Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Suppression of the Mechanisms of Stone Formation by a Flavonoid-enriched Ethyl Acetate Fraction of Aerial and Underground Parts of *Aerva lanata* (Linn.) Juss. Ex Schult

Bitasta Mandal[1], Swati Madan[1], Sayeed Ahmad[2], Sultan Zahiruddin[3]

[1]Department of Pharmacognosy, Amity Institute of Pharmacy, Amity University, Noida, Uttar Pradesh, [2]Department of Pharmacognosy and Phytochemistry, Jamia Hamdard, Hamdard University, [3]Bioactive Natural Product Laboratory, School of Pharmaceutical Education and Research, Jamia Hamdard, Hamdard University, New Delhi, India

Submitted: 06-04-2018 Revised: 31-05-2018 Published: 17-01-2019

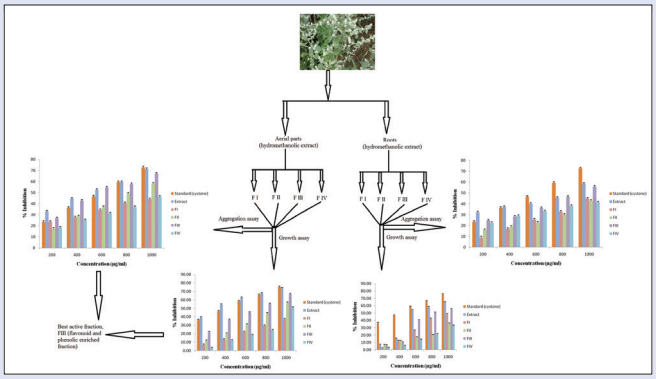
ABSTRACT

Background: *Aerva lanata* (Linn.) Juss. ex Schult.(AL), belonging to family Amaranthaceae, is a popular medicinal herb known for its immense antiurolithic properties. Several researchers have reported the antilithiatic efficaciousness of the extract. However, there does not exist any evidence regarding the bioassay-guided fractionation of the same as a route to identification of potential antiurolithic phytoconstituents. **Objective:** The present study aims to evaluate the antiurolithic competence of different extract based phytoconstituent enriched fractions (Fr) of the aerial parts and roots of AL under *in vitro* conditions to screen out the Fr possessing the best activity. **Materials and Methods:** Different Fr from the extracts of aerial parts and roots were prepared by solvent-solvent partitioning and were subjected to preliminary phytochemical screening to affirm the presence of intended phytometabolites. The Fr were analyzed for their antilithiatic efficacy by *in vitro* aggregation and growth assay to assess their ability to counteract calcium oxalate (CaOx) aggregation and growth. The phytochemical evaluation was also performed by high-performance thin layer chromatography (HPTLC). **Results:** Preliminary phytochemical screening ascertained the presence of expected phytochemicals in the Fr. Antiurolithic assays demonstrated that the flavonoid and phenolic enriched Fr III derived from the extract of aerial parts exhibited the maximum hindrance to CaOx aggregation and growth at 1000 µg/ml with a percentage inhibition of 67.14% ± 1.84% and 66.66% ± 1.65%, respectively. HPTLC analysis revealed the presence of flavonoids quercetin, kaempferol, and myricetin in the same. **Conclusion:** The antiurolithic ability of AL on the overall process of stone formation might be attributed to the presence of potent phenolic and flavonoid compounds in the aerial parts which may act as a source for isolation of powerful antiurolithic leads from the same.

Key words: *Aerva lanata,* antiurolithic, calcium oxalate, flavonoids, fraction, phenolics

SUMMARY

- The phenolic and flavonoid enriched fraction III from the aerial parts of *Aerva lanata* demonstrated maximum obstruction to *in vitro* calcium oxalate aggregation and growth
- Phenolics and flavonoids from the aerial parts might be accountable for the antiurolithic potency of *Aerva lanata*.



Abbreviations used: AL: *Aerva lanata*; Fr: Fraction(s); CaOx: Calcium oxalate; HPLTC: High-performance thin-layer chromatography

Correspondence:

Dr. Swati Madan,
Department of Pharmacognosy, Amity Institute of Pharmacy, Amity University, Noida,
Uttar Pradesh - 201 313, India.
E-mail: smadan3@amity.edu

DOI: 10.4103/pm.pm_140_18

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Urolithiasis is an urinary infirmity associated with the development of stones anywhere in the urinary tract which may include kidneys (nephrolithiasis), ureters (ureterolithiasis), or bladder (cystolithiasis).[1] About 5% of the population is afflicted with the incidence of urinary calculi and is associated with approximately 8%–10% possibility of passing a stone throughout their lifetime.[2] The formation of calculi is linked to several factors such as gender with males afflicted more than females, age, geography, genetics, eating habits, environmental conditions, climate, and season.[3,4] Stone formation may be related to meager drainage of urine, diet with surplus content of oxalates and calcium, insufficiency of Vitamin A, overload of Vitamin D, metabolism-related abnormalities such as hyperthyroidism, cystinuria, gout, intestinal malfunction, the presence of foreign bodies in the urinary system, and bacterial infections.[5]

Several surgical advancements and allopathic medications exist in the scenario to combat the development of stones but intend to act on only one facet of urolithiatic pathophysiology, thereby the underlying cause of calculi is not erased resulting in the repeated episodes of stones.[6] However, nature has bestowed us with a pool of medicinal herbs which

Cite this article as: Mandal B, Madan S, Ahmad S, Zahiruddin S. Suppression of the mechanisms of stone formation by a flavonoid-enriched ethyl acetate fraction of aerial and underground parts of *Aerva lanata* (Linn.) Juss. ex Schult. Phcog Mag 2018;14:S630-7.

have been of immense significance since antiquity on account of the presence of potent phytotherapeutic agents as well as better safety, efficacy, cultural acceptability, and lesser side effects over their conventional counterparts.[7] In this direction, Ayurveda lists a number of herbs under the name Pashanbheda having the ability of rupturing and disintegrating the stones.[8]

Aerva lanata (Linn.) Juss. ex Schult.(AL), belonging to family Amaranthaceae, is one of such potent antiurolithic herb well known as Gorakhabooti or Kapurijadi and is a prostrate dioecious herb flourishing up to a summit of 900 m being innate to Asia, Africa, and Australia.[9] It possesses a multitude of phytochemicals such as canthin-6-one alkaloids, β-carboline alkaloids, flavonoids such as kaempferol, quercetin and isorhamnetin, β-sitosteryl acetate, phenolic acids such as syringic acid and vanillic acid.[10] An extensive pharmacological spectrum of AL prevails in the literature including an excellent anti-urolithic efficacy of the plant-derived extract.[11-14] In addition, there exist reports on various solvent based extracts exhibiting antilithiatic activity,[15,16] but an extract is usually a mixture of several phytocomponents which does not guarantee the biological efficacy of a particular class of phytochemicals.

However, a comprehensive and methodical bioactivity-guided fractionation of the aerial parts and roots of AL has not been reported earlier which involves extraction of the plant material followed by step by step separation of those extracted phytoconstituents in the form of fractions (Fr) according to the variation in their physicochemical nature and analyzing them for their biological efficaciousness *in vitro* followed by *in vivo* studies of the best active Fr. Therefore, the current investigation is an effort to perform the same which aims at providing an appropriate route to the isolation of potent antiurolithic components from this medicinal herb. We have reported in our earlier studies about the effect of various extract based Fr of AL on *in vitro* calcium oxalate (CaOx) nucleation.[17] However, to confirm their antiurolithic ability further, we have evaluated the various Fr for their efficacy on CaOx aggregation and growth *in vitro* in the current study. As calculi development proceeds through the stages of nucleation, aggregation, and growth of stone forming constituents, any pharmaceutical entity capable of hindering all these stages will have the potential to counteract the overall process of stone formation. This will also assist in finding out the botanical part having greater anti-urolithic activity compared to the other. It also aims to identify and quantify the phytoconstituents present in the best active Fr by high-performance thin-layer chromatography (HPTLC). In addition, it objects to carry out the preliminary phytochemical screening of various extract derived Fr for confirming the presence of expected phytoconstituents.

MATERIALS AND METHODS

Chemicals and reagents

Calcium chloride dihydrate was procured from Central Drug House Pvt. Ltd., (New Delhi, India) and sodium oxalate was obtained from Qualigens Fine Chemicals (Mumbai, India). Sodium chloride was obtained from Thermo Fisher Scientific India Pvt. Ltd., (Mumbai, India) and Tris was procured from Ubichem Plc. (Hampshire, UK). Glacial acetic acid was purchased from HiMedia Laboratories Pvt. Ltd., (Mumbai, India) and sodium acetate trihydrate was purchased from Qualikems Fine Chem Pvt. Ltd., (New Delhi, India). Cystone was purchased from Himalaya Drug Company. Ethyl acetate and toluene were obtained from Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India). Methanol was obtained from Rankem (Gujarat, India) and formic acid was procured from Central Drug House Pvt. Ltd. (New Delhi, India). Standard flavonoids kaempferol and myricetin were procured from Cayman Chemical (Michigan, USA) and quercetin and rutin were obtained from Sigma-Aldrich (New Delhi, India). All other solvents and other chemicals used were of analytical grade.

Plant material

Dried whole plant of AL was collected during March 2015 from Balaji Traders, Tamil Nadu, India. The authenticity of the material was certified by Dr. Sunita Garg, Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi. A voucher specimen (NISCAIR/RHMD/Consult/2014/2792/171) was submitted at the Herbarium of National Institute of Science Communication and Information Resources, New Delhi, for future reference. Later, the roots were detached from the aerial parts, sun-dried and grinded into a fine powder.

Preparation of extract

Extracts from aerial parts and roots were prepared by overnight maceration of approximately 200 g of the powdered aerial parts and roots separately in a solvent mixture of water:methanol (2:1) after which they were subjected to refluxing for about 5 h. This was followed by evaporation of the refluxed blends to one-fourth of their original volume.

Preparation of fractions

The extracts obtained each from aerial parts and roots were segregated into three parts of which one-fourth portion was regarded as the mother extract which was dried <60°C. Out of the remaining volume, one-half was rendered acidic and subjected to partitioning with equal volume of ether followed by segregating the ether layer and evaporating it to dryness giving rise to Fr I which was expected to be composed of various nonpolar compounds including fatty material, steroidal aglycones, terpenoids, and coloring matter. The aqueous portion left from the partitioning was made alkaline and fractionated with equal volume of chloroform thrice followed by its separation and drying leading to Fr II supposed to contain alkaloids. The alkaline aqueous part left behind was made acidic and subjected to refluxing for 2 h. Thereafter, it was partitioned with ethyl acetate thrice in equal volume and evaporated to dryness generating Fr III expected to be rich in flavonoid and phenolic aglycones. The one-fourth volume of the extract which was left was fractionated with equal volume of ether and the remaining aqueous layer was partitioned with ethyl acetate thrice followed by evaporating it to dryness leading to Fr IV expected to possess glycosides.[18,19]

Preliminary phytochemical screening

To ascertain the presence of various bioactive constituents in the hydromethanolic extract derived Fr of aerial parts and roots, different qualitative phytochemical tests were carried out to ensure the presence of steroids, terpenoids, phenolics, flavonoids, glycosides, and alkaloids.[20,21]

Aggregation assay

CaOx crystals were prepared by mixing 50 mMol/l each of calcium chloride and sodium oxalate solutions followed by equilibration on a water bath for 1 h at 60°C followed by cooling to 37°C overnight. The CaOx thus formed was collected and allowed to dry at 37°C. Buffer consisting of Tris 0.05 mol/L and sodium chloride 0.15 mol/L at pH 6.5 was prepared in which a slurry of CaOx crystals at a final concentration of 0.8 mg/ml was made. The study was conducted in the absence (control) and presence of inhibitor (standard/extract/Fr). Stock solutions of standard (Cystone) and samples (extracts and Fr) were used at a concentration of 10 mg/ml. CaOx slurry could incubate in the absence of sample using distilled water as well as with 1 ml of different sample dilutions (200,400, 600, 800, and 1000 µg/ml) at 37°C for 1 h. The percentage inhibition of aggregation was computed from the following formula by comparing the turbidity observed in the presence of inhibitor with that observed in the control set: percentage inhibition = (1-[turbidity of sample/turbidity of control]) × 100.[22]

Growth assay

50 mMol/L of calcium chloride and sodium oxalate solutions were combined and equilibrated for 1 h in a water bath at 60°C and allowed to cool followed by filtration. The resulting crystals were washed with water and dried for 24 h at 50°C. 50 mMol/L sodium acetate buffer with pH 5.7 was prepared in which a slurry of CaOx was constituted with a concentration of 1.5 mg/ml. 4 mMol/L solutions of calcium chloride and sodium oxalate were made in a buffer composed of 10 mMol/L Tris and 90 mMol/L sodium chloride with pH 7.2. The experiment was carried out in the absence (control) and presence of sample (standard/extract/Fr). 1 ml of calcium chloride was taken to which 30 µl of CaOx slurry was added. Thereafter, 1 ml of distilled water was added to the control. However, in the sample set 1 ml of various sample dilutions were added (200, 400, 600, 800, and 1000 µg/ml) instead of water. This was followed by addition of 1 ml sodium oxalate to all the sets resulting in the immediate consumption of free oxalate ions from the system which was monitored for 10 min for the decrease of absorbance at 214 nm. The percentage inhibitory activity was calculated from the following formula: percentage inhibitory activity = ([reduction rate of free oxalate with the control-reduction rate of free oxalate with sample]/reduction rate of free oxalate with control) × 100.[23]

Phytochemical analysis by high-performance thin-layer chromatography

The identification and quantification of quercetin, kaempferol, and myricetin in the best active Fr III from aerial parts was performed by HPTLC using CAMAG HPTLC system. A sample of hydromethanolic extract of aerial parts was also run as a reference. Standard solutions of flavonoids quercetin, kaempferol, and myricetin were prepared at a concentration of 1 mg/ml in methanol and the samples at 2 mg/ml in methanol respectively. Precoated aluminum sheet (10 cm × 10 cm, Merck, Darmstadt, Germany) with silica gel 60 F_{254} was used on which 2 µl of the standards and 8 µl of the samples were applied in the form of bands of 6 mm length with the help of Linomat 5 applicator. The system was programmed through winCATS software installed with the apparatus. The development of chromatogram was done in CAMAG twin trough TLC chamber using the solvent system toluene: ethyl acetate: formic acid (6:4:0.5, v/v/v).[24] A concentration range of 100–1000 ng/spot for standard solution of quercetin and a range of 25–1250 ng/spot for standard solutions of kaempferol and myricetin were selected and run to establish the calibration plot. The developed chromatogram was scanned using CAMAG TLC Scanner 3 at 254 nm and 366 nm. The peak areas were recorded, and the percentage content of the flavonoids were calculated. The content of flavonoids present were calculated from the equations of calibration plot for various standards, y = mx + c, where, y is the peak area and x is the concentration of the flavonoid present.

Statistical analysis

Three sets of experiments were conducted, and the data were represented as mean ± standard deviation. Significance testing between the groups was performed using one-way analysis of variance followed by Tukey's multiple comparison test. The results were considered statistically significant at $P < 0.05$.

RESULTS

Yield of fractions and preliminary phytochemical screening

The results for percentage yield of Fr and phytochemical tests are presented in Tables 1 and 2. Qualitative chemical tests gave the evidence for the presence of intended phytoconstituents in the prepared Fr from aerial parts and roots namely, steroids, terpenoids, glycosides, alkaloids, phenolic compounds, and flavonoids.

Aggregation assay

In vitro aggregation assay indicated Fr III from the aerial parts, composed of flavonoids and phenolics, to be the most efficacious among various extract derived Fr (67.14 ± 1.84%) at 1000 µg/ml. Although, the flavonoid consisting Fr III from roots showed the highest efficacy (55.98 ± 1.81%) among the other Fr derived from the same, its potency was much lower compared to the one from aerial parts. Moreover, the extract from aerial parts demonstrated comparable potential (71.42% ± 3.25%) in relation to standard (72.85% ± 1.30%) but higher potential as compared to that from roots (58.54% ± 1.10%). Fr I from aerial parts composed of non-polar moieties and Fr IV from roots composed of glycosides showed the least ability to counteract CaOx aggregation with a maximum percentage inhibition of 44.23% ± 0.61% and 41.34% ± 0.25% at 1000 µg/ml, respectively. A significant difference was observed between the standard (cystone), extracts and various Fr derived from the aerial parts and roots ($P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$). The effect was seen in a dose-dependent manner with all the groups. The results for aerial parts and roots are shown in Tables 3 and 4. The graphical representations of the same are presented in Figures 1 and 2.

Growth assay

Assessment of *in vitro* inhibition of various samples on CaOx growth also justified the possible antilithiatic role of flavonoids and phenolics over other phytoconstituents. A dose-dependent increase in growth inhibition was demonstrated by various groups. The standard (Cystone), extracts and various Fr exhibited significant differences between them ($P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$). The percentage inhibitory activity of aerial parts and roots is presented in Tables 5 and 6. The graph of percentage inhibition of growth against concentration for aerial parts and roots is represented in Figures 3 and 4. Fr III composed of phenolic compounds and flavonoids from aerial parts exhibited the highest inhibitory activity among various other Fr (66.66% ± 1.65%) at 1000 µg/ml. Similarly, the flavonoid-rich Fr III derived from roots exhibited maximum inhibition (55.23% ± 0.96%) as compared to other Fr though the activity was much less than that from aerial parts. The extract from aerial parts displayed a remarkable activity (73.81% ± 2.18%) which was comparable to standard (75.26% ± 2.90%) and higher relative to roots (64.44% ± 1.10%) The lowest inhibition was shown by Fr I from aerial parts (37.14% ± 1.91%) containing non-polar compounds and glycoside rich Fr IV from roots (32.69% ± 1.10%) at 1000 µg/ml.

Phytochemical analysis by high performance thin layer chromatography

Phytochemical analysis confirmed the presence of flavonoids quercetin, kaempferol, and myricetin in the best active Fr III from aerial parts and were present in quantities of 0.095, 0.270, and 0.058%w/w, respectively. Standard rutin was also run with other standards at a concentration of 1 mg/ml in methanol but was found

Table 1: Percentage yield of fractions from aerial parts and roots of *Aerva lanata* (Linn.)

	Percentage yield (% w/w)	
Fr	Aerial parts	Roots
F I	28.79	20.24
F II	20.35	18.28
F III	16.00	13.16
F IV	15.55	12.09

FI: Fr I; FII: Fr II; F III: Fr III; F IV: Fr IV. Fr: Fractions

Table 2: Preliminary phytochemical screening of various extract-based Fractions of aerial parts and roots of *Aerva lanata* (Linn.)

Phytoconstituent	Chemical test	Aerial parts				Roots			
		F I	F II	F III	F IV	FI	FII	FIII	FIV
Steroids and terpenoids	Salkowski test	+	-	-	-	+	-	-	-
	Sulphur powder test	+	-	-	-	+	-	-	-
Glycosides	Fehling's test	-	-	-	+	-	-	-	+
Alkaloids	Mayer's test	-	-	-	-	-	-	-	-
	Dragendorff's test	-	-	-	-	-	-	-	-
	Wagner's test	-	-	-	-	-	-	-	-
	Hager's test	-	+	-	-	-	+	-	-
Phenols	Ferric chloride test	-	-	+	-	-	-	+	-
Flavonoids	Shinoda test	-	-	-	-	-	-	-	-
	Zinc-HCl test	-	-	+	-	-	-	-	-
	Alkaline reagent test	-	-	+	-	-	-	+	-

FI: Fr I; FII: Fr II; F III: Fr III; F IV: Fr IV. +: Presence; -: Absence; HCl: Hydrochloric acid

Table 3: Aggregation assay of aerial parts of *Aerva lanata* (Linn.)

Dose (µg/ml)	Percentage inhibition of treatment					
	CYS	Extract	F I	F II	F III	F IV
200	23.63±0.41	33.07±0.44^{c}	23.52±0.66^{g}	17.34±1.16^{b,h}	26.82±3.85^{f}	18.40±0.52^{a,h}
400	36.33±1.24	44.53±2.86^{c}	27.51±1.21^{c,h}	28.53±0.72^{c,h}	42.77±0.89^{b}	25.13±1.66^{d,h}
600	46.61±0.34	52.69±0.97^{d}	34.50±0.73^{d,h}	37.17±0.72^{d,h}	54.70±0.52^{d,e}	31.39±0.50^{d,h}
800	59.49±1.34	59.45±0.72	40.54±1.27^{d,h}	49.32±1.83^{d,h}	57.70±1.06	37.06±1.08^{d,h}
1000	72.85±1.30	71.42±3.25	44.23±0.61^{d,h}	58.21±0.46^{d,h}	67.14±1.84^{a}	46.54±1.44^{d,h}

Values are expressed as mean±SD, n=3. Significant differences between groups is evaluated by one-way ANOVA followed by Tukey's multiple comparison test. [a]Significantly different from standard (P<0.05); [b]Significantly different from standard (P<0.01); [c]Significantly different from standard (P<0.001); [d]Significantly different from standard (P<0.0001); [e]Significantly different from extract (P<0.05); [f]Significantly different from extract (P<0.01); [g]Significantly different from extract (P<0.001); [h]Significantly different from extract (P<0.0001). SD: Standard deviation; Fr: Fraction(s); FI: Fr I; FII: Fr II; F III: Fr III; F IV: Fr IV; ANOVA: Analysis of variance; CYS: Cystone

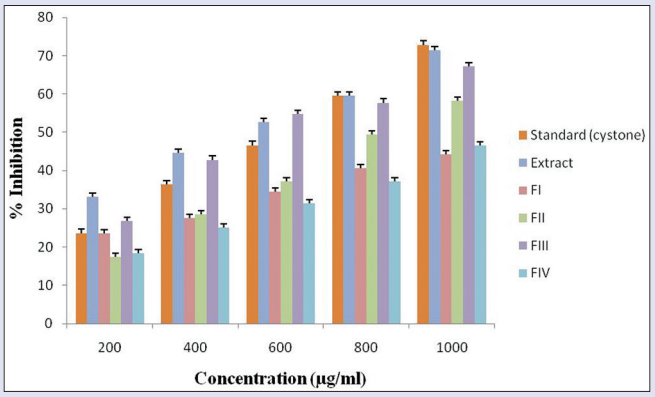


Figure 1: Aggregation assay of extract derived fractions of aerial parts of *Aerva lanata*

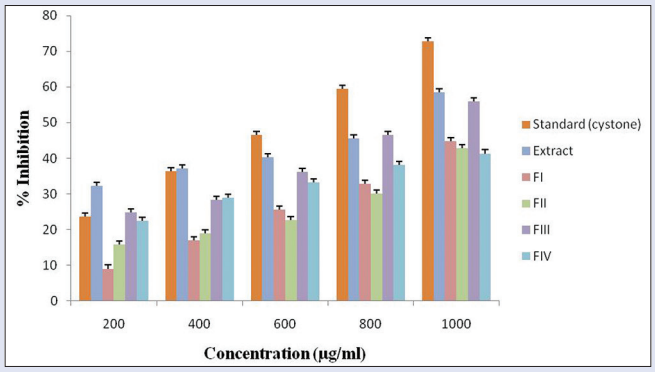


Figure 2: Aggregation assay of extract derived fractions of roots of *Aerva lanata*

to be absent in the samples. The results are represented in Table 7. The image of the developed plate viewed under daylight, 254 nm and 366 nm is presented in Figure 5 and the scanned chromatograms of standard quercetin, kaempferol, and myricetin along with the hydromethanolic extract and best active Fr III from aerial parts is depicted in Figure 6.

DISCUSSION

Phytochemical screening is a mandatory step in the evaluation of botanicals for ensuring the presence of bioactive phytoconstituents which may lead to the discovery and development of novel leads with enhanced pharmacological benefits.[25] The preliminary phytochemical screening of different Fr confirmed that the plant is loaded with phytochemicals which may account for its wide pharmacological spectrum.

The mechanism of stone formation proceeds through urinary saturation followed by supersaturation with stone forming constituents resulting in crystal nucleation and aggregation leading to crystal retention by the urinary epithelium over which the growth of calculi progresses.[26] The initial phase in the conversion from a liquid to a solid phase in the presence of a supersaturated solution is called nucleation which gets initiated with the amalgamation of stone salts in solution into loose clusters which grow in size by the inclusion of new components.[27] Crystal aggregation implies the binding of crystals to one another leading to the development of larger clusters.[28] For crystal aggregation to take place, there must be collision of crystals to form clusters which are driven using diffusion through Brownian motion and sedimentation which plays a vital role in accretion of crystals on walls of renal tubules.[29] Eventually, these clusters develop into crystal embryos followed by their growth into the nucleus of critical size which

Table 4: Aggregation assay of roots of *Aerva lanata* (Linn.)

Dose (µg/ml)	Percentage inhibition of treatment					
	CYS	Extract	F I	F II	F III	F IV
200	23.63±0.41	32.19±3.07^{c}	9.03±1.50^{d,h}	15.76±0.73^{c,h}	24.73±0.66^{f}	22.46±1.93^{g}
400	36.33±1.24	37.06±0.61	17.01±0.50^{d,h}	18.95±0.88^{d,h}	28.24±0.44^{d,h}	28.83±0.27^{d,h}
600	46.61±0.34	40.21±0.34^{d}	25.57±0.79^{d,h}	22.72±0.95^{d,h}	36.22±0.67^{d,g}	33.29±0.98^{d,h}
800	59.49±1.34	45.62±0.34^{d}	32.78±1.06^{d,h}	30.03±0.61^{d,h}	46.57±1.16^{d}	38.12±0.55^{d,h}
1000	72.85±1.30	58.54±1.10^{d}	44.82±0.96^{d,h}	42.88±0.83^{d,h}	55.98±1.81^{d}	41.34±0.25^{d,h}

Values are expressed as mean±SD, *n*=3. Significant differences between groups is evaluated by one-way ANOVA followed by Tukey's multiple comparison test. ^{a}Significantly different from standard (P<0.05); ^{b}Significantly different from standard (P<0.01); ^{c}Significantly different from standard (P<0.001); ^{d}Significantly different from standard (P<0.0001); ^{e}Significantly different from extract (P<0.05); ^{f}Significantly different from extract (P<0.01); ^{g}Significantly different from extract (P<0.001); ^{h}Significantly different from extract (P<0.0001). SD: Standard deviation; Fr: Fraction(s); FI: Fr I; FII: Fr II; F III: Fr III; F IV: Fr IV; ANOVA: Analysis of variance; CYS: Cystone

Table 5: Growth assay of aerial parts of *Aerva lanata* (Linn.)

Dose (µg/ml)	Percentage inhibition of treatment					
	CYS	Extract	F I	F II	F III	F IV
200	36.18±1.65	39.04±3.43	6.98±3.96^{d,h}	11.74±3.97^{d,h}	21.86±2.47^{b,g}	2.85±2.52^{d,h}
400	46.66±2.86	54.28±0.95^{b}	13.01±1.98^{d,h}	19.90±0.81^{d,h}	36.27±1.09^{c,h}	12.06±2.40^{d,h}
600	58.41±1.46	62.53±1.45	22.22±2.91^{d,h}	30.79±1.46^{d,h}	44.76±2.52^{d,h}	18.41±1.98^{d,h}
800	66.02±1.98	67.61±1.91	29.52±3.81^{d,h}	44.12±1.46^{d,h}	55.23±0.96^{c,g}	24.12±1.10^{d,h}
1000	75.26±2.90	73.81±2.18	37.14±1.91^{d,h}	56.57±3.67^{d,h}	66.66±1.65^{a}	50.87±3.81^{d,h}

Values are expressed as mean±SD, *n*=3. Significant differences between groups is evaluated by one-way ANOVA followed by Tukey's multiple comparison test. ^{a}Significantly different from standard (P<0.05); ^{b}Significantly different from standard (P<0.01); ^{c}Significantly different from standard (P<0.001); ^{d}Significantly different from standard (<0.0001); ^{e}Significantly different from extract (P<0.05); ^{f}Significantly different from extract (P<0.01); ^{g}Significantly different from extract (P<0.001); ^{h}Significantly different from extract (P<0.0001). SD: Standard deviation; FI: Fr I; FII: Fr II; F III: Fr III; F IV: Fr IV; ANOVA: Analysis of variance; CYS: Cystone; Fr: Fraction(s)

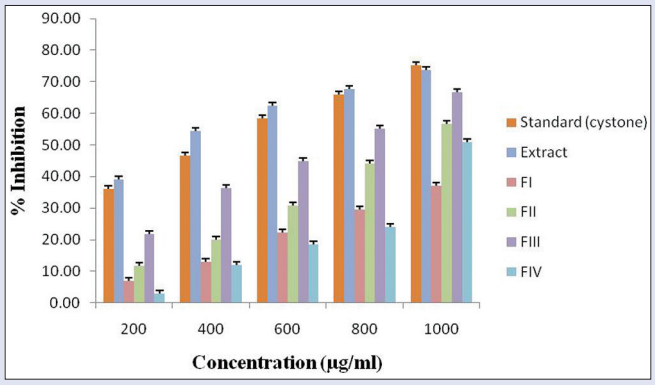


Figure 3: Growth assay of extract derived fractions of aerial parts of *Aerva lanata*

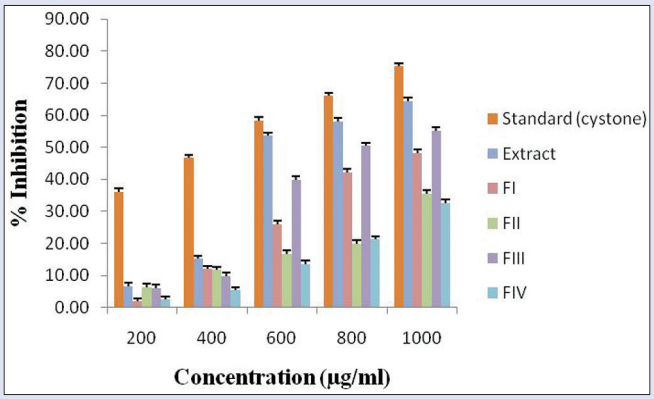


Figure 4: Growth assay of extract derived fractions of roots of *Aerva lanata*

further progresses to crystal growth whereby the constituents making up the crystals further combine with the nucleus, thereby decreasing the overall free energy with the supersaturation ratio being >1.[30] Any pharmacological entity obstructing the nucleation, aggregation and growth phases will ultimately hamper the process of stone formation and hence, act as a promising therapy in ruling out the occurrence of urolithiasis.

Earlier studies have demonstrated the antiurolithic efficacy of the extracts from the plant[11-14] which is not sufficient for an understanding of the major class of phytoconstituents chiefly contributing toward its antiurolithic ability as an extract is a combination of a diversity of phytoconstituents existing in a specific herb of interest. Moreover, a recent study has highlighted the immense antilithiatic efficacy of the aqueous extract out of the different solvent-based extracts (benzene, chloroform, and aqueous)[15] which does not support the antiurolithic role of any particular phytoconstituent as innumerable constituents are soluble in water. Similarly, another study revealed the tremendous activity of the methanolic extract among various solvent based extracts[16] which again confirms the overall efficacy of the plant regardless of the specific phytoconstituents as the methanolic extract was shown to possess a large number of constituents as was evident from the phytochemical screening. Therefore, the current study supports the stepwise separation of the specific phytoconstituents in the form of Fr and then analyzing them for their antilithiatic efficacy which leads to preliminary information regarding the possible role of flavonoids and phenolic components in this regard. We have earlier reported the potential of various extract based Fr from AL on CaOx nucleation *in vitro*.[17] However, to confirm their effect on the complete process of stone formation, the efficacy of Fr on aggregation and growth of CaOx needs to be observed and understood and the current work demonstrates the same.

In the current investigation, the phenolics and flavonoid-rich Fr III from aerial parts exerted the maximum *in vitro* antiurolithic efficacy

Table 6: Growth assay of roots of *Aerva lanata* (Linn.)

Dose (µg/ml)	Percentage inhibition of treatment					
	CYS	Extract	F I	F II	F III	F IV
200	36.18±1.65	6.66±1.91[d]	1.90±0.95[d,e]	6.34±1.45[d]	6.03±1.45[d]	2.53±1.10[d,e]
400	46.66±2.86	15.16±3.81[d]	12.06±1.46[d]	11.74±1.46[d]	9.84±1.45[d]	5.39±1.46[d,f]
600	58.41±1.46	53.65±2.40	26.03±1.45[d,h]	16.82±1.46[d,h]	39.84±2.44[d,h]	13.65±2.91[d,h]
800	66.02±1.98	58.09±0.95[b]	42.22±2.91[d,h]	20.00±0.96[d,h]	50.47±0.95[d,f]	21.26±2.40[d,h]
1000	75.26±2.90	64.44±1.10[c]	48.25±2.40[d,h]	35.55±2.91[d,h]	55.23±0.96[d,f]	32.69±1.10[d,h]

Values are expressed as mean±SD, n=3. Significant differences between groups is evaluated by one-way ANOVA followed by Tukey's multiple comparison test. [a]Significantly different from standard (P<0.05); [b]Significantly different from standard (P<0.01); [c]Significantly different from standard (P<0.001); [d]Significantly different from standard (P<0.0001); [e]Significantly different from extract (P<0.05); [f]Significantly different from extract (P<0.01); [g]Significantly different from extract (P<0.001); [h]Significantly different from extract (P<0.0001). SD: Standard deviation; FI: Fr I; FII: Fr II; F III: Fr III; F IV: Fr IV; ANOVA: Analysis of variance; CYS: Cystone; Fr: Fraction(s)

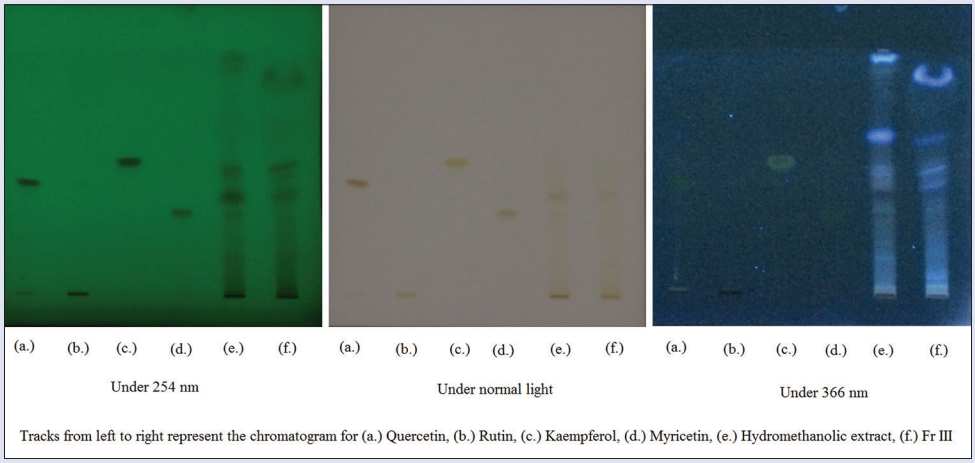


Figure 5: Image of the developed plate as viewed under 254 nm, normal light and 366 nm

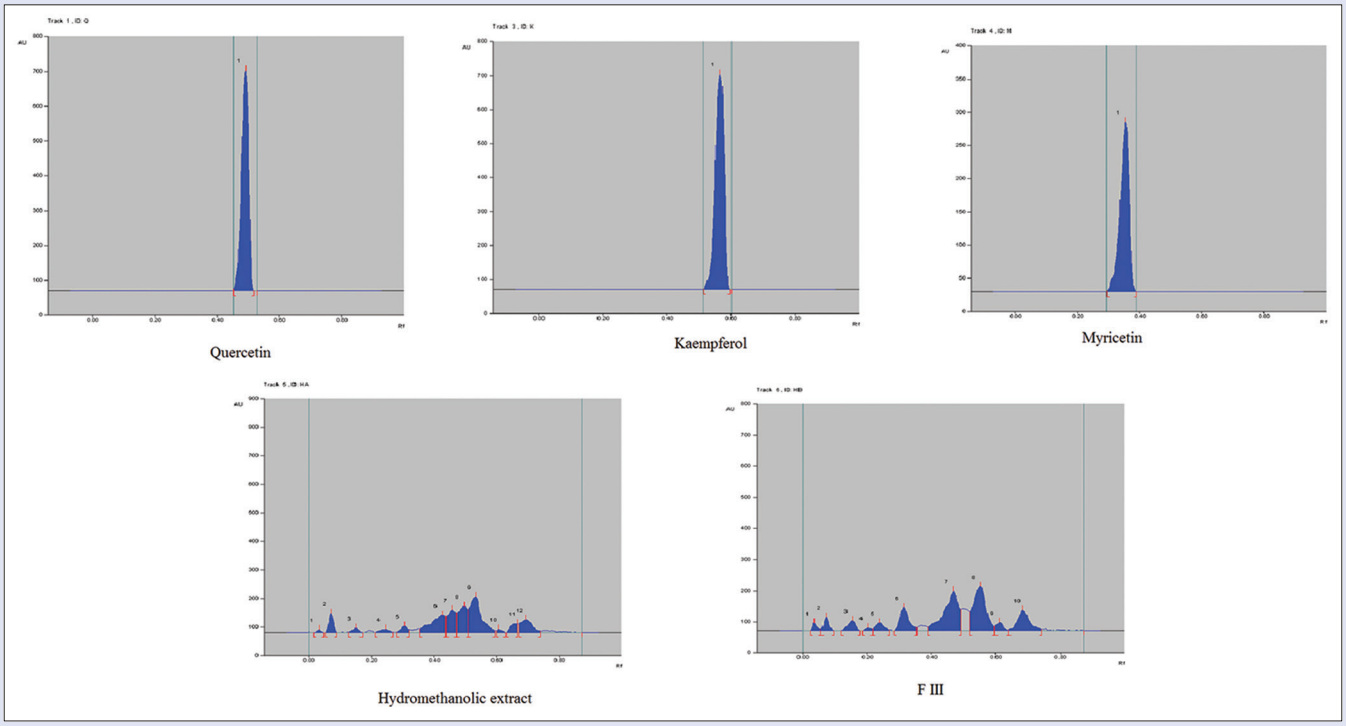


Figure 6: Images of the scanned chromatograms

Table 7: Quantification of flavonoids in fraction III of aerial parts through high performance thin layer chromatography

Flavonoid	Regression equation	Content present (% w/w)
Quercetin	10.55x + 1050	0.095
Kaempferol	15.50x + 248.1	0.270
Myricetin	17.89x+283.2	0.058

as was evident from its highest inhibition of CaOx aggregation and growth. The activity exerted by both the extract and flavonoid-rich Fr III from the aerial parts was very prominent and comparable to standard (Cystone) which is a well-established marketed polyherbal formulation. As the present study primarily focuses on finding out the best fraction with respect to its antiurolithic property, therefore, it becomes indispensable to compare it with a well acclaimed therapeutic preparation (Cystone) which is known to demonstrate excellent antiurolithic action. This will reveal the effectiveness of the fraction as compared to the standard (Cystone). Moreover, Fr III from aerial parts displaying significant antiurolithic effect is a blend of several phenolic and flavonoid components and not just composed of a single phenolic/flavonoid. In addition, literature highlights the use of Cystone as the standard for conducting antiurolithic studies on various Fr derived from different herbal sources.[31] However, the extract and flavonoid Fr III derived from roots exhibited lower efficacy compared to aerial parts and standard. The higher potential exhibited by aerial parts might be attributed to the symbiotic effect of different phytocomponents present in the extract and the existence of potent flavonoids and phenolics in the Fr III. Since polyphenolic compounds and flavonoids are well known as potent antioxidants, they have a possible role in alleviating urinary stones by impeding the oxidative distress caused to the renal tubular epithelium imparted by hyperoxaluria and thus, can block the attachment and accumulation of CaOx crystals which can otherwise evolve into clinical stones.[32] The confirmation of the existence of flavonoid compounds in the best active Fr III of aerial parts from HPTLC studies supports our study regarding their possible role in alleviating the process of stone formation.

CONCLUSION

The present study confirms that the flavonoids and phenolic components of the aerial parts of AL have an immense potential to counteract the aggregation and growth stages of calculi development confirming their antilithiatic potential. It also provides a guide to the isolation of potential antiurolithic moieties from the aerial parts. However, further, in-depth preclinical studies are necessary for observing the response of this flavonoid and phenolic containing Fr III on urinary and blood levels of stone promoters, inhibitors, nitrogenous substances, antioxidant enzymes and its effect on urinary tissues. Extensive clinical studies need to be performed for confirmation of efficaciousness in human subjects.

Acknowledgements

The authors are thankful to Dr. Sunita Garg, Head, Raw Materials Herbarium and Museum, NISCAIR, New Delhi, India, for identification of the plant material. The authors are highly grateful to Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy and Bioactive Natural Product Laboratory, School of Pharmaceutical Education and Research, Jamia Hamdard University, New Delhi, India, for providing the facilities and assistance for HPTLC analysis. The authors are thankful to Amity Institute of Pharmacy, Amity University, Noida, India, for providing the necessary chemicals and facilities required for the present work.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Panigrahi PN, Dey S, Jena SC. Urolithiasis: Critical analysis of mechanism of renal stone formation and use of medicinal plants as antiurolithiatic agents. Asian J Anim Vet Sci 2016;11:9-16.
2. Parmar MS. Kidney stones. BMJ 2004;328:1420-4.
3. Mehmet NM, Ender O. Effect of urinary stone disease and is effect on renal function. World J Nephrol 2015;4:271-6.
4. López M, Hoppe B. History, epidemiology and regional diversities of urolithiasis. Pediatr Nephrol 2010;25:49-59.
5. Joy JM, Prathyusha S, Mohanalakshmi S, Praveen Kumar AV, Ashok Kumar CK. Potent herbal wealth with litholytic activity: A review. Int J Innov Drug Discov 2012;2:66-75.
6. Pareta SK, Patra KC, Mazumder PM, Sasmal D. Establishing the principle of herbal therapy for antiurolithiatic activity: A review. J Pharmacol Toxicol 2011;6:321-32.
7. Alok S, Jain SK, Verma A, Kumar M, Sabharwal M. Pathophysiology of kidney, gallbladder and urinary stones treatment with herbal and allopathic medicine: A review. Asian Pac J Trop Dis 2013;3:496-504.
8. Chitme HR, Alok S, Jain SK, Sabharwal M. Herbal treatment for urinary stones. Int J Pharm Sci Res 2010;1:24-31.
9. Nagaratna A, Prakash LH, Harini A. A pharmacological review on Gorakha ganja (*Aerva lanata* (Linn) Juss. EX. Schult). J Pharmacogn Phytochem 2014;3:253-7.
10. Goyal M, Pareek A, Nagori BP, Sasmal D. *Aerva lanata*: A review on phytochemistry and pharmacological aspects. Pharmacogn Rev 2011;5:195-8.
11. Soundararajan P, Mahesh R, Ramesh T, Begum VH. Effect of *Aerva lanata* on calciumoxalate urolithiasis in rats. Indian J Exp Biol 2006;44:981-6.
12. Selvam R, Kalaiselvi P, Govindaraj A, Bala Murugan V, Sathish Kumar AS. Effect of *A. lanata* leaf extract and Vediuppu chunnam on the urinary risk factors of calcium oxalate urolithiasis during experimental hyperoxaluria. Pharmacol Res 2001;43:89-93.
13. Nirmaladevi R, Chandirika JU, Annadurai G, Kalpana S, Shrinidhi Rai T. Evaluation of *Aerva lanata* flower extract for its antilithiatic potential *in vitro* and *in vivo*. Int J Pharm Pharm Sci Res 2013;3:67-71.
14. Arthi I, Ravichandiran V, Sampath Kumar KP, Subburaju T. Antiurolithiatic effect of *Aerva lanata* Linn extract on ethylene glycol induced urinary calculi model in rats. Int J Pharm Sci Rev Res 2012;17:46-50.
15. Saravanasingh K, Ramamurthy M, Parthiban P. *In vitro* anti-urolithiatic activity of aerialparts of *Aerva lanata* (L.) Juss. Int J Curr Res Med Sci 2016;2:24-7.
16. Behera PC, Ghosh M. Evaluation of antioxidant, antimicrobial, and antiurolithiatic potential of different solvent extracts of *Aerva lanata* linn flowers. Phcog Mag 2018;14:53-7.
17. Mandal B, Madan S, Ahmad S. *In vitro* inhibition of calcium oxalate nucleation by extract-based fractions of aerial parts and roots of *Aerva lanata* (Linn.) Juss. ex Schult. Indian J Pharm Sci 2017;79:957-64.
18. Soni S, Anandjiwala S, Patel G, Rajani M. Validation of different methods of preparation of *Adhatoda vasica* leaf juice by quantification of total alkaloids and vasicine. Indian J Pharm Sci 2008;70:36-42.
19. Yadav S, Kumar P. Production, isolation and identification of flavonoids from aerial partsof *Hiptage benghalensis*. Int J Life Sci Pharm Res 2012;2:1-5.
20. Harborne AJ. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 3rd ed. Netherlands: Springer Science and Business Media; 1998.
21. Kokate CK, Purohit AP, Gokhale SB. Pharmacognosy. 13th ed. Pune: Nirali Prakashan; 2005.
22. Saranya R, Geetha N. Inhibition of calcium oxalate (CaOx) crystallization *in vitro* by the extract of beet root (*Beta vulgaris* L.). Int J Pharm Pharm Sci 2014;6:361-5.
23. Chaudhary A, Singla SK, Tandon C. *In vitro* evaluation of *Terminalia arjuna* on calcium phosphate and calcium oxalate crystallization. Indian J Pharm Sci 2010;72:340-5.
24. Forgacs E, Cserhati T. Thin-layer chromatography of natural pigments: New advances. J Liq Chrom Rel Technol 2002;25:1521-41.
25. Yadav M, Chatterji S, Gupta SK, Watal G. Preliminary phytochemical screening of six medicinal plants used in traditional medicine. Int J Pharm Pharm Sci 2014;6:539-42.

26. Aggarwal KP, Narula S, Kakkar M, Tandon C. Nephrolithiasis: Molecular mechanism of renal stone formation and the critical role played by modulators. Biomed Res Int 2013;2013:292953.
27. Boskey AL. Current concepts of the physiology and biochemistry of calcification. Clin Orthop Relat Res 1981;157:225-57.
28. Fleisch H. Inhibitors and promoters of stone formation. Kidney Int 1978;13:361-71.
29. Baumann JM, Affolter B. From crystalluria to kidney stones, some physicochemical aspects of calcium nephrolithiasis. World J Nephrol 2014;3:256-67.
30. Kanchana G, Sundaramoorthi P, Santhi R, Kalainathan S, Jeyanthi GP. Nucleation reductionstrategy of (Brushite) CHP crystals in SMS media and its characterization studies. J Miner Mater Charact Eng 2008;7:49-57.
31. Patel PK, Patel MA, Vyas BA, Shah DR, Gandhi TR. Antiurolithiatic activity of saponin rich fraction from the fruits of *Solanum xanthocarpum* schrad. & wendl. (Solanaceae) against ethylene glycol induced urolithiasis in rats. J Ethnopharmacol 2012;144:160-70.
32. Butterweck V, Khan SR. Herbal medicines in the management of urolithiasis: Alternative or complementary? Planta Med 2009;75:1095-103.