

Reduction of oxalate-induced renal tubular epithelial (NRK-52E) cell injury and inhibition of calcium oxalate crystallisation *in vitro* by aqueous extract of *Achyranthes aspera*

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Despite considerable progress in medical therapy, there is no satisfactory drug to treat kidney stones. Therefore, this study is aimed to look for an alternative treatment by using *Achyranthes aspera*. Here, the inhibitory potency of *A. aspera* was investigated on nucleation and the growth of the calcium oxalate (CaOx) crystals as well as on oxalate-induced cell injury of NRK 52E renal epithelial cells *in vitro*. Data are expressed as mean values of three independent experiments (each in triplicate) and analysed by the analysis of variance ($P < 0.05$) to estimate the differences between values of extracts tested. *A. aspera* extract exhibited a concentration-dependent inhibition of the growth of CaOx crystals but a similar pattern of inhibition was not observed with increase in the plant extract concentration for the nucleation assay. When NRK 52E cells were injured by exposure to oxalate for 72 hours, *A. aspera* extract prevented the injury in a dose-dependent manner. On treatment with the different concentrations of the plant, the cell viability increased and the lactate dehydrogenase (LDH) release decreased in a concentration-dependent manner. These studies indicate that *A. aspera* extract besides having a cytoprotective role also has a potential to inhibit both nucleation and the growth of the CaOx crystals and can prove to be a potent candidate for phytotherapy against urolithiasis.

Key words: *Achyranthes aspera*, calcium oxalate, NRK 52E, phytotherapy, urolithiasis

INTRODUCTION

Nephrolithiasis is common, affecting up to 10% of the population at some point during their life.^[1] Calcium-containing stones are the most commonly occurring ones to an extent of 75–90%, followed by magnesium ammonium phosphate (Struvite) to an extent of 10–15%, uric acid 3–10% and cystine 0.5–1%.^[2] Calcium oxalate (CaOx) stones are found in two different varieties, calcium oxalate monohydrate (COM) or Whewellite, and calcium oxalate dihydrate (COD) or Weddellite. COM, the thermodynamically most stable form, is observed more frequently in clinical stones than COD and it has a greater affinity for renal tubular cells, thus being responsible for the formation of stones in kidney.^[3]

Various workers have suggested the role of crystal-induced cell injury in the development of kidney stones by providing the sites for crystal attachment and retention within the kidneys.^[4,5]

Oxalate, a metabolic end product and a major constituent of the majority of renal stones, has been shown to be toxic to renal epithelial cells of cortical origin.^[6] It has been seen that exposure of renal epithelial cells to oxalate

which is a constituent of most kidney stones leads to a disruption of the normal activities of the renal epithelial cells such as altered membrane surface properties and cellular lipids, changes in gene expression, disruption of mitochondrial function, formation of reactive oxygen species and decreased cell viability.^[7]

Various mechanisms have been proposed to explain crystal retention.^[8] As a result of crystal growth and agglomeration, particles that are too large to freely pass the renal tubules may be formed. Alternatively, relatively small crystals could be retained by adhering to the surface of the urothelial lining and then increase in size.^[8,9]

The surgical methods available to treat kidney stones like extracorporeal shock wave lithotripsy (ESWL) have serious side effects and do not give satisfactory results. Therefore, it is worthwhile to look for an alternative for the management of urolithiasis. Many medicinal plants have been used since ages to treat urinary stones though the rationale behind their use is not well established through systematic and pharmacological studies, except for some composite herbal drugs and plants.^[10,11] Plant medicines are in great demand both in the developed

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as well as developing countries for the primary health care because of their wide biological and medicinal activities, higher safety margin and lesser costs.^[12]

Achyranthes aspera, commonly called as putkhanda, is being used in ayurveda as a herbal drug since ages. There are reports in the literature citing its antifertility,^[13,14] antimicrobial,^[15] anti-inflammatory role^[16] and also its role as an immune stimulator.^[17,18] It is an active component of various drug formulations for kidney stones though no scientific basis has yet been formulated for its diuretic potency.^[10]

The present study is aimed at investigating the efficacy of *A. aspera* on CaOx crystal nucleation and growth *in vitro* and further examining the potency of the same on oxalate-induced injury in NRK 52E (rat renal tubular epithelial) cells.

MATERIALS AND METHODS

Preparation of the *A. aspera* Extract

The dried roots of *A. aspera* were obtained from Natural Remedies Pvt. Ltd. at Bangalore in India. A collection of voucher specimen is available with the company.

The air-dried, fine powdered plant roots were boiled in distilled water. The extract was then filtered using Whatman No. 1 filter paper and the filtrate was evaporated *in vacuo* and dried using a rotary evaporator at 60°C.^[19] The final dried samples were stored in labelled sterile bottles and kept at -20°C. Various concentrations of the plant sample tested for their inhibitory potency were 25, 50, 100, 200, 400 and 1000 µg/ml which were prepared at the time of experiment and were referred to as aqueous extract of *A. aspera*.

For cell culture studies, a stock solution of the dried aqueous *A. aspera* extract was dissolved in dimethyl sulphoxide (DMSO) [final concentration of the DMSO in the highest concentration of plant extract tested did not exceed 0.4% (v/v) and did not affect the cell proliferation]. Further dilutions of the stock were done using serum free Dulbecco's Modified Eagle's Medium (DMEM) and filtered by 0.3 mm syringe filter.^[20]

Thin Layer Chromatography

Chromatography was performed on aluminium thin layer chromatography (TLC) silica gel 60 F₂₅₄ plates. The mobile phases were chloroform:methanol (9:1, v/v) for aqueous and methanolic extracts and butanol:acetic acid:water (12:1:2, v/v) for ethanolic extract. Ten microlitres of the sample was loaded and ascending development of the plates was performed in all-glass chamber. Spots were visualised using anisaldehyde:acetic acid:sulphuric acid (1:2:100, v/v) and heating the plates for 5 min at 110°C.

Nucleation Assay

The method used was similar to that described by Hennequin *et al.* with some minor modifications.^[21] Solutions of calcium chloride and sodium oxalate were prepared at the final concentrations of 3 and 0.5 mmol/l, respectively, in a buffer containing Tris 0.05 mol/l and NaCl 0.15 mol/l at pH 6.5. Both the solutions were filtered through a 0.22-µm filter. Thirty-three millilitres of calcium chloride solution was mixed with 3.3 ml of the aqueous extract at different concentrations. Crystallisation was started by adding 33 ml of sodium oxalate solution. The final solution was magnetically stirred at 800 rpm using a Polytetrafluoroethylene (PTFE)-coated stirring bar. The temperature was maintained at 37°C. The absorbance of the solution was monitored at 620 nm after every 1 min. The percentage inhibition produced by the herb extract was calculated as $[1 - (T_{si}/T_{sc})] \times 100$, where T_{sc} was the turbidity slope of the control and T_{si} the turbidity slope in the presence of the inhibitor.

Growth Assay

Inhibitory activity against CaOx crystal growth was measured using the seeded, solution-depletion assay described previously by Nakagawa and colleagues.^[22] Briefly, an aqueous solution of 10 mM Tris-HCl containing 90 mM NaCl was adjusted to pH 7.2 with 4 N HCl. Stone slurry (1.5 mg/ml) was prepared in 50 mM sodium acetate buffer (pH 5.7). COM crystal seed was added to a solution containing 1 mM CaCl₂ and 1 mM sodium oxalate (Na₂C₂O₄). The reaction of CaCl₂ and Na₂C₂O₄ with crystal seed would lead to deposition of CaOx (CaC₂O₄) on the crystal surfaces, thereby decreasing free oxalate that is detectable by spectrophotometry at wavelength 214 nm. When aqueous extract is added to this solution, depletion of free oxalate ions will decrease if the test sample inhibits CaOx crystal growth. Rate of reduction of free oxalate was calculated using the baseline value and the value after 30-second incubation with or without test sample. The relative inhibitory activity was calculated as follows: % relative inhibitory activity = $[(C - S)/C] \times 100$, where C is the rate of reduction of free oxalate without any test sample and S is the rate of reduction of free oxalate with a test sample.

Cell Culture

Normal rat epithelial derived renal tubular epithelial (NRK 52E) cells were obtained from National Centre of Cell Sciences (NCCS, Pune, India). The cells were maintained as monolayer in DMEM with 2.0 mM L-glutamine adjusted to contain 3.7 g/l sodium bicarbonate and 4.5 g/l glucose. Medium was supplemented with 1% penicillin (100 units/ml), streptomycin (10,000 µg/ml) and 10% fetal bovine serum. Cells were cultured in 25 cm² tissue-culture treated flasks at 37°C and 5% CO₂ in humidified chambers.

Oxalate-induced Cell Injury

NRK 52E cells were incubated in DMEM containing 1 mM sodium oxalate in the presence of different concentrations of the aqueous extract of the test sample (10, 25 and 50 µg/ml) for 72 hours.^[20,23] Cell injury was assessed by measuring the cell viability through trypan blue and monitoring the lactate dehydrogenase (LDH) leakage into the medium.

Cytotoxicity

Trypan blue assay

The cytotoxicity of the aqueous extract of *A. aspera* was assessed by determining the cell viability using trypan blue exclusion method. For the determination of cell viability, cells were plated at a density of 4×10^4 cells/well and cultured for 72 hours. The medium was replaced with serum-free medium and the cells were treated with various concentrations of the plant extracts (10, 25 and 50 µg/ml) for a further 72 hours. The percentage viability for the cells was calculated as (live cells/total cells) $\times 100$.

Lactate dehydrogenase leakage assay

LDH leakage assay was performed by the method of Wagner *et al.*^[24] Briefly, 6.6 mM NADH and 30 mM sodium pyruvate were prepared in Tris (0.2 M, pH 7.3). The reaction was initiated with the addition of 50 µl of the test sample and the disappearance of NADH was monitored at 340 nm for 5 min at an interval of 1 min. The percentage of LDH release was calculated by dividing the activity of LDH in the supernatant by the LDH activity measured after complete cell lysis achieved by sonication.

Statistical Analysis

Data were expressed as mean values of three independent experiments (each in triplicate) and analysed by the analysis of variance ($P < 0.05$) to estimate the differences between values of extracts tested.

RESULTS

Inhibition of Nucleation of Calcium Oxalate Crystals by *A. aspera* Extract

Figure 1 displays the effects of the different concentrations of the aqueous extract of *A. aspera* on the nucleation of CaOx crystals. With respect to control (with no plant sample), the percentage inhibition was at 71.4 ± 0.001 with 25 µg/ml of the plant extract which reduced to 42.9 ± 0.002 with increase in the concentration of *A. aspera* extract to 100 µg/ml. The percentage inhibition rose from 57.1 ± 0.002 to 100 ± 0.003 with the increase in concentration of *A. aspera* extract to the tune of 200, 400 and 1000 µg/ml of the extract.

Inhibition of Calcium Oxalate Crystal Growth by *A. aspera* Extract

Figure 2 demonstrates the percentage inhibition shown

by *A. aspera* on the CaOx crystal growth. *A. aspera* extract showed inhibition in a concentration-dependent manner. The percentage inhibition with 25 µg/ml of plant sample was 50.5 ± 0.001 . In the presence of the 50 µg/ml plant sample, the percentage inhibition increased to 58.2 ± 0.004 but remained almost constant in the range of 101–105% with 100 and 200 µg/ml. Inhibition increased significantly with 400 and 1000 µg/ml of *A. aspera* extract to 209.9 ± 0.002 and 249.5 ± 0.004 , respectively.

Diminution of Oxalate-Induced Renal Tubular Epithelial Cell Injury by *A. aspera* Extract

Figure 3 depicts the protective effect of the aqueous extract of *A. aspera* towards the renal tubular epithelial cells. The

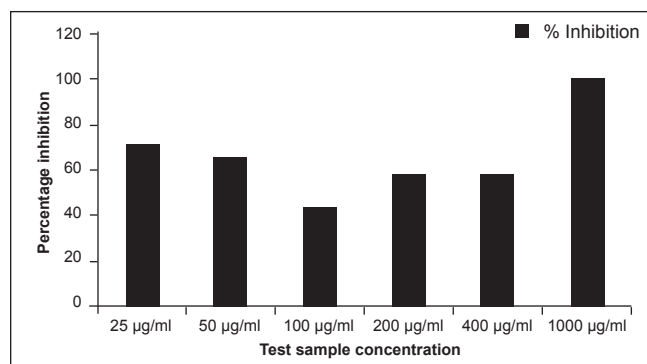


Figure 1: Effect of *A. aspera* on nucleation of CaOx.

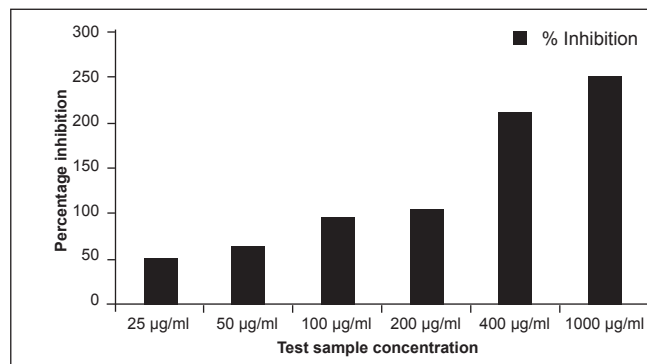


Figure 2: Effect of *A. aspera* on the growth of CaOx.

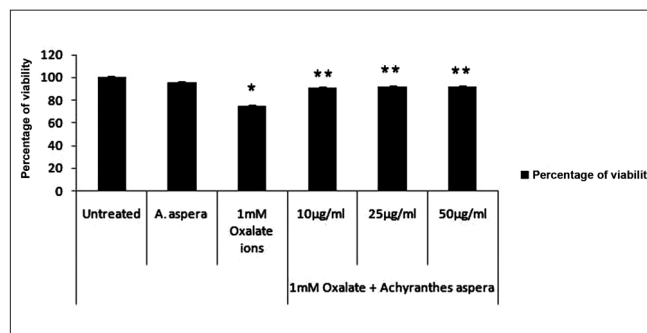


Figure 3: Effect of *A. aspera* on the viability of NRK 52E cells. Data are mean \pm standard error of mean (SEM) of three independent observations. * $P < 0.05$ versus untreated control. ** $P < 0.05$ versus oxalate control (the experiment was done thrice in triplicates each time)

oxalate induced a significant injury to the cells which could be ascertained by a decrease in viability from 100% in the control (untreated cells) to 74.7%. However, the injury due to oxalate was significantly reduced in those cells treated with the *A. aspera* extracts. As the concentration of the extract was increased from 10 to 50 $\mu\text{g/ml}$, the percentage viability improved showing that the plant shows an inhibitory activity towards the oxalate caused injury to the renal cells in a concentration-dependent manner. The plant extract alone (50 $\mu\text{g/ml}$, containing 0.4% DMSO) had no effect on the cell injury in the absence of oxalate, indicating that even at the highest concentration of DMSO used, there was no cytotoxicity to the cells. The percentage viability with 10, 25 and 50 $\mu\text{g/ml}$ was 91.2 ± 3.5 , 91.9 ± 2.7 and 92.1 ± 2.2 , respectively.

LDH is a stable cytosolic enzyme that is released when the cell is lysed or there is any injury on the cell membrane. A significant increase in LDH release was seen when the NRK 52E cells were exposed to oxalate alone. When NRK 52E cells were treated with the plant extract at varying concentrations (10, 25 and 50 $\mu\text{g/ml}$) along with oxalate (1 mM) for 72 hours, a reduction in oxalate-induced cell injury was observed as assessed by a decreased LDH release [Figure 4], emphasising the protective effect of the plant extract towards NRK 52E. It was observed that the plant extract alone had no significant effect on the measures of cell injury in the absence of oxalate. The percentage LDH release for 10, 25 and 50 $\mu\text{g/ml}$ was observed to be 152.8 ± 7.1 , 151.5 ± 8.5 and 149.0 ± 2.4 , respectively, after treatment with oxalate and the plant extract with respect to control.

Thin Layer Chromatography

Figure 5 displays the banding pattern of aqueous, ethanolic and methanolic extracts of *A. aspera*, formed after anisaldehyde reagent is sprayed.

DISCUSSION

Hyperoxaluria is a major risk factor for CaOx nephrolithiasis, which in turn is associated with renal injury. High level of oxalate causes a variety of changes in the renal epithelial

cells, such as an increase in free radical production and a decrease in antioxidant status, followed by cell injury and cell death. These changes are significant predisposing factors for the facilitation of crystal adherence and retention.^[5,20]

A dramatic advancement in using phytotherapy for urolithiasis treatments has been observed in the recent years and many investigators have proposed to implicate scientific study on its efficacy. Many medicinal plants have been used since ages to treat urinary stones though the rationale behind their use is not well established.

One such unexplored plant is *A. aspera*, commonly called as putkhandia and prickly chaff flower. The plant is said to be a divine medicine in Vedas and has a special mention in Charaka Samhita, though not much scientific study has been done to explore the therapeutic potency of *A. aspera*, which has been established through ethnobotanical studies.^[25]

In this study, the anticalcifying properties of *A. aspera* were explored *in vitro*. The inhibitory potency of the plant was tested on the nucleation and growth of the most commonly occurring kidney stones, COM. A concentration-dependent trend of inhibition by *A. aspera* extract was observed with maximum inhibition of 100% and 250% for CaOx nucleation and the growth assay, respectively, with 1000 $\mu\text{g/ml}$ of the extract.

In the study with NRK 52E cells, *A. aspera* proved to have a protective effect towards the renal epithelial cells, again in a concentration-dependent manner. When NRK-52E cells were injured by exposure to 1 mM oxalate for 72 hours, the plant extract prevented the injury in a dose-dependent manner. The mechanism of inhibition/reduction in the injury needs to be studied further. Studies have shown that inhibition of the inflammatory response induced by injury due to crystal formation helps in restoring normalcy.

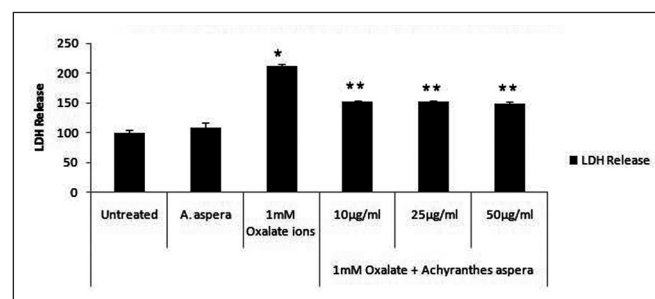


Figure 4: Effect of *A. aspera* on the % LDH release. Data are mean + SEM of three independent observations. * $P < 0.01$ versus untreated control. ** $P < 0.05$ versus oxalate control (the experiment was done thrice in triplicates each time)

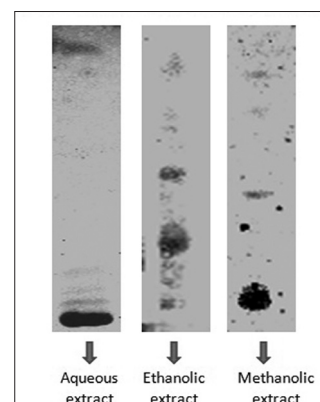


Figure 5: TLC profile of *A. aspera*

Beghalia *et al.*^[26] have suggested in studies using certain Algerian medicinal plants that the herb extract may contain substances that inhibit the growth of COM crystals. This property of plant extracts could be important in preventing kidney stone formation; the agglomeration of particles is a critical step in urinary stone formation, as larger crystals are less likely to pass spontaneously in urinary tract.^[8,27] They^[26] further postulated that the plant extracts may contain substances that inhibit CaOx crystal aggregation and also the binding of the crystal to the renal epithelial surface. This could explain a decrease in the LDH release seen in the cells treated with the plant extract as compared to those treated with oxalate alone.

The saponins extracted from the seeds of *A. aspera* have proved to show diuretic potency in rats in the study conducted by Gupta *et al.*^[28] *A. aspera* is an active component of Cystone, a drug to treat urolithiasis made by Himalaya Drug Co. Ltd., Mumbai^[10] and is being used by the folklore since a long time.

Recently, several plants including *Herniaria hirsuta*,^[29] *Phyllanthus niruri*^[30] and *Bergenia linguata*^[31] are being explored for their antiurolithiatic property on the basis of their usage in the traditional medicine. *H. hirsuta*, a plant from Morocco, is also known to exhibit the antilithiatic activity. The adhesion of the radioactive COM crystals to the Madin Darby canine kidney cells was studied in the presence and the absence of the aqueous extract. COM crystal binding to the cells was inhibited by the extract in a concentration-dependent manner.^[29] *B. ligulata* is a widely used plant in South Asia, mainly India and Pakistan, as a traditional medicine for the treatment of urolithiasis. The crude aqueous-methanolic extract of *B. ligulata* rhizome (BLR) was studied using *in vitro* and *in vivo* methods and the extract showed the antiurolithic activity through CaOx crystal inhibition, diuretic, hypermagneseuric and antioxidant effects.^[31] Very recently, in our lab, antilithiatic potency of *Dolichos biflorus*^[32] and *Trachyspermum ammi*^[33] has been evaluated *in vitro* and *in vivo*. Antilithiatic proteins were identified and characterised from these plants adding a new vista to study therapeutic proteins from plants.

In conclusion, the aqueous extract of *A. aspera* has been shown to possess an ability to inhibit CaOx crystallisation *in vitro*. Besides this, the extract also showed cytoprotective properties towards the NRK 52E cells by reducing the LDH leakage and increasing the cell viability. Our study thus put forth the possibility of using *A. aspera* as the therapeutic agent to treat urolithiasis and further characterisation of its active compound(s) could lead to a new candidate drug for the patients with urolithiasis.

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