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Role of *Tridax procumbens* Linn. in the management of experimentally induced urinary calculi and oxidative stress in rats

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Renal calculi formation is one of the common urological disorders. Hyperoxaluria and hypercalciuria are reported to be major risk factors for renal stone formation. Studies show that exposure to oxalate is toxic to renal epithelial cells and results in oxidative stress. In the present study, ethanolic extract of whole plant of *Tridax procumbens* Linn. was evaluated for antiurolithiatic activity against 0.75% v/v ethylene glycol and 2% w/v ammonium chloride induced calcium oxalate renal stones and also for antioxidant activity against hyperoxaluria promoted oxidative stress in male albino rats. In the control rats, ethylene glycol and ammonium chloride administration resulted in increased urinary calcium, oxalate and creatinine and renal deposition of calcium and oxalate. Significant increase in oxidative stress was also observed. Histopathology of kidneys revealed many calcium oxalate crystal depositions and extensive renal tubular damage. In the test rats, treatment with ethanolic extract of *T. procumbens* decreased the elevated levels of urinary calcium, oxalate and creatinine and significantly lowered renal deposition of calcium and oxalate. Substantial reduction in oxidative stress was also noticed. Renal histology showed considerable reduction in calcium oxalate crystal depositions. Our study results suggest that *T. procumbens* is endowed with antiurolithiatic and antioxidant activities.

Keywords: Ammonium chloride, Antioxidant, Antiurolithiatic, Ethylene glycol, Oxidative stress, *Patharchatti*, *Tridax procumbens*, Urolithiasis.

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Introduction

In humans, calcium oxalate (CaOx) stones are the most common type of urinary stones. Calcium stone patients excrete significantly more calcium and oxalate compared to normal subjects[1]. Studies show that renal cellular exposure to oxalate and/or CaOx crystals leads to the production of reactive oxygen species (ROS) and development of oxidative stress (OS) thus plays an important role in CaOx stone formation[2].

Surgical removal of stone is the general practice, but it is painful and recurrence is more. Alternatively, urinary stones can be treated with drugs but many of them produce metabolic adverse effects that limit their long-term use. Hence, it is vital to explore herbs for safe and effective treatment of urinary calculi. In the Ayurvedic system of medicine, group of plants with antiurolithiatic and diuretic property are referred to as *Pashanabhedhas*[3]. *Tridax procumbens* Linn. (Family: Asteraceae) is one among them. The plant is also known as *Patharchatti*, which means sucking and dissolving rocks and stones. Oral administration of the plant juice is claimed to remove stones in urinary bladder and kidney[4]. The leaves were reported to possess wound healing activity[5] and the plant possesses antidiabetic[6] and immunomodulator activities[7]. Literature survey revealed that antiurolithiatic studies were not conducted on the plant therefore, the present study was focused on the evaluation of the whole plant of *T. procumbens* for antiurolithiatic activity.

Materials and Methods

Chemicals

Urinary creatinine commercial kits from Med Source Ozone Biomedicals Private Limited, Haryana. DPPH and DTNB were purchased from Sigma (USA), TCA and Sulfosalicylic acid from Merck (India), TBA and all other chemicals used in the study from Sd fine chemicals or Merck (India).

Plant material

Whole plants of *T. procumbens* (Plate 1) were collected from Sri Padmavati Mahila Visvavidyalayam campus during March-April months. The plant was

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authenticated by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati. The voucher specimen (No. IPT/T-02) was preserved at the Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati.

Preparation of the extract

The whole plants were washed thoroughly; shade dried and then pulverized coarsely. Ethanol was selected for extraction, being suitable solvent to extract both polar and non polar chemical constituents from the plant. The ethanolic extract of *T. procumbens* (ETP) was prepared using ethanol (95%) by cold maceration (12 h) followed by refluxing over a water bath for 4 h at 70°C. The extract was concentrated under reduced pressure and was refrigerated. A 10% w/v extract was prepared by suspending the extract in water daily before administration to the rats. The extract was subjected to preliminary phytochemical screening.

Experimental animals

In the present study, Wistar strain male albino rats weighing 150-200 g were used. The rats were housed in polypropylene cages under hygienic conditions and maintained on standard pellet diet (Gold Mohur, Bangalore) and water *ad libitum*. Animal ethical norms were followed during all experimental procedures and the study was approved by Institutional Animal Ethical Committee (CPCSEA No: 1016/a/06/ CPCSEA /002/2007).

Plate 1—*Tridax procumbens* plant

Acute toxicity studies

The extract was subjected to acute toxicity studies as per OECD Guidelines 420. The rats were divided into five groups containing five animals in each group and were fasted overnight with free access to drinking water. Group 1 animals served as normal and received distilled water (10 ml/kg orally). Group 2 to 5 animals received a single dose of 5, 50, 300 and 2000 mg/kg body weight of the ethanolic extract, respectively, orally by gastric intubation using a soft catheter. After administration of the extract, the animals were observed continuously for 4 h and then intermittently at one hour interval up to 24 h and daily up to 14 days to record the number of deaths.

Dose selection of ethanolic extract

In acute toxicity studies, the extract was found to be safe as no animal died up to 2000 mg/kg body weight orally. Thus, the lowest dose that produced maximum volume of urine and the next highest dose were selected for the antiurolithiatic study. The doses selected for the study were 0.5 and 1.0 g/kg, oral.

Induction of urinary stones

Calcium oxalate urinary stones were induced in rats by providing free access to drinking water containing 0.75% v/v ethylene glycol (EG) and 2% w/v ammonium chloride (AC) for 15 days[8]. Rats were divided into four groups consisting 6 per group and were put on treatment as indicated in study design (Table 1).

Assessment of antiurolithiatic activity

Rats were hydrated with 5 ml of distilled water orally, placed in separate metabolic cages and 24 h urine samples were collected from overnight fasted rats on day 30. The samples were centrifuged at 2,500 rpm at 30 ±2°C for 5 min. The supernatant was used to determine *p*H and quantitative estimation of calcium[9], oxalate[10] and creatinine[11].

Table 1—Study design

Group	Treatment	Treatment	Purpose
I	Normal	-	Control for groupII
II	Control	Free access to EG and AC in drinking water from day 1-15 and distilled water from day 16-30	(i) Control for group III & IV (ii) To study the effect of EG and AC on renal stone formation and to study the effect of vehicle on dissolution of preformed stones
III	ETP treated	Free access to EG and AC in drinking water from day 1-15 and ETP (0.5 g/kg, oral) from day 16-30	To study the effect of ETP in dissolving preformed urinary stones
IV	ETP treated	Free access to EG and AC in drinking water from day 1-15 and ETP (1.0 g/kg, oral) from day 16-30	To study the effect of ETP in dissolving preformed urinary stones

Rats were sacrificed by cervical decapitation on day 31. Kidneys were perfused with ice-cold saline and were carefully incised. One kidney from each rat was washed with ice-cold 0.15M KCl and weighed. Then the kidney was sliced into two equal portions and one portion was homogenized using 10% HCl. The homogenate was centrifuged at 2,500 rpm at 30±2°C for 3 min and the supernatant was used to estimate renal deposition of calcium[9] and oxalate[10].

Histopathological studies

The other half of the kidney was transferred to 10% neutral formalin solution. Kidney sections were stained with hematoxylin, eosin and were observed under phase contrast microscope (Leica DM 1000). The studies were carried out at GKS histopathology lab, Thummalagunta, Tirupati.

Assessment of oxidative stress

Second kidney from each rat was homogenized with chilled phosphate buffer (*p*H 7.4). The homogenate was centrifuged at 800 rpm by using REMI-C24 laboratory centrifuge at 4°C for 5 min to separate nuclear debris. Then the resultant supernatant was centrifuged at 10,000 rpm at 4°C for 20 min to get post mitochondrial supernatant[12] which was used to determine malondialdehyde content (MDA)[13], reduced glutathione (GSH)[14] and catalase (CAT)[15].

***In vitro* antioxidant studies**

For *in vitro* antioxidant studies, ascorbic acid was used as reference standard. The concentrations of ascorbic acid were 10, 20, 40, 60, 80, 100 (μg/ml) and that of extract were 100, 200, 400, 600, 800 and 1000 (μg/ml).

DPPH and Nitric oxide free radical scavenging activities

1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was used to determine *in vitro* free radical scavenging activity of the extract[16]. Sodium nitroprusside with Griess reagent was used to determine nitric oxide (NO) free radical scavenging activity of the extract[17]. IC_{50} values for the extract, towards DPPH free radicals and NO induced free radicals were determined.

Statistical analysis

The results were expressed as mean ± SEM. The inter-group variation was measured by one way analysis of variance followed by Scheffe's test for multiple comparisons and statistical significance was considered at $P \leq 0.01$.

Results

On phytochemical screening, ETP was found to contain flavonoids and saponins. In acute toxicity studies, the ethanolic extract was found to be safe as no animal died up to 2000 mg/kg body weight orally. In control rats, on EG and AC ingestion for 15 days, a significant increase in urinary calcium, oxalate, creatinine (Fig. 1) and renal deposition of calcium and oxalate (Fig. 2) were noticed. A significant increase in kidney weight (Fig. 3) and decrease in urine *p*H

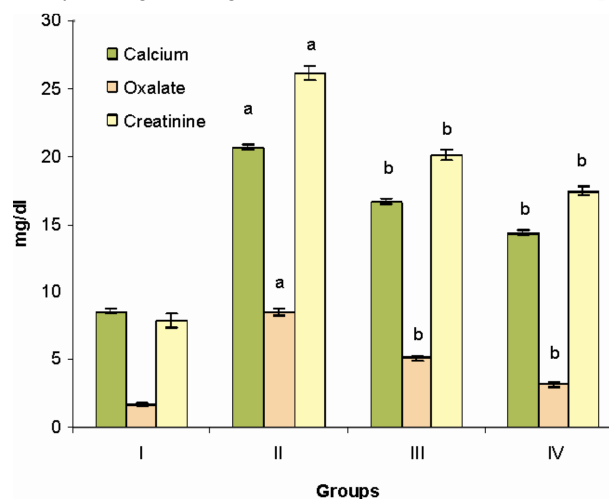

Fig. 1—Effect of ETP on urinary excretion of calcium, oxalate and creatinine

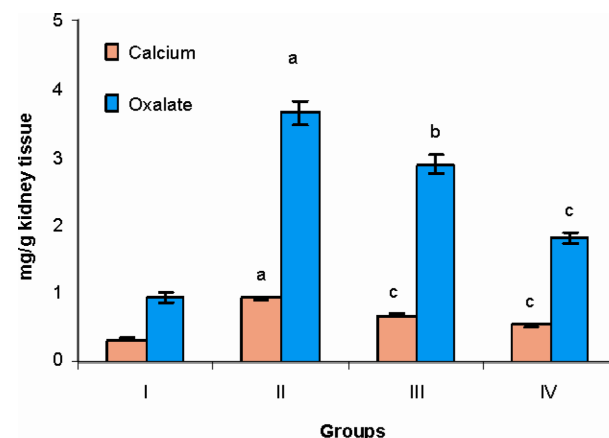

Fig. 2—Effect of ETP on renal deposition of calcium and oxalate

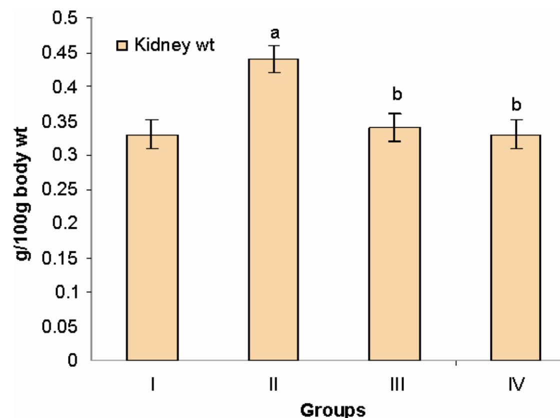

Fig. 3—Effect of ETP on kidney weight

Table 2 — Effect of ETP on *in vivo* lipid peroxidation and antioxidant parameters

Group	Treatment	MDA*	GSH*	Catalase**
I	Normal	67.00 ± 4.28	74.99 ± 4.14	365.70 ± 14.15
II	Control	93.20 ± 4.03[a]	26.95 ± 2.57[a]	109.8 ± 2.66[a]
III	ETP 0.5g/kg	54.39 ± 1.50[b]	58.33 ± 3.98[b]	180.40 ± 5.75[b]
IV	ETP 1.0g/kg	40.72 ± 1.88[b]	90.68 ± 4.32[b]	219.9 ± 7.02[b]

*(nM/mg tissue) **µM of H_2O_2 decomposed/ min/ mg tissue
a-*P*<0.001 control group compared to normal; b-*P*<0.001 treated groups compared to control

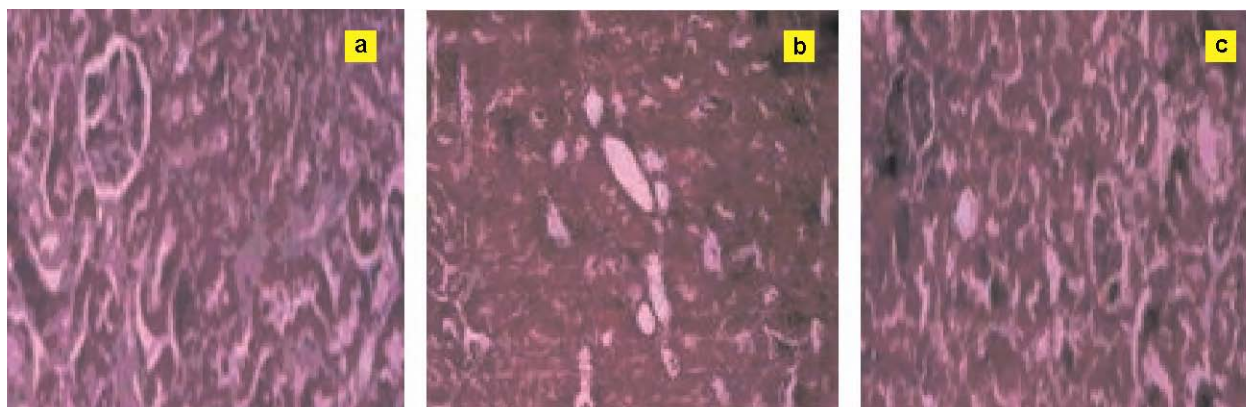

Plate 2 (a-c) — a- Section of normal rat kidney showing normal tubular epithelial cells and glomeruli (40x), b- Section of control rat kidney showing many CaOx crystal depositions and atrophy of glomeruli (40x), c-Section of ETP (1g/kg) treated rat kidney showing regenerative changes and a few CaOx crystals (40x)

(from 7.0-7.5 to 5.5-6.0) were observed. A significant raise in renal MDA, drop in GSH and Catalase levels were observed (Table 2). Histopathological studies of the kidney sections (Plate 2) revealed the presence of tubular congestion, inflammation of tubules and glomerular atrophy accompanied by intra-tubular and interstitial CaOx crystal depositions (Plate 2b).

On ETP administration, in group III (0.5 g/kg, oral) and group IV (1.0 g/kg, oral) rats, from day 16-30, a significant reduction in urinary excretion (Fig. 1) and renal deposition of calcium and oxalate (Fig. 2) were observed. ETP administration resulted in decreased urinary creatinine (Fig. 1) and kidney weight (Fig. 3) and restored urinary *p*H (6.5-7.5). A significant decrease in kidney MDA content and increase in GSH and catalase levels were observed (Table 2). In histopathological studies, a few CaOx crystal depositions associated with minimum renal damage and regenerative changes in renal tubules and glomeruli were noticed in the kidney sections (Plate 2c). In rats that received 1 g/kg body weight of the extract, the decrease in urinary excretion and renal deposition of calcium and oxalate were more. The reduction in urinary creatinine and kidney weight were also more apparent. Further, in these rats, the increase in renal GSH and Catalase levels were more and lipid peroxidation was less compared to the rats that received 0.5 g/kg body weight of the extract. ETP exhibited a concentration dependent DPPH free radical scavenging (IC_{50}-742.25 µg/ml compared to IC_{50} of ascorbic acid 58.45µg/ml) and NO induced free radical scavenging (IC_{50}-935.17 µg/ml compared to ascorbic acid (IC_{50} 77.04 µg/ml) activities.

Discussion

Oxalate is normally excreted in urine unchanged. But, hyperoxaluria promotes calcium oxalate renal calculi formation[18], as urinary oxalic acid has the tendency to complex with calcium and form insoluble CaOx crystals in the kidney[19]. Therefore, conditions that promote oxalate absorption from food or endogenous oxalate production can cause CaOx stone formation[20].

It was reported that EG when ingested, is converted to oxalic acid by the liver enzyme glycolate oxidase[21] and AC increases urinary acidification[22] thereby favour CaOx depositions in the kidney. In the present study, in the control

group, enhanced urinary excretion and renal deposition of calcium and oxalate shows that EG and AC ingestion induced hyperoxaluria and promoted CaOx stone formation. The lithogenic effects of EG and AC administration were also evident through increased kidney weight.

Elevated urinary creatinine levels are indicators of renal impairment. In the control group, the significant increase in 24 h urinary creatinine indicates that hyperoxaluria promoted renal impairment which might have led to decreased urine output and subsequent supersaturation of lithogenic promoters. Presence of CaOx crystal depositions and degenerative changes in kidney sections substantiated hyperoxaluria promoted renal impairment and stone formation. Urinary *p*H influences crystaluria. Thus by changing urinary *p*H, dissolution of calculi can be attained. *p*H of 5.0-6.5 promotes mostly CaOx type of stone formation[23]. In the present study, in the control group, the decrease in urine *p*H from 7.0-7.5 to 5.5-6.0 supports the formation of CaOx type of stones.

Mucoproteins have significant affinity for CaOx crystal surface and promote the growth of crystals and cement them[24]. Saponins prevent calcium and oxalate deposition and excretion by disintegrating mucoproteins[25]. In the present study, the considerable decrease in elevated urinary calcium, oxalate, creatinine and renal deposition of calcium and oxalate indicates alleviation of hyperoxaluria induced renal damage by the extract. Restoration of urinary *p*H (6.5-7.5) supports the dissolution of preformed CaOx crystals. Observation of limited CaOx crystal depositions associated with minimum renal damage and regenerative changes in renal tubules and glomeruli indicates that the extract favoured the dissolution of preformed CaOx crystals and reconstitution of renal histology. The decrease in kidney weight also substantiated these results. When compared to group III rats, the more decrease in urinary and renal parameters in group IV rats, with increased dose of the extract indicating that the extract possesses dose dependent curative effect on calcium oxalate renal calculi.

Oxidative stress mediates the pathogenesis of kidney stone disease[26]. Exposure to oxalate has been shown to be toxic to renal epithelial cells and results in free radical mediated lipid peroxidation (LPO)[27]. MDA is reported to be one of the most common indicators of ROS induced oxidative stress[2].

In our study, in the control group, enhanced MDA levels reflect that the hyperoxaluria promoted extensive generation of ROS. A marked decline in the levels of antioxidants GSH and catalase indicate impaired antioxidant protection. This oxidative stress may have favoured the accumulation and retention of oxalate and subsequent deposition of CaOx crystals.

Studies show that oxalate-induced peroxidative renal injury is involved in the nucleation aggregation and deposition of CaOx stones[28]. Therefore, treatments that favour the scavenging of and/or preventing the generation of ROS attenuate or prevent the OS and thereby mitigate the subsequent renal damage. Studies using rats showed that treatment with antioxidants reduced EG induced oxalate/CaOx crystal promoted OS and prevented CaOx precipitation in the kidney[29]. In the present study, in treated groups, the significant decrease in kidney MDA content reflects marked suppression of LPO. The significant increase in the levels of the antioxidants GSH and catalase indicate that the extract attenuated hyperoxaluria induced LPO and alleviated OS. When compared to group III rats, the more decrease in lipid peroxidation and increase in GSH and Catalase in group IV rats kidneys indicating that the extract possesses dose dependent antioxidant effect. The concentration dependent DPPH free radical scavenging and NO induced free radical scavenging activities of the extract also support the dose dependent antioxidant activity of the extract.

Flavonoids[30] exhibit antioxidant activity by quenching ROS and also by chelating metal ions like iron and copper[31]. Earlier researchers isolated lupeol and a flavonoid, procumbenetin from the plant[32,33]. In the present study, the decrease in renal deposition of calcium and oxalate in treated groups may be attributed to the presence of saponins and flavonoids in the extract as ETP was found to contain saponins and flavonoids in preliminary phytochemical study. The i*n vitro* DPPH and NO free radical scavenging activities of ETP also revealed that the extract is rich in antioxidants. Saponins may have favoured the dissolution of EG and AC promoted CaOx stones by disintegrating mucoproteins. Flavonoids may have decreased hyperoxaluria promoted oxidative stress through minimizing ROS by free radical scavenging and preventing their further generation by metal chelation.

Conclusion

The results indicate that *T. procumbens* (ETP) is effective in dissolving EG and AC induced preformed calcium oxalate renal calculi in rats. The curative effect of ETP on calcium oxalate stones may be credited to its saponin and flavonoid principles.

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