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Research Article

Antiurolithiatic Activity of Natural Constituents Isolated from *Aerva lanata* Flowers

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ABSTRACT

A well-known traditional herb *Aerva lanata,* broadly used in India for treatment of different ailments such as urolithiasis. *Pashanabheda* is used as antiurolithiatic in *Ayurveda*. In the present study, flowers of *A. lanata* were selected for isolation of active constituents and screening for *in vitro* antiurolithiatic potentials, as literature supports that flowers have the highest quantity of natural components when compared with the other parts. Hydroalcoholic (80%-water, 20%-alcohol) extract of *A. lanata* flowers was prepared and fractionation with different organic solvents.. The two fractions (ethyl acetate and n-butanol) were subjected to isolation of active constituents using column chromatography technique, followed by purification of the isolated constituents by preparative high performance thin layer chromatography (HPTLC)and then the individual components were characterized by IR spectrophotometery. Finally, *in vitro* antiurolithiatic activity was screened by nucleation and aggregation assay. In the aggregation assay, gradual decrease in the calcium oxalate (CaOx) crystal nucleation as well as growth was observed by light microscopy. The findings of the nucleation assay indicate that phytoconstituents inhibited the crystallization of CaOx in solution. The size and the number of calcium oxalate crystals decreased with increasing concentration of the phytoconstituents. The increasing concentrations of Quercetin and betulin (100, 200, 300, 400 and 500 µg/mL) inhibited the CaOx crystal growth. The isolated quercetin and betulin from *A. lanata* have shown antiurolithiatic effect by significantly reducing the CaOx crystal growth.

Introduction

Pashanabheda (stone breaking) plants are a group of medicinal plant which is used in Indian traditional medicinal system by *ayurvedic* practitioners as antiurolithiatic drugs. Traditionally *A. lanata* (L) is also known as *Pashanabheda*, belonging to the family Amaranthaceae, used for various medicinal uses including both antiurolithiatic and diuretic activities.[1-4] Kidney stones are potential risk factor for chronic kidney disease. The impact of different urinary stone components on renal function is unknown. Patients with urolithiasis have decreased renal function, and the impact of renal function varies depending on the stone components.

Nephrolithiasis or renal stone disease remains a significant health problem in the adult population, with serious medical consequences, throughout a patient's lifetime. The worldwide incidence of urolithiasis is quite high, and more than 80% of urinary calculi are calcium oxalate stones alone or calcium oxalate mixed with calcium phosphate.[5-7] The present day medical management of nephrolithiasis is either costly or not without side-effects. Invasive procedures for the treatment of nephrolithiasis

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may cause serious complications and also impose a great load of costs on the healthcare system. Kidney stones are a potential risk factor for chronic kidney disease.[8, 9]

The traditional medicine system of India is a rich source of valuable medicinal plant but there is no scientific data reported to establish the activity of these plants. Hence these plant parts need to be evaluated, based on their biological efficacy and chemical constituents for drug development.[10-14] So, we have selected *A. lanata* flowersof India for the present study. The flowers were subjected to bioactivity guided isolation and screening for antiurolithiatic activity to investigate and justify the traditional claim.

The reported phytochemical constituents present in *A. lanata* are responsible for various biological activities. These constituents include alkaloids (ervine, methylervine, ervoside, aervoside, ervolanine, and aervolanine), flavonoids (kaempferol, quercetin, isorhamnetin, persinol, persinosides A and B), methyl grevillate, lupeol, lupeol acetate benzoic acid, b-sitosteryl acetate and tannic acid.[15-16]

Although *A. lanata* is used traditionally by ayurvedic physicians, however, there is no report on the antiurolithiatic potentials by any of the active constituents isolated from *A. lanata* (L).[17-20] flowers. Hence, this *A. lanata* (L) flowers were selected to isolate and develop new lead molecules for treating urolithiasis.

Materials and Methods

Collection and Authentication of Plant Part

The flowers of *A. lanata* were collected from Medchal district, Hyderabad, Telangana. The plant was authenticated by Dr. K. Madhava Chetty, Department of Botany, Osmania University

Physico-Chemical Characterization of *A. lanata*

Percentage of moisture in the dry powder is calculated using the formula-

(LoD) (%) = [Loss in weight Moisture/Weight of powder(g)] × 100

The calculated percentage of moisturewas compared with the pharmacopoeial range.

Ash value percentage was calculated from the dry powder using the formula-

Ash value = [Weight of total of ash/Weight of powder (g)] × 100

The calculated percentage ash value (total ash, water soluble ash and acid insoluble ash) was calculated with the pharmacopoeial range.

Percentage Yield (Extractive Values) for he Fractions

A total of 200 g of dried powder was taken for extraction and the total weight of hydro alcohol extract obtained (dried).

Extraction and Fractionation

The flowers of *A. lanata* were shade dried and then it was grounded to a coarse powder. The powdered dried material was used for the extraction using hydroalcoholic (80%-water, 20%-alcohol) and followed by fractionation with different organic solvents such as dichloromethane (fraction I), ethyl acetate (fraction II), n-butanol (fraction III) to separate different groups of polar compounds like flavonoids, triterpenoids and saponins following the technique of liquid- liquid separation. As polar compounds would come in polar solvents, these two fractions (ethyl acetate fraction and n-butanol fraction), which were found to be rich with flavonoids and triterpenoids were subjected to isolation, characterization and *in vitro* analysis for antiurolithiatic potential.[13-19]

Qualitative Phytochemical Tests

Preparation of Test Solution

The extract was refluxed with alcoholic solution of potassium hydroxide till complete saponification. The saponified matter extracted with water and unsaponifiable matter extracted with diethyl ether. The ethereal extract was evaporated and the residue (unsaponifiable matter) was subjected to test for steroids, carbohydrates, proteins, flavonoids, glycosides, tannins, phenolic compounds, organic acids, alkaloids, carbohydrates and proteins by dissolving the residue in the chloroform.[21-23]

Isolation and Characterization

Ethyl acetate and n-butanol fractions were subjected to isolation of active constituents using column chromatography technique, followed by purification of the isolated components. The fraction (II) and fraction (III) were loaded separately in the glass column by dissolving in chloroform (5 g in 500 mL each fraction) and separation was carried out. The fraction II and III were purified by HPTLC technique, followed by the characterization. The individual components were characterized by IR spectrophotometery.[24,25]

Column Chromatographic Separation

A total of 175 g of silica gel (60–120 mesh) was activated in hot air oven at 110 ± 1°C for one hour. The glass wool was fixed at the bottom of column. The slurry of silica gel was made in the chloroform and loaded in the glass column in small portions with gentle tapping after each addition in order to ensure uniform packing. The small quantity of solvent system was allowed to remain on the top of the column to avoid the drying or cracking of the column. The air bubbles present in the column were removed by gentle tapping to get a uniform bed of adsorbent. The sample was added as a slurry by dissolving in non polar solvent like chloroform on top of the column.different solvents were added in varying proportions above the sample bed to continuously to ensure isolation of active constituents at the bottom.[26-29]

Thin Layer Chromatography (TLC)

In this technique, the Silica gel-GF254 was used as an adsorbent and plates were prepared by spreading technique, then air dried and activated for one hour at 110±5°C and used. Separation of components from fractions was carried out using different mobile phases such as methanol, hexane, ethyl acetate, chloroform and water to confirm thepresence of different compounds in the fractions.[28]

High Performance Thin Layer Chromatography analysis of fractions (HPTLC)

HPTLC analysis of fractions was carried out on CAMAG instrument. Sample solutions of fractions II and III were prepared by dissolving 50 mg of sample in 2 mL of n-hexane and chloroform (1:1). Fractions applied separately on HPTLC plates. Six bands of sample were applied using Linomat V sample applicator on plates (E. Merck, size 10 × 10 cm) with the help of Win CATS software programme.[29]

Plates were scanned at speed of 20 mm/sec using TLC Scanner four at different wavelengths viz. 200, 254, 366 and 430 nm. The chromatograms and R_f data were collected.

IR Spectroscopic Analysis

IR spectra of fractions were recorded on Shimadzu IR Affinity-1 (FTIR). The spectra were interpreted for presence of functional groups like hydroxy, keto, epoxy, cyclopropane and cis/trans configuration.

In-Vitro Antiurolithiatic Activity

Aggregation Assay

The rate of aggregation of the calcium oxalate crystals was determined by a spectrophotometric assay and by comparing the microscopic Figs of the CaOx crystals. The calcium oxalate monohydrate (COM) crystals were prepared by mixing both the calcium chloride and sodium oxalate solution of 50 mm each. Both solutions were then equilibrated. The solutions were then cooled to 37°C and then evaporated. The COM crystals were then dissolved with 0.5ml of 0.05mM Tris buffer and 0.5ml of 0.15mM NaCl solution at pH 6.5 to a final concentration of 1 mg/mL. Absorbance at 620 nm recorded. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract against control. CaOx crystals, were observed under light microscope (100X)[30, 31].

Nucleation Assay (Turbidity Method)

A spectrophotometric assay determined the inhibitory activity of the extracts on the nucleation of calcium oxalate crystals. Crystallization was initiated by adding 100µl of 4 mM calcium chloride and 100µl of 50 mM sodium oxalate solutions to 0.5ml of human normal urine, both prepared in a buffer containing 0.5ml of 0.05 mM Tris buffer and 0.5ml of 0.15mM NaCl solution at pH 6.5 and 37°C and adjusted to volume by adding 1.5mL of distilled water. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus became optically detectable) in the presence of the extract and that of the control with no extract. The optical density (OD) was recorded at 620nm, and the percentage inhibition calculated as (1-OD (experimental)/ OD (control))×100.[30,31]

Results and Discussion

Ash value percentage was calculated from the dry powder of the flower, the calculated percentage ash value (total ash, water soluble ash and acid insoluble ash) was found to be < 5%. Hence the ash value is in the pharmacopoeial range.[32]

Percentage of moisture in the dry powder was calculated. The calculated percentage of moisture was found to be < 10%. Hence the moisture is in the pharmacopoeial range.[32]

Percentage yield (Extractive values) for the fractions- Total weight of hydro alcohol extract obtained (dried) 12 g (6%) and for fractionation 12 g dried extract was taken.

Weight of DCM fraction (dried) -2.5g. Percentage yield is 20.8%.

Weight of ethyl acetate fraction (dried)-3.36 g and percentage yield is 28%.

Weight of n-butanol fraction (dried)-3.61g and percentage yield is 30.1%.

Weight of Aqueous extract-2.5g and percentage yield is 20.83%

Phytochemical Investigations by Qualitative Analysis of Extract and Fractions

Phytochemical investigation showed the presence of primary and secondary plant metabolites in flower extract and fractions. Fraction II and III (ethyl acetate and n-butanol) revealed the presence of steroids, carbohydrates, proteins, flavonoids, saponin glycosides,and phenolic compounds and DCM fraction (I) revealed the presence of alkaloids, carbohydrates, proteins and absence of steroids and flavonoids (Table 1).

Chromatographic Analysis of The Fractions

HPTLC chromatogram of n-butanol fraction at 366 nm has showed presence of four peaks with maximum R_f value 0.51, 0.53, 0.89, 0.94. This indicates the presence of probably 4 compounds in the fraction. The chromatograms and R_f data were collected. HPTLC chromatogram of ethyl acetate fraction at 366nm showed presence of two peaks with max R_f value 0.54, 0.92 which shows the presence of probably 2 compounds.[33](Table 2; Fig. 1)

Infrared Radiation Spectral Analysis of Fractions

Infra Red Radiation analysis of fractions II and III (based on phytochemical study results) was carried out using FT-IR Shimadzu IR Affinity-1 (FT IR) spectrophotometer using KBr pellets technique (Table 3 and Fig. 2).

Table 1: Various extracts subjected for various phytochemical analysis

	Different Solvents					
Phytochemicals	*Ethanol*	*Methanol*	*Chloroform*	*Hexane*	*Ethyl acetate*	*Water*
Flavonoids	-	+	+	+	-	-
Alkaloids		-	-	-	-	-
Phenol compounds	-	-	-	-	+	+
Tannins	+	-	-	-	-	-
Steroids	-	-	-	-	-	-
Saponins	-	+	+	+	+	+
Terpenoids	-	-	-	-	-	-
Glucosides	-	-	-	+	+	-

+: The phytochemical is present in the extract; -: The phytochemical is absentin the extract

Table 2: Thin layer chromatography of fractions

Fraction	*Mobile phase*	*Obsrvation*	*Groups*	*Rf*
Fraction I	Methanol: hexane: ethyl acetate: water (3.5:2:4:2)Ethyl acetate: Methanol:Water(3:1.5:1)	Two spots (Yellow)	Flavanoids	0.7
			Terpenoids	0.45
Fraction II	Choroform: methanol:water (6:3:0.5) Ethyl acetate: Methanol: Water(3:1.5:1)	Two spots (pink)	Saponins	0.35
			Terponoids	0.6
Fraction III	Ethyl acetate: Methanol: Water (3:1.5:1)	Single spot (Pink)	Terpinoids	0.3

Spraying reagents: Iodine and vanillin (5%)

Table 3: Results of Infrared Radiation Spectral analysis of fractions

Fraction	*Groups/bonds present*
Fraction II	Alc OH group Alkane C-H structure carbonyl group Alkene C=C bond
Fraction III	Alkane C-H structure carbonyl group Alkene C=C structure

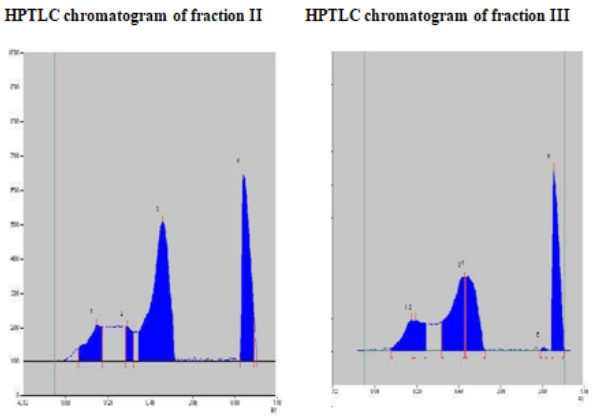


Fig.1: HPTLC chromatograms of Fraction II and III

Aggregation Assay

Calcium oxalate crystals begins to grow; aggregate with other crystals and retained in the kidney. This is aggregation process which causes renal injury. The extracted components of *A. lanata* demonstrated slightly better results compared to cystone standard solution

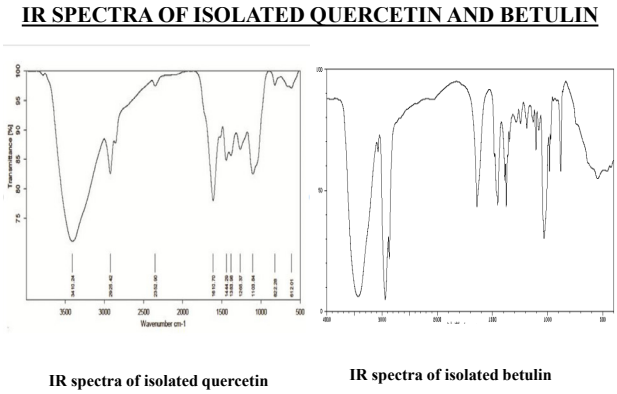


Fig.2: Charaterization of quercetin and betulin by IR

in inhibiting the promoted formation of COD crystals[33] (Figs 3 and 4).

Nucleation Assay

Extracted components of *A. lanata* flowers inhibited the crystallization by inhibiting nucleation of calcium oxalate through disintegrating into smaller particles with increasing concentrations of the fraction. The nucleation assay results confirmed that the extract contained nucleation-preventing agents[31-33](Fig. 5).

Discussion

In kidney stones formation, calcium oxalate and calcium phosphate or other chemicals in the urine form crystals on kidneys' inner surfaces. This stage is called as initial

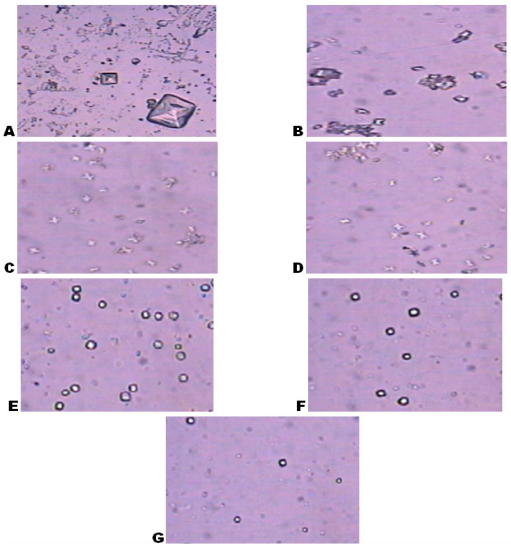


Fig.3: CaOx crystals, observed under light microscope(100X), formed in the metastable solution of CaOx in the absence (A) which are larger and the presence of saponin rich fraction of quercetin (B)10 mg/mL, (C)20 mg/mL, (D)40 mg/ml, (E) 60 mg/ml, (F)80 mg/mL and(G)100 mg/mL gradually decreases the CaOx crystals nucleation as well as growth.

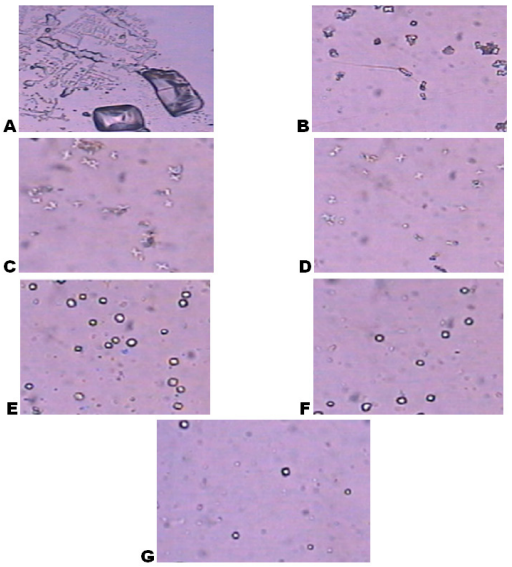
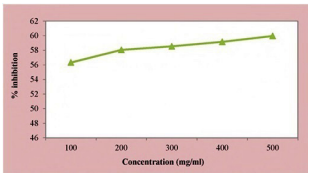
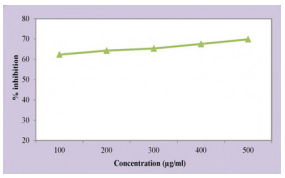


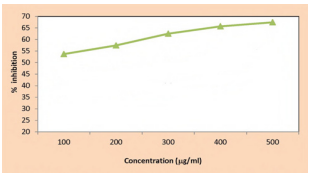
Fig.4: CaOx crystals, observed under light microscope(100X), formed in the metastable solution of CaOx in the absence (A) which are larger and the presence of saponin rich fraction of betulin (B)10 mg/mL, (C)20 mg/mL, (D)40 mg/ml, (E)60 mg/mL, (F)80 mg/mL and(G)100 mg/mL gradually decreases the CaOx crystals nucleation as well as growth.



Effect of Cystone on CaOx crystal nucleation



Effect of quercetin on CaOx crystal nucleation



Effect of Betulin on CaOx crystal nucleation

Fig. 5: Effect of Cystone, quercetin and Betulin on CaOx crystal nucleation

mineral phase formation. Over time, crystals may combine to form a small, hard mass called stones and stage is referred to as crystal growth. Calcium oxalate stones have been classified in two types i.e., calcium oxalate monohydrate stones (COM) and calcium oxalate dihydrate stones (COD). Kidney stones are reportedly affecting mankind since long time and have been one of the causes for renal failure.[12,18] As there is no single effective drug available for urolithiasis today, surgery is considered to be the best option especially when other alternatives fail. However it is expensive and not affordable for common man. Hence the natural drugs are considered to be next alternative.

Calcium oxalate crystals begin to grow; aggregate with other crystals and retained in the kidney. This is aggregation process which causes renal injury. The extracted components of *A. lanata* flowers demonstrated slightly better compared to cystone standard solution to inhibit promoted the formation of COD crystals. COM has a stronger affinity with cell membranes; it may lead to become higher potential risk for renal calculi formation.[13,17] This may be due to high content of saponins present *A. lanata*. The flowers have several polyphenolics, e.g; alkaloids, saponins, phenolics, flavonoids, and other phytoconstituents.

Saponins are well known to have anticrystallization properties by disaggregating the suspension of mucoproteins as crystallization promoters. The isolated components proved to inhibit initial mineral phase formation of calcium phosphate and growth of COM crystals.

Urine supersaturation attributes to calcium oxalate particles crystallization within the urinary tract.[12,17,20] This is nucleation process where stone forming salts begins to unite into clusters with addition of new constituents.

As *in vitro* crystallization study was performed, since nucleation is an important first step for crystals initiation, then grow and form aggregates. Extracted A. lanata flowers' components inhibited the crystallization by inhibiting nucleation of calcium oxalate by disintegrating into smaller particles with increasing concentrations of the fraction. From the results of the nucleation assay confirmed that the extract contained nucleation-preventing agents.

The present investigation will be supportive as additional information to the scientific evidences regarding *in-vitro* studies. Since the mechanism of anti-urolithiatic activity in the extract is unknown to date, correlation between *in-vitro* and *in-vivo* studies should be further

investigated to reveal the phytochemicals of the extract which are responsible for dissolving or disintegrating renal calculi and to know better understanding in the molecular mechanism of litholysis.[27,28]

This study examined the antiurolithiatic efficacy of the two isolated constituents of *A. lanata* flowers. Based on the bioactivity guided fractionation followed by isolation, these active constituents were characterized as quercetin and botulin.[30-32] These isolated constituents were proved to have potent *in vitro* anti-urolithiatic activity.

Conclusion

A. lanata flower extracted phytoconstituents, Quercetin and betulin exhibited significant *in-vitro* anti-urolithiatic activity. The present study conclusively demonstrates that *A. lanata* flowers is a good source of various phytochemicals like flavanoids, triterpenoids and saponins. This study evaluates that antiurolithiatic activity of extracts of *A. lanata* flower is significant. It has given primary evidence for *A. lanata* flower as the plants which possess lithotriptic property. *A. lanata* flower used in the above study also showed good activity when it was compared with the standard drug cystone.

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