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Original Article

A modified TLC bioautographic technique for the detection of antilithiatic potential of therapeutic plants from Indigenous Ayurvedic System

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ABSTRACT

Introduction: Urolithiasis is the most painful disorder associated with formation of stone in the urinary system. In Indian Ayurvedic systems, medicinal plants are preferred as natural drug resources. In the present study aqueous extract of *Bryophyllum pinnatum* (L.), *Tribulus terrestris* (L.), *Phyllanthus niruri* (L.), *Abutilon indicum* (L.) and positive control tri-sodium citrates were used to investigate their antilithiatic potential in the modified dot blot assay. **Methods:** In the present study aqueous extract of all four plants in variable concentrations were tested for antilithiatic potential using dot blot method. In this method glass plates were overlaid by 0.8% agar containing 0.2 M $CaCl_2$. These plates were deepening in 0.2 M Ammonium chlorite and then deepen in Alizarin Red S and Arsenzo III. **Results:** After removal of plate from the coloring solutions, zones of inhibition were observed, which shows the maximum CaOx crystallization percent inhibition of *B. pinnatum* (73.16 ± 2.05 %), followed by *Phyllanthus niruri* (L.), *Abutilon indicum* (L.) and *Tribulus terrestris* (L.) on TLC bioautography based dot blot assay. The lowest IC_{50} (70.163 mg/ml) of *B. pinnatum* confirms its higher antilithiatic potential as compared to rest of three plants. **Conclusion:** We conclude that aqueous B. *pinnatum extract* (100 mg/ml) showed maximum CaOx crystallization percent inhibition (73.16 ± 2.05 %), which was followed by *Phyllanthus niruri* (L.), *Abutilon indicum* (L.) and *Tribulus terrestris* (L.) on TLC bioautography based dot blot assay. The lowest IC_{50} (70.163 mg/ml) of *B. pinnatum* the confirms its higher antilithiatic potential as compared to rest of the three plants.

Key words: Urinary system, Antilithiatic potential, Therapeutic plants, Dot blot assay, Percent inhibition.

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INTRODUCTION

Urolithiasis is common urinary tract disorder, distributed world widely, which is reported to affecting all geographical, cultural or racial groups. The major causes of urolithiasis are positive family history, nutrition deficiency and unbalanced diet. It has been estimated about 5-12% of world's population develops kidney stones during their lifetime, which is high in people living in high-risk zones up to 40% in 2000. Such critical situation results into 10 per cent of renal failure cases can be attributed to the kidney stones.[1] Kidney stones are aggregates of poly crystals and organic matrix.[2-4]

In India, use of medicinal plants for treatment of various diseases is from ancient times, which is nowadays a fascinating area of research. Now these plant drugs are in the demand world widely because of their higher potential to treat diseases, safety margin and less cost.[5-8] We confirmed the antiurolithiatic activity of *Tribulus terretris*, *Phyllanthus niruri*, *Abutilon indicum* and *Bryophyllum pinnatum* in the urine assay.[9] These plants were used in folk medicine for the treatment of urolithiasis.[7-8,10-12]

Previously, therapeutic plants for their antilithiatic potential were screened by various techniques, including, Slide gel method,[13] Hydrogel method,[14] Crystallization assay[15] and Urine assay.[9] These techniques supposed to be significant with pure drug and can revert back with crude extractsdue to interfering metabolites. In continuation of this, we propose the Dot Blot assay technique, which have been reported earlier for assessment of antioxidant activity and antimicrobial activity for screening the antilithiatic potential. This method is based upon the diffusion of herbal drug in between the gel beads similar like Radial immune diffusion,[16] supposed to be the basic phenomenon of the positive dot blot assay. The above assay has been developed first time for screening antilithiatic potential of medicinal plant extracts on TLC plate for qualitative and quantitative assessment.

MATERIALS AND METHODS

Extraction and preparation of plant extract

In the present study, the four plants were selected assuming ethno pharmacological as well as folklore based therapeutic importance was collected from Melghat forest region, Amravati (M.S.) India. The herbarium specimen was prepared, authenticated by Prof. S.R. Manik taxonomists from Department of Botany and submitted to Department of Biotechnology, Sant Gadge Baba Amravati University, Maharashtra; India. The accession number allotted to *B. pinnatum*-SGBAU-DBT-03, *P.niruri*-SGBAU-DBT-04; *T. terrestris*-SGBAU-DBT-05 and *A.indicum*-SGBAU-DBT-06.

The fresh leaves of *B. pinnatum* extracted in grinder; the mixture was filtered through muslin cloth. Similarly, *P. niruri* (leaves and fruits), *T. terrestris* (fruits) and *A. indicum* (leaves) were collected, thoroughly washed in tap water and then shade dried. About 20 g powdered material was crushed in mortar and pestle in 100 ml distilled water. All extracts were filtered through muslin cloth in 50 ml centrifuge tubes, centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was allowed to evaporate under the vacuum conditions at 50 ± 2°C. During the experimentation stock solution (100 mg/ml) of tri-sodium citrate (positive control) and each plant extract was diluted to working range 20, 40, 60, 80, 100 mg/ml.

Development of TLC plate

The TLC plate was prepared on a clean glass plate (8X12 cm) using silica gel, allowed to dry and activated in the oven about 1 hour at 110^{0}C. After activation, the TLC plates were loaded in spot type with 20 µL of tri-sodium citrate (positive control), and all plant extract in working range from 20 mg to 100 mg. Keep the TLC plates for 30 min at room temperature to evaporate from the water base of sample, which further used for TLC bioautographic method.

Agar overlay onto the test TLC plate

The ready TLC plates were then over layered with 40 ml of 0.8% of bactoagar prepared in $CaCl_2$ (0. 2 M). The agar gel was allowed to cool about 55°C and subsequently poured onto TLC plate (care should be taken not to dislodge the silica and also to prevent TLC plate from cracks) and allowed to solidify. The experimental plates were kept in the moist chamber for 6-8 hrs. During incubation the bioactive antilithiatic fractions form crude extract diffusion into over the layered agar gel.

Calcium oxalate Inhibition

The detection of crystal inhibition can be done by direct TLC bioautographic method.[17] TLC agar gel plate was then removed from moist chamber and carefully immersed in 0.2 M ammonium oxalate solution (care should be taken for uniform flooding of agar over layered TLC plate). Calcium chloride (0.2 M) present in agar gel and ammonium oxalate (0.2 M) solution will form CaOx crystals by reacting with each other on the gel surface. The region supposed to be possessed antilithiatic or Crystal Inhibition potential can show clear zone due to CaOx crystallization inhibition depends on the metabolic capacity of extract. Since silica gel and CaOx crystals are white in colour, thus it is difficult to visualize CI zone. This situation has been overcome by using staining with 0.2% Alizarin red (S) or Arsenazo III stain (prepared in D/W) that specifically impart pink or blue colour to calcium oxalate crystals respectively (Figure 1). Clear zone of inhibition can be seen by this technique, which confirms the antilithiatic potential of the therapeutic drug as it inhibited CaOx crystal formation. The results were recorded in triplicate and statistically analyzed shown in Table 1.

The results were recorded in the form of Inhibitory area and calculated in the terms of percent inhibition (PI) using the following formula;

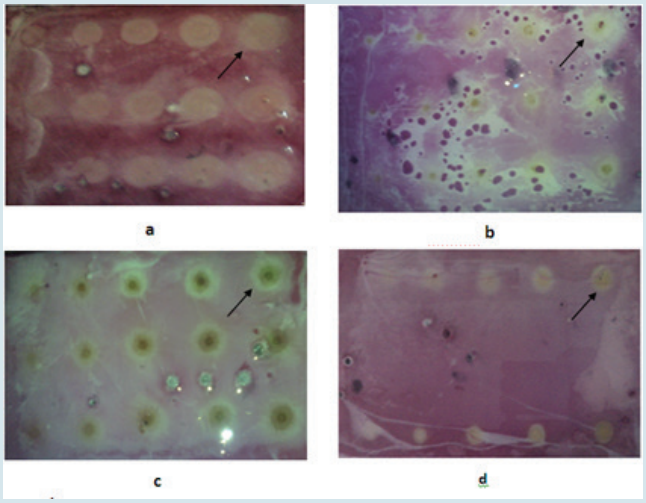

Figure 1: Screening of antilithiatic activity of selected medicinal plants using Dot blot assay a) *B. pinnatum* b) *T. terrestris* c) *P. niruri* d) *A. indicum.*

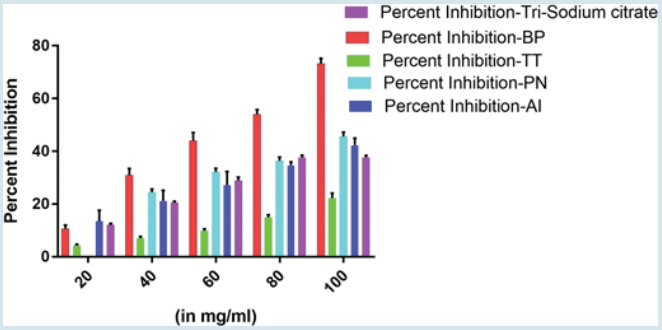

Figure 2: Increase in crystal Percent inhibition with increasing concentrations of selected plant extracts. A: *B. pinnatum*, B: *T. terrestris*, C: *P. niruri* and D: *A. indicum*. E. Tri-sodium citrate. Error bars represent ±SEM (n=3).

Table 1: Percent Inhibition (PI) of CaOx crystals using Dot blot assay technique

Concentration (mg/ml)	*B. pinnatum*		*T. terrestris*		*P. niruri*		*A. indicum*		*Tri-sodium citrate*	
	PI	±SEM	PI	±SEM	PI	±SEM	PI	±SEM	PI	±SEM
20	10.66	1.35	4.21	0.466	0.000	0.000	13.5	4.16	12.1	0.5
40	30.83	2.61	7.05	0.65	24.45	1.166	21.16	4.0	20.5	0.5
60	44	3.13	9.83	0.733	32.13	1.35	27.16	5.16	28.83	1.33
80	54	1.78	14.9	0.9	36.35	1.43	34.66	1.33	37.66	0.83
100	73.16	2.05	22.18	1.95	45.6	1.60	42.33	2.66	37.66	0.75
IC_{50} (mg/ml)	70.163		235.17		103.34		122.77		126.49	
Y-equation	Y = 0.740x - 1.921		Y = 0.219x - 1.503		Y = 0.515x - 3.224		Y = 0.355x + 6.414		Y = 0.341x + 6.866	
R^2	0.986		0.952		0.891		0.998		0.943	

$$\% \text{ Percent inhibition} = (As/Ac) \times 100$$

As = Area of zone of inhibition on TLC bioautographic plate; Ac = Maximum diffusing area for negative control [Ac was calculated as 6 cm^2 for these Technique which is constant]

RESULTS

Medicinal plants are playing the key role in treatment of kidney stones and to screen the antilithiatic potential can be important steps in drug discovery. The proposed Dot Blot technique provides such an opportunity to study the potential of antilithiatic drugs. The significant qualitative and quantitative data can be obtained by measuring the area of the zone of inhibition, which provides the opportunity to compare 10 samples on a single plate. In this study, aqueous extracts were used to screen antilithiatic activity by dot blot assay technique.

The inhibitory areas are visible as white circular patches surrounded by red/pink area were statistically analyzed using data analysis software Past 3. The mean percent inhibition values were recorded in triplicate, and data was statistically studied for ±SEM. The zones of inhibition area for all four plants were calculated, using the repressive area PI values for all four therapeutic plants extracts and tri- sodium citrate (positive control) as generated by Graph Pad PRIZM software (Figure 2).

The assay showed the dose-dependent inhibition of CaOx inhibition. The results confirms, *B. pinnatum* at 100 mg/ml possess the highest antilithiatic potential with higher PI (73.162.05 cm^2) as compare to rest of the three plants, i.e. *T. terrestris* (22.18 1.95 cm^2), *A. indicum* (42.33 2.66 cm^2),*P. niruri* (45.6 1.60 cm^2)and Tri-sodium citrate (37.660.75 cm^2).

The IC_{50} value of all experimental plant extracts along with positive control was calculated by Y-equation as shown in Table 1. The value suggests that minimum IC_{50} value of *B. pinnatum* 70.163 mg/ml can be the best antilithiatic drugs, followed by *P. niruri* 103.34 mg/ml and *A. indicum* 122.77 mg/ml. The positive control Tri-sodium citrate seems to be slightly more potential as compared as it showed IC_{50} value 126.49 mg/ml followed by *T. terrestris* 235.17 mg/ml. The results also confirm that presence of water extractable antilithiatic drug is higher in *B. pinnatum* as compared to *T. terrestris*.

DISCUSSION

In the present study, the experimental extracts were prepared in water, which is universally accepted solvent and is considered to be safe for human use. But as reported the major drug valued bioactive components, are aromatic or saturated organic compounds in nature are usually extracted in ethanol or methanol.[18] Thus the potential of such a medicinal plant can be improved and modified with stringent solvent extraction procedures.[19] As extract with higher saponins and terpenoid content, results into potent antimicrobial activity due to the less solubility of its active ingrediaents.[20]

As in this assay thin layer chromatography (TLC) is used, which permits the separation of plant extract or mixture of metabolites with little expense and higher effectiveness. Silica gel can hold the plant drug compounds without damaging its properties supposed to be used in various bioassays.[21-22] As a result, the proposed modified bioassay can be the best system for the screening of antilithiatic potential of plants as well as chemically synthesized drugs.

The proposed method is similar to the bioautographic method which is based on agar diffusion methods. The only difference is that the active metabolites were identified assuming diffusion into the agar over layered, where as in Dot blot method, diffusion is via silicagel.[23] According to earlier reports, the Dot Blot methods were available for antibacterial [24-25] and antifungal studies,[26-27] but the proposed modified method gives the opportunity for antilithiatic studies.

CONCLUSION

In the present study, it was concluded that the aqueous extract of *B. pinnatum* possesses the higher antilithiatic potential with lowest IC_{50} value. The results reconfirm and validate the used of these plants in Ayurvedic preparations to treat kidney stones.

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CONFLICT OF INTEREST

The author has no conflict of interest.

ABBREVIATIONS

PI: Percent inhibition; **CaOx:** Calcium Oxalate; **cm:** centimeter; **Mg/ml:** Milligram/milliliter; **IC_{50}:** Percent inhibition upto 50%; **SEM**: Standard Error Mean; **Min**: Minutes; µL: Microliter.

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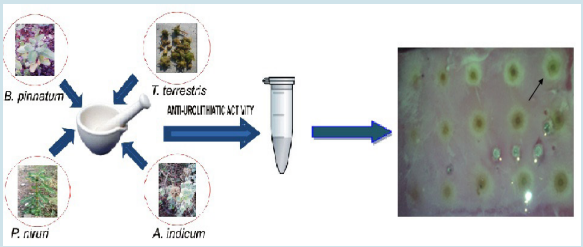
PICTORIAL ABSTRACT



SUMMARY

- Present method is rapid and cost effective method for detection of antilithiatic activity of medicinal plants.
- This method provides qualitative as well as quantitative data based on inhibition zone. TLC plate were prepared and overlaid by 0.8% agar gel in 0.2M CaCl2.
- Samples were loaded and the plates were deep in 0.2M ammonium oxalate followed by alizarin red S.
- The clear white zone indicates the inhibition of formation of Calcium oxalate crystals.
- Results obtained were statistically validated

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