In Vitro Antilithiasis Activity and Cytoprotective Properties of *Acalypha indica* Extracts

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Abstract

Kidney stone is a major health problem occurring in many individuals, especially men, and it is caused by dietary intake and excess excretion of chemicals in the urine. The aim of this study was to evaluate the inhibitory potential of *Acalypha indica* Linn. against calcium oxalate stones. Various solvent extracts of *A. indica* Linn. were prepared using cold extraction method. These extracts were then used for analyzing inhibitory action against three stages of crystal formation: nucleation, growth, and aggregation. The ethanolic extracts were found to have maximum inhibitory potential of approximately 90±0.0011%, 99.4±0.002%, and 93±0.002% against the crystal nucleation, growth, and aggregation of calcium oxalate. The inhibitory potential of calcium oxalate precipitation in artificial urine by the ethanolic extract was found to be approximately 99.231±0.0001%. The ethanolic extract was fractionated by column chromatography and the bioactive compound was identified as an aliphatic group from the structural characteristics of the ethanolic extract obtained from ^{13}C nuclear magnetic resonance. Finally, the effectiveness of partially purified extract was tested on calcium oxalate stones, which were highly dissolved by the ethanolic extract (61.82±0.133%) compared to calcium phosphate stones (35.71±0.06%). It was also tested on oxalate induced Vero cell lines and the cell viability was found to be approximately 70.5±2.99%. On the basis of the present study, it was concluded that the crude extract had higher activity against calcium oxalate stones than a partially purified extract.

Keywords: aggregation; artificial urine; calcium oxalate; crystal growth; kidney stone; nucleation; Vero cell lines
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1. Introduction

Renal calculi are generally called kidney stone. Being able to successively treat kidney stone these days is a milestone because it eventually leads to the death of the affected individuals. Kidney stone affects approximately 4-8% of population in the UK, 15% in the USA, and 11% in India [1]. Different forms of kidney stones that are present worldwide are calcium-containing stones of approximately 75-90%, followed by struvite crystals (10-15%), uric acid (3-10%), and cysteine (0.5-1%) [2, 3]. Lithiasis is a process in which calcium oxalate (CaC_2O_4 or CaOx) is formed in the kidney. Calcium oxalate stones are present in two forms: calcium oxalate monohydrate (COM) or whewellite, and calcium oxalate dihydrate (COD) or weddellite. COM stones are thermodynamically more stable than COD and have more affinity for the formation of renal calculi in the kidney [4-6]. It is noteworthy that the lifestyle and dietary intake of individuals also play a role in the formation of stones in the kidney. The major causes are high intake of calcium- and phosphorus-rich cereals and oxalate-rich vegetables, as well as lack of animal proteins [7]. There are several ways to treat renal stones. Some of the practices such as surgical approaches are endoscopic stone removal lithotripsy and extracorporeal shock wave lithotripsy, which cause traumatic effect and reduction in renal function with acute renal injury [8]. The alternative resource for the treatment of renal stones is the use of herbal extracts. The plants used to prepare these extracts are commonly found and the technique is also a cost-effective [9, 10]. This study deals with assessing the inhibitory effects of *Acalypha indica* Linn. leaves compared to *in vitro* analysis and on Vero cell lines.

2. Materials and Methods

2.1 Collection and authentication of plant materials

Acalypha indica Linn. plants were collected from nearby K. S. Rangasamy College of Technology, Tiruchengode, Tamil Nadu, India, from March to April 2014. The validation of plants was accomplished by Botanical Survey of India, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. Renal stone formation can be identified by three stages, via calcium oxalate crystal nucleation, growth and aggregation, and these can be analyzed by the following *in vitro* techniques in the presence and absence of leaf extracts.

2.2 Preparation of plant extracts and nucleation assay

The plant leaves of *A. indica* Linn. were washed with distilled water, dried, and powdered. The powdered leaves were used for cold extraction process with different solvents such as chloroform, ethyl acetate, acetone and ethanol in the ratio of 1:10 based on their polarity. After 48 h of interval, the extracts were filtered through Whatman Grade 40 filter paper and air dried. Different concentrations of dried leaf extracts were prepared (1%, 3%, 5%, 7%, and 10%) in respective solvents for *in vitro* analysis. The nearness of nuclei prompts the emergence of kidney stones, and this was studied using the modified method [11] in which a 1.5 ml aliquot of 3.5 mmol/l calcium chloride (in TBS pH 6.5) and 150 µl of the extracts were mixed, then a 1.5 ml aliquot of 6 mmol/l sodium oxalate (in TBS pH 6.5) was included, and absorbance was estimated at 620 nm for different time intervals. Rate restraint was determined using the equation below.

$$\% \, Inhibition = \frac{C-S}{C} * 100 \quad (1)$$

where *C* is the turbidity without leaf extracts and *S* is the turbidity with leaf extracts.

2.3 Growth assay

The hindrance of the development of COM crystal was contemplated with small alterations [12] where 1.5 ml of each calcium chloride and sodium oxalate solutions of 1 mmol were prepared in the TBS buffer (pH 7.2) and mixed with 2.5 mg/ml COM crystal slurry (in acetate buffer). The absorbance was estimated at 214 nm with and without leaf extracts, and inhibition percentage was determined as follows:

$$\%\ Inhibition = \frac{C-S}{C} * 100 \quad (2)$$

where *C* is the oxalate reduction rate without leaf extracts and *S* is the oxalate reduction rate with leaf extracts.

2.4 Aggregation assay

At the point when crystals are present in the solution, they bunch together and form large particles as aggregates. The formation of aggregates can be inhibited with the help of leaf extracts with some changes in the method [13]. For this, a COM crystal can be shaped with solutions of calcium chloride and sodium oxalate at 75 mmol/l. These solutions were blended and kept in a water bath at 60°C for 1 h and then dried to collect the COM crystals. Then, 2 mg/ml COM crystals were prepared in the TBS buffer (pH 6.5). On the basis of the turbidity with and without leaf extracts, the rate of aggregation was analyzed. The rate of aggregation inhibition (Ir) was broken down by estimating the slope of turbidity with the leaf extracts and control, as follows.

$$Ir\ =\ 1\ -\frac{Turbidity\ in\ sample}{Turbidity\ in\ control} * 100 \quad (3)$$

2.5 Precipitation of calcium oxalate in artificial urine

Artificial urine (AU) was prepared with compositions containing sodium chloride (105.5 mmol/l), sodium phosphate (32.3 mmol/l), sodium citrate (3.21 mmol/l), magnesium sulfate (3.85 mmol/l), sodium sulfate (16.95 mmol/l), potassium chloride (63.7 mmol/l), calcium chloride (4.5 mmol/l), ammonium hydroxide (17.9 mmol/l), and ammonium chloride (0.0028 mmol/l) [14]. The AU was prepared freshly each day and the pH was adjusted to 6.0. The examination was directed by adding 2 ml AU, 0.5 ml extract, and 1.5 ml sodium oxalate (0.01 M), and absorbance was estimated at 620 nm. Percentage inhibition was determined using the following equation:

$$\%\ Inhibition\ =\ 1\ -\frac{Si}{Sc} * 100 \quad (4)$$

where S_i is the slope of the graph with leaf extracts and *S* is the slope of the graph without leaf extracts.

2.6 Separation of bioactive compounds by column chromatography

The potential leaf extract was identified by the above methods, and the extract was subjected to column chromatography. The column was packed with activated silica gel of 60-120 mesh and filled with respective solvent. Then, approximately 3 g extract was added and fractions were eluted out at constant time intervals and stored for further analysis.

2.7 TLC Analysis

The collected fractions were analyzed by thin-layer chromatography, and the mobile phase selected was chloroform/ethanol/methanol (8:2:1) [9, 15, 16]. This helps to identify the bioactive compound containing fractions. The plates were observed under UV light and the spots were identified.

2.8 Fourier Transform-Nuclear Magnetic Resonance (FT-NMR) analysis

The bonding regions and type of carbons present in the bioactive compound were analyzed using FT-NMR. The ^{13}C nuclear magnetic resonance (NMR) was used for the distinguishing proof of the carbon atoms in the bioactive compounds. The sample was prepared with DMSO and the spectra were read on a Bruker Advance Ultra Shield 400 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), working at 400.13 MHz, using a broadband inverse probe head. The raw data points were obtained from the spectra.

2.9 Kidney stone analysis

Precisely expelled kidney stones were obtained from Senthil Multispeciality Hospital, Erode, Tamil Nadu, India. These stones were categorized into calcium oxalate stones and calcium phosphate stones (Figure 7 (a-b)). The initial size and weight of the stones were noted. The stones acted towards various concentrations of plant extracts and control (NaCl 10 g/l) for a period of 4 weeks with constant stirring. The stones were removed from the extracts at the end of each week and weighed after drying at 100°C. The parameters determined were decrease in weight and size of the stone, rate weight decrease, and disintegration rate [7].

$$\%\, Reduction\ in\ stone\ weight\ =\ 1\ -\frac{Final\ weight\ (\mathrm{g})}{Intial\ weight\ (\mathrm{g})} * 100 \tag{5}$$

$$Dissolution\ rate\ =\ \frac{Intial\ weight\ (\mathrm{g})\ -\ Final\ weight\ (\mathrm{g})}{Time\ (\mathrm{h})} \tag{6}$$

2.10 MTT assay

African Green Monkey Kidney cell lines (Vero cells) were acquired from the National Culture for Cell Sciences (NCCS, Pune). The cells were maintained in minimal essential medium (MEM) attuned with penicillin (100 units/ml), streptomycin (1 mg/ml), and 10% fetal bovine serum, and kept in a 25 cm^2 tissue-culture-treated flasks at 37°C and 5% CO_2 in humidified chambers. For oxalate induced cell injury, cells were refined with MEM medium alongside with 100 µg/ml sodium oxalate and various concentrations of plant extracts for 72 h [17, 18]. Cytotoxicity was measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

The viability of the cells was evaluated and estimated by the measure of decrease of MTT to purple formazan and it was evaluated by an altered method [19, 20]. The 96-well plates were used for culturing the Vero cells (1 × 105/wells). The cells were hatched at various concentrations of extracts and hatched at 37°C for 48 h. In the wake of gathering, the cells were cultured with MTT (5 mg/ml) in phosphate buffer (pH 7.4) and incubated at 37°C for 2 h. The absorbance was estimated at 540 nm by adding 0.2% DMSO to the treated cells and wells containing cells without extracts. Cell viability (%) was calculated from the following equation:

$$\%\, Cell\ viability\ =\ \frac{A540\ of\ treated\ cells}{A540\ of\ control\ cells} * 100 \tag{7}$$

2.11 Statistical analysis

All experiments were done in triplicates. These were presented as mean ± SD of three independent filters. Statistical analysis was performed by using the Analysis of Variance (ANOVA) with GraphPad Prism, V5.0, software. The distinction in the experiments was viewed significant if *P*-value was <0.05.

3. Results and Discussion

3.1 Inhibition of calcium oxalate crystal nucleation

The inhibitory potential of leaf extracts of *A. indica* Linn. was measured against crystal nucleation, which is the most critical stage in kidney stone development. Figure 1 shows the inhibitory activity in nucleation formation. It is observable that the ethanolic extracts of 1% and 5% concentration, and 1% of ethyl acetate had higher inhibition of approximately 90±0.0011% compared to all the other extracts. There is a deviation and decrease in the inhibitory activity against nucleation of the CaOx crystals, even after increasing the concentration of extracts. The results obtained by studies in *Bergenia ciliata* [5] show approximately 32%-92% inhibition in the nucleation formation.

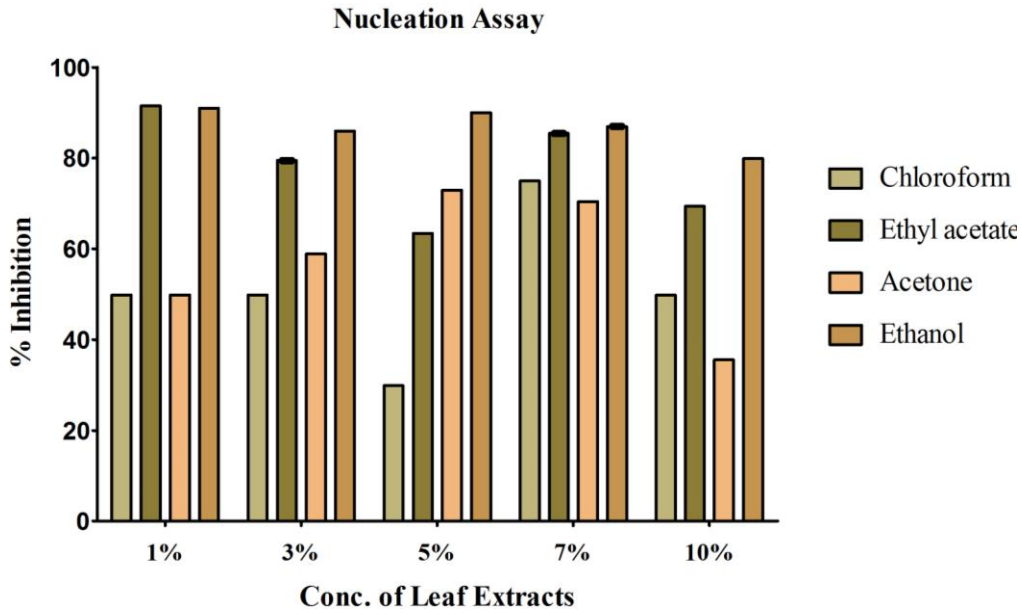

Figure 1. Effect of *Acalypha indica* Linn. leaf extracts on CaOx nucleation. Statistical analysis was depicted as p value < 0.0001.

3.2 Calcium oxalate crystal growth inhibition

Figure 2 illustrates that the concentration of ethyl acetate and ethanolic extracts has a comparable inhibition rate. The 5% concentration of both ethyl acetate and ethanolic extracts yielded 99.4±0.002% ($P < 0.0001$). The percentage inhibition of growth of CaOx crystals was comparable on increasing the concentration of leaf extracts. In banana cultivator Monthan [8] and in *Tribulus terrestris* [2], the inhibitory activity was found to increase in the inhibition of growth about 84% and 85.7% in ethanol extract respectively.

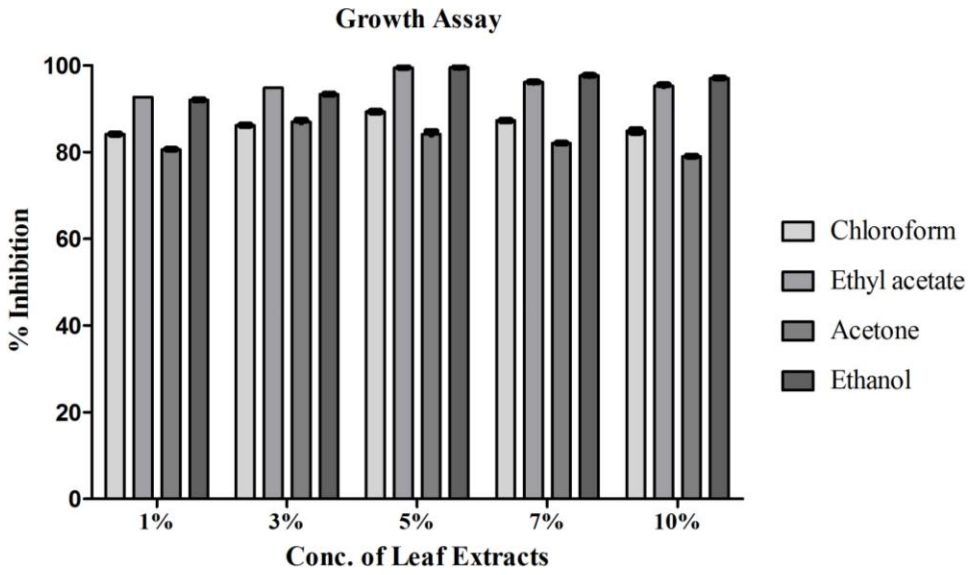

Figure 2. Effect of *Acalypha indica* Linn. leaf extracts on CaOx growth. Statistical analysis was depicted as p value < 0.0001.

3.3 Inhibition of calcium oxalate aggregation

The process of binding of kidney stone crystals to one another, called aggregation, is induced by a strong electric field. This is important in the lithiasis process. The inhibitory activity of different extracts is shown in Figure 3, which depicts that the ethanolic extracts at 3% and 5% concentrations have higher activity (93±0.002%) than other extracts. On increasing the concentration of ethanolic extract, a fall in the inhibitory activity against CaOx aggregation was observed. *Aerva lanata* [21] and *Bergenia ciliata* (Haw.) [22] showed considerable inhibition of 58%-97% against aggregation and decreased inhibition with increased concentration, and the results obtained in plants such as *Tachyspermum ammi*, [23] *Terminalia arjuna* [9], and *Tetraclinis articulate* [24] showed approximately 28%-78% inhibition.

3.4 Inhibition of calcium oxalate precipitation in artificial urine

In general, dissolved stones can be eliminated and excreted via urine. The inhibitory potential of CaOx precipitation in AU was studied with the help of leaf extracts of *A. indica* Linn. An increasing percentage of inhibition was observed for increasing concentration of ethanolic extracts and reached 99.231±0.0001% at 5% concentration (Figure 4). This is far greater compared to that of all the other

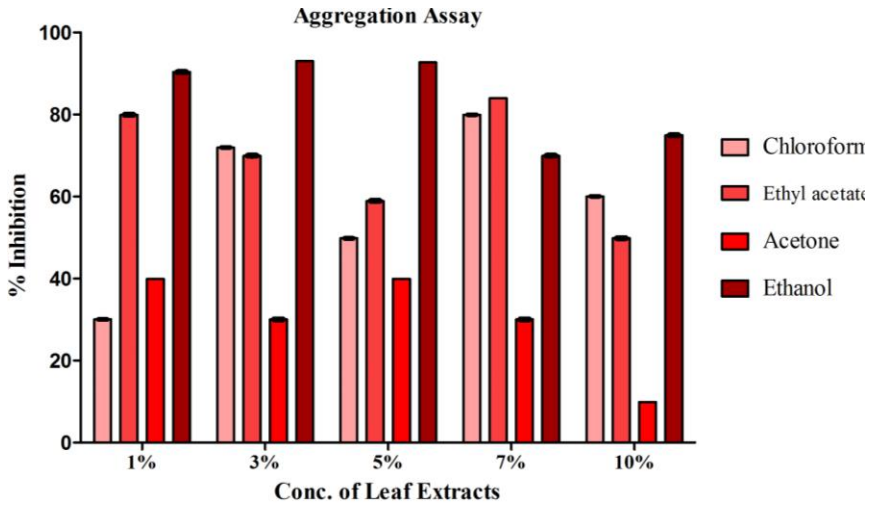

Figure 3. Effect of *Acalypha indica* Linn. leaf extracts on CaOx aggregation. Statistical analysis was depicted as p value < 0.0001.

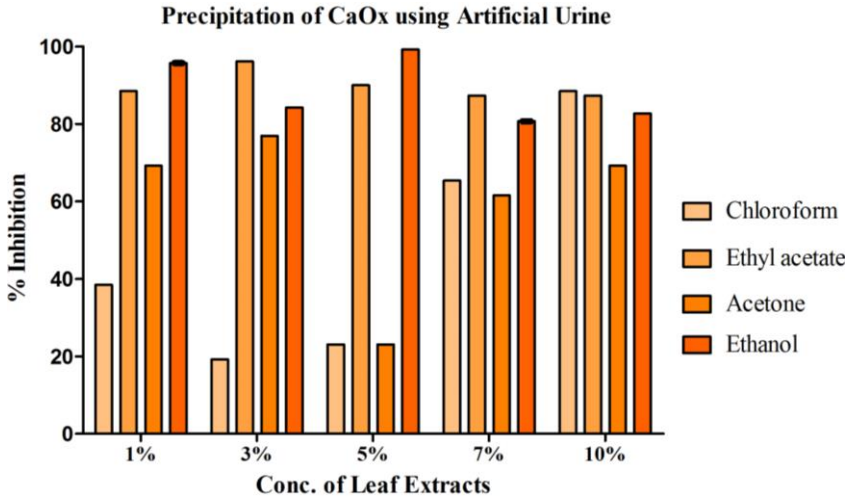

Figure 4. Effect of *Acalypha indica* Linn. leaf extracts on CaOx precipitation in artificial urine. Statistical analysis was depicted as p value < 0.0001.

extracts. A small increase in the concentration will have a negative effect on the inhibition rate. The formation of CaOx crystals in AU was considerably reduced by 99.23% with the ethanolic extract of *A. indica* Linn. The results acquired in *Achyranthes indica* Linn [10] and *Achyranthus aspera* [25] showed reductions of approximately 67%-95%.

3.5 Analysis of bioactive compounds

The ethanolic extract was found to have higher potential than other extracts based on the *in vitro* studies performed. The crude extract was subjected to column chromatography and purified fractions were obtained. The fractions were collected at definite time intervals and 53 fractions were obtained after elution. The fractions were analyzed by thin-layer chromatography for identifying the potential fractions and single band was observed in fractions 17-23 (Figure 5). The fractions 17-23 were pooled together and were analyzed using ^{13}C NMR for the carbon shift data and the results are shown in Figure 6. The peak shift data are shown in Table 1, which illustrates the compounds present in the extract. The peak 74.58 represents C=N group. Peaks 58.49-53.86 denote the presence of OCH3 group; DMSO is indicated from 38.91 to 38.33 and 17.21 shows the occurrence of the CH2 group. From these data, it can be inferred that the bioactive compound has aliphatic nature. The ethanolic extract was subjected to column chromatography and 53 fractions were obtained. Then, the single-band fractions were identified by TLC and pooled together for further process [15].

3.6 Analysis of efficacy of ethanolic extracts on kidney stone

The reduction in various properties of surgically removed stones was tested using ethanolic extracts on calcium oxalate and calcium phosphate stones. The results, tabulated as reduction in weight of the stone (g), % solubility, size reduction (cm) and mean dissolution rate (g/h), are depicted in Table 2. Figure 7(c-e) shows the kidney stones after treatment with ethanolic extract for 4 weeks. This shows the weight of the calcium oxalate and calcium phosphate stones and control stone reduced after 4 weeks (61.82±0.133%, 35.71±0.06%, and 58.75±0.03%, respectively). Similarly, the reduction in size of the kidney stones for calcium oxalate and calcium phosphate stones and control stones was 0.23±0.002, 0.30±0.017, and 0.28±0.0006, respectively. The solubility percentage and mean dissolution rate of the kidney stones after 4-week treatment with ethanolic extracts in calcium oxalate and calcium phosphate stones and control were 35.55±0.03 and 25±0.173, 15±0.115 and 0.018±0.016, and 0.04±0.06 and 0.02±0.01, respectively. From these results, it can be inferred that ethanolic extracts have less inhibitory potential against calcium phosphate stones.

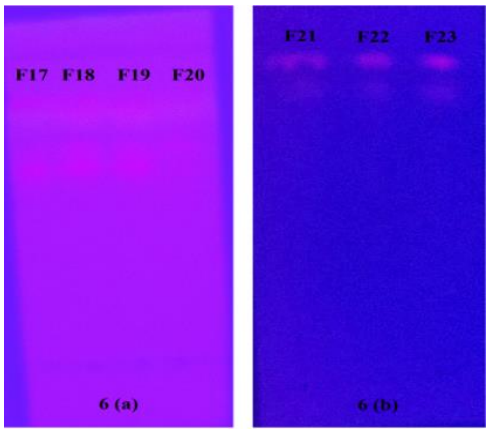

Figure 5. TLC plates showing bands of ethanolic extracts viewed under UV light
(a) Fractions 17-20, and (b) Fractions 20-23

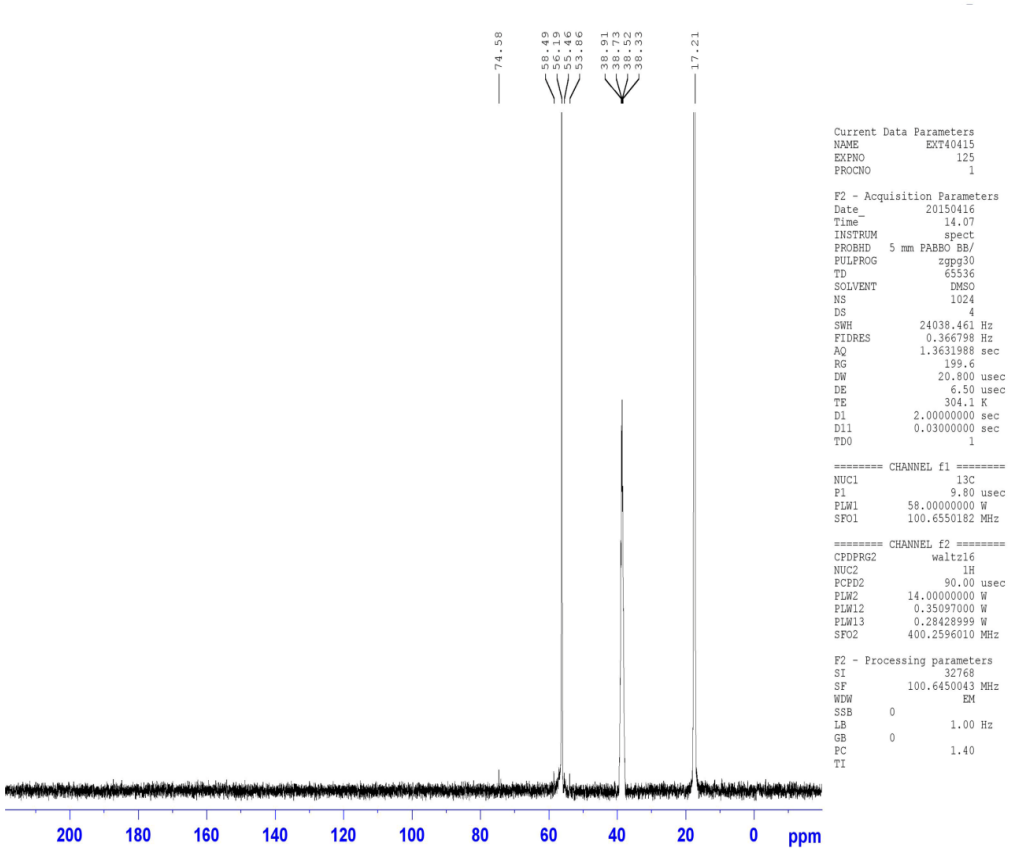

Figure 6. ^{13}C NMR spectrum of ethanolic extract of *Acalypha indica* Linn. at 400 MHz

Table 1.^{13}C NMR chemical shift data and their carbon types

S. No.	Chemical Shift (ppm)	Type of Carbon
1.	74.58	-C=N
2.	58.49	-OCH_3-
3.	56.19	-OCH_3-
4.	55.46	-OCH_3-
5.	53.86	-OCH_3-
6.	38.91	DMSO Solvent
7.	38.73	DMSO Solvent
8.	38.52	DMSO Solvent
9.	38.33	DMSO Solvent
10.	17.21	-CH_2-

Table 2. Weight reduction, % solubility, size reduction, and mean dissolution rate of calcium oxalate and calcium phosphate stones after treatment with ethanolic extracts

Particulars	Control				Calcium oxalate stone				Calcium phosphate Stone			
Initial weight of the stone (g)	0.08				0.11				0.56			
Initial Size of stone (cm)	0.6				0.6				1.1			
Week	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Weight of the stone (g)	0.07	0.006	0.045	0.033	0.103	0.1	0.08	0.042	0.56	0.56	0.5	0.36
% in weight reduction after 4th week	58.75±0.03				61.82±0.133				35.71±0.06			
Solubility of stone (g)	0.01	0.01	0.015	0.012	0.007	0.003	0.02	0.038	0	0	0.06	0.14
% Solubility of stone after 4th week	15±0.115				35.55±0.03				25±0.173			
Size of stone (cm)	0.55	0.53	0.44	0.32	0.59	0.57	0.45	0.37	1.1	1.1	1	0.8
Reduction in size of stone (cm) after 4th week	0.28±0.006				0.23±0.002				0.3±0.017			
Dissolution rate (g/h)	0.006	0.012	0.021	0.028	0.0042	0.006	0.018	0.04	0	0	0.036	0.12
Mean Dissolution rate (g/h) after 4th week	0.02±0.01				0.018±0.016				0.04±0.06			

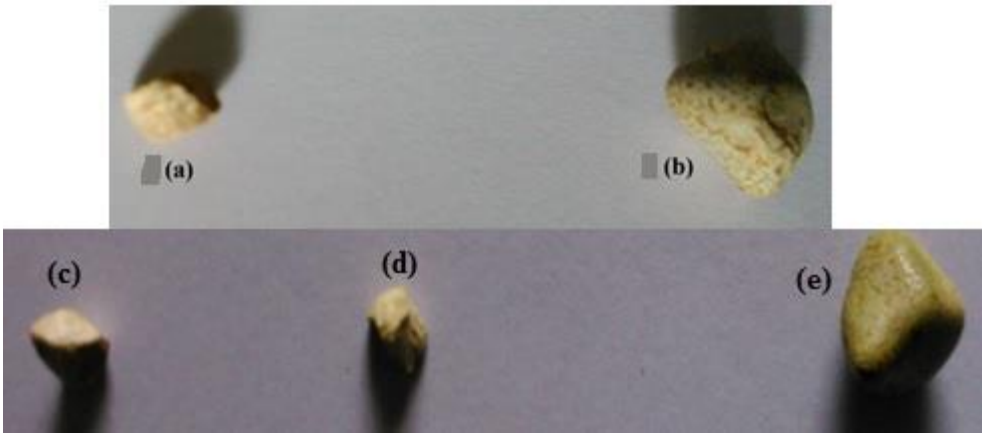

Figure 7. Stones before and after treatments with ethanolic extracts.
Before treatment with ethanolic extract; (a) calcium oxalate stone,
(b) calcium phosphate stone, and (c) control,
After Treatment with ethanolic extract; (d) calcium oxalate stone,
and (e) calcium phosphate stone

Comparable results were produced with food extracts having 9.09%-39.9% activity in dissolving the kidney stones [7] and in Phy and Art having inhibitory activity of 40.5%-52.1% [26]. *Solanum xanthocarpum*, *Rhamnus prushinae*, *S. granulate* and *Tribulus terrestris* extracts dissolved the kidney stones by approximately 15%-76.5% [27].

3.7 MTT Assay

The cytoprotective activity of ethanolic extracts of *A. indica* Linn. on oxalate-induced Vero cell lines (renal epithelial cell lines) was studied and results are shown in Figure 8. It was clearly visible that the purified extract with 1000 µg/ml concentration showed some white spots of viable cells along with untreated cells and with crude extract and all other concentration of the extracts showed little variations in the viable cells. Cell viability (%) was measured and shown in Figure 9. It was revealed that the crude extract alone can maintain the viability up to 77.4%, whereas 1000 µg/ml of purified extract can only retain the cell viability up to 70.5±2.99% compared to crude extracts and untreated cells. The viability of cells was measured and found to be high at 1000 µg/ml concentration of the ethanolic extracts. Similar results were observed in other studies [2, 28].

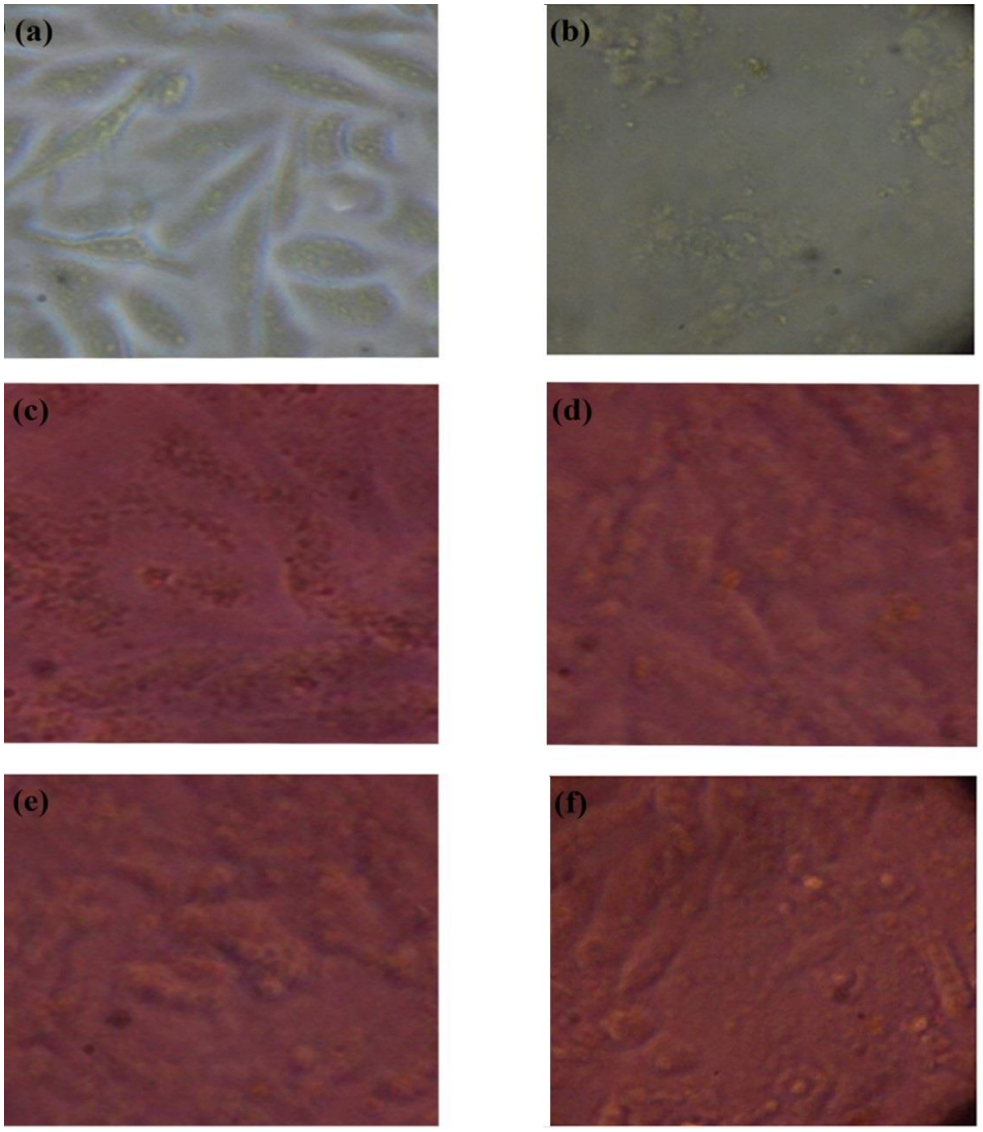

Figure 8. Viability of oxalate induced Vero cells treated with purified ethanolic extract of *Acalypha indica* Linn. (a) untreated cells, (b) extract alone, (c) 125 µg/ml extract, (d) 250 µg/ml extract, (e) 500 µg/ml extract, and (f) 1000 µg/ml extract.

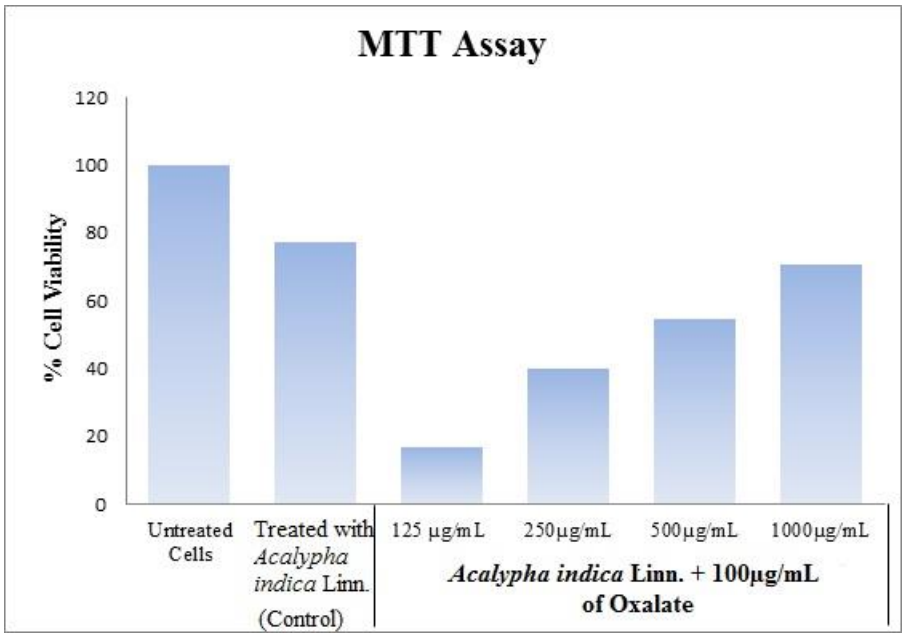

Figure 9. Effect of purified ethanolic extract of *Acalypha indica* Linn. on the viability of Vero cells

4. Conclusions

From the present study it can be concluded that *Acalypha indica* Linn. leaves show potential inhibitory activity against calcium oxalate stones. The three stages of formation and occurrence of kidney stones are nucleation, aggregation and growth of the CaOx crystal, and formation of calcium oxalate in the artificial urine was highly inhibited in the presence of the ethanolic extracts at an optimum concentration of about 5%. The efficiency of the partially purified and pooled fractions of ethanolic extracts was tested against surgically removed stones and oxalate-induced Vero cells (renal epithelial cell lines). The calcium oxalate stones were highly dissolved in the ethanolic extracts, whereas the calcium phosphate stones showed small amount of inhibition. This shows that the crude extract had higher activity compared to the partially purified extracts. Furthermore, this can be efficiently tested against animal models for the analysis of the inhibitory activity against calcium oxalate stones.

5. Acknowledgements

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