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RESEARCH ARTICLE

IN-VITRO EVALUATION OF ANTI-UROLITHIATIC ACTIVITY OF LEAVES OF *ALSTONIA SCHOLARIS*

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ABSTRACT

Background: Urolithiasis has become a global problem with increased prevalence over last few years. Renal calculi formed usually comprises of calcium oxalate as a major constituent. Conventional treatments include use of vasodilators in early stage of urolithiasis, whereas, staghorn calculi requires Extracorporeal Shockwave Lithotripsy (ESWL) intervention which carries risk of renal injury and recurrence. They are also expensive, time bound and associated with side effects. Literature reports medicinal plants rich with multi-functional bio-active constituents, possessing lithotriptic potential which needs to be scientifically explored.

Objective: Pentacyclic triterpenoids viz. lupeol, ursolic acid and betulin are reported in literature for their anti-urolithiatic effect. As the plant *Alstonia scholaris* (L.) R. Br. Apocynaceae is rich with these phyto-constituents, this study was conducted with the objective to find out *in-vitro* lithotriptic activity of four extracts of leaves of *Alstonia scholaris,* in comparison with standard poly herbal preparation Cystone (Cys).

Method:-Leaves of *Alstonia scholaris* were extracted with Methanol (MeAS), Chloroform (ChAS), Ethanol (EtAS) and Hydro-ethanol (HeAS) solvents using soxhlet apparatus. Preliminary phytochemical screening of the extracts was performed to identify presence of triterpenoids; which was further confirmed by HPTLC. *In-vitro* lithotriptic effect of four extracts was evaluated by incubating each extract (50,100,200,400 and 800mg) at different time intervals (8, 18 and 24 hours) with calcium oxalate crystals (10mg) in semi-permeable membrane isolated from chicken eggs.

Result:-Class of compound determination by HPTLC confirmed presence of pentacyclic triterpenoids in all four extracts with the highest content in EtAS. The order of extent of dissolution of calcium oxalate crystals observed was EtAS>ChAS>HeAS>MeAS.

Conclusion:-Maximum lithotriptic activity of leaves of *Alstonia scholaris* was observed with extract EtAS which is comparable with standard Cystone. Thus, anti-urolithiatic effect exhibited by leaves of *Alstonia scholaris* can be attributed to the presence of pentacyclic triterpenoids detected in the plant.

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INTRODUCTION

Urolithiasis is characterized by the formation of stones in the kidneys or urinary tracts. Nearly 4–15% of the human population is suffering from urolithiasis all over the globe (Khare *et al.,* 2014). It is characterized by high morbidity and low mortality but with significant socio-economic impact and serious consequences (Bansode *et al.,* 2015) like severe pain in the back or belly, pain and burning during urination, blood in urine, fever or chills. Currently, open renal surgery for urolithiasis is unusual and is rarely performed due to the introduction of Extracorporeal Shockwave Lithotripsy (ESWL), which has become a standard treatment to eliminate kidney stones.

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Shockwaves are used in ESWL to break stones but traumatic effects of these shockwaves leads to acute renal injury, decline in the renal function and increase in stone recurrence. At the same time the cost involved in treatment is also high (Bouanani *et al.,* 2010). The allopathic treatments with calcium channel blockers, α-adrenergic blockers, corticosteroids are also associated with side effects like allergic reaction, severe hypotension and congestive heart failure. Also, these drugs just ease out passage of small to medium sized stones by relaxing the renal tubules and do not act by dissolving renal calculi. Herbs have been documented in Ayurveda for their kidney stone dissolving activity. Few of them to name are *Bryophyllum pinnatum* (Shukla *et al.,* 2014), *Pedalium murex* (Patel *et al.,* 2016), *Biophytum sensitivum* (Pawar and Vyawahare, 2015), etc. Also, various phytoconstituents has been reported for their anti-urolithiatic activity such are catechin (Grases *et al.,* 2015), epigallocatechin-3-gallate (Kanlaya *et al.,* 2016), diosmin (Noorafshan *et al.,* 2013), rutin

(Ghodasara *et al.,* 2010), quercetin (Zhu *et al.,* 2014). Similarly, Pentacyclic triterpenoids of plant origin have been reported in literature to be efficient in minimizing crystal induced renal per oxidative changes. Presence of lupeol, in other plants have been claimed to prevent renal calculi induced tissue damage along with dissolution of urinary stone forming constituents (Yadav *et al.,* 2011). Leaves of *Alstonia scholaris* (L.) R. Br. Apocynaceae is reported to be store houses of pentacyclic triterpenoids like lupeol, betulin, ursolic acid (El-Askary *et al.,* 2012). Hence in this study plant *A.scholaris* is selected to probe its lithotriptic effect. In addition it possess immense pharmacological effects like anti-bacterial, anti-oxidant, anti-inflammatory and analgesic (Pratap *et al.,* 2013) which can further reduce associated complications of Urolithiasis. Thus efforts have been made in this study to probe efficiency of leaves of *A.scholaris* to dissolve calcium oxalate crystals (which constitutes 80% of analyzed calculi (Prien and Prien, 1968)) by *in-vitro* method in comparison with standard poly herbal preparation Cystone.

MATERIALS AND METHODS

Plant: The fresh leaves of *A.scholaris* were procured from Joginder Nursery, Delhi. Leaves were shade dried, crushed and ground to obtain a coarse powder. This powder was subjected to soxhlet extraction using methanol (MeAS), chloroform (ChAS), ethanol (EtAS) and hydro-alcohol (HeAS) (70% ethanol+ 30% water) as solvents. Extraction was continued till the siphon tube showed colorless solvent, after which extracts obtained were collected. Excess of solvent from each extract was evaporated to obtain semi-solid mass. All extracts were stored in air tight container in refrigerator at 2-8°C. Fresh leaves of *A.scholaris* with flowers were authenticated at St Xavier's Blatter Herbarium under specimen number NI-1417 of N. A. Irani.

Chemicals: All the chemicals and solvents used were of analytical grade and purchased from Loba chemie Ltd (Mumbai, India). Standard poly herbal preparationcystone (Cys) was procured from Himalaya Co. Chicken eggs were procured from local market.

Preliminary phytochemical screening of extracts: Extracts MeAS, ChAS, EtAS and HeAS were subjected to qualitative phytochemical screening (Palve *et al.,* 2015)

1. **Alkaloids (Dragendorff's Test):** To each extract dilute HCl was added. Shaken and then filtered. To 2-3ml of this filtrate few drops of Dragendorff's reagent was added and observed for color of ppt.
2. **Glycosides (Borntrager's test):** To each extract dilute H_2SO_4 was added, boiled and filtered. To cold filtrate, equal volume of benzene and chloroform was added. Shaken well. Organic layer was separated, ammonia was added and change in color of ammoniacal layer was observed.
3. **Flavonoids (NaOH Test):** Extracts were treated with few drops of sodium hydroxide solution. Change in color after the addition of acid was recorded.
4. **Terpenoids (Salkowski Test):-** Each extract was mixed with 0.4ml of chloroform and 0.6ml of concentrated H_2SO_4 was added carefully to form a layer at the interface. Color formed at the interface was observed.
5. **Steroids (Liebermann-Burchard Test):** 2ml of each extract was mixed with chloroform. To this solution 1-2ml of acetic anhydride was added and concentrated H_2SO_4 was added from side of test tube. Sequence of the color formed was observed.
6. **Saponins (Foam Test):** The small quantity of each extract was diluted with 4ml of distilled water. The mixture was shaken vigorously. Persistence of foam was observed for 10 minutes.
7. **Phenols (Ferric chloride Test):** The small quantity of each extract was treated with a few drops of ferric chloride solution. Formation of color was observed.

Identification of Triterpenoids using HPTLC: Each of the extract was dissolved in methanol and sonicated for 900 seconds. Mobile phase was optimized by hit and trial to obtain maximum separation of bands on plate. Aluminium silica gel 60 F_{254} TLC plates (Merck) were used as stationary phase. 8mm bands of sample aliquot of each extract was applied using Camag Linomat 5 as applicator. Twin trough developing chamber was saturated with solvent vapors of optimized mobile phase (n-Hexane: Ethylacetate (5:5 v/v)) for 20 minutes. Plate was developed, air dried and derivatized using Anisaldehyde Sulphuric acid Reagent (ASR) and heated at 110°C for 3-5 minutes. After derivatization images were captured at 254nm, UV-Visible and 366nm and scanned at 540nm using Camag TLC Scanner III to record densitogram.

***In-vitro* anti-urolithiatic study (Atodariya *et al.,* 2013; Saravanasingh *et al.,* 2016)**

(A) Preparation of experimental kidney stones by homogenous precipitation method:

1. Equimolar solution of calcium chloride dihydrate (AR) dissolved in distilled water and sodium oxalate (AR) dissolved in 10ml of 2N H_2SO_4 were mixed in a beaker and allowed to react. Distilled water was added drop by drop till the precipitate of calcium oxalate (CaOx) is formed
2. Precipitate was freed from the traces of sulphuric acid with the help ammonia solution. Further, precipitate was washed with distilled water and dried at 60°C for 4 hours.

(B) Optimized method for isolation of semi-permeable membrane from chicken eggs:

1. The semi-permeable membrane (SPM) of eggs lies in between the outer calcified shell and the inner contents like albumin and yolk.
2. Shell was removed chemically by placing the eggs in 3M HCl for 1-2 hours, which caused complete decalcification.
3. Decalcified egg was washed with distilled water and with a sharp pointer a hole was made on the top to squeeze out inner contents completely.
4. The outer egg membrane was washed thoroughly with distilled water and placed in ammonia solution in the moistened condition for a while and was rinsed again with distilled water. This isolated SPM was stored in refrigerator at pH of 7-7.4 in phosphate buffer.

(C) Incubation of extracts with calcium oxalate crystals for estimation of lithotriptic effect:

1. 10mg of CaOx crystals and various concentrations (50, 100, 200, 400, 800mg) of extracts MeAS, ChAS, EtAS, HeAS and standard Cys were accurately weighed and packed together in the isolated SPM pouch. Separately, only 10mg of CaOx was packed in SPM pouch which was used as blank.
2. SPM pouch was suspended in a conical flask containing 100ml of 0.1M TRIS buffer. (pH = 7.)
3. All conical flasks containing SPM pouches were placed in incubator, preheated to 37°C for 2 hours, for different time intervals viz 8hours, 18hours and 24hours.
4. The contents of SPM pouches were removed into conical flask after each time interval.
5. 20ml of 1N H_2SO_4 was added to each conical flask and titrated with 0.9494N $KMnO_4$ till a light pink color end point was obtained.
6. Following factor was used for calculation of undissolved CaOx crystals:1ml of 0.9494N $KMnO_4$ is equivalent to 0.1898mg of calcium.
7. The amount of calcium in undissolved CaOx was subtracted from blank reading to know quantity of CaOx dissolved by test extract.

RESULTS

Physicochemical and Phytochemical testing: Extraction of dried leaf powder of *A.scholaris* with various solvents produced 17.23%, 12.53%, 13.42%, 19.71% w/w yields for extracts MeAS, ChAS, EtAS, and HeAS respectively. All four extracts were weakly acidic. Presence of various phytoconstituents was confirmed using battery of chemical tests. Qualitative phytochemical screening of extracts revealed presence of phytoconstituents like alkaloids, flavonoids, triterpenoids, steroids and phenols in all four extracts. Along with these phytoconstituents additional presence of glycosides was detected in ChAS and EtAS and saponins were found in MeAS, EtAS and HeAS extracts.

HPTLC class of compound determination for triterpenoids: Mobile phase optimized by using hit and trial method was n-Hexane: Ethylacetate (5:5 v/v). Presence of triterpenoids in all extracts was confirmed by observing violet-blue coloration after derivatization with ASR. R_f values found were 0.46, 0.53, 0.7, 0.81 and 0.87. However among all four extracts, EtAS showed more intense color bands for triterpenoids. Percent areas of triterpenoid bands obtained of EtAS at R_f values 0.46, 0.53, 0.7, 0.81 and 0.87 were found to be 28.24%, 13%, 14.87%, 17.46% and 24% respectively; which is higher as compared to other extracts. Thus, indicating higher content of triterpenoids in EtAS as compared to other extracts.

***In-vitro* lithotriptic effect:** All four extracts of leaves of *A.scholaris* were found to produce lithotriptic effect. The order of dissolution of CaOx crystals observed after incubation at various time intervals (8, 18 and 24hours) for varied range of concentrations (50, 100, 200, 400 and 800mg) of each extract was found to be EtAS>ChAS>HeAS>MeAS. Incubation period optimized to produce maximum crystal dissolution was 24 hours. After incubation for 24 hours EtAS produced 32%, 40%, 50%, 60% and 63% dissolution of calcium oxalate crystals at concentrations 50, 100, 200, 400 and 800mg respectively. Among four extracts extent of dissolution after incubation period of 24 hours exhibited by EtAS (63%) at concentration 800mg was comparable with marketed formulation preparation Cys (65.6%).

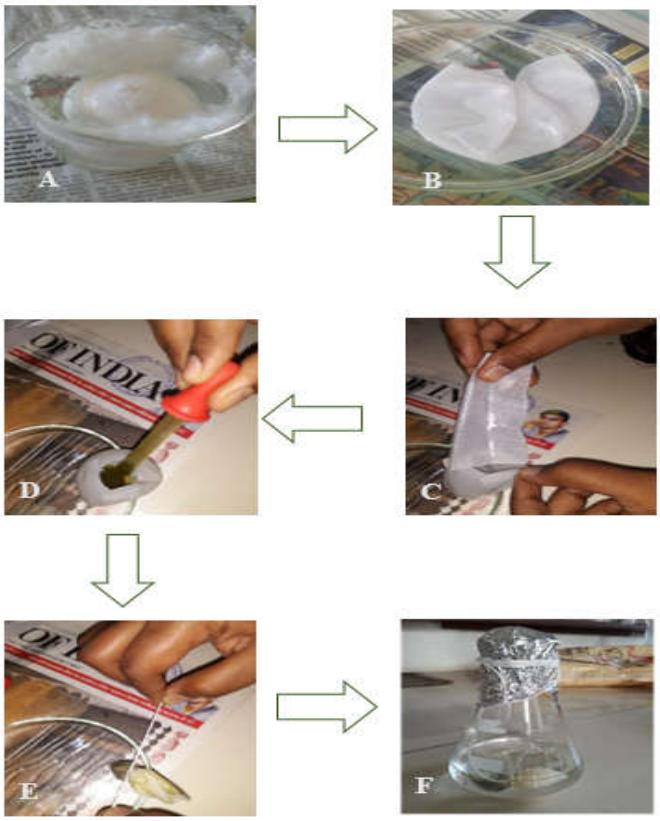
Table 1. Physicochemical properties of extracts

Extract	% Yield (% w/w)	Appearance	Consistency	pH
MeAS	17.23%	Dark green	Thick, sticky	6
ChAS	12.53%	Light green	Solid, Dry	5
EtAS	13.42%	Dark green	Solid, Dry	5
HeAS	19.71%	Dark green	Thick, sticky	5

Table 2. Qualitative phytochemical screening of each extract

Phytochemicals	MeAS	ChAS	EtAS	HeAS
Alkaloids	+	+	+	+
Glycosides	-	+	+	-
Flavonoids	+	+	+	+
Triterpenoids	+	+	+	+
Steroids	+	+	+	+
Saponins	+	-	+	+
Phenols	+	+	+	+

(+) =Present, (-) =Absent.



A:-Decalcification of egg in HCl;B:-Isolation of SPM from egg; C:-Filling of CaOx crystals in SPM pouch; D:-Mixing of extract with CaOx crystals in SPM pouch; E:-Suturing of SPM pouch; F:-Incubation of filled SPM pouch in 0.1M Tris buffer

Fig 1:- *In-vitro* lithotriptic effect

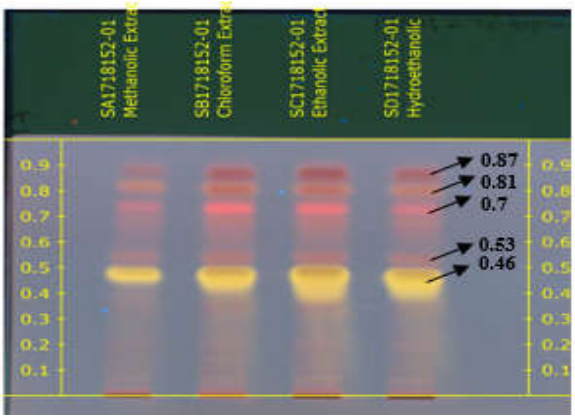
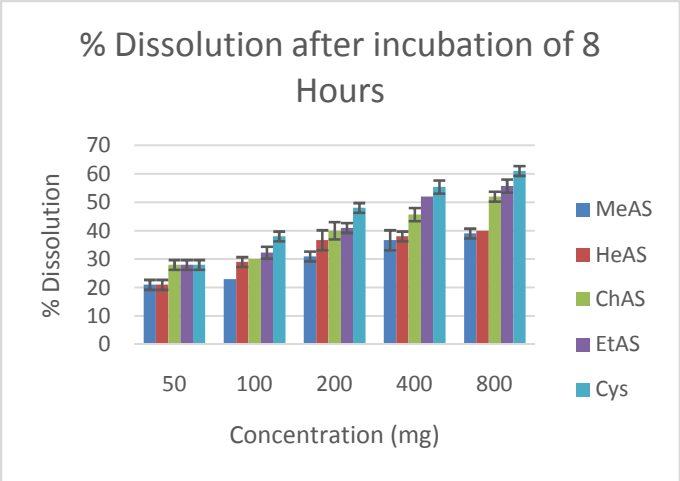
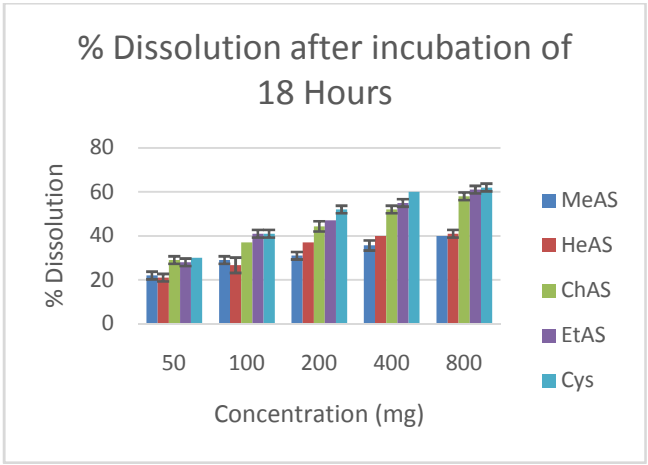


Fig 2. HPTLC chromatogram of MeAS, ChAS, EtAS and HeAS at 366nm showing different bands of separated triterpenoids with their R_f values 0.46, 0.53, 0.7, 0.81, 0.87 respectively



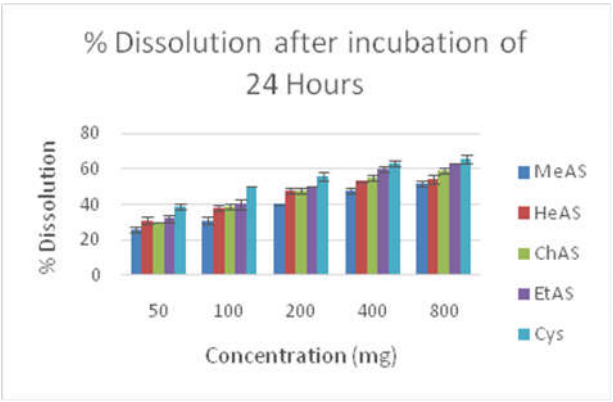
X axis represents concentration in mg; Y-axis represents % dissolution; each column represent mean of 3 readings (n=3); Vertical error bar represents ±SD

Figure 3. Percent dissolution of CaOx crystals after incubation period of 8 Hours



X axis represents concentration in mg; Y-axis represents % dissolution; each column represent mean of 3 readings (n=3); Vertical error bar represents ±SD

Figure 4. Percent dissolution of CaOx crystals after incubation period of 18 Hours



X axis represents concentration in mg; Y-axis represents % dissolution; each column represent mean of 3 readings (n=3); Vertical error bar represents ±SD

Figure 5. Percent dissolution of CaOx crystals after incubation period of 24 Hours

DISCUSSION

Kidney stones have afflicted mankind since a long time. Medical conditions which increase the risk for urolithiasis are idiopathic hypercalciuria, hyperoxalosis Dent's disease, medullary sponge kidneys, polycystic kidney disease, hyperparathyroidism, irritable bowel disease (IBD) and renal tubular acidosis or sarcoidosis (Han *et al.,* 2015). Patients with a family history of nephrolithiasis have a 2.5 times greater risk of stone formation (Curhan *et al.,* 1997). Other reasons for urolithiasis include modifications in lifestyle such as increase intake of caffeine, sodium, sugar, alcohol and low intake of fluids, etc. Current therapies available are costly, time bound and cannot be prolonged because of associated side effects (Segura *et al.,* 1997). Hence, there is need to probe a therapeutic approach which is economical, safe and effective. Since antiquity medicines of plant origin have been found to be safe, effective and free from side effects. One such medicinal plant with multiple therapeutic potentials is *A.scholaris*. Leaves of *A.scholaris* are rich with pentacyclic triterpenoids like lupeol, ursolic acid, betulin (El-Askary *et al.,* 2012Wang *et al.,* 2017). These triterpenoids are known for their urinary stone diluting activity. Hence, in this study leaves of *A.scholaris* were selected to probe lithotriptic effect. *In-vitro* testing of lithotriptic effect was performed by incubating CaOx crystals with extracts MeAS, ChAS, EtAS, HeAS and standard Cys at various time intervals. Among various incubation periods tried, 24 hours incubation period exhibited maximum percent dissolution for extract EtAS (63%) and standard Cys (65.6%). In addition our study also optimized duration of in-vitro method. Although literature reports overnight immersion of egg in 2M HCl for complete decalcification of the shell our study optimized duration of immersion to 1-2 hours in 3M HCl thereby, reducing duration of experiment and allowing processing of more number of test samples in short time leading to increase productivity of method. It was observed that on incubation of 24 hours the order of dissolution of CaOx crystals for extracts was MeAS<HeAS<ChAS<EtAS. The degree of dissolution of CaOx crystals exhibited by EtAS was similar to that produced by standard Cys. EtAS produced dose dependent increase in rate of dissolution of CaOx crystals till 400mg (60%) whereas, ceiling effect was observed at 800mg (63%). HPTLC studies revealed presence of triterpenoids in all extracts but greater % area of bands of triterpenoids were observed in EtAS indicating their highest content. Thus, the observed lithotriptic activity exhibited by EtAS might be due to pentacyclic triterpenoids like lupeol, betulin, ursolic acid present in it in major amount. In addition our study also optimized time parameter of *in-vitro* method. As literature reports overnight immersion of egg in 2M HCl for complete decalcification of the shell. This study optimized duration of immersion to 1-2 hours in 3M HCl thereby, reducing duration of experiment and thus more number of test samples can be processed in short time leading to increase productivity of method. Anti-inflammatory and analgesic effects have been reported for ethanolic extract of leaves of *A.scholaris* (Luo and Shang, 2010). The association between inflammation and urolithiasis have been reported (Tang, and Lieske, 2014) Inflammatory responses are involved in renal injury which upregulates deposition of renal calculi. Reported anti-inflammatory effect of EtAS in addition to dissolution of preformed calculi can obscure inflammation triggered by these calculi, thus, in turn preventing deposition and accumulation of renal calculus. Another classical feature of urolithiasis is renal colic, excruciating pain due to dislocation of renal calculi across urinary tract. Analgesic potential of EtAS might be useful to overcome renal colic thus minimizing the use of

painkillers such as corticosteroids and narcotic analgesic which carries risk of severe side effect. Oxidative stress is another ailment in pathogenesis of urolithiasis which can be counter acted by anti-oxidant potential of plant (Antony *et al.,* 2011; Arulmozhi *et al.,* 2011). Microbial infection is also very common with this condition, for which antibiotics are prescribed in conventional treatment. These antibiotics are associated with side effects like constipation or diarrhea, nausea and vomiting, decrease in gut flora, etc. Leaves of *A.scholaris* have been known for its anti-microbial (Parcha *et al.,* 2013) and anti-bacterial (Antonty *et al.,* 2014) effects, thus can replace antibiotics in pharmacotherapy of urolithiasis. Thus multiple drug therapy required for treatment of urolithiasis to get analgesic, anti-inflammatory, anti-oxidant and anti-microbial effects can be obtained by administration of one single extract of *A. scholaris*. In addition present *in vitro* study demonstrated kidney stone dissolving potential of *A. scholaris.* Thus *A.scholaris* can prove potential herbal candidate for treatment of urolithiasis if explored further.

Conclusion

Thus our study unfolds lithotriptic activity of leaves of *Alstonia scholaris*. The observed effect might be due to presence of rich content of pentacyclic triterpenoids. In future, EtAS can be further explored *in-vivo* to elucidate mode of action for observed lithotriptic effect.

Glossary of Abbreviations:

A.scholaris - *Alstonia scholaris*
AR – Analytical Reagent
ASR – Anisaldehyde Sulphuric acid Reagent
CaOx – Calcium Oxalate
ChAS – Chloroform extract of leaves of *Alstonia scholaris*
Cys – Cystone
ESWL – Extracorporeal Shockwave Lithotripsy
EtAS – Ethanolic extract of leaves of *Alstonia scholaris*
HPTLC – High Performance Thin Layer Chromatography
HeAS – Hydro-ethanolic extract of leaves of *Alstonia scholaris*
HCl – Hydrochloric acid
H_2SO_4 – Sulphuric acid
IBD – Irritable Bowel Disease
$KMnO_4$ – Potassium permanganate
MeAS – Methanolic extract of leaves of *Alstonia scholaris*
NaOH – Sodium hydroxide
R_f – Retention factor
SPM – Semi-permeable membrane
TLC – Thin Layer Chromatography

Conflict of Interest: The authors of this research article declare no potential conflict of interest.

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