



## ANTILITHIATIC ACTIVITY OF *KALANCHOE PINNATA* PERS. ON 1% ETHYLENE GLYCOL INDUCED LITHIASIS IN RATS

Umesh Kumar Gilhotra<sup>1</sup>, Christina A.J.M.<sup>2\*</sup>

<sup>1</sup> NIMS Institute of Pharmacy, NIMS University Jaipur, Rajasthan, India.

<sup>2</sup> AIMST university, Kedah, Malaysia.

\*Corresponding author's E-mail: [tinatina38@rediffmail.com](mailto:tinatina38@rediffmail.com)

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### ABSTRACT

*Kalanchoe pinnata* Pers. (Crassulaceae) is a perennial herb growing wildly and used in folkloric medicine in throughout India. The leaves of *Kalanchoe pinnata* have been widely used in many biological activities such as anti-inflammatory, antileishmaniasis, antiulcer activities etc. Currently *Kalanchoe pinnata* leaves are also used traditionally in lithiasis (kidney stone) treatment. Lithiasis was induced by oral administration of 1% ethylene glycolated water to male Wistar rats for 28 days. The ionic chemistry of urine was altered by ethylene glycol, which elevated the urinary concentration of crucial ions viz. oxalate, calcium, phosphate, thereby contributing to renal lithiasis formation. The ethanolic extract of *Kalanchoe pinnata* was administered (200 mg/ kg) and compared with standard drug Cystone (100 mg/ kg) for 28 days along with 1% ethylene glycolated water. The ethanolic extract of *Kalanchoe pinnata* significantly ( $p < 0.05$ ) reduced the elevated level of ions such calcium, oxalate, phosphate, protein, creatinine, uric acid in urine of treated animals compared to untreated animal. Also, it elevated the urinary level of magnesium, which is considered as one of the inhibitors of crystallization. The high serum creatinine level observed in ethylene glycol treated rats was also reduced. The histopathological studies also showed signs of improvement after treatment with the extract. All these observations provided the basis for the conclusion that plant extract inhibits lithiasis induced by ethylene glycol treatment.

**Keywords:** Antilithiatic, *Kalanchoe pinnata* Pers., Urolithiasis.

### INTRODUCTION

Lithiasis formation in the kidney is one of the oldest and most wide spread diseases known to man. Urinary calculi have been found in the tombs of Egyptian mummies dating back to 4000 BC.<sup>1</sup> and in the graves of American Indians from 1500-1000BC.<sup>2</sup> Lithiasis is a male-predominant disorder, with a recurrence rate of 70-80% in males and 47-60% in females.<sup>3</sup>

The present day medical management of lithiasis includes lithotripsy and surgical procedures. Unfortunately, these techniques do not correct the underlying risk factors; therefore, continued medical supervision and therapy for preventing stone recurrence are mandatory.<sup>4</sup> In addition, these techniques cause side effects such as hemorrhage, hypertension, tubular necrosis and subsequent fibrosis of the kidney.<sup>5</sup>

Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of various ailments. Medicinal plants are used from centuries due to its safety, efficacy, cultural acceptability and lesser side effects as compared to synthetic drugs.<sup>6</sup> *Kalanchoe pinnata* Pers. (Crassulaceae) commonly known as Patharchatta, it is a perennial herb growing widely and used in folkloric medicine in tropical Africa, America, India, china and Australia. A no. of active compounds including flavanoids, glycosides, steroids, bufadienolides and organic acids have been identified in *Kalanchoe pinnata*.<sup>7</sup>

In the present study, an effort has been made to establish the scientific validity for the antilithiatic activity of *Kalanchoe pinnata* leaves extract using ethylene glycol induced lithiasis model in rats.

### MATERIALS AND METHODS

#### Animals

Male albino Wistar rats (250-300 gm) were obtained from HAU, (Hisar India). They were housed in well-ventilated cages (5 to 6 cage), maintained at 25± 2°C under 12 h light-dark cycle. They were fed with commercial pellet diet (Sanjay biological museum, Amritsur India) and had free access on water. The animals were maintained in these conditions for 1 week before the experimental session. This study was approved by our institutional animal ethical committee.

#### Preparation of the extract

Leaves of *Kalanchoe pinnata* were collected from the medicinal farm at Hanumangarh (Rajasthan India) and identified by Dr. G.C. Abraham, Reader; Department of Botany, American College Madurai (India). A voucher specimen (MAC 16672) was deposited in our laboratory. The leaves were shade dried and coarsely powdered in such a way that it passed through sieve 20 and was retained on sieve 40. About 1Kg of the dry powder was Soxhlet- extracted with 70% ethyl alcohol for 72 h. The solvent was evaporated in vacuo yielding dried extract (20 g).



## Analysis of Plant Extracts by GC-MS

Analysis of ethanolic extracts of *Kalanchoe pinnata* by GC-MS was carried out at IICPT, Thanjavur (India), showed the presence of various components such as Glycerin, Levulinic acid, Itaconic anhydride, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 2-Furancarboxaldehyde, 5-(hydroxymethyl)-, Hydroquinone, Pyrogallol, Tyrosol, Levoglucosan, 1,6 anhydro- $\alpha$ -D-glactofuranose, 1,2,3,5-Cyclohexanetetrol, (1a,2a,5a)-, 2,5-Furandione, 3-methyl-4-propyl-, n-Hexadecanoic acid, 1-Heptadecanol, Coumarin, 7,8-dihydro-7-hydroxy-6-methoxy-8-oxo-, Hexanedioic acid, bis(2-ethylhexyl)ester, 1,2-Benzenedicarboxylic acid, dilsocetyl ester and Squaline.

### Ethylene glycol induced lithiasis model

Ethylene glycol induced lithiasis was used to assess the antilithiatic activity in Wistar albino rats.<sup>8</sup> Animals were divided into four groups containing six animals in each. Group I served as control and received regular rat food and drinking water ad libitum. Ethylene glycol (1%) in drinking water was fed to Group II to IV for induction of renal calculi till 28 days. Group III received ethanolic extract suspended in water (200 mg/kg b.w.)<sup>9,10</sup> of *Kalanchoe pinnata* leaves and Group IV received standard antiurolithiatic drug, Cystone suspended in water (100 mg/kg b.w.).<sup>11</sup> All extracts were given once daily by oral route.

### Collection and analysis of urine

Animals were kept in separate metabolic cages and urine samples of 24 h were collected on 28 day. A drop of concentrated hydrochloric acid was added to the urine before being stored at 4°C. Urine was analyzed for calcium, creatinine, uric acid, phosphate, protein, oxalate, and magnesium content.<sup>12-18</sup>

### Serum analysis

After the experimental period, blood was collected from the retro-orbital under anesthetic conditions and the animals were sacrificed by cervical decapitation. Serum was separated by centrifugation at 10,000 rpm for 10 min and analyzed for calcium, creatinine, uric acid, phosphate, oxalate, and magnesium content.

### Histopathological Studies

Kidney samples were weighed and fixed rapidly with 10% neutralized formalin (pH 7.4). Sections of kidney fixed in paraffin were prepared and stained with hematoxylin and eosin and observed for histopathological changes.

### Statistical analysis

The results are expressed as Mean  $\pm$  SEM. Data were evaluated using ANOVA; Single factor test followed by Newman – Keuls multiple range tests. Probability values less than ( $p < 0.05$ ) were considered significant.

## RESULTS

### Urinary Analysis

In the present study, chronic administration of 1% (v/v) ethylene glycol aqueous solution to male Wistar rats resulted in hyperoxaluria. Urinary concentration of the various ions investigated varied drastically, following ethylene glycol treatment. The oxalate excretion was  $20.33 \pm 1.59$  mg/24hr/rat on 28<sup>th</sup> day respectively for G1. It increased significantly to  $54.13 \pm 2.04$  mg/24hr/rat ( $P < 0.001$ ) on day 28<sup>th</sup> day in G2, following ethylene glycol treatment; treatment with KPE (G3), reduced the oxalate excretion significantly on 28<sup>th</sup> day, treatment with this extract to  $38.49 \pm 1.57$  mg/24hr/rat, ( $P < 0.05$ ). However, treatment with standard drug Cystone (G4) oxalate excretion was  $29.93 \pm 1.76$  mg/24hr/rat, restored to near normal limits. The results are shown in the table 1.

The urinary calcium excretion was  $7.15 \pm 0.33$  mg/24hr/rat on day 28<sup>th</sup> respectively for G1. It increased significantly to  $18.43 \pm 0.39$  mg/24hr/rat ( $P < 0.001$ ) on day 28<sup>th</sup> day in G2. The calcium excretion was significantly reduced in following ethylene glycol treatment with KPE (G3), on 28<sup>th</sup> day calcium excretion was significantly reduced to  $13.92 \pm 0.19$  mg/24hr/rat, ( $P < 0.05$ ) in G3, respectively. Treatment with standard drug Cystone (G4) calcium excretion was  $9.23 \pm 0.23$  mg/24hr/rat, restored to near normal limits. The results are shown in the table 1.

Likewise phosphate  $86.51 \pm 2.03$  mg/24hr/rat and creatinine  $82.10 \pm 2.31$  mg/24hr/rat excretion values gradually increased in G2 on the 28<sup>th</sup> day. However in G3 & G4 these elevated values were brought down to Phosphate on 28<sup>th</sup> day  $65.53 \pm 1.88$  mg/24hr/rat,  $55.15 \pm 2.00$  mg/24hr/rat respectively, in KPE extracts & Cystone treatment (table 1). Likewise, creatinine value reduced in G3 and G4 such as on 28<sup>th</sup> day  $36.93 \pm 1.73$  mg/24hr/rat and  $35.41 \pm 1.62$  mg/24hr/rat respectively.

Likewise urinary protein and uric acid concentration increased following ethylene glycol treatment in G2 and it reached maximum of  $170.51 \pm 2.99$  mg/24hr/rat and  $17.36 \pm 0.58$  mg/24hr/rat respectively on the 28<sup>th</sup> day, on treatment with KPE extracts and Cystone treatment, the protein and uric acid excretion was restored to near normal limits in G3, and G4 for protein  $127.74 \pm 2.00$  mg/24hr/rat,  $83.94 \pm 1.53$  mg/24hr/rat; on 28<sup>th</sup> day ( $p < 0.001$ ) and for uric acid on 28<sup>th</sup> day  $9.96 \pm 0.40$  mg/24hr/rat,  $07.24 \pm 0.23$  mg/24hr/rat; ( $P < 0.001$ ).

In G1 normal rats the magnesium excretion was estimated as  $4.97 \pm 0.25$  mg/24hr/rat on 28<sup>th</sup> day. Contrary to this, in G2 lithiatic control rats, the magnesium level in urine gradually decreased to  $1.66 \pm 0.16$  mg/24hr/rat following ethylene glycol treatment on the 28<sup>th</sup> day. Subsequent administration of the extract enhanced the magnesium excretion significantly to  $3.58 \pm 0.27$  mg/24hr/rat in G3,  $4.10 \pm 0.13$  mg/24hr/rat, in G4;  $P < 0.001$  respectively on 28<sup>th</sup> day treatment with plant extract and Cystone treatment.



**Table 1:** Effect of ethanolic extract of *Kalanchoe pinnata* pers. leaves in experimental hyperoxaluria

Parameter (unit)	Group I Control	Group II Calculi induced	Group III Plant extract Treated	Group IV Cystone Treated
<b>Urine (mg/dl)</b>				
Protein	71.93 ± 2.23	170.51 ± 2.99 <sup>*a</sup>	127.74 ± 2.00 <sup>#a, b</sup>	83.94 ± 1.53 <sup>*a, b, c</sup>
Magnesium	4.97 ± 0.25	1.66 ± 0.16 <sup>*a</sup>	3.58 ± 0.27 <sup>#a, b</sup>	4.10 ± 0.13 <sup>*a, b, c</sup>
Calcium	7.15 ± 0.33	18.43 ± 0.39 <sup>*a</sup>	13.92 ± 0.19 <sup>#a, b</sup>	9.23 ± 0.23 <sup>*a, b, c</sup>
Uric Acid	3.96 ± 0.34	17.36 ± 0.58 <sup>*a</sup>	9.96 ± 0.40 <sup>#a, b</sup>	7.24 ± 0.23 <sup>*a, b, c</sup>
Creatinine	15.63 ± 0.48	82.10 ± 2.31 <sup>*a</sup>	36.93 ± 1.73 <sup>#a, b</sup>	35.41 ± 1.62 <sup>*a, b, c</sup>
Oxalate	20.33 ± 1.59	54.13 ± 2.04 <sup>*a</sup>	38.49 ± 1.57 <sup>#a, b</sup>	29.93 ± 1.76 <sup>*a, b, c</sup>
Phosphate	34.68 ± 1.79	86.51 ± 2.03 <sup>*a</sup>	65.53 ± 1.88 <sup>#a, b</sup>	55.15 ± 2.00 <sup>*a, b, c</sup>
<b>Serum (mg/dl)</b>				
Magnesium	6.1 ± 0.32	1.98 ± 0.24 <sup>*a</sup>	3.47 ± 0.38 <sup>#a, b</sup>	6.02 ± 0.26 <sup>*a, b, c</sup>
Calcium	10.25 ± 0.68	31.73 ± 1.54 <sup>*a</sup>	23.18 ± 0.93 <sup>#a, b</sup>	14.02 ± 0.76 <sup>*a, b, c</sup>
Uric Acid	4.28 ± 0.21	17.56 ± 1.52 <sup>*a</sup>	12.13 ± 0.63 <sup>#a, b</sup>	6.85 ± 0.22 <sup>*a, b, c</sup>
Creatinine	1.16 ± 0.14	3.60 ± 0.28 <sup>*a</sup>	2.37 ± 0.23 <sup>#a, b</sup>	1.52 ± 0.17 <sup>*a, b, c</sup>
Oxalate	8.73 ± 0.28	21.12 ± 1.26 <sup>*a</sup>	15.01 ± 0.70 <sup>#a, b</sup>	9.15 ± 0.29 <sup>*a, b, c</sup>
Phosphate	16.31 ± 1.43	35.32 ± 1.56 <sup>*a</sup>	24.33 ± 1.47 <sup>#a, b</sup>	15.18 ± 0.89 <sup>*a, b, c</sup>

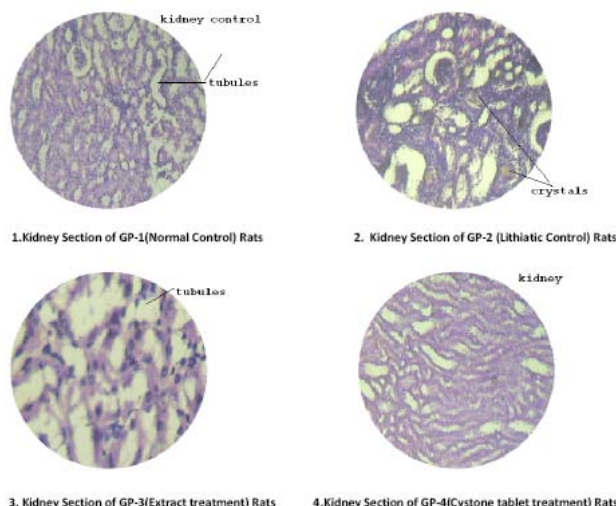
Values for urine parameters are assessed in 24 h urine sample  
 All values are expressed as mean ± SD for six animals in each group.  
 Comparisons are made: <sup>a</sup> with Group I; <sup>b</sup> with Group II; <sup>c</sup> with Group III  
 Symbols represent statistical significance: #  $p < 0.05$ , \*  $p < 0.001$

**Serum Analysis**

The data on serum analysis (Table 1) showed significant increase in level of serum ions except magnesium ion levels in G2 rats when compared to that of normal control rats. In G3, treatment with KPE extract and G4, Cystone treatment restored all the parameters to normal limits.

**Histopathological Data**

Section of kidney from animals treated with ethylene glycol showed that deposition of micro-crystals (Figure-2). There was marked dilatation of tubules, tubular damage and infiltration of inflammatory cells into the interstitial space. However, kidney section of animals treated with extract (G-III), showed improvement of the above symptoms and reduced crystal deposition as shown in (Figure 3) but significantly improved with Cystone treatment in G-IV as shown in (Figure-4).



1. Section Show structure of kidney with glomeruli and tubules which appear normal
2. Section Show structure of kidney with glomeruli and tubules which appear normal. Tubules show crystals in the lumen indicating stone formation.
3. Section Show structure of kidney with glomeruli and tubules which appear normal. No evidence of crystal deposition seen.
4. Section Show structure of kidney with glomeruli and tubules which appear normal.

## DISCUSSION

In the present study, male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans<sup>19</sup> and also earlier studies have shown that the amount of stone deposition in female rats was significantly less.<sup>20</sup>

Urinary supersaturation with respect to stone-forming constituents is generally considered to be one of the causative factors in calculogenesis. Evidence in previous studies indicated that in response to 14 day period of ethylene glycol (1% v/v) administration, young male albino rats form renal calculi composed mainly of calcium oxalate.<sup>21,22</sup> The biochemical mechanisms for this process are related to an increase in the urinary concentration of oxalate. Stone formation in ethylene glycol fed animals is caused by hyperoxaluria, which causes increased renal retention and excretion of oxalate. Similar results have been obtained when rats were treated with ethylene glycol. Therefore, this model was used to evaluate the antilithiatic effect of *Kalanchoe pinnata* against urolithiasis.<sup>23,24</sup>

In the present study oxalate and calcium excretion progressively increased in calculi-induced animals (G2), since it is accepted that hyperoxaluria, is a far more risk factor in the pathogenesis of renal stones than hypercalciuria<sup>25</sup>, the changes in urinary oxalate levels are relatively much more important than those of calcium<sup>26</sup>. Increased urinary calcium is a factor favoring the nucleation and precipitation of calcium oxalate (or) apatite (calcium phosphate) from urine and subsequent crystal growth<sup>27</sup>. However *Kalanchoe pinnata* lowered the levels of oxalate as well as calcium excretion.

An increase in urinary phosphate is observed in calculi induced rats (G2). Increased urinary phosphate excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which is epitaxially, induces calcium oxalate deposition<sup>28</sup>. Treatment with *Kalanchoe pinnata* restored phosphate level, thus reducing the risk of stone formation.

The increases in urinary uric acid excretion were observed in urolithiatic rats. Increased excretion of uric acid has been reported in stone formers and hyperoxaluric rats. Uric acid interferes with calcium oxalate solubility and it binds and reduces the inhibitory activity of glycosaminoglycans. The predominance of uric acid crystals in calcium oxalate stones and the observation that uric acid binding proteins are capable of binding to calcium oxalate and modulate its crystallization also suggests its primary role in stone formation. Treatment with *Kalanchoe pinnata* lowered the excretion of uric acid and reduces the risk of stone formation.

Supersaturation, a step in the pathogenesis of nephrolithiasis, occurs when substances that make up the stone are found in the high concentration in urine, when urine volume decreases, and when urinary concentration

of chemicals that inhibit stone formation decreases. Inhibitors of crystallization include citrate, magnesium, phosphate; nephrocalcin etc.<sup>29,30</sup> Low urinary magnesium content is a common feature in stone formers.<sup>31</sup> A similar condition was observed in the (G2) rats. *Kalanchoe pinnata* extract elevated the urinary magnesium level, and thus, reduced the propensity to crystallize, thereby creating an ambience unfavorable for precipitation.

Increased excretion of proteins has been noted in hyperoxaluric rats and stone formers.<sup>32</sup> A high urinary colloidal concentration favors crystal growth.<sup>33</sup> Such a condition was observed with ethylene glycol treated rats, in this study. Administration of the extract reduced the urinary protein excretion in the G3 rats, and hence minimizes the conditions favorable for crystal growth.

In urolithiasis, the glomerular filtration rate (GFR) decreases due to the obstruction to the outflow of urine by stones in the urinary system. Due to this, the waste products, particularly nitrogenous substances such as creatinine and uric acid get accumulated.<sup>34</sup> Also increased lipid peroxidation and decreased levels of antioxidant potential have been reported in the kidneys of rats supplemented with a calculi-producing diet (CPD)<sup>35,36</sup>. Elevated oxalate has been reported to induce lipid peroxidation and to cause renal tissue damage by reacting with poly unsaturated fatty acids in the cell membrane.<sup>37</sup> In calculi-induced rats (G2), marked renal damage was seen as indicated by the elevated serum levels of creatinine and uric acid. However, treatment with *Kalanchoe pinnata* causes diuresis and hastens the process of dissolving the preformed stones and prevention of new stone formation in the urinary system.

Increase in calcium and oxalate levels in the renal tissue of EG-treated rats were observed. Treatment with *Kalanchoe pinnata* suppresses this increase in intracellular calcium. Several studies reported that flavanoids, polyphenols and triterpenes have anti-inflammatory and antioxidant effects.<sup>38-40</sup> It can be expected that antilithiatic activity might be through an antioxidant activity and free radical scavenging principle(s).<sup>41,42</sup>

In GC-MS Spectra Tyrosol, Pyrogallol and Squaline identified in ethanolic extract of *Kalanchoe pinnata* are having antioxidant properties,<sup>43-47</sup> so these components might be responsible for antilithiatic activity.

Microscopic examination of kidney sections derived ethylene glycol induced urolithiatic rats showed polymorphic irregular crystal deposits inside the tubules which causes dilation of the proximal tubules along with interstitial inflammation that might be attributed to oxalate. Treatment with the *Kalanchoe pinnata* decreased the number and size of calcium oxalate deposits in different parts of the renal tubules and also prevented damages to the tubules and calyces.



## CONCLUSION

In conclusion, the presented data indicate that administration of the *Kalanchoe pinnata* extracts to rats with ethylene glycol induced lithiasis, reduced and prevented the growth of urinary stones, supporting the folklore claim regarding antilithiatic activity of the plants. The mechanism underlying this effect is still unknown but is apparently related to diuresis and lowering of urinary concentrations of stone forming constituents. The protective effect against oxalate-induced lipid peroxidation may be contributory to the recovery of renal damage.

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