

# *In vitro* Inhibition of Calcium Oxalate Nucleation by Extract-based Fractions of Aerial Parts and Roots of *Aerva lanata* (Linn.) Juss. ex Schult

B. MANDAL, SWATI MADAN\* AND S. AHMAD

Department of Pharmacognosy, Amity Institute of Pharmacy, Amity University, Noida-201 313, India

## Mandal, *et al*: Antiurolithiatic Efficacy of Extract Based Fractions of *Aerva lanata*

The current investigation is an attempt to explore the comparative *in vitro* antiurolithic potential of various phytoconstituent-rich fractions derived from the hydroalcoholic extract of aerial parts and roots of *Aerva lanata* (Linn.) Juss. ex Schult (Amaranthaceae). The antiurolithic assay was performed by nucleation method. The analysis revealed the phenolic and flavonoid containing fraction from aerial parts to be the most active (71.01±1.13 %) amongst the different extract-derived fractions compared to the one obtained from roots (54.61±2.30 %). Besides, fluorescence analysis and physicochemical evaluation were carried out according to the official guidelines and supported the quality control of the plant material. Preliminary phytochemical screening of the fractions was also performed and confirmed the presence of various metabolites. Additionally, the content of tannins, total phenolics and flavonoids were also determined spectrophotometrically and found to be comparatively higher in aerial parts (tannin content of 4.34±0.63 mg tannic acid equivalent/g extract, total phenolic content of 127.84±1.50 gallic acid equivalent/g extract and total flavonoid content of 77.61±3.78 rutin equivalent/g extract) than roots (tannin content of 3.03±0.63 mg tannic acid equivalent/g extract, total phenolic content of 98.09±1.10 gallic acid equivalent/g extract and total flavonoid content of 62.81±5.69 rutin equivalent/g extract). Hence, the present study aids in screening out the best active extract derived fraction exerting litholytic efficacy, which provides a route to further isolation of lead bioactive compounds contributing to significant antiurolithic potency of this traditionally employed herb of immense pharmacological spectrum.

**Key words:** *Aerva lanata* (Linn.) Juss. ex Schult, antiurolithiatic, fraction, phenolics, flavonoids

Urolithiasis refers to the formation of calculi anywhere in the urinary tract afflicting the kidneys, ureters, bladder or urethra<sup>[1]</sup>. It is the third most prevalent urological disorder after urinary tract infection and prostate conditions<sup>[2]</sup>. Approximately 12 % of the world population is affected by the incidence of urinary stones with a comparatively higher frequency of recurrence in males (70-81 %) than in females (47-60 %)<sup>[3]</sup>. Depending upon the chemical nature, there are various types of stones such as calcium oxalate monohydrate, calcium oxalate dihydrate, basic calcium phosphate (brushite), magnesium ammonium phosphate (struvite), uric acid and cystine stones<sup>[4]</sup>. Manifestations include colicky pain, vomiting, dysuria, haematuria, pyuria and oliguria<sup>[5]</sup>. These calculi arise as an outcome of supersaturation of urine with stone forming constituents resulting in crystallization followed by crystal nucleation, aggregation and growth leading to their adherence to the renal tubules<sup>[6]</sup>.

Numerous surgical strategies exist in scenario for management of urinary calculi such as shock wave lithotripsy (SWL), ureteroscopic lithotripsy (URS), digital endoscopy, percutaneous nephrolithotomy (PNL) and robotic surgery<sup>[7]</sup>. However, many of these techniques bring along with them several drawbacks such as renal casualties in the long run, hypertension and repeated episodes of stones<sup>[8]</sup>. Several allopathic interventions are available but are increasingly being overtaken by herbal therapeutics on account of their safety, minimal side effects, comparatively greater efficacy in dissolving stones and preventing the

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\*Address for correspondence

E-mail: smadan3@amity.edu

chances of recurrence<sup>[9]</sup>. Moreover, the applications of medicinal plants in combating urinary stones have been extensively mentioned in the ancient medical literature as well as Ayurvedic system of medicine in addition to their relevance as folk remedies<sup>[10]</sup>.

*Aerva lanata* (Linn.) Juss. ex Schult. (family: Amaranthaceae) is one such efficacious medicinal plant amongst the diverse pool of litholytic herbs bestowed by nature. It is commonly referred as Mountain knotgrass in English and *Gorkhabundi* or *Kapurijadi* in Hindi, which is an erect or prostrate herb with a long tap-root and many woolly-tomentose branches<sup>[11]</sup>. It grows up to an elevation of 900 m and is indigenous to India, Africa and Australia and considered as *Pashanbheda* in Ayurveda, which is a collective term assigned to a group of medicinal herbs with a potential for dissolving kidney stones<sup>[12]</sup>. It is a reservoir of diverse phytoconstituents such as canthin-6-one (aervine, aervoside) and  $\beta$ -carboline alkaloids (aervolanine), flavonoids (kaempferol, quercetin),  $\beta$ -sitosterol, phenolic acids (syringic acid, vanillic acid), betulin<sup>[13]</sup>. Literature elaborates a tremendous *in vitro* and *in vivo* antiurolithic potency of the extract from whole plant<sup>[14-18]</sup>. However, evaluating the extracts for biological activity provides generalized information regarding the efficacy of a particular medicinal plant regardless of the specific phytoconstituents. The current era of phytopharmaceuticals has led to an emerging curiosity towards an in depth scrutiny of bioactive phytoconstituents contributing to the therapeutic efficacy of herbs and to achieve this bioassay-guided fractionation is a mandatory step, which implies step-by-step separation of extracted components based on the differences in their physicochemical properties using solvents of different polarities and assessing the biological activity. A recent study reported about the efficacy of various extracts derived from aerial parts using different solvents<sup>[19]</sup>. The research highlighted that the aqueous extract exhibited the best activity which however, does not establishes the basis regarding the contribution of phenolics and flavonoids towards antiurolithic ability of *A. lanata* as a large number of phytocomponents are soluble in water apart from phenolic and flavonoid compounds. Additionally, the activity was performed at only one dose level, which cannot ascertain the degree of efficacy as a dose range is more reliable in analysing the maximal efficacy of a particular pharmaceutical agent. Statistical analysis was missing in the study, which is a mandatory step while assessing the biological activity to assure any

significant differences among the different extract groups. Another protocol was conducted with the objective of *in silico* antiurolithic screening of the isolated components<sup>[20]</sup>. But, the study did not reported a systematic *in vitro* screening of different fractions for bioactivity followed by an in-depth evaluation of various altered biochemical parameters *in vivo*.

From the literature, it is evident that a systematized bioactivity guided fractionation of *A. lanata* has not been reported to provide a scientific basis for identification of actual active principles responsible for its antiurolithic activity. Keeping this in view, we have reported the *in vitro* nucleation assay of various extract derived fractions of aerial parts and roots at various dose levels in our present study to screen out the best bioactive fraction contributing towards the antiurolithic potency of this miraculous herb of nature. This gives a better evidence for antiurolithic activity of various fractions of *A. lanata* at different dose levels. Hence, the present study constitutes a novel research, which has not been reported earlier. The current research also aims at carrying out the fluorescence analysis as well as physicochemical assessment, which is a mandatory step to ensure fulfilment of the plant material towards the fundamental criteria for identity, quality and purity. Additionally, it also objects towards the preliminary phytochemical screening of the fractions derived from them to ensure the presence of intended phytochemicals and spectrophotometric estimation of the content of tannins, total phenolics and flavonoids to correlate their influence on the antiurolithic efficacy of this medicinal plant.

## MATERIALS AND METHODS

Folin-Ciocalteu reagent, calcium chloride dihydrate and aluminium chloride were procured from Central Drug House Pvt. Ltd., New Delhi. Methanol was purchased from Rankem, Gujarat. Diethyl ether was procured from High Purity Laboratory Chemicals Pvt. Ltd., Mumbai. Ethyl acetate, chloroform, sodium chloride and sodium hydroxide were obtained from Thermo Fisher Scientific India Pvt. Ltd., Mumbai. Tris was purchased from Ubichem Plc., Hampshire. Sodium carbonate was obtained from Loba Chemie Pvt. Ltd., Mumbai. Sodium oxalate and sodium nitrite were procured from Qualigens Fine Chemicals, Mumbai. Tannic acid and gallic acid were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai. Rutin was purchased from Search Chem, Mumbai. All other chemicals employed were of analytical grade.

### Collection, identification and authentication of plant material:

Dried whole plant of *A. lanata* was procured from Balaji Traders (Tamil Nadu, India) during the month of March, 2015. The identity of the plant was validated at the Raw Materials Herbarium and Museum, NISCAIR, New Delhi and a voucher specimen number NISCAIR/RHMD/Consult/2014/2792/171 for the same was deposited at the Herbarium of National Institute of Science Communication and Information Resources, New Delhi. Subsequently, the roots and aerial parts were separated, dried and powdered.

### Fluorescence analysis:

A small portion of the powdered aerial parts and roots was placed on a microscopic slide and examined under short wave UV light (254 nm), long wave UV light (366 nm) and day light as such as well as following their treatment with various reagents<sup>[21]</sup>.

### Physicochemical evaluation:

The physicochemical parameters were performed as per the official guidelines and included foreign matter, extractive values (water soluble, alcohol soluble, ether soluble), ash values (total, water soluble, acid insoluble), loss on drying, foaming index and swelling index<sup>[22,23]</sup>.

### Extraction of plant material:

About 200 g of the powdered aerial parts and roots were weighed and macerated separately in a solvent mixture of water:methanol (2:1) overnight followed by refluxing the hydromethanol blend for 5 h. The extracts thus obtained from aerial parts and roots were then evaporated to dryness to one-fourth of their original volume and further divided into various portions for the generation of fractions.

### Preparation of fractions:

The hydromethanol extract, separately for both roots and aerial parts, was divided into three portions out of which one-fourth part was evaporated to dryness below 60° and considered as the mother extract. One-half of the volume was made acidic and partitioned with equal volume of ether in a separating funnel followed by separating the ether layer and evaporating it to dryness which led to fraction I, which was supposed to contain fatty material, steroidal aglycones, terpenoids and coloring matter. The remaining aqueous layer was rendered alkaline with ammonia solution and partitioned with equal volume of chloroform thrice

after which it was separated and evaporated to dryness resulting in fraction II expected to be composed of alkaloids. The remaining alkaline aqueous layer was made acidic with dilute hydrochloric acid. Thereafter, it was refluxed for two hours followed by further fractionation with equal volume of ethyl acetate thrice, which was evaporated to dryness consequently leading to fraction III supposed to contain flavonoid and phenolic aglycones. Finally, the remaining one-fourth volume of the extract was passed with equal volume of ether and the aqueous layer left behind was passed with ethyl acetate thrice, which was evaporated to dryness giving rise to fraction IV expected to be composed of glycosides<sup>[24,25]</sup>.

### Preliminary phytochemical screening:

Various qualitative tests were performed to confirm the presence of steroids, terpenoids, phenolics, flavonoids, glycosides and alkaloids in the distinctive fractions generated from the hydromethanolic extracts of roots and aerial parts<sup>[26,27]</sup>.

### Nucleation assay:

*In vitro* antiurolithiatic assay was conducted by the procedure as mentioned in literature<sup>[28,29]</sup>. The assay was carried out in the absence (control) and presence of inhibitor (standard/extract/fraction). About 5 mM and 7.5 mM calcium chloride and sodium oxalate solutions were made in a buffer composed of 50 mM Tris and 150 mM sodium chloride at pH 6.5. Stock solutions of standard (cystone) and samples (extracts of aerial parts and roots along with their fractions) were prepared at a concentration of 10 mg/ml. About 1 ml of calcium chloride was added in both the control and sample sets. Additionally, 1 ml of distilled water was added in the control set. On the contrary, 1 ml of various dilutions of sample (200, 400, 600, 800, 100 µg/ml) were added in the sample set instead of distilled water. The onset of crystallization was achieved by the addition of 1 ml of sodium oxalate solution to all the sets. The tubes were incubated at 37° for 30 min after which the absorbance was read at 620 nm using Perkin Elmer Lambda 25 UV/Vis spectrophotometer. Percent inhibition of nucleation was calculated as follows: percent inhibition =  $[(C-S)/C] \times 100$ , where, C = turbidity of control set, S = turbidity of sample set.

### Determination of tannins:

Tannin content was assessed by Folin-Ciocalteu reagent method<sup>[30]</sup>. About 0.5 g each of powdered aerial parts and roots were extracted by boiling in

water, filtered and concentrated on a water bath and dried in an oven below 60°. Stock solutions of the extracts were prepared (1 mg/ml) in distilled water. Thereafter, 0.1 ml of the extracts were mixed with 0.5 ml of Folin-Ciocalteu reagent, 1 ml of 35 % sodium carbonate solution and the volume was made up to 10 ml with distilled water. The samples were left for colour development for 30 min and absorbance was read at 725 nm using Perkin Elmer Lambda 25 UV/Vis spectrophotometer. Stock solution of tannic acid was prepared in water (100 mg/100 ml) from which appropriate dilutions were made (2, 4, 6, 8 and 10 µg/ml) and standard curve was achieved. Tannin content was expressed as mg of tannic acid equivalents per g extract (mg TAE/g). The experiment was carried out in triplicate.

#### Determination of total phenolics:

Total phenolic content was analysed by spectrophotometric method<sup>[31]</sup>. Stock solutions of the extracts of roots and aerial parts were made in distilled water (1 mg/ml). About 1 ml of the extracts from stock was admixed with 1 ml of Folin's reagent. After 5 min, 4 ml of 7 % sodium carbonate solution was added along with 4 ml of distilled water. The samples were prepared in triplicate. The mixtures were incubated for 60 min in dark and the absorbance was taken at 750 nm against a reagent blank. Stock solution of gallic acid in water was prepared (100 mg/100 ml) from which appropriate dilutions were obtained (2, 4, 6, 8 and 10 µg/ml) and standard curve was established. Total phenolic content was expressed as mg of gallic acid equivalents per g extract (mg GAE/g).

#### Determination of total flavonoids:

The content of total flavonoids was evaluated by aluminium chloride colorimetric assay<sup>[32]</sup>. Stock solutions of extracts were prepared (1 mg/ml) in distilled water. About 0.5 ml of the extracts from stock solutions was mixed with 2 ml distilled water to which 0.15 ml of 5 % sodium nitrite solution was added. After 6 min, 0.15 ml of 10 % aluminium chloride solution was added to the mixture and placed for another 6 min. About 2 ml of 4 % sodium hydroxide solution was added to this and final volume was made up to 5 ml by adding 0.2 ml of distilled water. The experiment was conducted in triplicate. All the reaction mixtures were mixed well and left to stand for 15 min and the absorbance was recorded against a reagent blank at 510 nm. Stock solution of rutin in water was prepared (100 mg/100 ml) from which appropriate dilutions

were made (2, 4, 6, 8 and 10 µg/ml) and standard curve was obtained. Total flavonoid content was expressed as mg of rutin equivalents per g extract (mg RE/g).

#### Statistical analysis:

The experiments were carried out in triplicate and data were expressed as mean±standard deviation. Detection of significant differences between the groups was done by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The results were recognized significant when P<0.05.

## RESULTS AND DISCUSSION

The results of fluorescence analysis of the powdered aerial parts and roots are mentioned in Table 1 and Table 2. It served as a relevant criterion for their identification on account of the characteristic fluorescence displayed by the phytoconstituents in them. The values for physicochemical evaluation are given in Table 3. The foreign matter was slightly higher in roots (0.54±0.04 %) than aerial parts (0.48±0.03 %). The water soluble, alcohol soluble and ether soluble extractives were found to be 13.41±0.46, 4.02±0.42 and 1.62±0.06 %, respectively in aerial parts while in roots they were found to be 13.20±0.82, 5.01±0.51 and 5.61±0.17 %, respectively. The moisture content (loss on drying) was within the limit in both the aerial parts (4.80±0.30 %) and roots (4.32±0.26 %), which assures the acceptable quality of the plant material. The levels of total ash, acid insoluble ash and water-soluble ash values were also agreeable and reported to be

**TABLE 1: FLUORESCENCE ANALYSIS OF AERIAL PARTS OF AERVA LANATA**

Treatment	Day light	UV light	
		254 nm	366 nm
Powder as such	Light green	Light green	Light brown
Powder+1 N NaOH (aq.)	Dark brown	Light green	Dark green
Powder+1 N NaOH (alc.)	Greenish brown	Light green	Yellowish brown
Powder+1 N HCl	Greenish yellow	Light green	Light yellow
Powder+ammonia	Light brown	Light green	Dark brown
Powder+5 % iodine	Dark brown	Light green	Black
Powder+5 % FeCl <sub>3</sub>	Light green	Light green	Greenish brown
Powder+acetic acid	Light green	Light green	Light brown
Powder+1 N H <sub>2</sub> SO <sub>4</sub>	Light yellow	Yellowish green	Deep yellow
Powder+1 N HNO <sub>3</sub>	Light yellow	Light green	Yellowish brown

**TABLE 2: FLUORESCENCE ANALYSIS OF ROOTS OF AERVA LANATA**

Treatment	Day light	UV light	
		254 nm	366 nm
Powder as such	Light brown	Yellowish green	Whitish yellow
Powder+1 N NaOH (aq.)	Dark brown	Light green	Greenish brown
Powder+1 N NaOH (alc.)	Light brown	Yellowish green	Brownish yellow
Powder+1 N HCl	Light brown	Light green	Deep yellow
Powder+ ammonia	Light brown	Light green	Yellowish brown
Powder+5 % iodine	Dark brown	Light green	Dark green
Powder+5 % FeCl <sub>3</sub>	Light brown	Light green	Dark brown
Powder+acetic acid	Light brown	Light green	Light brown
Powder+1 N H <sub>2</sub> SO <sub>4</sub>	Light green	Light green	Light yellow
Powder+1 N HNO <sub>3</sub>	Light brown	Light green	Brownish yellow

**TABLE 3: PHYSICOCHEMICAL PARAMETERS OF AERVA LANATA**

Physicochemical parameter (in % w/w)	<i>Aerva lanata</i>	
	Aerial parts	Roots
Foreign matter	0.48±0.03	0.54±0.04
Water soluble extractive	13.41±0.46	13.20±0.82
Alcohol soluble extractive	4.02±0.42	5.01±0.51
Ether soluble extractive	1.62±0.06	5.61±0.17
Loss on drying	4.80±0.30	4.32±0.26
Total ash	9.50±0.47	7.50±0.46
Acid insoluble ash	2.23±0.21	3.15±0.08
Water soluble ash	8.51±0.12	7.48±0.18
Foaming index	<100	<100
Swelling index	4.11±0.66	7.65±0.72

Values are expressed as Mean±SD (n=3). SD: Standard deviation

9.50±0.47, 2.23±0.21 and 8.51±0.12 %, respectively in aerial parts whereas, 7.50±0.46, 3.15±0.08 and 7.48±0.18 %, respectively in roots. The foaming index was found to be less than 100 in both the aerial parts and roots but, the swelling index was comparatively higher in roots (7.65±0.72 %) as compared to aerial parts (4.11±0.66 %). Preliminary qualitative tests of the fractions obtained from aerial parts and roots revealed the presence of various phytoconstituents such as steroidal compounds, terpenoids, flavonoids, phenolic substances, alkaloids and glycosides thereby, supporting the phytochemical diversity of the plant material. The results are shown in Table 4.

*In vitro* nucleation assay demonstrated a significant dose-dependent enhancement of the percentage inhibitory activity as exhibited by the extracts from aerial parts, roots and diverse fractions derived from them compared to standard (cystone). The percentage

inhibition shown by aerial parts and roots is mentioned in Table 5 and Table 6. Fraction III from both aerial parts and roots exerted the maximum inhibition on calcium oxalate nucleation at the concentration of 1000 µg/ml amongst the various generated fractions (71.01±1.13 % for aerial parts and 54.61±2.30 % for roots). Moreover, the extract from aerial parts and roots gave a reasonable activity compared to standard at 1000 µg/ml (74.18±2.57 %) with the aerial parts exhibiting a higher inhibition (72.96±1.48 %) than roots (67.82±0.43 %). The least hindrance to nucleation of calcium oxalate was demonstrated by fraction I in case of aerial parts (36.37±2.11 %) and fraction IV in case of roots (27.42±2.12 %).

The quantity of tannins, total phenolics and total flavonoids were found to be 4.34±0.63 mg TAE/g extract, 127.84±1.50 mg GAE/g extract and 77.61±3.78 mg RE/g extract, respectively in aerial parts, which were comparatively higher than roots being 3.03±0.63 mg TAE/g extract, 98.09±1.10 mg GAE/g extract and 62.81±5.69 mg RE/g extract, respectively. The results are reported in Table 7.

The fluorescence characteristic displayed by the aerial parts and roots either as such or after treatment with various reagents are emanated by the specific phytochemicals contained in them and is an exclusive feature of the crude drug thereby, serving as a crucial factor for pharmacognostic identification<sup>[33]</sup>. The results of fluorescence behaviour from the current study were unique to the plant material.

Physicochemical inspection is an imperative tool to ascertain the identity, quality and purity of a crude drug.

**TABLE 4: PRELIMINARY PHYTOCHEMICAL SCREENING OF VARIOUS FRACTIONS OF AERIAL PARTS AND ROOTS OF AERVA LANATA**

Phytoconstituent	Chemical test	Result	
		Aerial parts	Roots
Steroids and terpenoids	Salkowski test	+ve	+ve
	Sulphur powder test	+ve	+ve
Glycosides	Fehling's test	+ve	+ve
	Mayer's test	-ve	-ve
Alkaloids	Dragendorff's test	-ve	-ve
	Wagner's test	-ve	-ve
	Hager's test	+ve	+ve
Phenols	Ferric chloride test	+ve	+ve
	Shinoda test	-ve	-ve
Flavonoids	Zinc-HCl test	-ve	+ve
	Alkaline reagent test	+ve	+ve

'+' Presence, '-' absence

**TABLE 5: NUCLEATION ASSAY OF AERIAL PARTS OF *AERVA LANATA***

Dose ( $\mu\text{g/ml}$ )	Percentage inhibition of treatment					
	Standard (cystone)	Extract	F I	F II	F III	F IV
200	15.86 $\pm$ 2.12	24.11 $\pm$ 1.61 <sup>a</sup>	10.15 $\pm$ 0.22 <sup>ab</sup>	9.30 $\pm$ 1.16 <sup>ab</sup>	41.04 $\pm$ 3.01 <sup>a<sup>o</sup>b<sup>o</sup></sup>	6.95 $\pm$ 1.65 <sup>ab</sup>
400	30.53 $\pm$ 1.95	41.38 $\pm$ 2.69 <sup>a</sup>	12.08 $\pm$ 1.49 <sup>a<sup>o</sup>b<sup>o</sup></sup>	14.20 $\pm$ 2.21 <sup>a<sup>o</sup>b<sup>o</sup></sup>	53.69 $\pm$ 3.03 <sup>a<sup>o</sup>b<sup>o</sup></sup>	30.94 $\pm$ 2.26 <sup>ab</sup>
600	43.90 $\pm$ 0.62	65.17 $\pm$ 2.78 <sup>a</sup>	15.80 $\pm$ 3.36 <sup>a<sup>o</sup>b<sup>o</sup></sup>	39.57 $\pm$ 3.67 <sup>ab</sup>	62.00 $\pm$ 0.70 <sup>a<sup>o</sup>b<sup>o</sup></sup>	36.31 $\pm$ 0.25 <sup>ab</sup>
800	59.34 $\pm$ 1.58	65.14 $\pm$ 1.61 <sup>a</sup>	22.19 $\pm$ 2.05 <sup>a<sup>o</sup>b<sup>o</sup></sup>	53.38 $\pm$ 1.36 <sup>ab</sup>	66.09 $\pm$ 0.56 <sup>ab</sup>	38.11 $\pm$ 0.67 <sup>a<sup>o</sup>b<sup>o</sup></sup>
1000	74.18 $\pm$ 2.57	72.96 $\pm$ 1.48 <sup>a</sup>	36.37 $\pm$ 2.11 <sup>a<sup>o</sup>b<sup>o</sup></sup>	60.23 $\pm$ 0.99 <sup>a<sup>o</sup>b<sup>o</sup></sup>	71.01 $\pm$ 1.13 <sup>ab</sup>	44.19 $\pm$ 1.80 <sup>a<sup>o</sup>b<sup>o</sup></sup>

Values are expressed as mean $\pm$ SD (n=3, P<0.05), significant differences at 5 % level. F I: Fraction I, FII: fraction II, F III: fraction III, F IV: fraction IV. Comparisons are made: <sup>a</sup>with standard (cystone), <sup>b</sup>with extract. Symbols represent statistical significance when compared to standard (cystone): \*P<0.05. Symbols represent statistical significance when compared to extract: <sup>o</sup>P<0.05. Comparisons a or b not followed by any symbol are not statistically significant

**TABLE 6: NUCLEATION ASSAY OF ROOTS OF *AERVA LANATA***

Dose ( $\mu\text{g/ml}$ )	Percent inhibition by treatment					
	Standard (cystone)	Extract	F I	F II	F III	F IV
200	15.86 $\pm$ 2.12	32.87 $\pm$ 1.85 <sup>a</sup>	10.27 $\pm$ 2.28 <sup>ab</sup>	13.88 $\pm$ 1.19 <sup>ab<sup>oooo</sup></sup>	10.02 $\pm$ 2.18 <sup>ab</sup>	0.81 $\pm$ 0.69 <sup>a<sup>o</sup>b<sup>o</sup></sup>
400	30.53 $\pm$ 1.95	39.55 $\pm$ 2.80 <sup>a</sup>	30.53 $\pm$ 1.21 <sup>ab</sup>	17.28 $\pm$ 1.25 <sup>a<sup>o</sup>b<sup>o</sup></sup>	22.28 $\pm$ 2.08 <sup>ab</sup>	3.99 $\pm$ 2.35 <sup>a<sup>o</sup>b<sup>o</sup></sup>
600	43.90 $\pm$ 0.62	59.81 $\pm$ 1.39 <sup>a</sup>	41.90 $\pm$ 0.69 <sup>ab</sup>	29.48 $\pm$ 1.32 <sup>a<sup>o</sup>b<sup>o</sup></sup>	31.88 $\pm$ 3.69 <sup>a<sup>o</sup>b<sup>o</sup></sup>	15.85 $\pm$ 1.50 <sup>a<sup>o</sup>b<sup>o</sup></sup>
800	59.34 $\pm$ 1.58	64.91 $\pm$ 1.08 <sup>a</sup>	44.21 $\pm$ 0.31 <sup>a<sup>o</sup>b<sup>o</sup></sup>	35.96 $\pm$ 2.19 <sup>a<sup>o</sup>b<sup>o</sup></sup>	42.64 $\pm$ 2.59 <sup>a<sup>o</sup>b<sup>o</sup></sup>	22.33 $\pm$ 1.76 <sup>a<sup>o</sup>b<sup>o</sup></sup>
1000	74.18 $\pm$ 2.57	67.82 $\pm$ 0.43 <sup>a</sup>	47.78 $\pm$ 3.53 <sup>a<sup>o</sup>b<sup>o</sup></sup>	43.21 $\pm$ 2.10 <sup>a<sup>o</sup>b<sup>o</sup></sup>	54.61 $\pm$ 2.30 <sup>a<sup>o</sup>b<sup>o</sup></sup>	27.42 $\pm$ 2.12 <sup>a<sup>o</sup>b<sup>o</sup></sup>

Values are expressed as Mean $\pm$ SD (n=3, P<0.05), significant differences at 5% level. F I: Fraction I, FII: fraction II, F III: fraction III, F IV: fraction IV. Comparisons are made: <sup>a</sup>with standard (cystone), <sup>b</sup>with extract. Symbols represent statistical significance when compared to standard (cystone): \*P<0.05. Symbols represent statistical significance when compared to extract: <sup>o</sup>P<0.05. Comparisons a or b not followed by any symbol are not statistically significant

**TABLE 7: TANNIN, TOTAL PHENOLIC AND TOTAL FLAVONOID CONTENT OF *AERVA LANATA***

<i>Aerva lanata</i>	Tannin content (mg TAE/g)	Total phenolic content (mg GAE/g)	Total flavonoid content (mg RE/g)
Aerial parts	4.34 $\pm$ 0.63	127.84 $\pm$ 1.50	77.61 $\pm$ 3.78
Roots	3.03 $\pm$ 0.63	98.09 $\pm$ 1.10	62.81 $\pm$ 5.69

Values are expressed as Mean $\pm$ SD (n=3). SD: standard deviation, TAE: tannic acid equivalent, GAE: gallic acid equivalent, RE: Rutin equivalent

It plays a vital role in the detection of adulteration, substandard variety and discrepant handling of crude drugs<sup>[34]</sup>. Failure to perform physicochemical studies is associated with deterioration of the potency and stability of medicinal herbs. The values of foreign matter, moisture content, ash values, extractive values, foaming index and swelling index in the present work were in accordance with the official standards thus supporting the purity and quality of the plant material. Hence, the physicochemical parameters of roots and aerial parts of *A. lanata* complied with the official limits, which can serve as a reference for carrying out standardization of the plant material for future studies.

*In vitro* antiurolithiatic activity was conducted through solvent-solvent partitioning based fractionation with the objective of achieving the concept regarding the category of phytoconstituents responsible for the litholytic efficacy of this herb. Nucleation refers to the formation of a solid crystal state in a solution and is

the initial stage in crystal formation<sup>[35]</sup>. The process of nucleation involves either homogenous nucleation or heterogenous nucleation. The former occurs in pure solution whereas, the latter takes place in divergent chemical environment such as the human urinary system and refers to epitaxial accumulation of crystals on pre-formed urinary crystals, cellular material or urinary casts<sup>[36]</sup>. Stone formation commences with the urinary supersaturation with stone forming components such as calcium, oxalate and phosphate leading to the formation of crystals, which serve as a nidus for crystal nucleation, growth, epitaxial growth and aggregation progressively leading to development of calculi<sup>[37]</sup>. Therefore, a phytopharmaceutical agent capable of impeding the nucleation phase will also intervene with further stages of stone formation thereby preventing lithiasis. Restoration of the balance between urinary stone promoters (calcium, oxalate, uric acid, phosphate) and inhibitors (citrate and magnesium) needs to be attained by the intended antiurolithiatic agent as the imbalance contributes to lithiasis<sup>[38]</sup>. Besides this, raising the urine volume, pH and anticalcifying activity, enhancing renal function and oxalate metabolism, lessening the oxidative stress on renal tissues are other mechanisms exhibited by medicinal herbs for alleviating the incidence of calculi<sup>[39]</sup>.

In the current investigation, fraction III demonstrated the maximum inhibition on nucleation of calcium

oxalate as compared to other extract-derived fractions of aerial parts and roots. This fraction was chiefly composed of flavonoids and phenolic compounds as evidenced from phytochemical screening. Flavonoids and phenolics chiefly exhibit antioxidant activity and hence, can serve as antiurolithiatic agents by averting the crystal adhesion and consecutive formation of urinary stones by alleviating the injury caused by crystal accumulation in the urinary system that otherwise enhances the formation of free radicals resulting in destruction of membrane surface of kidney tubules via lipid peroxidation<sup>[40,41]</sup>. In the present research, the extract and the flavonoid enriched fraction of aerial parts exerted a better efficacy in comparison to those from roots, which is likely attributed to the existence of highly efficacious antiurolithiatic moieties in aerial parts compared to roots. This can be further ascertained by the isolation and quantification of active phytoconstituents from aerial parts. Moreover, these phytometabolites exist in varying concentrations in different parts of a medicinal plant and thus, exert variable degrees of activity because of which it becomes indispensable in exploring the botanical part majorly contributing towards the remedial effect. Both the extracts from aerial parts and roots exhibited a higher impediment to nucleation, which was comparable to that by the standard cystone, which is a polyherbal blend and an established potent marketed formulation. Fraction III from aerial parts demonstrated an immense hindrance to nucleation which was as high as the standard formulation and the extract while fraction III from roots exhibited a lower activity than the standard and the extract. This highlights the potency and tremendous efficacy of various antiurolithic flavonoid and phenolic aglycones in the aerial parts compared to roots. However, the greater antiurolithic ability of the extract as a whole is likely associated with the synergistic effect of various phytoconstituents present. On the contrary, fraction I composed of steroidal moieties as well as terpenoidal compounds derived from aerial parts and fraction IV from roots possessing glycosidal compounds displayed the least inhibition amongst the various fractions. This is reasonably attributed to the lower concentration and limited antiurolithic efficiency of those specific metabolites in aerial parts and roots, respectively. Fraction II from aerial parts and roots composed of alkaloidal compounds also demonstrated lower activity compared to flavonoid and phenolic containing fraction. This implies that flavonoids and phenolics have a dominating contribution in lessening the incidence of stone formation as compared to

other metabolites. An earlier study reported that both flavonoids (quercetin) and terpenoids (betulin) have a significant role in antiurolithic activity of *A. lanata*<sup>[20]</sup> unlike the present study, which shows the fraction rich in steroids and terpenoids exhibiting much inferior activity compared to the flavonoid rich fraction displaying the best activity.

As evident from the current study, the amount of tannins, total phenolics and total flavonoids in aerial parts were also relatively greater than roots. This accounts for the higher antiurolithic efficacy of aerial parts over roots. Hence, the present work provides the concept regarding the role of flavonoids and phenolics towards the litholytic efficacy of this medicinal plant of immense pharmacological scope. Further investigation is mandatory with respect to *in vivo* studies of the fraction followed by thorough phytochemical evaluation and isolation of the phytoconstituents from the same by column chromatography, flash chromatography, HPLC and other techniques to establish the validity of the current finding. This miraculous herb can be a source of novel leads, which can exhibit a crucial role in alleviating the prevalence of this affliction amongst the general population.

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There are no conflicts of interest.

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