

## Preventive and curative effects of *Achyranthes aspera* Linn. extract in experimentally induced nephrolithiasis

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The present study was undertaken to evaluate the efficacy of *Achyranthes aspera* in preventing and reducing the growth of calcium oxalate stones in ethylene glycol induced nephrolithiatic model. Hyperoxaluria was induced in rats using ethylene glycol (EG, 0.4%) and ammonium chloride (1%) for 15 days and was then replaced with EG (0.4%) only. Upon administration of cystone (750 mg/kg body wt.), aqueous extract of *A. aspera* (500 and 1000 mg/kg body wt.), levels of renal injury markers (lactate dehydrogenase and alkaline phosphatase) were normalized with a decrease in serum urea and serum creatinine. Concurrent treatment reduced changes in the architecture of renal tissue and also decreased the size of crystals thereby helping in quick expulsion of the crystals. The present results indicated that *Achyranthes aspera* had an ability to maintain renal functioning and reduced renal injury.

**Keywords:** *Achyranthes aspera*, Calcium oxalate, Cystone, Nephrolithiasis, Urolithiasis

Urinary stone disease occurs worldwide with some geographical and racial variation and is constantly rising in parallel with socio-economic development<sup>1</sup>. It is largely a recurrent disease with an approximate relapse rate of 50% in 5–10 years and 75% in 20 years. Urinary tract stones composed of calcium oxalate (CaOx), either alone or mixed with calcium phosphate, are hitherto the most common uroliths accounting for more than 80% of the stones<sup>2</sup>. The crystallization inhibiting capacity of urine does not allow urolithiasis to happen in most of the individuals, whereas, this natural inhibition is in deficit in stone formers<sup>3</sup>. Studies have also shown that tubular cell injury facilitates calcium oxalate crystal formation and deposition in the renal tubules<sup>4</sup>. Animal and tissue culture studies have demonstrated that both oxalate and calcium oxalate crystals directly induce renal epithelial cell injury mediated through lipid peroxidation and involve oxygen free radical generation<sup>5,6</sup>. Endoscopic stone removal and extracorporeal shock wave lithotripsy have revolutionized the treatment of nephrolithiasis, but do not avoid the possibility of new stone formation<sup>7,8</sup>. Various therapies including thiazide diuretics and

alkali-citrate are being used in an attempt to prevent the recurrence of hypercalciuria- and hyperoxaluria induced calculi, but scientific evidence for their efficacy is less convincing<sup>9</sup>. Medicinal plants have played a significant role in various ancient traditional systems of medication. Even today, plants provide a cheap source of drugs for majority of world's population. Several pharmacological investigations on the medicinal plants used in traditional antiurolithiatic therapy have revealed their therapeutic potential in the *in vitro* or *in vivo* models<sup>10,11</sup>. Researchers are also trying to isolate potent phytoconstituents and antiurolithiatic potency is being evaluated. The most active protein fraction was isolated from *Dolichos biflorus*<sup>12</sup> and *Trachyspermum ammi*<sup>13</sup> and their therapeutic use as antilithiatic proteins was established.

*Achyranthes aspera* commonly called as “*puthkanda*” in hindi, is being used in ayurveda as an herbal drug since ages. There are reports on its antifertility<sup>14,15</sup>, antimicrobial<sup>16</sup>, anti-inflammatory<sup>17</sup>, antinociceptive role<sup>18</sup>, and also as an immune stimulator<sup>19,20</sup>. It is an active component of various drug formulations for kidney stones<sup>21</sup>; however, no scientific basis has been formulated for its antiurolithiatic potency. The present study was undertaken to examine both preventive and curative effects of the aqueous extract of *Achyranthes aspera* in rat urolithiatic model.

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## Materials and Methods

**Preparation of *Achyranthes aspera* extract**—Dried roots of *Achyranthes aspera* were obtained from “Natural Remedies Pvt. Ltd.” Bangalore, India. A collection of voucher specimen is available with the company. The air dried fine powdered roots were extracted in autoclaved distilled water. The extract was then filtered using Whatman No. 1 filter paper and the filtrate was evaporated *in vacuo* and dried using a rotary evaporator at 40°C<sup>22</sup>. The final dried samples were stored in labelled sterile bottles and kept at -20°C.

**Animals**—Healthy male rats of the Wistar strain weighing between 150 and 175 g of equivalent age groups were obtained from central animal house of Panjab University, Chandigarh, India. Rats were acclimatized for one month in polypropylene cages under hygienic conditions and provided with standard animal feed and water *ad libitum*. All procedures were done in accordance with ethical guidelines for care and use of laboratory animals and were approved by the local care of Experimental Animals Committee.

**Dosage**—Plant extract was suspended in distilled water and was administered (*i.p.*) at doses of 500, 1000 mg/kg body wt. based on preliminary experimentation<sup>23</sup>.

**Experimental procedure**—Ethylene glycol induced hyperoxaluria model was used to assess the antilithiatic activity in albino rats following procedures as under.

**Prophylactic regimen (PR)**—Animals were divided into 5 groups containing 6 animals in each. Group A served as a vehicle treated control and maintained on regular rat food and drinking water *ad libitum*. All remaining groups (Groups B – E) received calculi inducing treatment, comprised of ethylene glycol (EG, 0.4% v/v) with ammonium chloride (NH<sub>4</sub>Cl, 1% w/v) in drinking water *ad libitum* for 15 days to accelerate lithiasis. Groups C, D and E were administered cystone (750 mg/kg body wt.) and extract at doses of 500 and 1000 mg/kg body wt. from day 1 to day 15 of calculi induction, respectively. Extract and standard drug were suspended in distilled water and given intraperitoneally once daily<sup>24</sup>.

**Curative regimen (CR)**—Animals were divided into 5 groups containing 6 animals in each. Group A served as a vehicle treated control and maintained on regular rat food and drinking water *ad libitum*. All remaining groups (Groups B – E) received calculi inducing treatment, comprised of ethylene glycol (EG, 0.4% v/v) with ammonium chloride (NH<sub>4</sub>Cl, 1% w/v) in drinking

water *ad libitum* for 15 days to accelerate lithiasis, followed by only EG (0.4% v/v) for next 13 days. Groups C, D and E were given cystone (750 mg/kg body wt.) and extract at doses of 500 and 1000 mg/kg body wt. from day 16 to day 28 of calculi induction respectively. Extract and standard drug were suspended in distilled water and given intraperitoneally once daily<sup>25</sup>.

After the treatment, the rats were placed in metabolic cages and urine was collected in a glass bottle having 20 µl of 20% sodium azide as a preservative for 24 h. The urine was frozen at -20°C and used for determination of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) and creatinine content. The rats were anaesthetized with diethyl ether and sacrificed by decapitation after 24 h of above treatment. Before sacrificing, the blood was taken from orbital sinus into a centrifuge tube without anticoagulant and allowed to clot at room temperature to collect serum. Urine from the urinary bladder was directly obtained by puncturing with a needle (5/8 in.) attached to a 1 mL. tuberculin syringe. After dissection both kidneys were removed and transverse section from both the kidneys was fixed for histological analysis.

**Biochemical assays in urine and serum**—Serum urea level was estimated by diacetylmonoxime method<sup>26</sup>. The creatinine in both serum and urine was estimated by the method of Bonsnes and Tausky<sup>27</sup>. Creatinine clearance was calculated<sup>28</sup>. Urinary LDH was measured by decrease in absorbance at 340 nm resulting from the oxidation of NADH. The activity of ALP was determined by measuring the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol at 405 nm<sup>29</sup>.

**Polarization microscopy of urinary crystal**—A drop of urine obtained from bladder was spread on a glass slide and visualized under polarized light using Leica DM3000 light microscope<sup>30</sup>.

**Histopathological studies**—Transverse sections of kidney tissue were fixed in formaldehyde (10%). The tissues were then dehydrated and embedded in paraffin wax. The paraffin sections (8 µ) were then cut and stained in H & E staining and viewed under light microscope.

**Statistical analysis**—Data were expressed as mean ± SD and analyzed by ANOVA.

## Results

In the present study, administration of EG (0.4%) along with NH<sub>4</sub>Cl (1%) induced hyperoxaluria. As a result of stone induction, there was a gross increase in

the activity of kidney injury markers (LDH and ALP) and also in the levels of serum urea and serum creatinine significantly (Tables 1, 2). However, on treatment with aqueous extract of *Achyranthes aspera*, levels of LDH and ALP decreased significantly along with normalization in levels of serum urea and serum creatinine in a dose dependent manner in both prophylactic and curative regimen as compared to control and cystone (positive control).

To estimate injury in the kidney tissue, the levels of renal injury marker enzymes (LDH and ALP) were estimated in urine. LDH was found elevated in calculi induced rats, which was restored to 51% in PR and 77% in CR on administration of aqueous root extract of *A. aspera* (1000 mg/kg body wt.) compared to the

control rats (Group A). ALP was also increased in urolithiatic group rats by 140% in both PR and CR as compared to control group rats. On supplementation with aqueous extract of *A. aspera* (1000 mg/kg body wt.), the activity of ALP was decreased to 78% in PR and 52% in CR in comparison to control group rats (Tables 1, 2).

The serum urea was remarkably increased in calculi induced rats (63% in PR and 79% in CR) as compared to control group rats, while, serum creatinine levels were almost doubled in both PR and CR compared to control rats. Treatment of urolithiatic rats with *A. aspera* presented a significant reduction in serum urea content (30% in PR and 41% in CR) as compared to control group rats. Similar pattern was

Table 1—Alterations in the level of urinary enzymes (LDH and ALP), serum urea and serum creatinine in various test groups in prophylactic regimen (PR)

Parameters	Group A	Group B	Group C	Group D	Group E
Urinary LDH (units/ml/mg prt)	7.89 + 0.8	20.12 + 1.1** (155%)	9.15 + 0.9### (15.9%)	15.75 + 1.8*#ψ (99.6%)	11.94 + 1.5*# ψ (51.3%)
Urinary ALP (IU/L)	33.45 + 5.1	80.15 + 3.4** (139.6%)	40.89 + 4.4### (22.2%)	77.92 + 7.8** ψ (132.9%)	59.66 + 6.9*# ψ (78.4%)
Serum urea (mg/dl)	218.37 + 8.9	355.48 + 9.4** (62.8%)	245.45 + 11.8*### (12.4%)	307.15 + 19.8*# ψ (40.7%)	284.77 + 14.7*# ψ (30.4%)
Serum creatinine (mg/dl)	0.429 + 0.07	0.915 + 0.05** (113.3%)	0.551 + 0.08*### (28.4%)	0.842 + 0.04** ψ (96.3%)	0.787 + 0.04*# ψ (83.4%)
CrCl (ml/min)	4.47 + 0.4	2.07 + 0.5** (-53.7%)	3.94 + 0.7### (-11.9%)	2.58 + 0.4*# ψ (-42.3%)	3.15 + 0.3*# ψ (-29.5%)

Group A – Control; Group B – Hyperoxaluric; Group C – Treated with Cystone (750 mg/kg body wt.); Group D – Treated with Plant extract (500 mg/kg body wt.); Group E – Treated with Plant extract (1000 mg/kg body wt.)

Values in brackets are percentage increase (+) or percentage decrease (-) compared to control (Group A).

[\**P*<0.05, \*\**P*<0.01 - Indicate significant change in comparison to control group (Group A); #*P*<0.05, ##*P*<0.01 - Indicate significant change in comparison to hyperoxaluric group (Group B); ψ *P*<0.05, ψ ψ *P*<0.01 - Indicate significant change in comparison to positive control groups (Group C)]

Table 2—Alterations in the level of urinary enzymes (LDH and ALP), serum urea and serum creatinine in various test groups in curative regimen (CR)

Parameters	Group A	Group B	Group C	Group D	Group E
Urinary LDH (units/ml/mg prt)	11.15 + 2.8	34.89 + 5.6** (212.9%)	14.1 + 3.4### (26.5%)	27.28 + 6.4*# ψ (144.7%)	19.75 + 4.6*# ψ (77.1%)
Urinary ALP (IU/L)	43.23 + 9.4	105.42 + 15.4** (143.9%)	51.48 + 8.4### (19.1%)	89.56 + 11.6*# ψ (107.2%)	65.78 + 11.7*# ψ (52.2%)
Serum urea (mg/dl)	198.54 + 13.7	355.23 + 15.4** (78.9%)	230.19 + 11.8*### (15.9%)	340.87 + 15.4** ψ (71.7%)	280.14 + 19.5*# ψ (41.1%)
Serum creatinine (mg/dl)	0.478 + 0.07	0.945 + 0.04** (97.7%)	0.569 + 0.06*### (19%)	0.845 + 0.03* ψ (76.8%)	0.678 + 0.04*# ψ (41.8%)
CrCl (ml/min)	5.05 + 0.5	1.98 + 0.6** (-60.8%)	4.95 + 0.4### (-1.9%)	2.75 + 0.9*# ψ (-45.5%)	3.45 + 0.5*# ψ (-31.7%)

Group A – Control; Group B – Hyperoxaluric; Group C – Treated with Cystone (750 mg/kg body wt.); Group D – Treated with Plant extract (500 mg/kg body wt.); Group E – Treated with Plant extract (1000 mg/kg body wt.)

Values in brackets are percentage increase (+) or percentage decrease (-) compared to control (Group A).

[\**P*<0.05, \*\**P*<0.01 - Indicate significant change in comparison to control group (Group A); #*P*<0.05, ##*P*<0.01 - Indicate significant change in comparison to hyperoxaluric group (Group B); ψ *P*<0.05, ψ ψ *P*<0.01 - Indicate significant change in comparison to positive control groups (Group C)]

observed with decrease in serum creatinine levels (83% in PR and 42% in CR) on supplementation with aqueous *A. aspera* (1000 mg/kg body wt.; Tables 1, 2).

The normal creatinine clearance (CrCl) was found to be close to 5 mL/min in control group rats. However, in EG + NH<sub>4</sub>Cl exposed rats, there was a significant decrease ( $P < 0.01$ ) in CrCl. Increase in CrCl was observed in a dose dependent manner on

treatment with aqueous *A. aspera* in both PR and CR as compared to control group rats (Tables 1, 2).

*Evaluation of severity of renal damage and crystals in urine*—Analysis of haematoxylin and eosin stained kidney section also supported the serum and urine biochemistry results showing normal structure and architectural intactness without any apparent damages in control group rats (Figs 1, 2). On histopathological

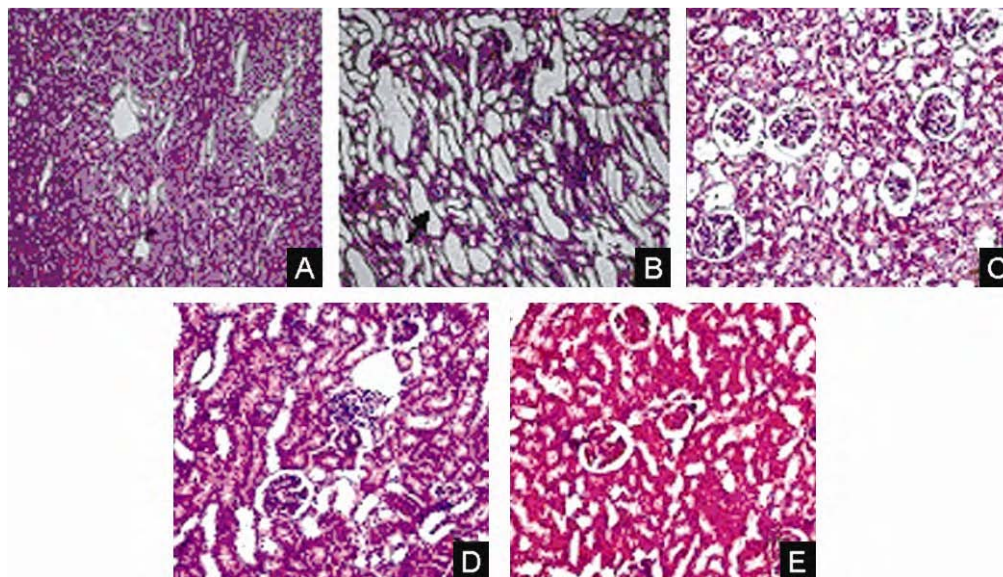


Fig. 1—Photomicrographs of kidney tissue sections of rats under prophylactic regimen (PR). [(A):-control rats showing normal renal architecture; (B):-ethylene glycol induced urolithiatic rats showing dilatation of proximal tubules; (C):-cystone treated (750 mg/kg body wt.); (D) and (E):-treated with different concentration of aqueous root extract of *A. aspera* (500 mg/kg body wt. and 1000 mg/kg body wt. respectively); Magnification: 100×]

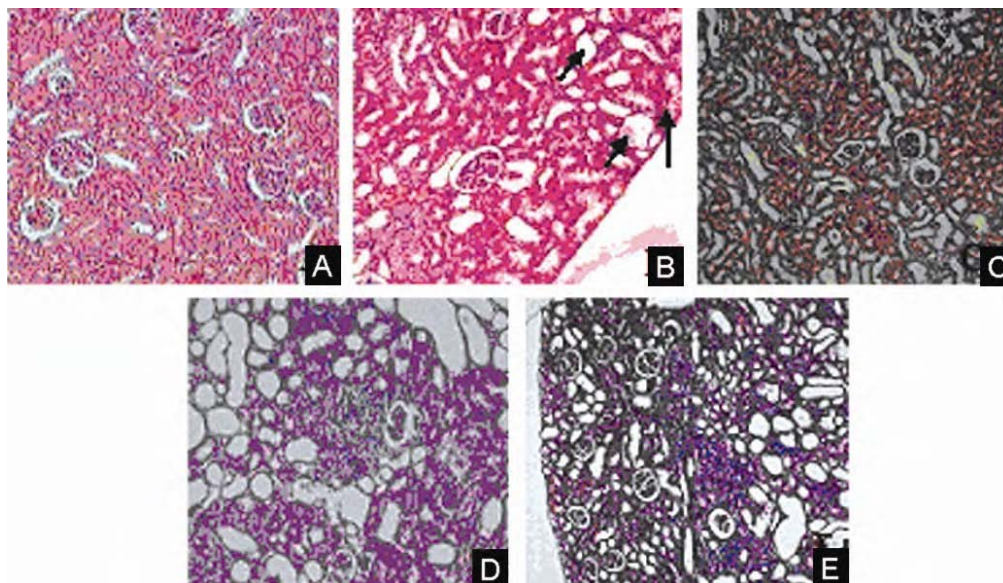


Fig. 2—Photomicrographs of kidney tissue sections of rats under curative regimen (CR). [(A):-control rats showing normal renal architecture; (B):-ethylene glycol induced urolithiatic rats showing dilatation of proximal tubules, glomerular damage along with interstitial inflammation; (C):-cystone treated (750 mg/kg body wt.); (D) and (E):-treated with different concentration of aqueous root extract of *A. aspera* (500 mg/kg body wt. and 1000 mg/kg body wt. respectively); Magnification: 100×]

examination, EG induced urolithiatic groups showed the presence of polymorphic irregular CaOx crystals in the lumina of tubules along with interstitial infiltration. On administration of the aqueous extract of *A. aspera* roots to urolithiatic rats in both PR and CR, moderate and few crystals are observed along with mild edema and dilatation in tubules.

Polarisation light microscopic observation of the rat bladder urine revealed that urine of the control

group rats was more or less devoid of any crystal whereas, in EG + NH<sub>4</sub>Cl exposed rats, the urine samples revealed presence of abundant crystals of large sizes. In the extract treated groups, a drastic decrease in size of urinary crystals was observed along with a decrease in number of crystals too. In curative regimen too, a similar pattern was observed with decrease in number and size of crystals on supplementation with aqueous *A. aspera* in a dose dependent manner (Figs 3, 4).

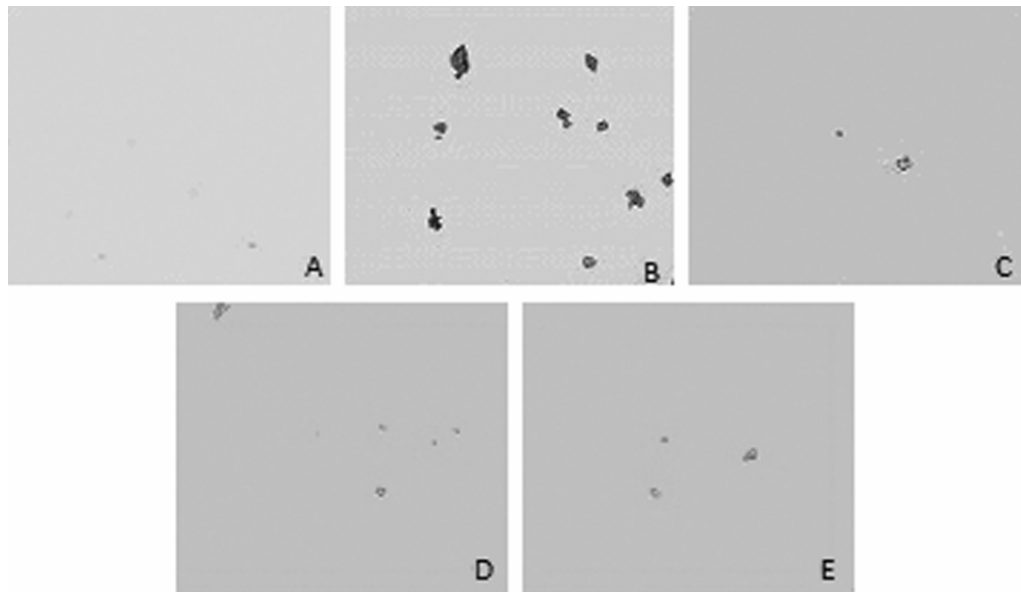


Fig. 3—Micrograph of crystalluria of rat's bladder urine under prophylactic regimen (PR). [(A):-control rats devoid of any crystals; (B):-EG induced urolithiatic rats showing large number of crystals; (C):-cystine treated (750 mg/kg body wt.); (D) and (E):- treated with different concentration of aqueous root extract of *A. aspera* (500 mg/kg body wt. and 1000 mg/kg body wt. respectively); Magnification: 100×]

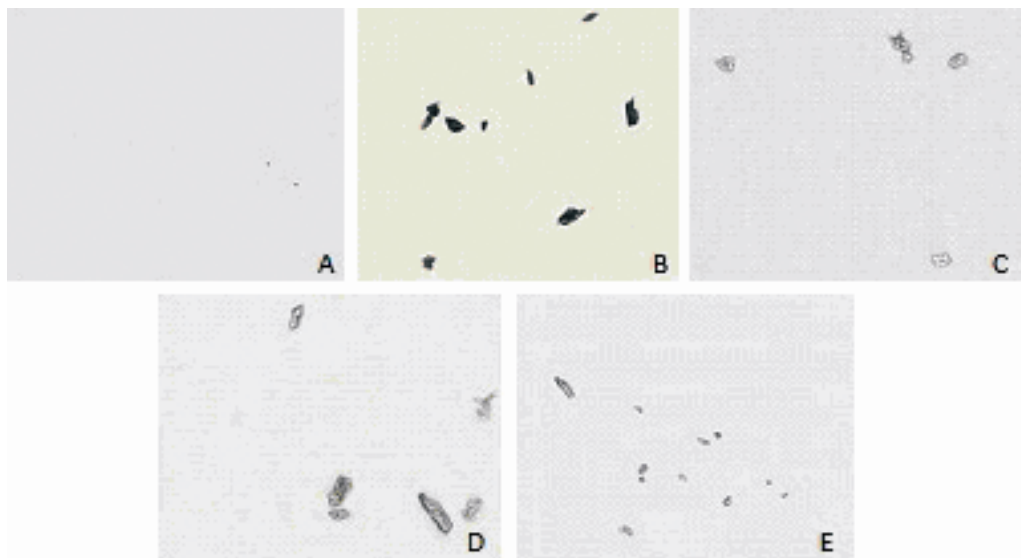


Fig. 4—Micrograph of crystalluria of rat's bladder urine under curative regimen (CR). [(A):- control rats devoid of any crystal; (B):-EG induced urolithiatic rats showing large number of crystals; (C):-cystine treated (750 mg/kg body wt.); (D) and (E):-treated with different concentration of aqueous root extract of *A. aspera* (500 mg/kg body wt. and 1000 mg/kg body wt. respectively); Magnification: 100×]



## Discussion

Despite being an active component in various marketed herbal formulations to treat urolithiasis like Cystone (Himalaya Herbal Healthcare), Neeri (AIMIL Pharmaceuticals) and Uriflow (BIONEUTRIX Labs), *Achyranthes aspera*, a widely distributed plant, is less explored for its antiurolithiatic potency. The aqueous extract of *A. aspera* was found to be effective in inhibiting CaOx nucleation and growth *in vitro* and also exhibited reduction in oxalate induced injury on renal epithelial cells, NRK 52E<sup>31</sup>. Hence, *Achyranthes aspera* root extract was studied to evaluate its potential to prevent CaOx urolithiasis *in vivo* in prophylactic and curative regimen. In the present study, male rats were selected to induce urolithiasis by induction of ethylene glycol because the urinary system of male rats resembles that of humans<sup>32</sup> and also earlier studies have shown that amount of stone deposition in female rats was significantly less<sup>33</sup>. Ethylene glycol disturbs oxalate metabolism by way of increasing the substrate availability thus, leading to hyperoxaluria while ammonium chloride accelerate lithiasis through urinary acidification<sup>32,10</sup>. Intraperitoneal administration of the extract was used as it may lead to adequate absorption of active compounds of the plants<sup>34,9</sup>.

A positive correlation between urinary oxalate levels and renal tubular epithelial cell injury has been discovered in experimental urolithiasis rats<sup>35</sup> and patients with kidney stones<sup>36</sup>. Several studies have revealed that injury to renal epithelium is associated with deposition of crystals in renal tubules<sup>37</sup>. Due to renal injury, enzymes like LDH and ALP are released in urine<sup>38</sup>. Consistent with previous reports, there was an increase in activity of both LDH and ALP in calculi induced rats, which was normalised following treatment of *Achyranthes aspera* in prophylactic and curative regimen in a dose dependent manner.

Lithogenic treatment impaired glomerular filtration rate (GFR), as evident from increased serum urea and serum creatinine due to obstruction of the outflow of urine by stones in the urinary system<sup>39</sup>. Similar to previous reports, the EG + NH<sub>4</sub>Cl exposed rats showed significant increase in serum urea and creatinine which was decreased on treatment with aqueous extract of *A. aspera*, supporting its antiurolithiatic activity. Clinically, creatinine clearance (CrCl) is a useful measure for determining renal functioning<sup>40</sup>. Renal dysfunction diminishes the ability to filter creatinine and increases serum creatinine levels, thus decreasing CrCl. The impairment of renal functioning after

exposure to EG and NH<sub>4</sub>Cl is an outcome of CaOx crystals deposition in renal tissue which was restored by plant doses in both the regimens.

The histopathological studies showed renal tubular damage consisting of tubular obstruction due to crystal formation, dilatation and interstitial inflammation which corroborates the results of other studies reporting that crystal deposition majorly occurs in tubules<sup>41</sup>. Several studies have shown that, crystal formation results in cell damage and cell detachment from the basement membrane and the released degradation products further promote nucleation of crystals<sup>42</sup>. Decreased renal injury further decreases sites for calcium oxalate deposition. Administration of aqueous extract of *A. aspera* to EG and NH<sub>4</sub>Cl exposed rats, prevents supersaturation of CaOx and thus decreased their deposition in renal tubules.

EG administration resulted in the development of persistent crystalluria in all rats showing abundant and larger crystals in untreated animals as compared to treated rats. Crystalluria can occur in both healthy and stone forming subjects though, agglomeration of particles is considered to be a crucial step in urinary stone formation because agglomerates tend to retain in kidney by getting trapped in renal tubules and develop into renal stones<sup>10</sup>. Based on all modifications observed in crystalluria after the treatment of urolithiatic rats by *A. aspera* extract, two effects of this plant can be attributed. First, the extract induced more crystals in urine thereby it reduced supersaturation which is an initial and prerequisite step for lithogenesis. Second, the size of crystals was reduced and thus can simply be swept by urine flow out off the kidneys.

In conclusion, the presented data indicated that administration of aqueous extract of *A. aspera* roots to rats prevented urolithiasis induced with ethylene glycol and reduced the growth of calcium oxalate stones. The extract was effective in reducing the renal tissue injury, decreasing the crystal size and thus, facilitating easy expulsion and restoring normal kidney architecture. Further, experimental and clinical studies are required to elucidate the chemical constituents of the extract and mechanism(s) responsible for the pharmacological activities.

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