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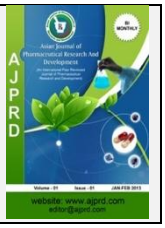

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Research Article

Evaluation of Anti-Urolithiasis Activity of Leucasaspera By*in Vitro* Method

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A B S T R A C T

Objective: Urolithiasis is the accumulation or development of stone in any component of urinary system including kidney, ureters and urinary bladder. The aim of the current research is to perform anti- urolithiasis activity of ethanolic extract of *leucasaspera* can be evaluated *in vitro* by utilize the nucleation assay, aggregation assay, growth assay and titrimetric method.

Methods: The leaf of leucas aspera is subjected to ethanolic extract by cold maceration method. anti urolithiasis action can be evaluated in vitro by utilize the nucleation assay, aggregation assay, growth assay and titrimetric method.

Results: The result of this study shows that an ethanolic extract of *leucasaspera* leaf may have therapeutic benefits for reducing of kidney stone through *in vitro* models. The study findings of nucleation assay shows that the extract and cystone also reduced the size and number of caox crystals. the percentage reduction in size of caox crystals obtained for the extract (60.57%) was comparable to that produced by cystone (62.97%). in growth assay, the percentage of reduction in growth in the presence of this fraction was found to be 79±2.2% comparable to that of cystone 82.7±2.6% at 1000µg/ml was demonstrated.

Conclusion: The study findings indicate that *leucasaspera* ethanolic extract has anti urolithiasis capability when used in *in-vitro* methods. Mostly all extracts showed marked crystal size reduction and morphological modifications, thus ethanolic extract of leaf part of *leucasaspera* could be further analyzed and further characterization of its active compound lead to the discovery of a new drug for urolithiasis.

Keywords: Urolithiasis – Extraction - *Leucasaspera*– *In-Vitro* Assays

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INTRODUCTION

Plants are a significant source of medication is essential to maintaining global health. It has been long been that medicinal herbs or plants can be significant sources of therapeutic or curative help [1].Recently, WHO(world health organization) estimation that 80% of people worldwide on herbal medicine for some aspect of their primary health care needs [2]. In this study selection of herbal plant is *Leucas aspera*, often called as “thumbai” is found all over India,from the Himalayas to Ceylon. The plant has historically has been employed as a pesticide and antipyretic. It has been demonstrated to have a variety of pharmacological effecs in the terms of medicines including antifungal, antioxidant, antibacterial and cytotoxic activity. These investigations shows that *Leucas aspera* is a source of chemical compounds that medicinal activity and a variety of pharmacological effects, which supports the development of new therapeutic application of this plant. It includes nicotine, sterols, glycoside, diterpenes, oleanolicacid, triterpenoids and β-sitosterol [3].

Urolithiasis is a kidney stone production is one of the most common and ancient human disorders. In India, people from various plants to treat urolithiasis. Urolithiasis is the accumulation or development of stone in any components of

the urinary system, including the kidney and ureters [4]. It is a complicated mechanism that results from an imbalance between kidney promoters and inhibitors. Many phytochemical processes, including crystals nucleation, aggregration and retention within the urinary system, all contribute to the buildup kidney stones. The calculi causing urinary obstruction occurred more commonly in males may be due to smaller diameter and increased length of urethra and also bacterial infection may causes stone formation by crystal adherence [5].urolithiasis is an extremely painful disease that afflicts the human population since ancient times. Hyperoxaluria is one of the main risk factors of humans idiopathic calcium oxalate disease [6]. Renal stone are composed of mucopolysaccharide, urates, calcium oxalate, calcium phosphate and calcium carbonate [7,8].

Epidemiology: It was found that the incidence of upper urinary tract stones was 2.4 fold greater in men than in women [9]. The ratio of incidence of calcium oxalate stone was reported as 74.9% in males and 63.1% in females [10]. This disease affected all age group from less than 1 year old more than 70, with male to female ratio 2:1. Urolithiasis is longerly a recurrent disease with a relapse rate of 50% in 5-10 year and 75% in 20years [11].

Etiology: Dehydration from reduced fluid intake or exercise without adequate fluid replacement increases the risk of kidney stone and also there is decrease in urine volume [12].

Sign and symptoms: Pain on urination, severe pain in the side and the back, genitalia pain, blood in urine, burning sensation at the time of urination, increased frequency of urination and cloudy urine fever along with chills [13].

The main aim of the study is to evaluate of the anti-urolithiasis activity of *Leucasaspera* by *in-vitro* methods.

MATERIALS AND METHODS

Taxonomical Classification:

Kingdom:	Plantae
Plant Subkingdom:	Tracheobionta
Super division:	Spermatophyta
Division :	Angiosperma
Class:	Gamopetala
Series:	Bicarpellatae
Order :	Tubiflorae
Family :	Labiatae
Genus :	*Leucas*
Species :	*Aspera*[14]

PREPARATION OF PLANT EXTRACT:

The leaves of *Leucasaspera* are subjected to ethanolic extraction of cold maceration method. Maceration is an extractive technique that is conducted on room temperature. It consists of immersing of plant in a liquid inside air tight container. Before being processed, the plant materials must be washed properly to remove unwanted substances. The fresh and dried leaf materials are used based on the desired product. The solvent must be chosen based on the chemical nature of compounds within the plant [15]. In this process, the coarsely powdered leaves in a placed in a conical flask with the solvent hydroethanol (80:20) and allowed to stand at room temperature continuously for 72 hours with frequent agitation until the soluble matter has dissolved. The mixture is strained, the marc is pressed and the solvent are clarified by filteration. The filterate is concentrated using heating mantile maintained at 30 to 40ºC until the solvent gets evaporated.The resultant residue was kept in a refrigerator till further use [16].The percentage yield of *Leucasaspera* was 92.5 %.

PHYTOCHEMICAL STUDIES ON THE ETHANOLIC EXTRACT OF LEAF OF *LEUCASASPERA*

The crushed of leaf of *Leucas aspera* was subjected to cold maceration. The extracted material weighed about 100gm used for identification of phytochemical consistutents. Ethanolic extract of *Leucasaspera* were subjected to preliminary phytochemical screening qualitative test for the identification of various active consistutents[17,18,19,20,21,22]..

IN VITRO MODELS OF ANTI UROLITHIASIS ACTIVITY

NUCLEATION ASSAY

Principle: The nucleation assay is based on the crystal dissolution present, absorbance increases with increase in the crystal dissolution which is measured at 620nm spectrophotometrically. Reagents - Calcium chloride, Sodium oxalate,Tris buffer, Sodium chloride.

Procedure: This model includes the study of crystallization without inhibitor and with it, in order to assess the inhibiting capacity of the plant extract used. Solution of calcium chloride and sodium oxalate were prepared at the final concentrations of 5mmol/L and 7.5mmol/L respectively in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5.950 Ml of calcium chloride solution mixed with 100ml of calcium chloride solution mixed with 100ml of extracts at different concentrations. Crystallization was started by adding 950 ml of sodium oxalate solution. The temperature was maintained at 37ºC. The OD of the solution was monitored at 620nm using spectrophotometer (systronics digital spectrophotometer 166) after 30 minutes. The rate of nucleation was estimated by comparing the induction time in the presence of the extract with that of control. Data was represented in percentage inhibition [23]. The growth of crystal was expected due to the following reaction: CaCl2+ Na2C2O4 CaC2O4 + 2NaCl.

AGGREGATION ASSAY

Principle: The aggregation assay is based on the crystal dissolution per cent as turbidity increases in with increase in the crystal dissolution and measured at 620nm. Reagents Calcium chloride Sodium oxalate Tris buffer Sodium oxalate.

Procedure: The aggregation of the calcium oxalate (CaOx) crystals was determined. The CaOx monohydrate (COM) crystals were prepared by mixing calcium chloride (50 mmol/L) and sodium oxalate (50 mmol/L). Both solutions were equilibrated in a water bath for 1 hour at 60degree celsius for the formation of calcium oxalate monohydrate crystal. The crystals were cooled to 37ºC prior to evaporation. The COM crystals were prepared at a final concentration of 0.8mg/ml in a tris buffer (Tris 0.005 mol/L and NaCl 0.15 mol/L) at pH 6.5. A 100µL of extracts were added to the COM crystals solution and incubated at 37ºC for

360 min. The presence of the extract compared to the control by measurement at 620 nm for 0, 30, 60, 180 and 360 min [24]. The percentage inhibition of nucleation and aggregation were calculated as below: % of inhibition = [(OD control /OD Test)] *100.

GROWTH ASSAY

Growth of CaOx crystals was examined with or without the plant extract/standard drug following the method of 4 mM CaCl2 solution and 4 mM Na2C2O4 solution (1 ml each) were added to 1.5 ml of solution containing NaCl (90 mM) buffered with Tris-HCl (10 mM) at pH 7.4. To this 30µl of CaOx crystal slurry (1.5 mg/ml CaOx slurry was prepared in a 50 mM sodium acetate buffer at pH 5.7) was added. Growth of CaOx crystals was then determined by measuring the rate of oxalate depletion from the solution at 214 nm wavelength for 600s effect of each concentration of extracts on crystal growth was determined by the addition of1 ml of extract (100 µg/mL, 500 µg/mL, and 1000 µg/mL) to the reaction mixture and change in the optical density was recorded with an UV-visible spectrophotometer. Percentage inhibition of crystal growth was calculated. Relative inhibitory activity (%) = C – S × 100 C Where C is the rate of reduction of free oxalate without any extract and S is the rate of reduction of free oxalate in the presence of extract [25].

TITRIMETRY METHOD

As per the experimental design, dissolution studied of calcium oxalate were carried out in six groups; one group severed as negative control [Group I] which consists of 1mg of calcium oxalate alone, weighed exactly 1 mg of calcium oxalate and packed along with 10mg of cystone (removing color coating and crushed into powder form) as standard group [Group II]. All groups were packed as above in separate egg based semipermeable membranes tied with thread by suturing at one end. Now, the materials packed in semi permeable membranes were allowed to suspend in separate conical flask containing 100ml of 0.1M Tris buffer. Another end of the thread was tied on the mouth of conical flak and covered with aluminium foil. The conical flask of all groups was kept in an incubator, preheated to at 37±1ºC for 7 hour. After 7 hours, flasks with membrane were kept in magnetic stirrer for 5 minutes. Finally, all the membranes were taken out of the flask and content of each membrane was collected in different test tube by removing sutures. 2 ml of 1N sulfuric acid was added to each test tube and titrated with 0.9494 N KMNO4 till a light pink colour end point obtained. 1ml of 0.9494 N KMNO4 is equivalent to 0.1898 mg of calcium. The amount of undissolved calcium oxalate is subtracted from the total quantity used in the experiment in the beginning to known how much quantity of calcium oxalate was dissolved by the test substances there is inverse relationship between the dissolution of calcium oxalate and concentration of calcium ions in solutions. Each treatment was repeated three times and volume of standard KMNO4 was expressed as mean ± SD [26].

RESULTS AND DISCUSSION

Results of Phytochemical tests

The results of Preliminary phytochemical analysis of ethanolic extract of leaf part of *Leucasaspera* shown in the table1

Table 1: Phytochemicals constituents present in ethanolic extract of leaf part of Leucas aspera

S. No	Phytochemicals		Ethanolic Extract Of Leucas Aspera
1.	Alkaloids	Wager's test	+
		Mayer's test	+
		Hager's test	+
		Dragendroff's test	-
2.	Flavonoids	Alkaline reagent test	+
		Lead acetate test	+
3.	Glycosides	Modified Borntrager's test	+
		Legal's test	+
		Balget's test	+
4.	Phenolic compounds	Ferric chloride test	+
5.	Tannis	Gelatin test	+
		Basic lead acetate test	+
		Potassium ferric cyanide test	-
6.	Saponins	Froth test	+
		Foam test	+
7.	Pytosterol's	Salkowsi's test	+
		Liebermann Burchard test	+
8.	Protein and amio acids	Xanthoprotein test	-
		Ninhydrin test	-
9.	Terpenoids	Copper acetate test	+
10.	Carbohydrates	Molisch's test	+
		Benedict's test	+
		Fehling's test	+

NUCLEATION ASSAY

An *in vitro* crystallization study was performed, since nucleation is an important first step for the initiation of crystals. Incubation of synthetic urine that contains calcium chloride and sodium oxalate results in the formation of CaOx crystals. The results of the nucleation assay confirmed that the extract contained nucleation-preventing agents.The extract and Cystone also reduced the size and number of CaOx crystals. The percent reduction in size of CaOx crystals obtained for the extract (60.57%) was comparable to that produced by Cystone (62.97%).(Fig 1)

In the nucleation assay, addition of Na2C2O4 solution to the reaction mixture consisting of CaCl2 resulted in the formation of numerous CaOx crystals. Microscopic analysis showed that numerous large CaOx monohydrate (COM) crystals of either rectangular habit or dendrites with sharp edges were predominant in the control group. The extract at higher concentrations and Cystone at lower concentrations favored the formation of tetrahedral shaped calcium oxalate dihydrate (COD) crystals with smoother morphology.(fig 2)

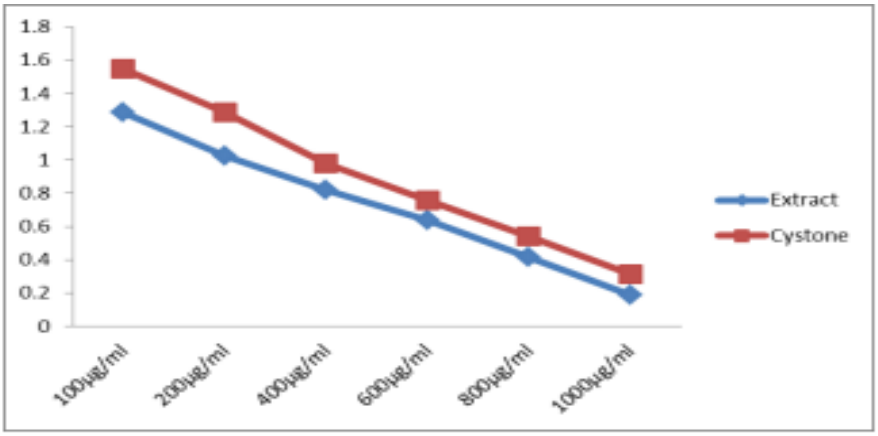

Figure 1: Nucleation assay showed that the various Concentration of leaf exctract of ***Leucasaspera*** and Cystone against absorbance at 620nm.

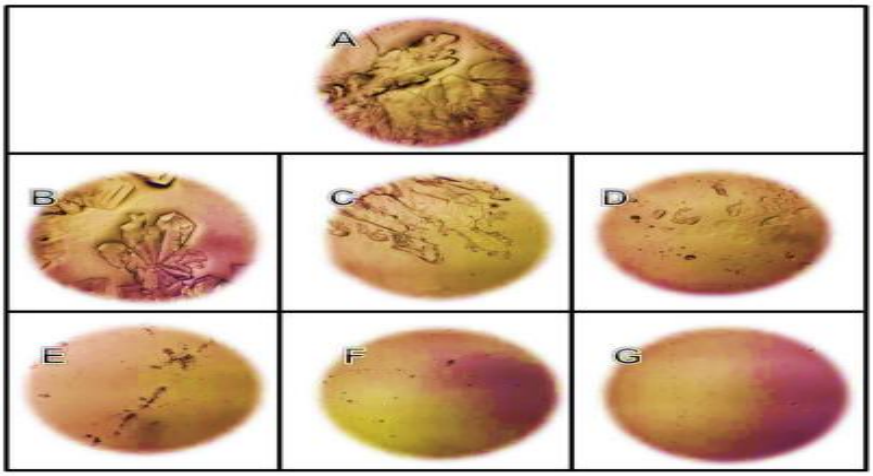

Figure 2: Representative photographs of CaOx crystals as observed under the microscope in the absence of extract (A) Presence of extract (B) 100µg/ml, (C) 200µg/ml, (D) 400µg/ml, (E) 600µg/ml, (F) 800µg/ml and (G) 1000µg/ml.

AGGREGATION ASSAY

The extract of EELA showed a significant reduction in the aggregation of preformed CaOx crystals, with a percentage reduction of 96.87% compared to Cystone. Aggregation, a key factor in the genesis of stones, is a key determinant of crystal retention. The extract's anti-crystallization, anti-aggregatory, and crystal growth defying activity is attributed to its phytoconstituents, including flavonoids with CaOx crystal dissolution potency and antioxidant activity. The limiting factors in stone formation could be processes that affect crystal growth, as particles may become large enough to occlude the urinary tract, leading to stone formation. The extracts may contain substances that inhibit the growth of CaOx crystals, which may be important in preventing kidney stone growth.

Table 2: Effect of Ethanolic extract of leaf part of ***Leucasaspera*** on aggregation of calcium oxalate crystals

S. NO	*DOSE(µg/ml)*	*STANDARD*	*EXTRACT*
1.	200µg/ml	24.11±1.61	15.86±2.12
2.	400µg/ml	41.38±2.69	30.53±1.95
3.	600µg/ml	65.17±2.78	43.90±0.62
4.	800µg/ml	65.14±1.61	56.34±1.58
5.	1000µg/ml	72.96±1.48	65.18±2.57

Values are expressed as mean± SD(n=3,P<0.05),significant different at 5% level.

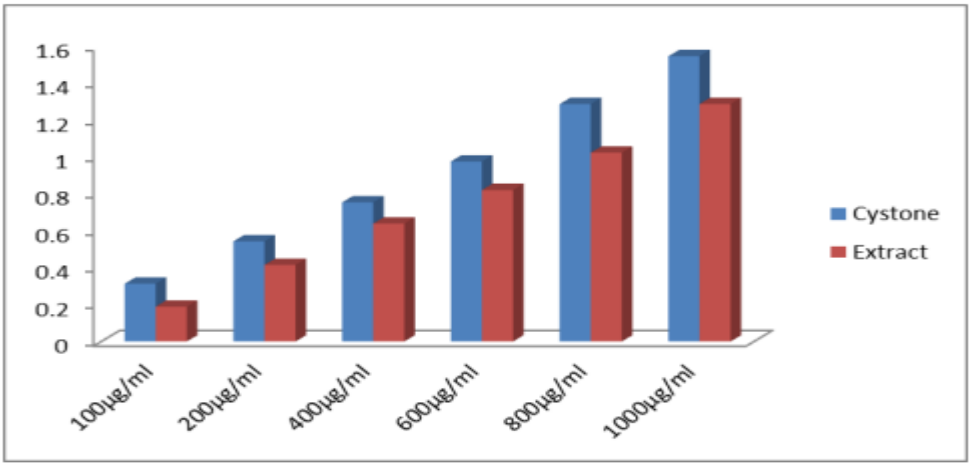

Figure 3: Aggregation assay showed that the various concentration of leaf part extract of ***Leucasaspera*** and Cystone against absorbance at 620nm

Titrimetry Method

The study analyzed the percentage dissolution of calcium oxalate stone in various extracts of EELA. The standard group showed a significant dissolution rate of 26.25%, 30%, and 38.75% at different concentrations. EELA showed a comparable rate of 21.2%, 25%, and 27.5% at different concentrations. The mean percentage dissolution of calcium oxalate increased with the concentration of extracts. The IC50 values for each case were estimated using linear regression. The response of EELA was found to be similar to that of Cystone. There was a positive correlation between extracts and the concentration used in the study, indicating that increasing the concentration of extract increases the percentage dissolution of calcium oxalate up to a certain limit, thereby increasing its efficacy.

Table 3: Percentage of Inhibition of calcium oxalate stone (10 mg) by various extracts of EELA.

S. No	Group	Concertration of Drug	Vol. of Standard Kmno4	Wt. of Calcium Estimate	Wt. of Calcium Reduced	Percentage of Inhibition
1.	Group I (Negative Control)	-	8ml	10mg	-	100%
2.	Group II(Standard as cystone)	50mg	5.9ml	7.3mg	2.6	73.7%
		100mg	5.6ml	7.0mg	3	70%
		150mg	4.9ml	6.1mg	3.9	61.2%
3.	GroupIII (Contains EELA)	50mg	6.3ml	7.8mg	2.1	78.7%
		100mg	6.0ml	7.5mg	2.5	75%
		150mg	5.8ml	7.2mg	2.7	72.5%

Table 4: Descriptive statistics for individual extracts with different concentrations of EELA.

S. No	*Group*	*Concertration of Drug(Mg)*	*N*	*Mean % ± Sd*	*Ic_{50}*
1.	Group II(Standard as cystone)	50mg	3	26.25±0.31	237
		100mg	3	30.02±2.39	
		150mg	3	38.75±1.90	
2.	GroupIII (Contains EELA)	50mg	3	17.15±0.82	274
		100mg	3	27.51±1.70	
		150mg	3	31.25±1.35	

GROWTH ASSAY

The standard fraction showed maximum inhibitory effect after 3 hours, leading to heterogeneous nucleation and crystal growth. In an in-vitro growth assay, COD crystals formed in CaOx solution, with a reduction in growth rate comparable to cystone (82.7±2.6%).

Table 5: Effect of Ethanolic leaf part extract of Leucas aspera and Cystone on growth assay

S. No	Group	% Inhibition		
		1 Hour	2Hour	3Hour
1.	Standard	52.0±3.5	65±3.2 8	82.7±2.6
2.	200µg/ml	20.0±1.9	21.2±1.8	30.9±0.8
3.	400µg/ml	39.2±0.7	48.7±2.1	53.4± 2.3
4.	600µg/ml	42.3±2.0	57.3±2.2	65.3±2.0
5.	800µg/ml	55.3±3.1	60.2±2.0	70.7±1.9
6.	1000µg/ml	58.1±2.7	62.2±2.2	79.4±2.2

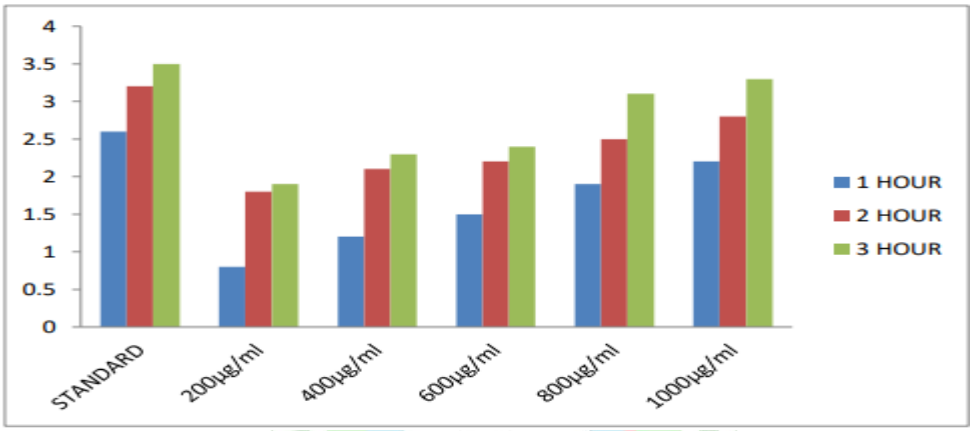

Figure 3: Effect of ethanolic leaf extract of ***Leucasaspera*** and cystone on growth assay

SUMMARY & CONCLUSION

The present investigation will be supportive as additional information to the scientific evidences regarding *in-vitro* studies. The correlation between *in-vitro* and *in-vivo* studies should be further investigated to reveal the phytochemicals of the extract are responsible for dissolving or disintegrating renal calculi and to know better understanding in the molecular mechanism of litholysis. The finding of the present study suggests that anti-urolithiatic potential ethanolic extract of leaf part of *Leucasaspera* by *in-vitro* methods. Mostly all extracts showed marked crystal size reduction and morphological modifications were observed through microscopically.The leaf part extract of *Leucasaspera* exhibited inhibition of calcium oxalate stone formation, nucleation, aggregation and growth of calcium oxalate crystals. The ethanolic extract of leaf part of *Leucasaspera* reveals that anti-urolithiatic activity by in vitro methods. Thus ethanolic extract of leaf part of *Leucasaspera* could be further analyzed *in-vivo* and further characterization of its active compound could lead to the discovery of a new drug for the patients suffering with urolithiasis.

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