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Research Article

In-vitro assessment of U*rginea indica* (R.) Bulb fractions against calcium oxalate crystallization: Role of Citronellal identified by GC-MS

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Abstract

Objectives: The present study aimed to investigate the in-vitro antiurolithiatic activity of the methanolic bulb extract of *Urginea indica* (R.) and its fractions, focusing on their ability to inhibit calcium oxalate (CaOx) crystal nucleation, aggregation, and oxalate degradation. The goal was to validate its traditional use in urolithiasis and assess its potential as a clinically relevant phytotherapeutic agent. Methods: *Urginea indica* bulbs were extracted using Soxhlet extraction with methanol, followed by column chromatography to yield five fractions (F1–F5). A factorial approach guided the optimization and separation of fractions using TLC profiling. GC-MS analysis identified key phytoconstituents. In-vitro assays for nucleation, aggregation, oxalate degradation, and titrimetric estimation of calcium oxalate were conducted to assess antiurolithiatic efficacy. Cystone was used as the standard reference. Results: Phytochemical screening revealed the presence of alkaloids, flavonoids, terpenoids, tannins, and saponins. GC-MS identified citronellal (49.43%), camphor (12.79%), and other active compounds. Among the fractions, F5 consistently showed the highest activity: 85% nucleation inhibition, 84% aggregation inhibition, 84% oxalate degradation, and 87% inhibition of CaOx content at 1000 µg/mL, closely matching the standard Cystone. F5 was thus selected as the optimized batch based on superior efficacy across all in-vitro parameters. Conclusion: *Urginea indica* fraction F5 exhibits significant antiurolithiatic activity, supporting its traditional use and potential clinical application. Its multi-targeted action and natural origin make it a promising candidate for future development. Further in-vivo studies are essential for clinical translation.

Keywords: *Urginea indica,* Urolithiasis, Calcium oxalate, Antiurolithiatic, GC-MS, Cystone, Herbal medicine, Phytoconstituents

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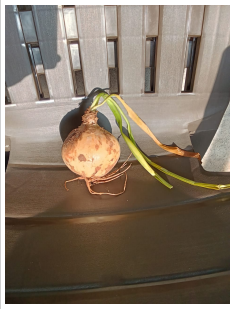
Introduction

Urolithiasis is an illness ailment experienced by approximately 12% of the global population and it is the third most prevalent urinary illness. There is more likelihood that males would recur compared to females (1). It has a multifaceted aetiology which is strongly influenced by genetic, epidemiological, biochemical risk factors (2). More than 80% of the stones contain calcium oxalates (CaOx), the concentration of which forms the primary component of most of the stones. The other 2% is mostly composed of uric acid, cysteine and struvite (3). The pathogenesis of urolithiasis is a complex course, the development of which is stimulated by a number of physicochemical processes. When supersaturation of the urine increases, CaOx crystallizes and this leads to the formation of solid crystalline particles within the urinary system. This is succeeded by nucleation which subsumes the aggregation of stone-forming salts in the supersaturated urinary solution into clusters that are later expanded in terms of size due to the introduction of new constituents (4). These. Such crystals are later deposited and collected in the urinary system after growing and clumping together with other crystals in solution. Along with promoting the formation of crystals at a lower supersaturation level, renal damage promotes crystal retention and nidus of a stone on renal papillary surfaces (2). Uroliths mainly occur due to supersaturation of the urinary salts in urine. The reason gives outward connection between the type of stone which is formed and the degree of urine supersaturation. Hence, stone recurrence prevention is applicable through the decrease of supersaturation (3).

Figure 1*: Urginea indica* (R.) bulb



Urolithiasis is managed on the basis of size and location of the stones. Alkali citrate and thiaiazide diuretics are also very common in preventing urolithiasis recurrence. An endoscopy procedure such as endoscopic lithotripsy to extract the stones with

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larger size beyond 5mm or those that fail in excretion should be undertaken through interventional means like endoscopic stone extraction, extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS) or percutaneous nephrolithotomy (PNL). These treatments can prove to be very expensive to many individuals and there are 60% likelihoods that kidney stones can rebuild. They must also be carefully observed to take action in case of problems such as acute renal damage many years afterwards (2). Thus, phytotherapeutic agent may be found as a considerable way of complementary and alternative therapy in multiple countries, such as India, in terms of urolithiasis management. Medicinal herbs have been widely used in many of the old time traditional kidney stone nursing routines. The plants provide an affordable alternative supply of drugs, are readily available, affordable, and considered to be relatively safe with limited or no adverse effects (3). In order to differentiate between systems that investigate the pathophysiology of renal stone disease and those that investigate the physical chemistry of stone formation, in vitro approaches to experimental nephrolithiasis are more often used. Analysis of the process involved in crystal nucleation, growth and aggregation is often undertaken in systems in vitro, in which the first requirement can be realized (5). It is noted that several medicinal herbs inhibit CaOx crystallizations (6).

Similar reports indicate that an extract from the herb *Herniaria hirsuta* L., traditionally used to treat lithiasis in Morocco, has led to an increase in the quantity of the calcium oxalate crystals, yet a reduction in size, subsequently fostering, in turn, its nucleation. H. hirsuta was subsequently demonstrated to inhibit crystal attachment to cultured kidney cell (7). Phyllanthus niruri L, a traditional medicine of Brazilian urolithiasis, had similar in vitro effects on calcium oxalate crystallization with its aqueous extract. According to these authors, the extract inhibited the process of CaOx crystallization, suppressing the growth of CaOx crystals and their assembly (8). Demonstrated that the presence of an extract made of the seeds of Vigna unguiculata (L.) Walp. used in traditional Indian ayurvedic medicine inhibited the precipitation of in vitro calcium and phosphate. It has also been demonstrated that some Kampo herbs and traditional Chinese medicines (TCM) could inhibit calcium oxalate crystallization (9). On balance, the in vitro crystallization experiments have shown that seeding miniature crystal nucleates and reducing supersaturation assisted in prevention of kidney stones (10). *Urginea indica*(R.) plants used in this study belonging to family of Liliaceae are normally called Indian squill or sea onion (Figure 1) Perennial geophyte with fibrous roots up to eight to ten inches long, the rounded conical, pear shaped bulbs have white transparent scales on them of considerably big size, that of a large size of onion (11-14). Traditional ayurvedic medical practitioners in India use these plants as a treatment option in urolithiasis (15). Along with the antiurolithiatic properties, antidiabetic, anticancer, antimicrobial, and cardiac effects are treated with *Urginea indica* (R.) (16).

Cystone is a polyherbal product developed to cure urolithiasis. This formulation has been in clinical use since long and has been given approval of being ayurvedic formulation by Indian regulatory bodies in the management of urinary calculi (17). It is hence adopted regularly as a reference drug in many exploratory experiments about the screening of medicinal plants and as part of the preventive agent of urolithiasis against antiurolithiatic potential. As various phytochemicals are extracted using different polar solvents; the present study aimed at elucidating the in vitro antiurolithiatic property of methanol extract of bulb of *Urginea indica* (R.) nucleation, aggregation, growth and transformation of CaOx crystals relative to the popular polyherbal supplement, Cystone and to support the scientific rationale of using *Urginea indica* (R.) plant in the traditional systems of medicine.

Materials and Methods

Materials

The analysis was carried out in Cystone (Himalaya Herbal Healthcare, Bangalore, India) and sodium oxalate (Fine Chemicals, Mumbai).All other chemicals and reagents were of the analytical grade and were of approved suppliers. Instruments used by the researchers include the soxhelt apparatus, UV-spectrophotometer (INESA L6S) and Leica DM 2500 LED microscope.

Methods

Collection, authentication, preparation and extraction of plant material

This plant is *Urginea indica* (R.) and was collected in the month of August in region Dudheshwar Gadh, Nimgaon-Jali, Taluka Sangamner, District Ahilyanagar, Maharashtra, India. The plant material was herbarium-processed following regular botanical procedures and deposited to the Department of Botany, Padmashri Vikhe Patil College of Arts, Science and Commerce, Loni where it was authenticated. A taxonomic verification of the plant specimen was done by Dr. A.S. Wabale, and a reference number PVPC/Bot/2024-25/301 was given to the voucher specimen which has been conserved to be referred to later in the future. After identification to botanical species, the *U. indica* bulbs were thoroughly scrubbed with running tap water to wash surface impurities. After cleaning, the bulbs were chopped into small portions and shade dried within 15 days. The dry product was then coarsely ground up with a mechanical grinder. A 100 g of the powdered substance was Soxhlet-extracted in 500 mL of methanol as a solvent do so over a period of 8 hours. The resultant extract was filtrated to remove any remaining insoluble plant material or other solid impurities from the liquid extract and the methanolic extract was dried by evaporating the solvent under reduced pressure through a vacuum dryer to give an extremely concentrated, dried extract, which was kept at 4°C until further analysis of phytochemical and pharmacological properties (18-20).

Preliminary Phytochemical Screening

The methanolic extract of *Urginea indica* bulbs was subjected to preliminary phytochemical screening to detect the presence of various bioactive constituents. The extract was tested for the presence of alkaloids, flavonoids, glycosides, saponins, tannins, phenols, steroids, terpenoids, and carbohydrates using standard qualitative methods (21).

TLC profiling for solvent selection for column chromatography

An analysis was conducted on the best solvent system to be used on the column chromatography of methanolic extract of *Urginea indica*, using thin layer chromatography (TLC). This was done on Silica gel 60 F 254 TLC plates (Merck, Germany). A drop of extract was placed in the form of a spot on the plate of the TLC and development was performed through the following solvent concentrations: n-hexane, n-hexane:benzene (5:5), benzene, benzene:ethanol (9:1) and ethanol (23). The plates were examined under UV (254 and 366 nm), iodine vapor and vanillin sulfuric acid spray reagent after development. The system that has given sharp and distinct and well separated spots was chosen as the most

appropriate mobile phases to be used in the further column chromatography (22, 23).

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

Using Gas ChromatographyMass Spectrometry (GC-MS) analysis, the methanolic extract of *Urginea indica* bulbs was tested to determine volatile as well as semi-volatile phytoconstituents. An analysis was conducted by a GC-MS apparatus (Agilent Technologies, Model 7890B GC coupled with 5977A MSD, USA) containing an HP-5MS capillary column (30 m 0.25 mm i. d., 0.25 mm film thickness). The oven was first set to 60 °C followed by ramping up to 300 °C at a rate of 10 °C/min and maintained at this temperature after 10 minutes. The carrier gas was helium with a constant flow rate of 1.0mL/min. The temperature of the injector was set at 250 °C and 1 µL of the methanolic extract (dissolved in methanol) injected in splitless mode. Operated in the electron ionization (EI) mode, the mass spectrometer was set to 70 eV operating in a mass range of 50 to 600 m/z. The identification of the compounds was done with the help of the mass spectrum of the eluted peaks and comparison of it with NIST 2014 spectral libraries. It was noted that the retention time, molecular weight, as well as the area percentage of each of the identified compounds were also noted. This qualitative profiling has given details of chemical content of the extract some of which could be attributed to the activity observed in the extract as related to known bioactivities of phytochemicals identified (24).

Fractionation by column chromatography

A single glass column (60 cm length, 3 cm diameter), which was filled with activated silica gel (60 120 mesh, Qualigens, India) was used to fractionate mainly the methanolic extract of *Urginea indica*. The slurry-packing of silica gel was done with n-hexane to the formation of uniform bed package. The pre-adsorbed dried extract was placed only in a small amount of silica gel and carefully placed at the top of the column. The procedure of elution was done by using the solvent of increasing polarity; n-hexane, n-hexane:benzene (5:5), benzene, benzene: ethanol (9:0.5) and ethanol. The collection of fractions was done in a sequential manner total 28 fractions were collected each of 20 ml and followed by TLC on Silica gel 60 F 254plates monitored by ultra violet light and spray reagent vanillin-sulfuric acid. Fractions with similar TLC patterns were combined (25).

***In-vitro* anti-uroliathiatic activity evaluation**

Nucleation assay

The calcium oxalate nucleation assay was used to determine the changes in the in vitro anti-urolithiatic activity of the fractions of *Urginea indica* which are named F1, F2, F3, F4, and F5. The standard reference was Cystone (Himalaya Herbal Healthcare, India, Batch No. 11700361). Calcium chloride (5 mM) and sodium oxalate (7.5 mM) solutions were prepared in Tris-HCl buffer (0.5 mM) containing NaCl (0.15 mM), and brought to pH 6.5. F1 to F5 and Cystone were dissolved in distilled water to make a concentration of 200, 400, 600, 800, and 1000 µg/mL. In the assay, 1 mL of the test sample was combined with 3 mL of a $CaCl^2$ solution and 3 mL of a $Na^2C^2O^4$ solution and incubated at 37 °C for 30 minutes. The samples were then allowed to cool at room temperature and turbidity was measured at 620nm by INESA L6S UV- Visible spectrophotometer. The % inhibition of nucleation of calcium oxalate crystal was calculated with the following formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{OD test}}{\text{OD control}} \times 100 \quad (1)$$

where OD_test and OD_control represent the absorbance values of the test sample and control, respectively. Additionally, microscopic evaluation of calcium oxalate crystal number, size, and morphology was conducted for F5 and Cystone using a Leica DM 2500 LED microscope at 1000× magnification to assess morphological changes induced by treatment.

Aggregation assay

The calcium oxalate aggregation assay to further on determine in vitro anti-urolithiatic activity of the fractions was done by labelling the samples as F1, F2, F3, F4,F5 and Cystone used as the standard. The crystals of calcium oxalate were obtained by combining equal aliquots of 50 mM solutions of calcium chloride and sodium oxalate in Tris-HCl buffer (0.05 M, pH 6.5) supplemented with NaCl (0.15 M) and incubating it at 37 °C for 1 hour. The crystals were washed, centrifuged and resuspended in the buffer to the final concentration of 0.8 mg/mL. The crystal suspension was mixed with each test sample (F1 F5) and Cystone at a range of 100-1000ug/mL and left to incubate at 37 °C for 30 minutes. The absorbance was taken at 620 nm by employing INESA L6S UV-Visible spectrophotometer. The percentage of the inhibition of the crystal aggregation has been determined using equation 1.

Oxalate Degradation Assay

The oxalate degradation capacity of *Urginea indica* fractions was determined using an oxalate degradation assay and the test samples referred as F1, F2, F3, F4, and F5. The standard was Cystone. Potassium oxalate (10 and 50 mM) in 0.05 M potassium phosphate, pH 7.0, was prepared. All fractions and the standard were run in twofold dilution (100,500 and 1000 µg/mL). In the assay, a total of 1 mL of the test solution was combined with 3 mL of potassium oxalate solution and incubated at temperatures of 37 °C and 1 hour. Following incubation, the left-over oxalate concentration was determined spectrophotometrically at 214:nm on an INESA L6S UV- Visible spectrophotometer. To calculate the percentage of oxalate degradation a formula was utilized;

$$\% \text{ Oxalate Degradation} = 1 - \frac{\text{OD test}}{\text{OD control}} \times 100 \quad (2)$$

where OD_test represents the absorbance of the reaction mixture with the test fraction and OD_control corresponds to the absorbance of the control (without any sample). A reduction in absorbance indicated the ability of the test fractions to degrade or reduce oxalate content.

Titrimetric Estimation of Calcium Oxalate Content

A standard titrimetric method was used to estimate the content of calcium oxalate. Crystals were made by adding equal parts of 50 mM calcium chloride and 50 mM sodium oxalate in phosphate buffer (pH 6.5) together and allowing them to incubate at 37 °C for 1 hour after which thorough washing was done. The following amount was taken to estimate oxalate 10mL crystal suspension then 10mL 1N sulphuric acid added to it, to dissolve the crystals, and a titration carried out with 0.05N potassium permanganate at 60–70 °C until a faint pink endpoint gave. The volume of $KMnO_4$ used is directly proportional to the oxalate content and calculated with following formula (26):

$$\text{Amount of calcium oxalate (mg)} = \frac{\text{Vol.of } KMnO^4 \times N \times \text{Equi.wt.of oxalate}}{\text{VoL.of sample used}} \times 100 \quad (3)$$

Results and Discussion

Results

Phytochemical investigation

Methanolic extract of the bulbs of *Urginea indica* contained the presence of major phytoconstituents such as alkaloids, flavonoids, phenolics, tannins, glycosides, proteins and carbohydrates, saponin and terpenoids, whereas, starch was absent (Table 1). These findings signify presence of varied phytochemical composition, which is indicative of possible pharmacological potential that could be the source of its conventional application in urolithiasis and justify its investigation based on antiurolithiatic applications.

Table 1: preliminary phytochemical investigation of *Urginea indica* methanolic extract

Phytoconstituents	Test	Methanolic extract
Alkaloids	Dragendorff 's test	+
	Mayer's test	+
	Hager's test	+
Flavonoids	Alkaline reagent test	+
	Shinod's test	+
Phenolic compounds and tannins	Ferric chloride test	+
	Lead tetra acetic acid test	+
Proteins	Biuret test	+
	Ninhydrin test	+
Carbohydrates	Molish's test	+
	Benedict's test	+
	Fehling's test	+
Glycosides	Keller Killiani test	+
Saponins	Froth test	+
Terpenoids	Horizon test	+
	Salkowski test	+
Starch	Iodine test	-

(-) indicates the absence of phytoconstituents and (+) indicates their presence.

Pre-column TLC Profiling

TLC profiling of the methanolic extract of *Urginea indica* pre-column using various solvent systems exhibited each having a unique separation pattern with only one spot being common in all the systems (Table 2). Its Rf values were between 0.38 (in n-hexane) and 0.73 (in ethanol), which means that with more polar solvents, the polarity of the compound increases. Benzene:ethanol (9:0.5) and ethanol gave a better resolution and this led to the two being used in further column chromatography.

Table 2: TLC Profiling of *Urginea indica* Fractions Using Different Solvent Systems

Solvent System	No. of Spots	R_f Values
n-Hexane	1	0.38
n-Hexane : Benzene (5:5)	1	0.53
Benzene	1	0.61
Benzene : Ethanol (9:0.5)	1	0.69
Ethanol	1	0.73

Post column TLC profiling and collection of fractions

TLC profiling of *U. indica* fractions by post-column TLC led to the pooling of 28 column eluates into five different fractions as defined by the spot characteristics (Table 3). In each of the pooled groups, one spot provided F1 (0.41), to F5 (0.69) increasing Rf indicating sequential elution of more and less polar compounds. Such homogeneous separation validated successful fractionation to be used in subsequent in-vitro antiurolithiatic testing.

Table 3: TLC Profiling and Pooling of Column Chromatography Fractions of *Urginea indica*

Fraction Numbers Pooled	No. of Spots	R_f Value	Final Fraction Code
1–5	1	41	F1
6–10	1	49	F2
11–15	1	57	F3
16–21	1	63	F4
22–28	1	69	F5

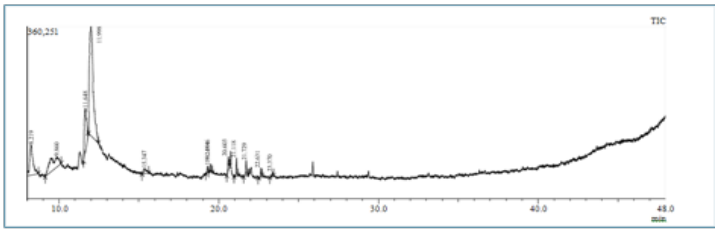
GC-MS analysis

Analysis of the methanolic extract using GC-MS demonstrated 14 significant phytoconstituents in bulbs of *Urginea indica* (Figure 2; Table 4). The most significant and key warfare agent- probing compound was identified as Citronellal (49.43% area), followed by camphor (12.79 %) and the cyclohexanol derivative (12.67%) recovered and found to be the main representatives of terpenoids. There were other key constituents such as flavonoid compounds which were considerable and some were dl-Isopulegol and derivatives of pyrazines; aromatic compounds and stereo-isomers. These bioactives validate the possibility of pharmacological activities of the extract.

Table 4: Compound identified in *Urginea indica* methanolic extract

Peak	R Time	Area %	Name	Identified Compounds
1	3.04	6.16	Pyrazine, 2,6-dimethyl-	Flavanol
2	5.91	1.56	gamma.-Terpinene	Terpenoid
3	8.21	12.79	Camphor	Terpenoid
4	9.86	12.67	Cyclohexanol, 5-methyl-2-(1-methylethenyl)	Terpenoid
5	11.6	10.57	dl-Isopulegol	Flavonoids
6	11.99	49.43	Citronellal	Terpenoid
7	15.34	1.65	Benzene, 2-methoxy-1-methyl-4-(1- methyleth	Aromatic Compound
8	19.28	0.92	Copaene	Terpenoid
9	19.49	0.66	alfa.-Copaene	Terpenoid
10	20.60	1.56	Caryophyllene	Terpenoid
11	21.11	1.65	cis-.alpha.-Bergamotene	Sterioisomer
12	21.72	1.83	1,4,7,-Cycloundecatriene, 1,5,9,9- tetramethyl-	Terpenoid
13	22.63	0.57	Naphthalene, decahydro-4a-methyl-1-methyle	Aromatic Compound
14	23.37	0.32	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dim	Aromatic Compound

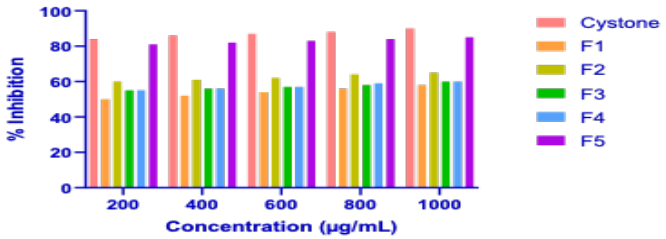
Figure 2: GC-MS spectra of *Urginea indica* methanolic extract



Nucleation Assay

Nucleation assay revealed that each fraction of *U. indica* (F1 to F5) was more effective in dose-dependently preventing the formation of calcium oxalate crystals and F5 was the most efficient one among them (Figure 3). At 1000 µg/mL, F5 showed 85% inhibition, which is next to the standard Cystone (90%). The inhibition values increased progressively with concentrations between 200 and 1000 µg/Ml in all the fractions implying that these fractions had a possible concentration-dependent capability to inhibit special nucleation of crystals.

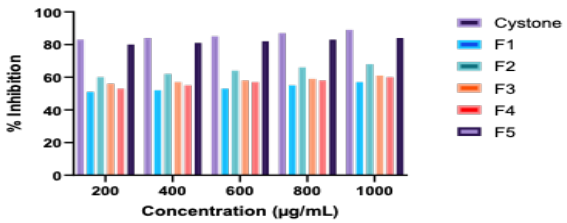
Figure 3: Effect of *Urginea indica* Fractions (F1–F5) and Cystone on Calcium Oxalate Crystal Nucleation at Varying Concentrations (200–1000 µg/mL)



Aggregation assay

The aggregation assay analysis on calcium oxalate system demonstrated that the fractions F1 to F5 of *Urginea indica* have the effect of inhibit crystal aggregation in a concentration dependent way and fraction F5 had the best activity among the tested samples (Figure 4). When the concentrations were tested to their maximum, F5 demonstrated 84% inhibition, which is very similar to Cystone with 89% inhibition. Each fraction had gradual increments in the inhibition between the lowest doses and highest doses indicating that they had the ability to minimize clumping of crystals, in which F2 and F5 were markedly stronger in its action.

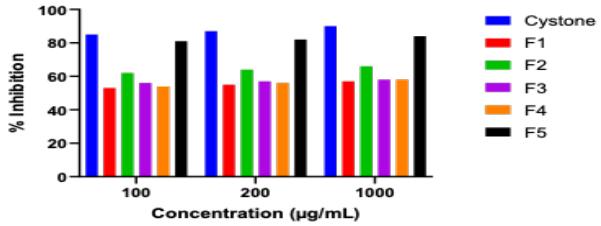
Figure 4: Effect of *Urginea indica* Fractions (F1–F5) and Cystone on Calcium Oxalate Crystal aggregation at Varying Concentrations (200–1000 µg/mL)



Oxalate degradation assay

Oxalate degradation assay revealed that all of the *Urginea indica* fractions (F1-F5) exhibited augmenting degradation of oxalate as the concentration (100, 200 and 1000 µg/mL) escalated as in Figure 5. F5 had comparatively high degradation activity with a range of 84% at 1000 µg/mL which approximated the standard Cystone (90%). The activity of F2 moderate and the relatively lesser degradation in F1 and F4 were observed. Such a trend answers the concentration-specific oxalate-reducing capacity of the extract.

Figure 5: Oxalate Degradation Activity of *Urginea indica* Fractions (F1–F5) Compared to Cystone at Different Concentrations



Titrimetric estimation of calcium oxalate

The phenol titration technique indicated a dose proportional decrease in the concentration of oxalate in all *Urginea indica* fractions with F5 fraction exhibiting the maximum inhibition of 87% even at the lowest concentration, 1000 µg/mL, bleeding well near the standard Cystone, 92% (Table 5). There was a positive correlation of $KMnO_4$ and calcium oxalate content and a linear decrease in their volume with an increase in concentration. F2 and F3 were the next last in terms of performance ranking among the test samples with F1 and F4 having relatively lower inhibition.

Table 5: Titrimetric Estimation of Calcium Oxalate with $KMnO_4$ Used and % Inhibition

Sample	Conc. (µg/mL)	$KMnO_4$ Used (mL ± SD)	CaC_2O_4 (mg)	% Inhibition
Cystone	400	6.22 ± 0.10	14.01	86
	600	5.33 ± 0.08	12.00	88
	800	4.44 ± 0.07	9.99	90
	1000	3.55 ± 0.05	7.99	92
F1	400	13.32 ± 0.11	29.99	70
	600	12.87 ± 0.09	28.99	71
	800	12.43 ± 0.10	28.00	72
	1000	11.98 ± 0.08	26.97	73
F2	400	10.21 ± 0.10	23.00	77
	600	9.77 ± 0.09	22.01	78
	800	9.32 ± 0.08	20.99	79
	1000	8.88 ± 0.09	19.99	80
F3	400	11.99 ± 0.09	27.00	73
	600	11.54 ± 0.10	25.98	74
	800	11.10 ± 0.08	24.98	75
	1000	10.66 ± 0.07	23.99	76
F4	400	12.87 ± 0.08	28.99	71
	600	12.43 ± 0.10	28.00	72
	800	11.98 ± 0.09	26.97	73
	1000	11.54 ± 0.08	25.98	74
F5	400	7.10 ± 0.09	15.98	84
	600	6.66 ± 0.08	14.99	85
	800	6.22 ± 0.07	14.01	86
	1000	5.77 ± 0.06	12.99	87

All values are expressed as mean ± SD

Discussion

Methanolic extract of the *Urginea indica* bulbs had a unique phytochemical profile with a generous mixture of secondary metabolites such as alkaloids, flavonoids, tannins, terpenoids, glycosides, saponins, proteins, and carbohydrates as illustrated in Table 1. The literature is well lined with occurrence of such compounds as was seen in their bioactivity against renal calculi formation due to their antioxidant, diuretic and calcium chelating strengths. The lack of starch and the occurrence of various phytoconstituents in the extract was a good reason for using it in urolithiasis as well as the reason to continue using it in the analysis (21). Initial profiling of the TLC using various solvent systems before subjecting to column chromatography depicts that components can be successfully run and separated using varied solvent systems. Table 2 depicts an upward trend in values of Rf of non-polar (n-hexane) to polar solvents (ethanol) which explains the chemical diversity of the extract. The post-column fractionation, TLC profile (Table 3) also demonstrated that extract components were separated into five distinct fractions (F1-F5) that actually exhibited different Rf values of 0.41-0.69. Such fractionation was necessary to purify and analyze bioactive components that bear particular antiurolithiatic potential (27,28).

Chemical composition The GC-MS chromatogram analysis of the methanolic extract further revealed more information on the chemical composition. Fourteen compounds were identified, and a prominent peak was detected of citronellal (49.43%) that is monoterpenoid with anti-inflammatory and antimicrobial properties, according to Figure 2 and Table 4. Other major constituents were camphor (12.79%) and cyclohexanol derivatives, pyrazine and isopulegol. Terpenoids as well as flavonoids predominance agrees well with the biological efficacy of the extract, since the two classes of compounds have been noted to interfere with crystal growth as well as oxidative stress, which are among the major pathogenesis pathways in urolithiasis. Nucleation assay was used to determine the first stage of crystal formation of calcium oxalate (29). All fractions were dose dependent by inhibiting nucleation as shown in Figure 3. Fraction F5 showed great activity with 85% inhibition at 1000 µg/mL, which is very near to Cystone of 90 % inhibition at the same concentration. The F2 and F3 fractions were moderately active and the least active ones were F1 and F4. One possible interpretation of this finding is that the bioactive molecules in F5 would disrupt the ionic interactions or trigger complexation of calcium/oxalate ions, thus preventing crystals to form initial crystal nuclei (30,31).

Additional testing using the aggregation assay gave an idea of the ability of fractions to inhibit the clumping of preformed crystals. Inhibitory activity was once more exerted F5 with has the best result in all the test fraction as illustrated in Figure 4, with 84% aggregation inhibition being achieved at 1000 µg/mL, versus 89% inhibition by Cystone. This tendency supports the possibility of F5 to not only avoid the early formation of CaOx crystals but to prevent subsequent aggregation, one important domain in stone formation and retention in renal tubules. The oxalate degradation experiment examined the capacity of the extract to lower the level of oxalate in the media, which is very important in supersaturation and crystal formation. As it can be seen in Figure 5, all fractions exhibited an oxalate-degrading capacity that improved with concentration. F5 was once more the greatest active degrading 84 % of oxalate at 1000 µg/mL whereas Cystone attained 90% destruction. The capacity to decrease the soluble oxalate underlines the potential of the extract reducing urinary oxalate levels and by extension, the danger of CaOx stone formation (32).

As to prove the biochemical significance of the findings, titrimetric technique was used as a direct estimation of calcium oxalate post-treatment. Table 5 showed reduction in $KMnO^4$ used to replace titration in all the fractions as the concentration of the fractions increased which was related to the decrease in oxalate content. Fraction F5 elicited the best inhibitory effect with maximum inhibitory activity at 1000 µg/mL and 87% as compared to that of Cystone at 92%. The results provide the evidence of the dose-dependent effect based on the reduction in the availability of oxalate to create crystals that confirms the results of spectrophotometric assays (33,34).

Conclusion

The current research established that the methanolic extract of *Urginea indica* bulbs and especially fraction F5 have substantial antiurolithiatic activities in vitro since they potently inhibited the nucleation of calcium oxalate crystals and aggregation and degradation of oxalate as well as showed a similar effect to the standard drug Cystone. This is due to the rich phytochemical profile which is confirmed by the GC-MS profiling results with evidence showing the presence of bioactive phytoconstituents such as terpenoids and flavonoids that may attribute to this effect. These revelations indicate that *Urginea indica* could be useful phytotherapeutic option in the prophylaxis as well as treatment of urolithiasis. The cost-effectiveness, natural nature and multi-mechanistic effect of the formulation reinforce its possible use in the medical setting. What unarguably needs to be done however, is more in-vivo studies and toxicological assessment of the same so as to confirm its efficacy and safety and as such bring it under development as one of the standardized antiurolithiatic herbal formulations.

Abbreviations

GC-MS: Gas Chromatography–Mass Spectrometry; TLC: Thin Layer Chromatography; CaOx: Calcium Oxalate; $KMnO_4$: Potassium Permanganate; OD: Optical Density; SD: Standard Deviation; µg/mL: Microgram per milliliter; Rf: Retardation Factor; EI: Electron Ionization; NIST: National Institute of Standards and Technology; F: Fraction; HCl: Hydrochloric Acid; NaCl: Sodium Chloride; C_2O_4: Oxalate Ion; Tris-HCl: Tris(hydroxymethyl)aminomethane Hydrochloride Buffer; m/z: Mass-to-charge ratio; °C: Degree Celsius; %: Percentage.

Conflict of interest

Authors declare no conflict of interest regarding this study

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