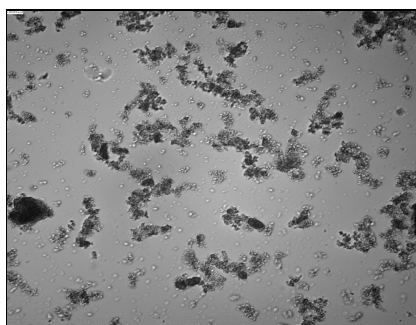


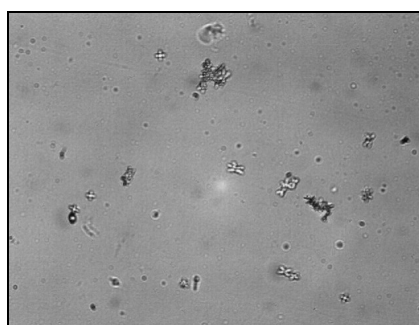
Plate 3. Effect of *Tridax procumbens* on CaOx crystal aggregation

(Light microscopy, 40x)

a. without extract



b. with extract



CONCLUSION

In the present study, in presence of plant extracts, the length and width of the crystals were reduced. The average length of the crystal grown in the presence of the inhibitors (extracts) was less than that of the control sample. It was

found that extracts used in this study inhibited potentially the nucleation, growth and aggregation phases of stone formation.

REFERENCES

- [1] Zaidi SMA, Jamil SS, Singh K, Asif M: Clinical evaluation of herbo-mineral Unani formulation in urolithiasis **2006**; Amala Ayurvedic Hospital Centre, 42.
- [2] Sailaja B, Bharathi K and Prasad K: *Pharmanest- An International Journal of Advances in Pharmaceutical Sciences* **2011**; 2 (1): 9-14.
- [3] Verkoelen CF, Romijn JC, de Bruijn, WC, Boever ER, Cao LC and Schroder FH: *Kidney International* **1995**; 48:129-138.
- [4] Prasad KV, Sujatha D and Bharati K: *Journal of Pharmacy Research* **2008**; 1: 175-179.
- [5] Oehlschlager S, Fuessel S, Meye A, Herrmann J, Froehner M, Albrechet S and Wirth MP: *Urology* **2009**; 73:480-483.
- [6] Tombolini P, Ruoppolo M, Bellorofonte C, Zatar C and Follini M: *Journal of Nephrology* **2000**, 13:71.
- [7] Sneha M and Ruchi S: *International Journal of PharmTech Research* **2010**; 2 (2): 1391-1394.
- [8] Hennequin C, Lalane V, Daudon M, Lacour B and Druke T: *Urological Research* **1993**; 21:101-108.
- [9] Chaudhary A, Tandon C and Singla SK, *Indian Journal of Urology* **2009**; 24:105-111.
- [10] Hess A: *World Journal of Urology* **1989**; 23: 302–303.
- [11] Atmani F and Khan SR: *BJU International* **2000**; 85:621-625.
- [12] Barros ME, Schor N and Boim MA: *Urological Research* **2003**; 30:374-379.
- [13] Beghalia M, Ghalem S, Allali H, Belouatek A and Marouf A: *Journal of Medicinal Plant Research* **2008**; 2: 66-70.
- [14] Pachana K, Wattanakornsire A and Nanuam J: *Science Asia* **2009**; 36:165-168.