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In Vitro Evaluation of Anti Urolithiatic Activity of *Bryophyllum Pinnatum* Lam

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***Abstract*- Background**: *Bryophyllum pinnatum* is a perennial herb, widely used in the treatment of several conditions in folklore medicine, including Urolithiasis**.** The ethanol extracts of *Bryophyllum pinnatum* stem, leaf and root were evaluated on preliminary phytochemical analysis by using standard protocol and the analysis of antiuroliathiasis potency of the plant extracts on *in vitro* crystallization of calcium oxalate through analyzing nucleation, aggregation assays and extracted kidney stone weight reduction assay. Bioactive ingredient such as alkaloids, flavonoids, steroids, phenols, terpenoids and proteins were detected in most of the plant parts tested. In uroliathiasis activity, the maximum inhibition of CaOx crystal formation and its aggregation were detected in the following sequential order; leaf > stem > root. Also, the leaf extract of *B. pinnatum* was significantly reduced the weight of extracted CaOx kidney stone (% reduction) when compared with stem and root extracts. This study has given primary evidence for *B. pinnatum* as the plant which possess antiurolithiatic property.

***Keywords*:** Urolithiasis, *Bryophyllum pinnatum*, CaOx, Nucleation assay.

I. INTRODUCTION

Urolithiasisis is the urinary calculi are formed in the urinary system or the process of formation of the stone in kidney, bladder and urinary tract[1]. Currently, 4-15% of the human populations suffer from urinary stone problem all over the globe[2]. Different factors including metabolic abnormalities, diet, heredity, and infection have been concerned in the pathobiology of stone formation in kidney[3]. Urolithiasisis are mainly composed of calcium salts, uric acid, cysteine, and struvite. Among these types, more than 80% of the stones in human are encompassed of CaOx[4] and calcium phosphate stones represent up to 15 – 25% of analyzed stones[5]. The formation of Calcium Oxalate stone occurs with crystal nucleation, aggregation and retention within urinary tract. If a stone blocks the flow of urine, excruciating pain may result[6]. Moreover, a disparity between promoters and inhibitors of calcification is considered as a significant trigger for calcium stone formation in the urinary tract[7].

Medical conditions that are related with increased risk of kidney stone formation include cystic fibrosis, hyperthyroidism, gout, hyperparathyroidism[8]. The abnormal urine colour, blood in urine, fever, nausea and vomiting are the symptoms of urolithiasis[9]. The increasing water intake, dietary restrictions, and urinary alkalizing and calcium-chelating agents i.e., sodium bicarbonate, potassium citrate, and sodium citrate are the current preventing measures of CaOx supersaturation in urine[10]. Now days, the surgical operation of lithotripsy and local calculus disruption using high-power laser are widely used to remove the calculi. However, these procedures are highly costly and with these procedures recurrence is quite common[11]. The different therapies including thiazide diuretics and alkali-citrate are being used in attempt to prevent recurrence but scientific evidence for their efficacy is less convincing[12].

The overall outcome gives clear cut indication for exploration of new remedy. In view of this, exploitation of natural sources has been assumed to be of greater potential. *Bryophyllum pinnatum* Lam. plant belongs to family Crassulaceae, commonly used as traditional medicines. The plant has been used for the treatment of a variety of illness in tropical America, India, China, Australia and Africa, including, rheumatism, body pain, arthritis, heartburn, skin ulcers, peptic ulcer, diabetes mellitus, microbial infections, and hypertension[13-17].

The wide range of active phytochemicals such as alkaloids, triterpenes, glycosides[18], flavonoids[19], steroids[20], bufadienolides[21], lipids and organic acids[22] are reported in this plant. These compounds have been considered to be responsible for the plant's diverse pharmacological activities. Previously, the majority of researchers used the leaf extracts of *B. pinnatum* for the investigation of antiuroliathiatic properties, *in vivo*[23-25]. But we focused on

the influence of ethanol extracts of stem, leaf and root of *B. pinnatum* on *in vitro* crystallization of calcium oxalate through analyzing nucleation, aggregation assays and extracted kidney stone weight reduction assay. *In vitro* methods are generally the scheme of selection of drugs for large-scale production by the pharmaceutical industry because of the ease of culture for production, compared with use of animals, and because of economic considerations[26].

II. MATERIALS AND METHODS

Preparation of plant extracts

The stem, leaf and root of *Bryophyllum pinnatum* Lam collected from the Tiruporur forest, Chennai, Tamil Nadu, India and was authenticated. The study was conducted during November 2018 to February, 2019 in PG and research Department of Biotechnology, Mohamed Sathak College of Arts and Science, Sholinganallur, Chennai - 119. The plant samples were shade dried and powdered in a mixer grinder and stored in air tight jar for further study. The extraction was carried out by hot percolation method using Soxhlet apparatus. The solvent used was ethanol. About 40 gm of powder was extracted with 200 ml of ethanol. The extract was concentrated to dryness under controlled temperature 40-50°C. The extract was preserved in refrigerator till further use.

Phytochemical screening

Phytochemical screening for major constituents was undertaken using standard qualitative methods as described by Fadeyi *et al*[27] and Harbone[28]. The plant extracts were screened for the presence of alkaloids, flavonoids, steroids, terpenoids, phenol, carbohydrates, proteins and amino acids

III. ANTIUROLITHIASIS ACTIVITY

Nucleation Assay

The inhibitory activity of the ethanol extracts of *B. pinnatum* on initiation of CaOx crystallization (nucleation rate) was performed according to method of Sasikala *et al*[29]. The solutions of calcium chloride (5 mmol/l) and sodium oxalate (7.5 mmol/l) were prepared in a buffer containing 0.05 mol/l Tris-HCl and 0.15 mol/l sodium chloride at pH range of 6, 8 and 10. An aliquot of calcium chloride (950 µl) was mixed separately with 500ul of stem, leaf and root extracts at different concentrations (100, 200, 300 and 400 µg/ml). Crystallization was started by adding 950µl of sodium oxalate (7.5mmol/l) at 37^0C. The optical density of the reaction mixture was monitored at 620nm using UV-visible spectrophotometer over 10min. All the experiments were performed triplicates and the rate of nucleation was determined comparing the induction time of control. The percentage inhibition was calculated by following formula:

% nucleation inhibition = [(^A Control - ^A test)/^A control] x 100

Aggregation assay

The rate of aggregation of the CaOx crystals was determined by method of Sasikala *et al*[29]. The CaOx monohydrate crystals were prepared by mixing equimilimolar concentrations of calcium chloride and sodium oxalate (50mmol/l) in 0.05 mol/Tris-Hcl and 0.15 mol/Nacl buffer) respectively. This mixture was equilibrated at 60^0C in a water bath for 1 h and then kept overnight on magnetic stirrer with hot plate (maintained at 37^0C, 200 rpm). Mixture was subjected to centrifugation at 4000 rpm for 15 min, and crystals were collected of 0.8mg/ml 0.05mol/l Tris-Hcl and 0.15mol/l Nacl buffer at pH range of 6, 8 and 10. The optical density of turbidity at 620nm was recorded at 60 min at 37^0C in the absence (control) or presence of plant extracts at different concentrations (100, 200, 300 and 400 ug/ml) after stopping the stirring. The concentration range in this assay was chosen based on the results of nucleation assay. The percentage aggregation inhibition was then calculated by comparing the turbidity in control and test groups using following formula:

% aggregation inhibition = [(ΔA TurbidityControl-ΔA TurbidityTest)/TurbidityControl] ×100

Extracted kidney stone weight reduction assay

Surgically removed human kidney stones are procured from Sri Sathya Sai hospital, Ammapettei, Tiruporur, Chennai. Assay was performed following method of Rao and Bano[30]. Large CaOx stone was cut into 12±2 mg pieces, and a piece was placed in 100ml of 50, 100, 150, 200 and 250ug/ml of leaf, stem and root extracts (at pH 6.5), in 0.05 mol/l Tris-Hcl and 0.15 mol/l NaCl buffer. A pinch of NaCl was added to each flask. After 24h, the stone pieces were carefully removed from the solution, was washed with distilled water, and dried in hot air oven at 100^0C for 30min. After drying, pieces were cooled to room temperature and weighed out carefully. The percentage of weight reduction was calculated using the following formula:

%dissolution = (Initial weight-final weight)/initial weight) x100

Statistics:

Data are expressed as mean ± standard error of the mean (SEM) (n=3).

IV. RESULTS

The phytochemical analysis carried out on the ethanolic extract of dried leaf, stem and root showed the presence of some bioactive compounds in the plant. Bioactive ingredient such as alkaloids, flavonoids, steroids, phenols, terpenoids and proteins were detected in most of the plant parts tested. Saponin was not detected in any of the plant extracts. However, flavonoid, terpenoids and proteins were absent in root, leaf and stem, respectively (Table 1).

TABLE 1: Phytochemical analysis of *B.pinnatum*

S.No	Phyto Chemical	Test	Result		
			Stem	Leaves	Root
1	Alkaloids	Mayer's test	+	+	+
		Wagner test	+	+	+
2	Flavonoids	Lead acetate test	+	+	-
3	Steroids		+	+	+
4	Terpenoids	Salkowakis test	+	-	+
5	Phenols	Ferric chloride test	+	+	+
		Lead acetate test	+	+	-
6	Saponins		-	-	-
7	Carbohydrates	Benetic's test	+	+	-
8	Protein & Aminoacids	Ninhydrin test	-	+	+

Crystallization in Nucleation Assay

The CaOx crystallization in nucleation phase of *B. pinnatum* stem, leaf and root was shown in Fig 1. Among the three plant parts tested, the leaf of *B. pinnatum* showed maximum inhibition of CaOx crystal formation in nucleation phase with a concentration of 100 µg/ml being most effective and produced 96.12% inhibition of crystal formation at pH 8. The minimum inhibition of CaOx crystal formation in nucleation phase was observed in 400 µg/ml root extracts at pH 10. The inhibition of CaOx crystal formation in nucleation phase were detected in the following sequential order; leaf > stem > root.

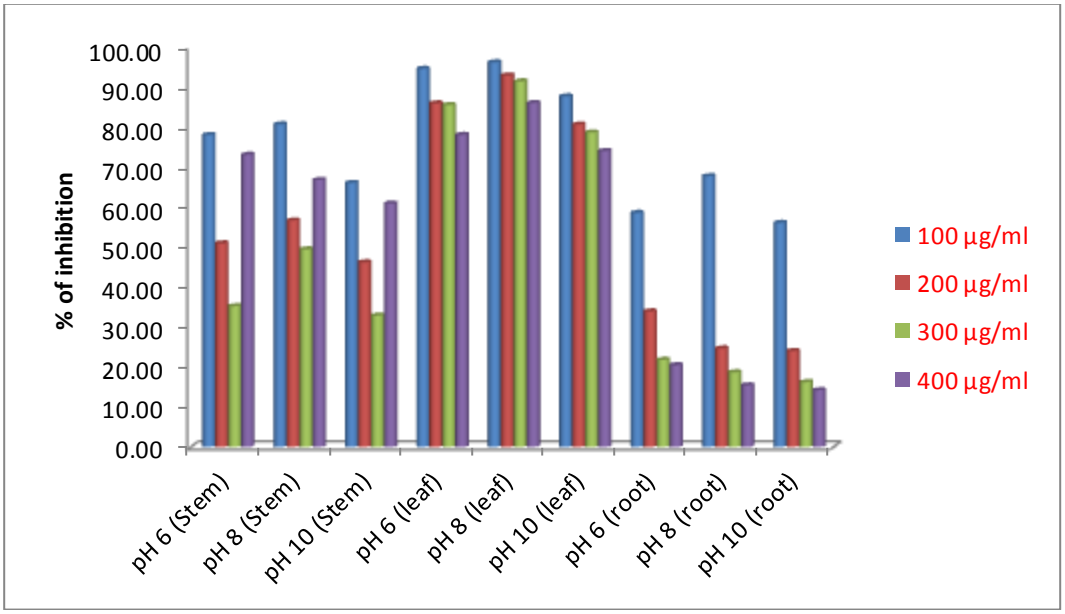

Fig 1: Effect of *B. pinnatum* on CaOx crystal formation in nucleation phase

Aggregation Assay

The reduction of CaOx crystals aggregation of *B. pinnatum* stem, leaf and root was shown in Fig 2. Among the three plant parts tested, the leaf of *B. pinnatum* showed maximum inhibition of CaOx crystal aggregation with a concentration of 100 µg/ml being most effective and produced 87.21% inhibition of crystal aggregation. The minimum inhibition of CaOx crystal aggregation (14.08%) was observed in 100 µg/ml root extracts at pH 8. The inhibition of CaOx crystal aggregation in nucleation phase were detected in the following sequential order; leaf > stem > root.

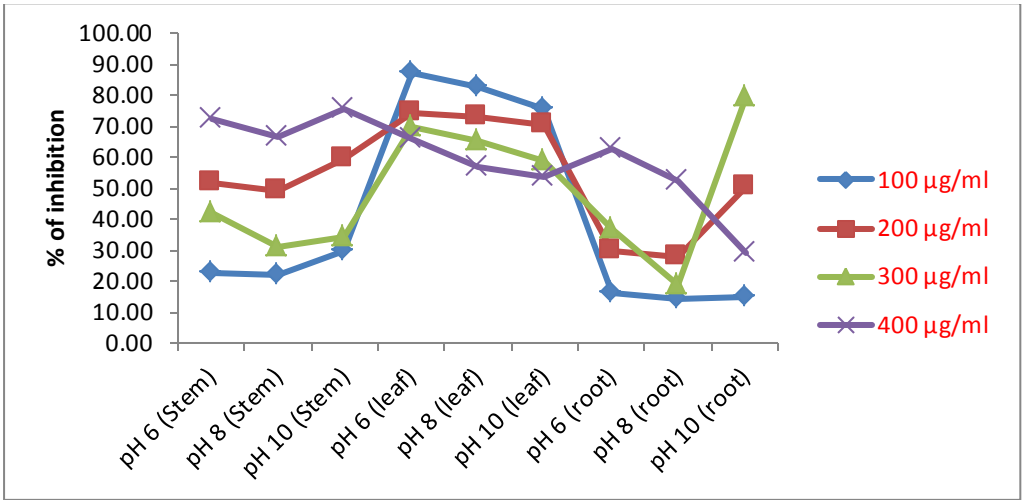

Fig 2: Effect of *B. pinnatum* on CaOx crystal aggregation

Extraction Kidney Stone Weight Reduction Assay

Fig 3 highlights the percentage weight reduction of human kidney CaOx stone when kept it in the solution of stem, leaf and root of *B. pinnatum* (50 - 250µg/ml) for 24 h. The final weight of stone recorded after 24 h revealed that the leaf showed maximum reduction of stone weight, followed by stem and root extract.

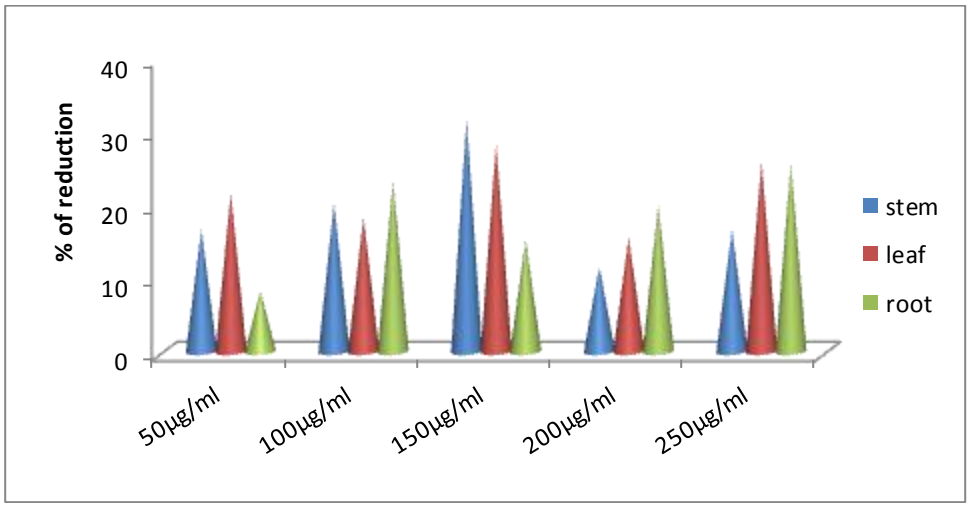

Fig 3: Effect of *B. pinnatum* on kidney stone weight reduction

V. DISCUSSION

Phytochemical screening of medicinal plants is very important in identifying new sources of therapeutical and industrial importance[31]. In the present study, using ethanol extracts of *B. pinnatum* stem, leaves and roots were tested for the presence of alkaloids, flavonoids, steroids, phenols, terpenoids and proteins. The results indicated that the presence of all the phytocompounds tested were present in all plant samples. Medicinal plants with proven antiurolithiatic activity were found to contain alkaloids, tannins, steroid and terpenoids as chief principles[32-34]. The antiurolithiatic mechanism is mediated possibly through diuretic and nephroprotective actions of the phytocompounds including phenols present in rhizomes of *Acorus calamus*[35,36].

Formation of kidney stones is a complex process and involves a series of biological events that are most likely triggered by genetic susceptibility together with dietary factors and lifestyle changes[37]. Nucleation is a prerequisite in the pathogenesis of CaOx urolithiasis. Nucleation basically marks a thermodynamically driven event of phase change wherein dissolved substances in a supersaturated solution spontaneously crystallize[38,39]. In the present investigation, similar phase change and formation of CaOx

crystals was witnessed while carrying out nucleation assay. This suggests the anticrystallization activity of *B. pinnatum* against CaOx crystallization. One possible mechanism of anticrystallization activity of *B. pinnatum* could be its ability to complex with free calcium and oxalate ions, thus preventing the formation of CaOx complexes, as has also been suggested for *Sarghassum wightti*[40]. Similarly, Niharika *et al*[41] reported that the highest percentage (87%) of calcium oxalate {CaOx} dissolution was observed in ethanolic extract of *Gossypium herbaceum*.

Aggregation of crystals marks the process wherein numerous crystals in the solution come together and adhere forming large crystal agglomerates. Aggregation is a key determinant of crystal retention as large crystal agglomerates are the ones that produce renal tubular obstruction thereby promoting stone formation[39]. In this study, the ethanolic leaf extrcat of *B. pinnatum* showed maximum inhibition of CaOx crystal aggregation with a concentration of 100 µg/. Similarly, Bawari, *et al*[42] noted that the ethanol extracts of *Daucus carota* L. roots produced a significant reduction in aggregation of preformed CaOx crystals. Percent reduction in aggregation produced by *D. carota* L. roots was found to be 49.94±2.07% comparable to that of Cystone (57.15±2.53%) at 1000 µg/ml concentration.

In the present study, also studied the efficacy of stem, leaf and root extracts on extracted CaOx kidney stone. The leaf extract was found to be most effective in dissolving CaOx stone followed by stem and root in terms of weight reduction. Like that of our study, Rao and Bano[30] have noted that weight reduction of extracted kidney stone, especially the oxalate containing calculi, showed a low dissolution rate compared to the non-oxalate stones.

VI. CONCLUSION

From the reported evidences along with above findings, it is asserted that *B. pinnatum* extract may reduce the availability of calcium in renal tubules to form CaOx crystals by virtue of their Ca^{2+} chelating ability and may eventually prevent aggregation of already formed CaOx crystals and subsequent stone formation. In addition, the plant extracts also increased dissolution and significantly reduced the weight of extracted CaOx kidney stone in *in vitro* studies. This study will help the researchers to uncover the critical areas of drug development of renal lithiasis that many researchers were not able to explore. The present study has shown also that the leaf, stem and root of *B. pinnatum* could be considered as potential alternatives for safer and effective management of CaOx-induced urolithiasis. However, in detail findings are needed to elucidate the long term benefit/side-effects of this extracts of *B. pinnatum*.

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