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Anti-urolithiasis potential of *Aerva lanata* metabolites investigated in synthetic urine and cell-free *in vitro* assays

Roopkatha Ghosh[1], Jeetu Kumar[1], Sourav Roy[1], Abhishek Kadahalli[1] and Anju Thangammal[1,*]

[1]Department of Biotechnology, School of Bioengineering, SRM Institute of Science Technology, Tamil Nadu, India

[*]Corresponding author: anjut@srmist.edu.in

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Abstract

Urolithiasis commonly referred to as kidney stone disease, is a painful urologic condition that can cause rapid deterioration of renal function. Though there are several options for anti-urolithiasis treatment there is a need to avoid stone recurrence, maintain renal functions and to get a treatment which is economical. In this study, we investigated the anti-urolithiasis potential of whole plant extracts of the medicinal herb *Aerva lanata*. We identified the potential phytochemicals in *A. lanata* with a majority of α-Tocopherol, γ-Sitosterol and campesterol being identified using gas chromatography-mass spectrometry (GC-MS). In the *in-vitro* study using nucleation, aggregation and oxalate depletion assays, the inhibition efficiency of the plant extract at different stages of stone development was tested, where the experiments were performed in triple runs. The results showed a maximum of average crystal area reduction of up to 88.73% and a 52.36% stone formation inhibition at a concentration of 3mg/mL during a 30 min incubation period. This pilot study thus highlights the anti-urolithiatic efficiency of *A. lanata* metabolites.

Keywords: Anti-urolithiatic activity, Ayurveda, Calcium oxalate, Gas chromatography-mass Spectrometry, Kidney stones, Siddha medicine

1. Introduction

Kidney stone disease (KSD) is steadily becoming more common, affecting 10-20 percent of men, 3-5 percent of women, and 12 percent of the world's population [1,2]. Studies show that the oversaturation of urine is the primary cause of renal stone crystallization [3]. Furthermore, calcium oxalate stones are the leading cause of urolithiasis [1]. KSD can cause many other conditions that affect the urinary system, including chronic kidney disease (CKD), chronic obstructive nephropathy and end-stage renal disease in certain circumstances [1,4]. Technological breakthroughs in the treatment of KSD have been shown to have downsides, including their high cost and high recurrence rate [5]. Numerous plant phytochemicals have been demonstrated to reduce stone recurrence and prevention by influencing several urolithiasis pathophysiologic features [6]. The use of natural, traditional medicines can have a significant impact on factors such as preventive actions adopted to reduce the incidence of KSD among high-risk populations and those with a history of stones or CKD. Given their accessibility, affordability, and few side effects, interest has grown in the quest for herbal medications [7].

Aerva lanata (AL) is a dicotyledonous plant belonging to the class Magnoliopsida and order Caryophyllales. Indian traditional medicine refers to *A. lanata* as a 'stone breaker' plant known as *pashanabheda* [8]. *A. lanata* is employed in Indian traditional medicine for headaches, burns and skin conditions. It is also used to dissolve kidney and biliary stones, clean the uterus after childbirth and stop lactation [9]. In recent research, *A. lanata* has been found to have diuretic, hypoglycemic, anti-inflammatory, antiparasitic, anti-diabetic, hepatoprotective, antimicrobial, hypolipidemic, antifertility, antiasthmatic, and anti-urolithiatic activities [10]. Several physicochemical processes, such as crystal supersaturation, nucleation, growth, aggregation, and retention within the renal tubules, cause urolithiasis. The methods of supersaturation, nucleation, development, and aggregation are frequently studied *in-vitro* [11]. By conducting *in-vitro* anti-urolithiatic studies at various stages of

crystallization, it is possible to determine the efficacy of the AL plant extract at distinct stages of stone formation. In this study, we utilized the cold maceration extraction technique to focus on energy-efficient, easy-to-use, sustainable, and high-quality plant extraction methods.

Henceforth, this study aimed to evaluate the anti-urolithiatic potential of the cold-macerated methanolic whole plant extracts of *A.lanata* at various stages of stone formation after the identification of the phytochemical constituents of the extract.

2. Materials and methods

2.1 Plant sample collection and preparation

From December 2021 to April 2022, we purchased *A. lanata* plants from internet retailers. The plants were identified and certified by Ms Kanimozhi, an agriculturist at the SRM Institute of Science and Technology, Kattankulathur (Figure 1). Shade-dried whole plants made into a coarse powder and cold macerated in 80% (v/v) methanol for ten days, with stirring performed every other day. Following cold maceration, the AL whole plant extract filtrate was concentrated in a rotary evaporator and dried at 40°C. The AL plant extract stock concentrations were made in methanol [12]. The extracts were further evaluated for their anti-urolithiatic efficacy and phytochemical screening.

Figure 1 Photograph of *A. lanata.*

2.2 Gas chromatography-mass spectrometry (GC-MS) analysis

GC-MS study of the methanolic extract of whole plant parts of AL was performed using 7890B GC interfaced to 5977A MSD of Agilent Technologies following the procedure reported by Thangavel et al. with slight modifications [13]. The carrier gas, helium, had a constant flow rate of 1 mL/min. The oven temperature was fixed at 70°C and increased at a rate of 6°C/min until it reached 260°C. The mass range was 50 to 600 (m/z) and the total running time was 42 min. The GC-MS chromatogram obtained was used for the analysis of phytochemicals obtained by their retention time, peak area, and mass spectrometry data. Identification of phytochemicals of AL was done by comparing the mass spectra of the peaks obtained with those stored in the NIST Mass Spectral Library.

2.3 Experimental design of in vitro anti-urolithiatic analysis

Supersaturation, nucleation, crystal development, and crystal aggregation are some of the physiochemical mechanisms that result in the formation of urinary stones [7]. We examined the anti-urolithiatic potential of methanolic AL extracts at three stages of the crystallization event by calculating the degree of inhibition of calcium oxalate crystal formation in various concentrations of the extract and comparing the results to those of the reference drug, cystone. A study on the ability of the extract to prevent the production of crystals in artificial urine (AU) was also conducted. The absorbance data from the nucleation, aggregation, oxalate depletion, and AU experiments were used to compute the percentage inhibition of crystal formation. GraphPad Prism charts were

created to depict the inhibitory trends. The crystal size and area were examined using a light microscope. The decrease in the average crystal area for the nucleation, aggregation, and AU assays were calculated using ImageJ.

2.3.1 Nucleation assay

Nucleation, the initial phase of crystal growth, was assessed in the current study both with and without the addition of the AL whole plant extract in 96 well plates [14]. Calcium chloride (0.005 M) and sodium oxalate (0.007 M) solutions were made in a buffer comprising 0.05 M Tris and 0.15 M NaCl at a pH of 6.5. Calcium chloride (95 µl) was added to the control and extract wells. A volume of 10 µl of the extract samples was added at different concentrations ranging from log2 to log10 mg/mL to the extract wells. Crystallization was initiated by 95 µl of sodium oxalate solution in all sample wells. The reaction was conducted at 37°C. The absorbance at 660 nm was measured using a spectrophotometer (Thermo Scientific Multiskan GO microplate reader). The decrease in nucleation was calculated by comparing the reaction rate with and without the extract. Percentage inhibition was calculated using the following formula [15].

$$\text{Percent inhibition} = \left[\frac{(Ab_C - Ab_S)}{Ab_C}\right] \times 100 \quad (1)$$

where, Ab_C = Absorbance of the control, Ab_S = Absorbance of sample

The formation of crystals was expected from the following reaction:

$$CaCl2 + Na2C2O4 \rightarrow CaC2O4 + 2NaCl \quad (2)$$

2.3.2 Aggregation assay

The aggregation assay was performed according to instructions described by Bawari et al. [16]. After 1 h in a water bath at 60°C, sodium oxalate and calcium chloride (each 0.05M) were mixed and incubated at 37°C for 48 h. After the mixture was evaporated at 45°C in a hot air oven, seed crystals of calcium oxalate were produced. The calcium oxalate seed crystals were mixed with Tris (0.05 M) and NaCl (0.15 M) with a pH of 6.5 to make a calcium oxalate solution with a concentration of 0.8 mg/mL. About 1.5 mL calcium oxalate solution was added to every 0.5 mL of extract or standard (Cystone) and incubated at 37°C for 30 min. The absorbance of the test samples and the standard were both measured at 660 nm and the percentage inhibition of calcium oxalate crystal aggregation was calculated, as stated in the nucleation assay (2.3.1).

2.3.3 Oxalate depletion assay

An oxalate depletion experiment was used to determine the effect of methanolic whole plant extracts of *A. lanata* on CaOx crystal formation [16]. Three strengths of AL extract were prepared in sterile distilled water: 1000 µg/mL, 1500 µg/mL, and 2000 µg/mL. At a concentration of 1.5 mg/mL and a pH of 5.7, calcium oxalate crystal slurry was prepared in sodium acetate buffer. About 1.5 mL of Tris (0.01 M) and NaCl (0.09 M) buffer were combined with 1 mL of 4 mM calcium chloride solution and 4 mM sodium oxalate solution, respectively. A volume of 30 µl of calcium oxalate crystal slurry was added to this mixture. The absorbance was then measured at 214 nm for 600 sec to determine the growth of the crystals. Next, the effect of AL extract at the concentrations on crystal development was tested by adding 1 mL of each concentration to the reaction mixture and measuring the change in absorbance. Finally, as described in the nucleation assay (2.3.1), the percentage inhibition of crystal development was calculated.

2.4 Urolithiasis efficiency of A. lanata using artificial urine

Artificial urine (AU) analysis was performed to investigate the impact of AL whole plant methanolic extracts on the CaOx crystallization development in a urine-like environment. With few modifications, the AU was made fresh according to the Burns and Finlayson procedure [17]. The composition of the AU prepared is given in Table 1. The effects of AL plant extract at concentrations of 1.0 mg/mL, 1.5 mg/mL, 2 mg/mL, 2.5 mg/mL and 3 mg/mL in artificial urine were compared to the same concentrations of the reference drug, cystone. To 1 mL of AU and 0.5 mL of different concentrations of AL plant extract were mixed with 0.5 mL of 0.01 M sodium oxalate solution. For a total of 30 min, the absorbance at 620 nm was measured at five-minute intervals [18]. After microscopic examination of the crystals generated, the level of inhibition was calculated as indicated in procedure 2.3.1.

Table 1 Composition of artificial urine.

Chemicals	Quantity (mM)
Ammonium chloride	0.0028
Sodium oxalate	0.32
Sodium citrate	3.21
Magnesium sulfate	3.85
Calcium chloride	4.5
Sodium sulfate	16.95
Ammonium hydroxide	17.9
Sodium phosphate	32.3
Potassium chloride	63.7
Sodium chloride	105.5

3. Results

3.1 Phytochemical analysis of A. lanata GC-MS data

The phytochemical components of the *A. lanata* whole plant methanolic extract were revealed by the results of the GC-MS spectrum [18]. The GC-MS chromatogram shows the presence of 22 peaks with a retention time range between 6.018 and 41.108 (Figure 2).

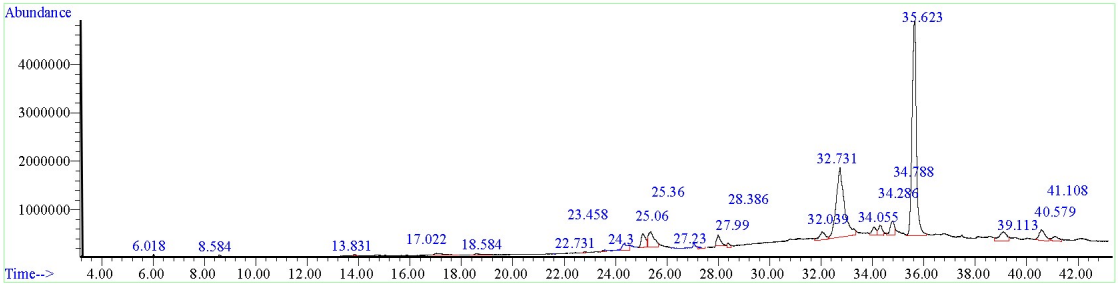

Figure 2 Gas chromatography-mass spectrometry chromatogram of methanolic extracts of the whole plant of *A. lanata*.

Table 2 Phytochemical constituents of methanolic whole plant extract of *A. lanata* from gas chromatography-mass spectrometry spectra.

Retention time (Min)	Compound name	Molecular weight	Molecular formula	Area
6.018	Eucalyptol	154	$C_{10}H_{18}O$	0.18%
8.584	L-camphor	152	$C_{10}H_{16}O$	0.28%
13.831	α-Elemene	204	$C_{15}H_{24}$	0.13%
17.022	Phenol, 2,4-bis(1,1-dimethylethyl)	206	$C_{14}H_{22}O$	0.95%
17.584	Carotol	222	$C_{15}H_{26}O$	0.51%
27.239	Phytol	296	$C_{20}H_{40}O$	0.87%
28.386	Heptadecanoic acid, 15-methyl-, ethyl ester	312	$C_{20}H_{40}O_2$	0.45%
32.039	β-Tocopherol	416	$C_{28}H_{48}O_2$	2.30%
32.731	γ-Sitosterol	414	$C_{29}H_{50}O$	26.20%
34.055	9-Hexadecenoic acid, eicosyl ester, (Z)-	534	$C_{36}H_{70}O_2$	1.47%
34.286	17-Pentatriacontene	490	$C_{35}H_{70}$	1.81%
35.628	α-Tocopherol	430	$C_{29}H_{50}O_2$	43.00%
39.113	Campesterol	400	$C_{28}H_{48}O$	3.35%
40.579	Stigmasterol	412	$C_{29}H_{48}O$	3.02%
41.108	(+)-α-Tocopherol, O-methyl	444	$C_{30}H_{52}O_2$	1.42%

Table 2 lists the compounds detected, along with the corresponding retention period, molecular weight, chemical formula, and concentration in terms of the percentage of peak area covered. Among the identified compounds, α-Tocopherol was found to be the major compound obtained with the largest peak (43%), and a retention time of 35.628 min. This was followed by γ-Sitosterol (26.2%) with a retention time of 32.731 min. The third-largest peak is due to the presence of campesterol, having a peak area of 3.35% with a retention time of

39.113 min. The compound stigmasterol showed a peak area of 3.02% with a retention of time of 40.579 min. The fifth less prominent peak (2.3%) was attained by the compound β-Tocopherol with a retention time of 32.039 minutes. The maximum retention time of 41.108 min was shown by the α-Tocopherol derivative, (+)-α-Tocopherol O-methyl which covered a peak area of 1.42%. The other phytochemicals showing the least prominent peaks are given in Table 2.

3.2 Anti-urolithiatic activity of A. lanata in in vitro analysis

3.2.1 Nucleation assay

The rate at which the methanolic AL extracts influenced the first step of urinary crystallization was investigated at various logarithmic concentrations (0.3,0.6,0.77,0.9 and 1 mg/mL) and was compared to the extent of nucleation in the control (without cystone /AL plant extract) and the standard drug, cystone. Formation of crystal nucleus was detected by turbidimetry, and the OD values help measurement of particle concentration per unit volume [19]. Here, the turbidity of the solution was observed to be lower in the samples containing AL extract than in the control. This indicates that a reduced extent of crystallization was achieved in the AL plant extract samples. The extent of inhibition at nucleation of the urinary crystals was observed to rise with an increase in the concentrations of the methanolic extracts of AL, as shown in Figure 3A.

Among the AL plant extract test concentrations used in the study, the least extent of inhibition (11%) was seen at a concentration of 0.3 mg/mL (log2) and the greatest magnitude of inhibition was observed at 1mg/mL with a percent inhibition value of 27%. However, an inhibitory percentage of 57% was observed in the samples containing 1mg/mL of cystone. The size of the crystals formed during the nucleation process showed a gradual decrease with the addition of the plant extract (Figure 3B). The control recorded a maximum average crystal area of 0.008 mm^2 and this decreased to a minimum average area of 0.001 mm^2 with the addition of 1 mg/mL of plant extract as shown in Table 3.

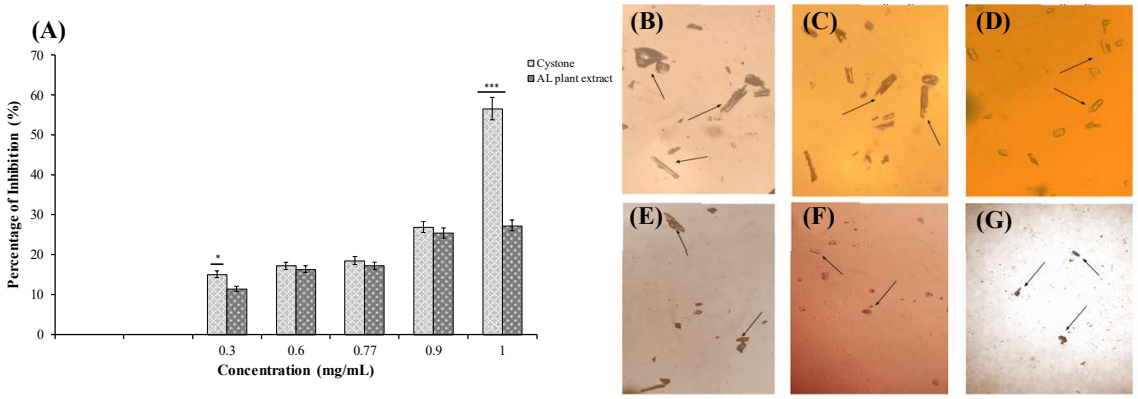

Figure 3 Graphical representation depicting percentage inhibition of crystal formation for various concentrations of methanolic *A. lanata* whole plant extract in the Nucleation assay (A). Microscopic images of nucleated calcium oxalate crystals as observed under a light microscope (X1000): Control (B), log2 mg/mL, log4 mg/mL, log6 mg/mL, log8 mg/mL, log10 mg/mL (C-G) concentrations of methanolic whole plant extract of *A. lanata*, respectively. All values expressed are mean ±standard deviation (n=3), $^{***}p<0.001$ compared to the standard drug. Statistical analysis of the data was done using Two-way analysis of variance (ANOVA) with Bonferroni post hoc correction tests. Calcium oxalate crystals depicted by black arrows. Elongated hexagonal crystal discs are the predominant nucleated forms observed. There was a dose-dependent decrease in crystal size and number.

Table 3 Percentage reduction in the area of nucleated calcium oxalate crystals at the 28th h of incubation in a dose-dependent manner in addition to different concentrations of methanolic whole plant extract of *A. lanata.*

Concentration (mg/mL)	Crystal area (mm^2)	Decrease in crystal area (%)
Control	0.008 ± 0.0018	0
0.301 (log2)	0.005 ± 0.0009	37.5
0.602 (log4)	0.004 ± 0.0026	50
0.778 (log6)	0.003 ± 0.0008	65.5
0.903 (log8)	0.002 ± 0.0015	75
1.000 (log10)	0.001 ± 0.0003	87.5

3.2.2 Aggregation assay

The aggregation of calcium oxalate crystals was investigated by comparing the extent of aggregation in the samples containing AL extract to those without any extract added. As significant inhibition effects for AL extract were observed at 1 mg/mL, the aggregation and oxalate depletion examinations were performed at different concentrations of 1 mg/mL, 1.5 mg/mL, 2 mg/mL, 2.5 mg/mL and 3 mg/mL. The crystal aggregation was studied at 660 nm absorbance. A direct relation was observed between the concentration of AL extract and the reduction in aggregation. The least extent of inhibition of 3.29% was seen with the addition of 1mg/mL of the extract whereas the sample containing 3 mg/mL of extract exhibited the highest percentage inhibition of 21.72%. The standard drug, cystone showed the highest inhibition of 46.3% at a concentration of 3mg/mL (Figure 4A). The amount of crystal aggregation observed was lesser in the presence of AL extract than in the absence of extract, both in terms of the number of aggregates and the size of individual aggregates (Figure 4B). This indicates the efficacy of methanolic whole plant extracts of AL in reducing the aggregation rate. The area of crystal aggregation was reduced significantly after the addition of plant extract, with a mean crystal area of 0.024 ± 0.014 mm^2 formed without adding the plant extract and a reduced mean area of 0.018 ± 0.007 mm^2 after the addition of AL extract after a 24-h incubation period, and a consequent reduction from 0.075 ± 0.033 mm^2 to 0.059 ± 0.025 mm^2 after 48 h of incubation. This decrease in area corresponded with the dose-dependent inhibition of stone formation, with the 3 mg/mL concentration showing the highest reduction in area. The inhibitory effects beyond the 3 mg/mL concentration were not statistically significant. Therefore, from our aggregation assay experiment, we found that the methanolic extract of *A. lanata* showed promising anti-urolithiatic potential, which is also reported by previously conducted experiments using different modes of extraction for *A. lanata* phytochemicals [20]. Our data is consistent with those reported in previous studies demonstrating lower absorbance related to reduced aggregation [21].

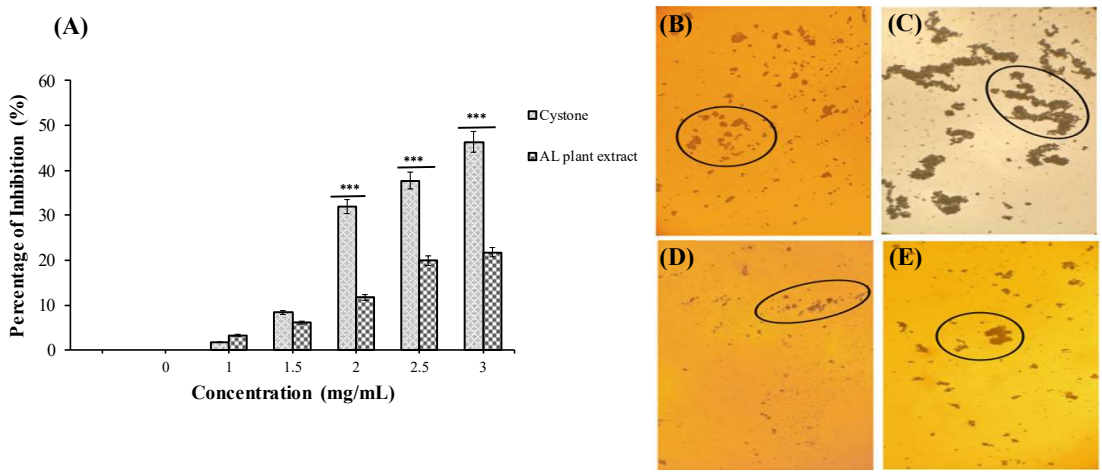

Figure 4 Percentage inh ibition of CaOx crystal aggregation for various concentrations of *Aerva lanata* whole plant extract in the Aggregation assay (A). Light microscope images of calcium oxalate crystal aggregates at 1000x magnification. (B) and (C) aggregation in control at the 24th h and 48th h, respectively. (D) and (E) aggregation in *A. lanata* methanolic whole plant extract (2 mg/mL) at 24th h and 48th h, respectively.

3.2.3 Oxalate depletion assay

The oxalate depletion assay experiment was used to establish the effects of methanolic *A. lanata* extracts on calcium oxalate crystal growth. The depletion of oxalate ions was observed to be higher in the presence of methanolic AL extract than in the absence of extract, indicating the possibility of the reduction of oxalate ions. An inhibition percentage of 8.14% was observed with the addition of 1000 µg/mL of the AL extract. The sample containing 2 mg/mL of AL extract exhibited the highest percentage inhibition of 14.09%. The standard drug cystone showed the highest inhibition of 36.04% at a concentration of 2 mg/mL of cystone. A comparative bar chart depicting the extent of calcium oxalate crystal growth inhibition in the sample and in the standard is shown in Figure 5.

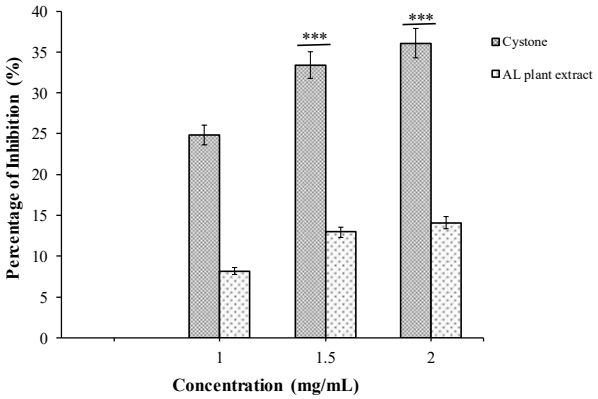

Figure 5 Percentage inhibition of calcium oxalate crystal growth for various concentrations of *A. lanata* whole plant extract.

3.2.3 Artificial urine assay

In the control sample, the size of urinary crystals was found to be the largest. The size of the crystals was reduced when varying quantities of plant extract were added in a dose-dependent manner (Figure 6A). The crystals that were formed due to the addition of various concentrations of the extract were on average smaller than the ones formed in the control sample. As shown in Table 4, AL extracts inhibited crystal development successfully, with a maximum average crystal area of 0.071 mm^2 formed in the control and a minimum crystal area of 0.008 mm^2 formed in the sample containing 3 mg/mL of extract, thus achieving a maximum of 88.73% reduction in the crystal area. The number of crystals formed as well as the percent inhibition of the formation of crystals decreased with an increase in the extract concentration, with the least extent of inhibition of 14.56% seen on the addition of 1.0 mg/mL of extract. The sample containing 3 mg/mL of extract exhibited the highest percentage inhibition of 52.36% which was slightly lower than the highest inhibition shown by the standard drug, cystone (56.04%). A comparative bar chart depicting the extent of inhibition in the sample and standard is shown in Figure 6B.

In comparison with the previous assays conducted in *in vitro* buffer solutions, there was a marked inhibitory efficiency of crystal inhibition in artificial urine. The percentage inhibitions at a concentration of 3 mg/mL for AL whole plant extract (52.36%) and the standard drug, cystone (56.04%) exhibited similar anti-urolithiatic activity with no significant variation in the percentage inhibition [22].

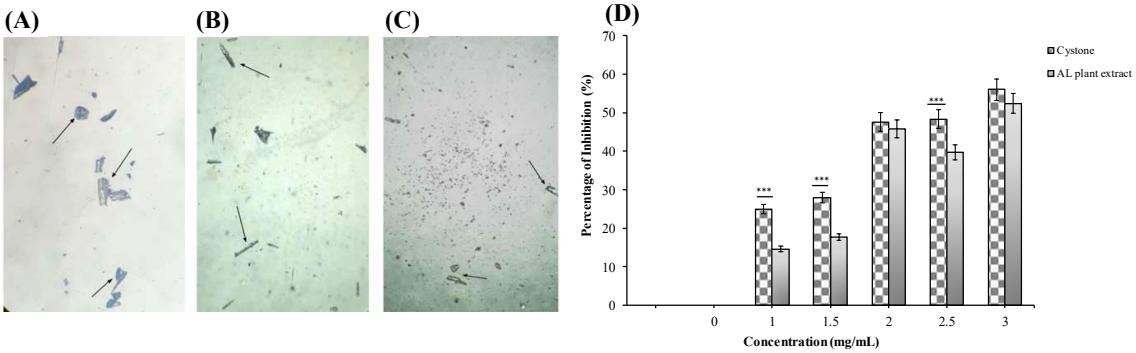

Figure 6 Photomicrographs of urinary crystals (depicted by black arrows) formed in artificial urine under a light microscope (X1000) at 1 mg/mL of methanolic whole plant extract of *A. lanata* (A); 2.0 mg/mL of methanolic whole plant extract of *A. lanata* (B); and 3 mg/mL of methanolic whole plant extract of *A. lanata* (C). Effect of whole plant extract of *A. lanata* against urinary crystals formation in artificial urine (D).

Table 4 Dose-dependent inhibitory effect of *A. lanata* plant extracts on calcium oxalate crystal development in artificial urine.

Concentration (mg/mL)	Crystal area (mm^2)	Decrease in crystal area (%)
Control	0.071 ± 0.009	0
1	0.039 ± 0.024	45.07
1.5	0.021 ± 0.017	70.42
2	0.016 ± 0.021	77.46
2.5	0.011 ± 0.003	84.5
3	0.008 ± 0.012	88.73

The greatest size reduction was achieved at a concentration of 3 mg/mL and considerable reductions of at least 45% were observed on incubating the urinary crystals with the plant extract.

4. Discussion

4.1 Interpretation of GC-MS data

The main groups of compounds identified were mainly terpenoids, phytosterols, plant alcohols, and stigmastanes. Studies on monoterpenoids such as eucalyptol and L-camphor obtained in Table 2 have shown antioxidant, antifungal, anticancer, antidiabetic and hepatoprotective activities [22,23]. Sesquiterpenes are cyclic or acyclic compounds having 3 isoprene units and 15 carbon atoms. Sesquiterpenoids such as α-Elemene and carotol obtained in Table 2, which are naturally found in plants as defensive agents [22], are related to sesquiterpenes that are produced through biochemical changes such as structural rearrangement or oxidation. Both monoterpenes and sesquiterpenes are good antioxidants [24]. Previous research on the prevention of CaOx urolithiasis by various medicinal plants reported the presence of the aforementioned terpenes and terpenoids in the different plant parts utilized for their anti-urolithiatic potential [25]. β-sitosterol, campesterol and stigmasterol are among the most common phytosterols and show hypocholesterolemic, antidiabetogenic and antioxidant activities [26,27]. Phytol is a diterpene alcohol and a precursor compound for the formation of Vitamin E and vitamin K_1. Phytol showed a retention duration of 27.239 min. Studies have established its antioxidant and anti-carcinogenic properties against epoxide-induced breast cancer [28]. Stigmastane is a class of steroids that has been reported to show anti-inflammatory and anti-parasitic activities [29].

4.2 Anti-urolithiatic activity of A. lanata

Calcium oxalate urolithiasis begins with nucleation, which is a crucial initial phase of stone formation [11]. In this study, methanolic whole plant extracts of *A. lanata* were shown to inhibit the CaOx crystal nucleation. This conclusion is supported by similar studies on *A. lanata* and other medicinal plants. A study of various fractions of the aerial parts and roots of AL showed that the highest inhibitory effect was achieved at a concentration of 1 mg/mL and that the aerial parts showed better anti-urolithiatic activity as compared to the root extracts [15]. This supports the claim that *A. lanata* plant extract has an anti-crystallization effect on CaOx crystallisation as seen by the decrease in the size of the crystals with plant extract as compared to the control. Anti-urolithiatic investigations with other medicinal plant extracts such as *Beta vulgaris* [26], *Daucus carota* roots [16] and *Herniaria hirsuta* [30] have reported similar phase changes in the inhibitory effects of calcium oxalate nucleation. It is likely that the plant extract forms complexes with free calcium and oxalate ions, thereby preventing or reducing the extent of nucleation [16].

Following the crystal nucleation and growth stages, aggregation of the crystals is the most prominent step in increasing the size of the urinary crystals and consequently results in cell injury, which is escalated by reactive oxygen species such as an increase in the oxalate concentration [20,21]. A dose-dependent reduction in CaOx aggregation was observed in a study of *L. prostrata* fractions with the highest percentage inhibition at 750 µg/mL [20]. In a study of *Beta vulgaris* leaf and root aqueous extracts, the highest inhibitory effects were observed at a concentration of 3 mg/mL and further microscopic examination revealed that the extract had successfully reduced the crystal size as compared to the control [21]. These findings are comparable to the present study in which a dose-dependent inhibition was observed over a period of 48 h, with 3 mg/mL of AL extract showing the highest inhibition. Microscopic analysis of the crystal size showed a similar decrease in area. The study thus supports the anti-urolithiatic potential of AL extracts during the aggregation stage of stone formation.

Cystone is an ayurvedic polyherbal preparation manufactured by Himalaya Drug Co. Ltd., Mumbai, India. The formulation predominantly consists of Didymocarpus pedicellata, Saxifraga ligulata, Rubia cordifolia, Cyperus scariosus, Achyranthes aspera, Onosma bracteatum, Vernonia cinerea, calcium silicate and other minerals. Studies have shown inhibitory effect of cystone on super saturation or deposition of calcium oxalate crystals, and drug induced renal toxicity [31]. Cystone is used as a reference drug to evaluate the anti-urolithiatic effect of putative anti-urolithiatic compounds, in addition to independent investigations on the efficacy of cystone in treating urolithiasis [11]. In the current study, have employed equal concentrations of cystone and methanolic

plant extracts to assess the anti-urolithiatic potential of the plant extract in comparison to similar doses of the reference drug.

The aqueous extracts of the barks of the medicinal plant *Terminalia arjuna* [32] showed promising anti-urolithiatic activity through inhibition of crystal growth and decreased free oxalate ion levels in a dose-dependent manner. *Achyranthes aspera* [33] also showed the inhibition of the growth of the calcium oxalate crystals. An *in-vitro* calcium oxalate crystal growth assay study of *Chenopodium album* leaf aqueous extracts showed a 51% growth inhibition in the presence of 1000 µg/mL of cystone and a 40% inhibition of growth [34]. Furthermore, a study of methanolic extracts of the aerial parts of *Mentha piperita* also showed that crystal growth inhibition took place due to the depletion of oxalate ions, with an inhibition percentage of about 48% at 1 mg/mL of extract. This was comparable to the 55% inhibition as observed with the same concentration of the standard, cystone [35]. In accordance with the methods and results of these studies, the present study concludes that methanolic whole plant extracts of *A. lanata* show similar inhibitory activity of crystal growth to the standard drug. This may occur due to an interference in the growth process by the activity of the secondary metabolites of the plant extract, as discussed by Aryal et al. [12] in a study of anti-urolithiatic activities of the methanolic extracts of five medicinal plants.

4.3 Anti-urolithiatic efficacy of A. lanata in artificial urine

Various *in-vitro* studies have utilized artificial urine (AU) to mimic physiologic human urine [36]. This is the first study to compare the anti-urolithiatic efficacy of whole AL plant extract in synthetic urine to that of other *in-vitro* tests. In a synthetic urine-like environment, AL whole-plant extracts exhibited anti-urolithiatic activity comparable to that of the standard drug cystone, which was greater than in cell-free *in-vitro* assays. This enhanced anti-urolithiatic efficacy of AL whole plant extracts in synthetic urine provides further evidence of its anti-urolithiatic potential under physiological conditions, which will be investigated further through animal testing.

Overall, our study shows that methanolic extracts of *A. lanata* have a therapeutic effect on urolithiasis. However, further studies in cell and animal models are required to establish its clinical implications. The highest degree of reversal of crystal formation was observed in artificial urine, where the effects shown by the study samples were comparable to the effects of cystone. The plant extracts also inhibit nucleation and crystal growth but to a lesser extent. Further studies into the pharmacokinetics of crystal formation will elucidate the effect of plant extracts on kidney stone formation.

5. Conclusion

In this work, methanolic whole plant extracts of *A. lanata* showed promising anti-urolithiatic potential, providing scientific proof for traditional medicine. Enhanced inhibitory action and calcium oxalate crystal size reduction during kidney stone formation were dose-dependent. Analyses of stone formation in artificial urine demonstrated the inhibitory potency of the plant extracts during the initiation of crystallization. The phytochemical analysis of the AL plant, identified by GC-MS, showed the presence of several therapeutic compounds. These significant phytoconstituents possibly could account for their anti-urolithiatic activity and prevention of stone reoccurrence. Further *in vitro* studies in cell lines and *in vivo* investigations in murine models are required to determine the potential of *A. lanata* as a preventive or alternative treatment for urolithiasis.

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