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In-vitro Calcium Oxalate Crystallization Inhibition by *Achyranthes aspera* L. and *Bryophyllum pinnatum* Lam

Kumkum Agarwal[1*] and Ranjana Varma[1]

[1]Department of Botany, Sarojini Naidu Govt. Girls P.G. (Auto.) College, Shivaji Nagar, Bhopal, M.P. India.

Authors' contributions

This work was carried out in collaboration between both authors. Author KA designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches, analyses of the study and performed the spectroscopy analysis. Author RV managed the overall checking of the manuscript. Both authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Introduction: Urolithiasis has plagued human kind since antiquity and in the present era, its frequency is increasing with maximum occurrence rate of calcium oxalate stones. Although dramatic changes in the methods of its treatment has undertaken, still need exists for search of antilithic drugs from the nature. Thus, the inhibition of *in-vitro* calcium-oxalate crystal formation by *Achyranthes aspera* L. and *Bryophyllum pinnatum* Lam. was investigated.
Methods: Leaf extracts of both the plants were screened by using nucleation assay. Different concentrations of both the extracts were screened.
Results: In the nucleation assay the% inhibition for calcium oxalate crystal formation was found to be directly proportional to the increase in concentration of the plant extracts. *Achyranthes aspera* showed maximum inhibition of 60.06±0.19% at 1000 mg/ml while *Bryophyllum pinnatum* showed

**Corresponding author: Email: atharva72013@gmail.com;*

maximum inhibition of 49.93±0.07% at the same concentration.
Conclusion: The present *in-vitro* study provides evidence that *Achyranthes aspera* is a potent anti-urolithiatic agent; however these *in-vitro* results should be confirmed *in-vivo*.

Keywords: Calcium oxalate stones; In vitro; nucleation assay; optical density; anti-urolithiatic agent; Achyranthes aspera L.; Bryophyllum pinnatum Lam.

1. INTRODUCTION

Urolithiasis is a urinary disorder, known to the mankind since ancient times. In India ancient Sanskrit documents have reference of stone formation. Among the various diseases of the urinary tract, it is considered to be the third most common disease [1]. In India, calcium oxalate remains the most predominant constituent of urolithiasis [2]. Several advancements have taken place in the methods for treating urolithiasis, but the costly expenditure and the re-occurrence of stone formation are some of the side effects which the general people have to undergo [3]. Thus, searching new antilithic drugs from natural sources could be of great help as drugs from plant origin are cheaper as well as they confer least side effects.

Achyranthes aspera L. (Amaranthaceae) commonly known as chirchita or aandhi jhadha, is a herb that grows as weed in rainy season. In the traditional system of medicine it serves as a cure for oedema, dropsy, piles, boils, skin disorders, asthma and cough. Leaves are emetic, hydrophobic, digestive, carminative, resolve swelling and expel phlegm. The crushed leaves are used to treat backache and its paste is used in cases of insect bites like wasp bite. The plant is also used as anti-allergic, antiparasitic, spermicidal, hypoglycaemic and as an analgesic and cardiovascular agent [4]. Leaf extracts are reported to possess hypoglycemic, thyroid stimulating, antiperoxidative [5], analgesic [6], antimicrobial [7], anticancer [8] and antipyretic activity [9] etc.

A. aspera has been documented for its therapeutic effects in treating urinary complaints and antilithic activity. The ethanolic extract of its leaves, were tested against ethylene glycol induced lithiasis in rats [10]. Its root paste is employed in urinary trouble in various parts of India [11]. Earlier reports have shown that the whole plant methanolic extract of *A. aspera* is also capable of providing protection against nephrotoxicity induced by lead acetate in rats [12]. The aqueous extract of its roots were found to inhibit nucleation and growth of calcium oxalate crystallization. In addition, *A. aspera* provided protective effect against oxalate induced renal tubular epithelial cell injury *in-vitro* and is also used as an active component of various drug formulations for treating kidney stone [13]. It is worth mentioning that its roots aqueous extract was also found to prevent urolithiasis in rats which was induced by ethylene glycol as well as it reduced the growth of calcium oxalate stones [14]. Report related to the use of whole plant of *A. aspera* in cases of kidney stones has been found in an ethnobotanical study [15]. Some other species of *Achyranthes* has been reported to inhibit crystallization of calcium oxalate in synthetic urine [16].

A. aspera (roots and leaves) were screened for triterpenoids content [17]. Similarly, the presence of alkaloids, flavonoids, glycosides, saponins, tannins and proteins has been reported [18]. The aqueous extract of its leaves has been reported to show the presence of alkaloids, flavonoids and resins [19,20]. Similar work has been reported on petroleum ether extract [21] and ethanolic and aqueous extract of its leaves [22]. Phytochemicals in its dried leaf powder has been studied [23]. Similarly, phytochemicals in methanolic extract of its leaves was reported [24].

Bryophyllum pinnatum Lam. (Crassulaceae) commonly known as patharchatta, is a perennial plant which is grown for ornamental and medicinal purposes in different parts of India. It is traditionally used for the treatment of: abdominal discomfort, boils, bruises, cholera, cuts, diabetes, diarrhea, dysentery, flatulence, headaches, kidney stones, indigestion, insect bites, scabies, sores, urinary insufficiency and wounds. Leaf extract is taken in empty stomach for treating bladder stones while leaf juice is used in cough, dysentery, leucoderma and in case of bleeding [25].

In a study alcoholic extract of leaf of *B. pinnatum* showed promising antiurolithiatic activity [26]. *B. pinnatum* (fresh leaf juice) along with some *Piper nigrum* powder is taken twice a day for 15 days to expel the stones [27,28] while it's aqueous leaf

extract was found to have nephroprotective effect against gentamicin induced toxicity in rats [29]. In a review, the anti-urolithiatic activity of hydroalcoholic extract [30] and ethanolic extract [26] of its leaves in rats has been reported. Other ethnobotanical studies have also shown its use in urinary disorders like kidney stone (1 cup of leaf juice is taken daily) [31].

B. pinnatum was found to be rich in alkaloids, triterpenes, glycosides, flavonoids, cardienolides, steroids, bufadienolides and lipids while its leaves were found to contain all these alongwith proteins, terpenoids, carbohydrates, phenols, tannins and saponins [32,33]. The preliminary phytochemical studies with the help of thin layer chromatography, revealed the presence of alkaloid in chloroform, acetone, and methanolic extract, while flavonoids were found in chloroform, acetone, and ethanolic extracts respectively [34]. Similarly, work on aqueous extract of its leaves was also reported [33,35,36,37]. Phytochemical screening of fresh and dried leaf was reported [38,39].

Several phytochemicals like flavonoids, triterpenes, saponins, tannins, alkaloids, glycoside derivatives, proteins, tannins and steroids are reported to be responsible for antiurolithiatic effect by either inhibiting the formation of calcium oxalate crystals, preventing their attachment to renal cells or by their calcium channel blocking activity [14,40-55].

The *in-vitro* nucleation assay was used to test the antilithic potential of aqueous extracts of leaves of *A. aspera* and *B. pinnatum* in this piece of research work.

2. MATERIALS AND METHODS

2.1 Chemicals

High grade chemicals were purchased from Sumeet Enterprises Pvt. Ltd, Bhopal and Burgoyne Reagents.

2.2 Plant Collection and Identification

The leaves of *A. aspera* and *B. pinnatum* were obtained from southern part of the Bhopal district during the month of September 2011. The plant materials were identified by BSI (Allahabad) where voucher specimens were kept for record: numbers 1310-130.01-629 and 1131-60.01-263 respectively [56].

2.3 Extraction

After collecting the fresh leaves of both the plants they were cleaned with tap water, followed by distilled water after which they were shade dried at room temperature. Aqueous extracts were prepared by boiling the fully dried and powered leaves (100 g) with distilled water. The dried extracts were filled in glass vials and kept in refrigerator until used for further analysis.

2.4 Nucleation Assay

The method as given by Atmani et al. [57] was chosen for the study of oxalate crystallization. In this method crystallization was studied without inhibitor (i.e. control) and with plant material (i.e. inhibitor), in order to assess the inhibiting ability of the plant extracts used. The solutions of calcium chloride and sodium oxalate were prepared at concentrations of 5 mmol/L and 7.5 mmol/L respectively. These solutions were prepared in the presence of buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5. The solution of plant extracts (100 ml) at different concentrations i.e.100 mg/ml to 1000 mg/ml was mixed with calcium chloride (950 ml) after which the process of crystallization was initiated by adding 950 ml of sodium oxalate solution. In case of control no inhibitor substance was added to the crystal forming solutions. The whole procedure was carried out at 37ºC. For each experimental solution, the optical density was monitored at 620 nm after 30 minutes of reaction using Systronics digital spectrophotometer 166. The induction time in the presence of the extract was compared with that of control in order to calculate the rate of nucleation. The results were expressed in percentage. The following reaction resulted in the growth of crystals: [58,59].

$CaCl_2 + Na_2C_2O_4 \rightarrow CaC_2O_4 + 2NaCl$ (Calcium chloride + Sodium oxalate → Calcium oxalate + Sodium chloride)

2.5 Statistical Analysis

Data were presented as mean ± standard deviation (S.D), and values were considered significant at $P<0.05$.

3. RESULTS

In the present study out of the two aqueous extracts, the extract of *A. aspera* showed maximum inhibition of 60.06±0.19% at 1000

mg/ml and minimum of 37.74±0.46% was obtained at 100 mg/ml. On the other side, the extract of *B. pinnatum* showed maximum inhibition of 49.93±0.07% at 1000 mg/ml and minimum of 29.96±0.04% at 100 mg/ml (Fig. 1). The control (without any inhibitor) was found to show no inhibition. These results clearly demonstrated that *A. aspera* extract is a better antilithic agent as compared to *B. pinnatum*. It was found that increasing the concentration of plant extracts resulted in the increase in percentage inhibition of calcium oxalate crystallization. Thus these results were found to be in accordance with the use of both these plants for treating lithiasis in the traditional medicine.

4. DISCUSSION

The crystals of calcium oxalate were formed as result of incubation of solutions of calcium chloride and sodium oxalate. The induction time in the presence of the extract was compared with that of control in order to calculate the rate of nucleation. When the optical density of each solution was monitored at 620 nm after 30 minutes of reaction it was found that the turbidity was lower in the presence of plant extracts than in the control solution. It showed that less amount of calcium oxalate crystals were formed in the presence of extracts. It was also found that increasing the concentration of plant extracts resulted in the increase in percentage inhibition of calcium oxalate crystallization. Thus, concentration and percentage inhibition were directly proportional to each other.

Previous report on inhibition of nucleation and growth of calcium oxalate crystallization by root of *A. aspera* (aqueous extract) was found [13], however, it is the first report on *in vitro* evaluation of antilithic potential [57] of the aqueous extract of its leaves.

A. aspera and *B. pinnatum* are reported to have alkaloids, flavonoids, glycosides, saponins, tannins, proteins and phenolic compounds which are reported to play significant role in inhibiting calcium oxalate crystallization. The phytochemical constituents isolated from these plants could be responsible for their observed antilithic potential. Based on the results of present study, detailed studies are warranted on the extracts as well as individual chemical constituents of these plants and their effect both *in vivo* and *in vitro* experimental models.

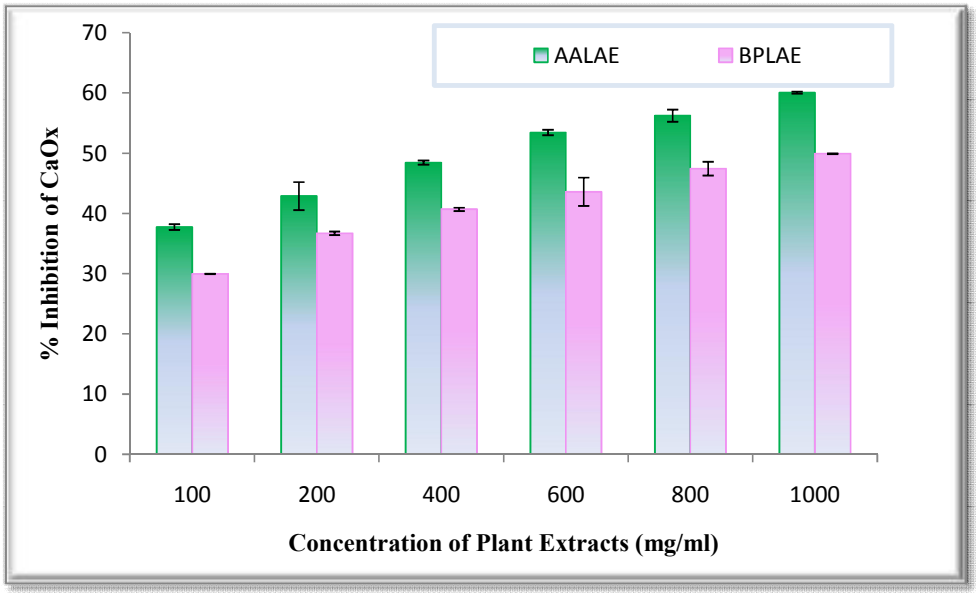

Fig. 1. *In vitro* antiurolithiatic activity of plant extracts in nucleation assay
Note: AALAE, Achyranthes aspera leaf aqueous extract; BPLAE, Bryophyllum pinnatum leaf aqueous extract

5. CONCLUSION

The results of the nucleation assay showed that both the plant extracts have antiurolithiatic potential, thus further *in vivo* studies should be undertaken to confirm these results. As reported earlier the presence of some of the phytochemicals may be considered responsible for this inhibitory action. Thus, phytochemicals in these extracts will be analysed in future studies.

However these *in-vitro* results should be confirmed in.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

The authors hereby declare that they have no competing interest or any other conflict of interest. The study reported is only for academic interest.

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