Antiurolithiatic activity of \textit{trans}-cinnamic acid against ethylene glycol induced renal calculi in rats

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Urolithiasis is a complex process characterized by supersaturation and retention of salts within the kidney and also a debilitating problem worldwide. Here, we have investigated antiurolithiatic effect of \textit{trans}-cinnamic acid (\textit{t}-CA) against ethylene glycol (EG) induced urolithiasis in rats. Urolithiasis was induced in Wistar albino rats using 0.75\% v/v EG in drinking water for 28 days. \textit{t}-CA was administered @200 and 400 mg/kg along with EG for 28 days. Biochemical, urine and histopathological analysis were performed to observe the calcium oxalate (\textit{CaC}_2\textit{O}_4) deposits and renal tissue damage. The EG group showed significant rise in urine oxalate, calcium, phosphate, and renal tissues oxalates, as compared to normal group. Serum creatinine and uric acid levels were also increased significantly in EG-treated group. Histopathological studies showed marked renal tissue damage and the presence of \textit{CaC}_2\textit{O}_4 crystals. Further, treatment of \textit{t}-CA significantly ameliorated oxalate, calcium, magnesium, phosphate (urine) and creatinine, uric acid (serum) in EG-induced urolithiasis after 28 days. Moreover, \textit{t}-CA-treated groups showed reversal of renal tissue damage and reduced level of \textit{CaC}_2\textit{O}_4. Interestingly, \textit{t}-CA @400 mg/kg, was more effective in preventing the urolithiasis and regeneration of renal tissues in rats.

\textbf{Keywords:} Antioxidant, Calcium oxalate, Diuretic, Kidney stone, Nephrolithiasis, Urinary oxalate, Uric acid, Urolithiasis

Urolithiasis, also known as kidney or renal stone, is a multi-factorial disorder and highly prevalent clinical problem that accounts for 12\% of the human population worldwide at some stage in their lifetime. Generally, it happens both in men and women, but the risk is high in men than women within the age of 20-49 years. In both developed and developing countries, the prevalence of urolithiasis has been increased in the past decades\textsuperscript{1}. It has been estimated that the overall probability of forming stones differ in various parts of the world and is estimated at 1-5\% in Asia, 5-9\% in Europe, 13\% in North America and the recurrence rate of renal stones is about 75\% in 20 years span\textsuperscript{2}. Approximately, 12\% of the Indian population is anticipated to have urinary stones and from the total, 50\% may conclude with a loss of kidney functions\textsuperscript{1}.

There are several options available in the management of ureteral stones. Treatment selection depends on stone size, location and composition, equipment available, physician skill, patient health and preference and costs. The surgical operation, lithotripsy and local calculus disruption using high-power laser are widely used to remove the calculi\textsuperscript{3,4}. However, these techniques are highly costly and with these procedures, recurrence is quite common. Moreover, surgical interventions may cause undesirable side effects such as tubular necrosis, hypertension, hemorrhage and subsequent fibrosis of the kidney leading to cell injury and recurrence of renal stone formation\textsuperscript{5,6}.

The animal models have played an important role in basic, etiological studies of stone diseases and the identification of herbal medication and other effective substances. \textit{CaC}_2\textit{O}_4 urolithiasis models have commonly been used to investigate the influence of urolithiasis on experimental rats\textsuperscript{7,8}. In recent years, interest is resurgent for the development and utilization of herbal drugs and traditional medicines for the benefit of the world population in terms of cost-effectiveness and low side effect of these drugs. The plant products are reported to be effective in decreasing the recurrence rate of renal calculi with no side effects. Since ancient times, herbal preparations have been used in urolithiasis therapy as medicine\textsuperscript{8,12}.

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t-CA is an organic acid occurring naturally in plants that has low toxicity and a broad spectrum of biological activities. t-CA derivatives, both isolated from plant materials and synthesized, showed diverse pharmacological activities such as antidiabetic, hepatoprotective, antibacterial, anti-inflammatory, antiviral and antifungal activity. Moreover, t-CA also exhibits antioxidant, antimalarial, and anticancer activities. Additionally, it is well documented that the plants which are a rich source of t-CA such as *Daucus carota* L., *Bergenia ligulate*, *Nigella sativa*, *Cynodon dactylon*, *Rotula aquatica* and *Raphanus sativus* have been proven to exhibit promising effects in the management of kidney stones. However, so far no scientific study has been reported concerning the antiurolithiatic activity of the t-CA. Hence, in the present study, we investigated the protective effect of the t-CA against EG-induced urolithiasis and its possible underlying mechanisms in experimental rats.

**Materials and Methods**

**Chemicals and apparatus**

TBA was purchased from Sigma-Aldrich Co., St. Louis, MO, USA. EG and t-CA were purchased from Molychem, Mumbai, India. Cystone tablets were purchased from Himalaya Drug Company, India. Calcium, uric acid, magnesium and creatinine kit were purchased from Anamol Diagnostics Ltd, Palghar, India. All other chemicals and reagents used were of analytical grade and procured from approved chemical suppliers. Equipment such as the metabolic cages (Tecnip-last, Italy), auto-analyzer (Merck, Mumbai, India), centrifuge and homogenizer (Remi Industries, Mumbai, India), UV-spectrometer (Shimadzu Scientific Instruments, UV-1800, Mumbai, India) were used for analysis in the present study.

**Animals**

Male Wistar albino rats (150-300 g; eight weeks) were used for the present study. The animals were housed five rats per cage under well-controlled conditions of temperature (25±1°C), humidity (55±5%) and 12 h light-dark cycle (lights on 07:00-19:00 h) in the central animal facility of Department of Pharmaceutical Sciences, Saurashtra University, Rajkot, Gujarat, India. Animals had free access to standard rat chow (Amrut, Pranav Agro Ind. Ltd., Vadodara, India) and water *ad libitum*. All the experimental protocols were approved (Protocol approval no IAEC/DPS/SU/1231) by the Institutional Animal Ethics Committee (IAEC), Department of Pharmaceutical Science, Saurashtra University, Rajkot, Gujarat, India. The experimental procedures were performed as per the guidelines laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Ministry of Environment Forest and Climate Change, Government of India, New Delhi. Six rats per cage were allowed to acclimatize for one week before the experiments commenced.

**Experimental design**

Fig. 1 depicts a schematic representation of the experimental design and treatment timeline. Briefly, EG-induced urolithiasis model was employed for the present study. Forty-two animals were randomly divided into seven groups (n = 6). The treatment groups were as follows: Group I (Vehicle control) received 1% CMC in drinking water for 28 days. Group II (Lithiatic control) received 0.75% v/v EG in drinking water for 28 days. Group III (Cystone) received cystone (750 mg/kg) from day 1 to 28. Groups IV and V (t-CA preventive) received t-CA at the doses of 200 and 400 mg/kg, respectively from day 1 to 28. Groups VI and VII (t-CA curative) received t-CA at doses of 200 and 400 mg/kg, respectively from day 15 to 28 days of calculi induction. Treatment groups III-VII received calculi inducing treatment for 28 days. All the treatment drugs and calculi inducing agent were suspended in 1% CMC and given once daily by oral route.

**Collection and analysis of urine**

Animals were kept in individual metabolic cages and 24 h urine samples were collected on 0, 7, 14, 21

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**Fig. 1** —Experimental design for urolithiasis study
and 28th day of calculi induction treatment. The volume and calcium content of urine were estimated. Calcium in urine was estimated using calcium diagnostic kit in a semi-autoanalyzer. Urine samples were also analysed for oxalate, magnesium, phosphate, and uric acid. Urine crystals were observed under light electron microscope at 40X.

Serum analysis
The blood was collected from the retro-orbital sinus plexus under the anaesthetic condition and serum was separated by centrifugation at 10000xg for 10 min and analysed for creatinine and uric acid. The creatinine and uric acid diagnostic kits were used to estimate serum creatinine and uric acid levels, respectively.

Preparation and analysis of tissue homogenate
Animals were sacrificed under deep anaesthesia and both kidneys were immediately removed. Subsequently, the kidneys were washed in cooled saline (0.9%), kept on ice and blotted on filter paper to remove excess saline. Immediately, the left kidney was finely minced and 20% homogenate was prepared in tris-HCl buffer (0.02 mol/L, pH 7.4). Kidney homogenate was used for assaying tissue calcium, oxalate, and LPO activity. Calcium in tissue homogenate was estimated using calcium diagnostic kit.

Histopathological evaluation
To confirm the incidences of lithiasis and its healing, isolated kidneys were subjected to histological study. After removal, the right kidney was cleaned off extraneous tissues and rinsed in ice-cold physiological saline and fixed in 10% v/v neutral buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 μM and stained with hematoxylin and eosin (H & E dye) for histological evaluation. The slides were examined under microscope (DM3000, Leic, Germany) for the evaluation of kidney architecture and CaC2O4 deposits.

Microscopy of urinary crystal
A drop of urine sample obtained was spread on a glass slide and visualized under polarized light using Leica DM3000 (Leica, Wetzlar, Germany) light microscope.

Statistical analysis
The results were expressed as mean ± standard error mean (SEM). The statistical significance was assessed using a one-way analysis of variance (ANOVA) followed by Dunnett’s comparison test and P < 0.05 was considered as statistically significant.

Results
Effect of t-CA on urinary output
The urinary output of the vehicle-treated group was found to be 7.53±0.53 mL/day/rat on the 28th day, which was significantly (P <0.001) decreased in the EG-treated group. t-CA (200 and 400 mg/kg, p.o.) treated groups significantly heightened (P <0.001) urine output as compared to the EG-treated group. Cystone (750 mg/kg) produced similar effects (Fig. 2).

Effect of t-CA on urinary oxalate, phosphate and uric acid
As shown in Fig. 3 (A-C), treatment of 0.75% v/v EG in drinking water to male rats for 28 days increased levels of oxalate, phosphate and uric acid concentration in the urine of lithiatic rats (Gr. II) showing hypercalciuria, hyperphosphaturia and hyperoxaluria, respectively, as compared to normal group. In contrast to these results, the treatment t-CA in preventive (Grs IV and V) and curative (Grs VI and VII) groups at the doses of 200 and 400 mg/kg, significantly (P <0.001) reduced the levels of oxalate, phosphate and uric acid in urine. A similar effect was observed in the rats treated with cystone (750 mg/kg).

Effect of t-CA on urinary calcium and magnesium
The results of different treatments on urinary calcium and magnesium levels are presented in Fig. 3 (D and E). As compared to control group, the excretion of calcium was significantly (P <0.001) increased whereas, excretion of magnesium was decreased gradually in lithiatic rats. Supplementation with t-CA (200 and 400 mg/kg) prevented these changes (P <0.001) and

![Fig. 2 — Effect of t-CA on urine output.](image-url)

Values are expressed as mean ± SEM, N = 6, ANOVA followed by Dunnett’s multiple comparison test, **P < 0.01 and ***P < 0.001. Comparisons are made against lithiatic control group]
restored it to near-normal values. The effect was well-comparable to cystone (750 mg/kg).

**Effect of t-CA on urinary pH**

Urine pH levels in different treatment groups shown in Fig. 3F. Urine pH analysis of EG-treated rats showed a significant ($P < 0.01$) decline in urine pH as compared to normal rats whereas, treatment with cystone (750 mg/kg) and t-CA (200 and 400 mg/kg) restored the pH values to normal.

**Effect of t-CA on serum and kidney homogenate analysis**

The renal function was assessed by measuring serum creatinine and uric acid in normal, lithiatic control and treated rats and the results are depicted in Fig. 4 A & B. The serum creatinine and uric acid levels were significantly ($P < 0.001$) elevated in lithiatic group because of obstruction of the urinary system due to renal papillary hypertrophy and crystal deposition causes a deterioration in renal function,
which results in the accumulation of uric acid in the blood (Gr. II) whereas, treatment with t-CA (200 and 400 mg/kg) significantly (P <0.001) reduced the levels of these markers. Interestingly, the effect of t-CA treatment (400 mg/kg) was well-comparable to the effect of standard drug cystone (750 mg/kg) on serum creatinine levels.

Furthermore, Fig. 4 (C-E) depicts the deposition of oxalate, a crystalline component, and % LPO in the renal tissue. Lithiatic group (Gr. II) showed increase incidences of deposition of the CaC₂O₄ crystals whereas, treatment with t-CA (200 and 400 mg/kg) significantly reduced the renal oxalate content and other stone forming constituents in both prophylactic and curative treatment groups. Renal stone induction was significantly aggravated (P <0.001) % LPO of kidney tissue in lithiatic rats, which was effectively prevented by simultaneous treatment with t-CA in the experimental animals.

**Effect of t-CA on histological evaluation of kidney**

In histopathological evaluation, we have found normal architecture of the kidney in the vehicle treatment group (Fig. 5). In contrast to this, extensive intratubular crystal depositions and degenerative tubular
structures were found in EG-treated rats. These pathological changes were reversed by cystone (750 mg/kg) and t-CA (200 and 400 mg/kg) treatments.

Effect of t-CA on urinary crystal

In the microscopic examination, after 28 days of EG-treatment, urine samples from Gr. I rats showed the absence of any crystal, whereas the presence of abundant CaC$_2$O$_4$ crystals (needle-shaped) and in many instances their aggregates were visible in the urine samples of Gr. II rats. In cystone (750 mg/kg)-treated group, a drastic decrease in several urinary crystals was observed. The CaC$_2$O$_4$ crystals observed in preventive groups were significantly smaller in size as compared to lithiatic-treated rats. Moreover, curative groups also exhibited less CaC$_2$O$_4$ crystals. Besides, certain CaC$_2$O$_4$ crystals were also found to be broken in a few instances (Fig. 6).

Discussion

The present study demonstrated antiurolithiatic effect of oral administration of t-CA on EG-induced urolithiasis in experimental rats. There were no signs of toxicity during the entire study duration i.e. four weeks of the treatment doses. Although calcium channel blockers, steroids, NSAIDs, and α$_1$ adrenergic receptor antagonists are the primary agents used in the therapeutic intervention and management of urolithiasis, these treatment regimens have numbers of side effects. Therefore, alternative treatments with phytoconstituents have become the mainstay of medical therapy.

Diverse animal models are used for induction of CaC$_2$O$_4$ crystals in experimental rats. The most commonly employed simplest method is to provide EG in drinking water. EG is readily absorbed along with the intestine, metabolized in the liver to oxalate causing hyperoxaluria, and precipitates in the urine as CaC$_2$O$_4$ as a result of its poor solubility. In particular, CaC$_2$O$_4$ in nephron leads to damage of epithelial cells, inducing heterogeneous crystal nucleation and causing aggregation of crystals. Male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans and earlier studies reported inhibitory effects of female sex hormones on kidney stone formation in experimental animals.

Urinary chemistry is one of the important factors in determining the type of crystal formed and the nature of macromolecules included on the surface of the crystals. Hence, the study of the urinary chemistry related to the calculi forming minerals may provide a good indication of the extent of stone formation. One previous study reported that urinary volume was decreased in EG-induced urolithic rats. The treatment with t-CA increased urine output in experimental rats and this may be due to the diuretic effect of t-CA which reduces the CaC$_2$O$_4$ supersaturation in the urine and thereby stone formation.

It is well-known that oxalate plays an important role in stone formation and has about a 15-fold greater effect than urinary calcium. In the present study, urinary oxalate levels were increased in EG-induced urolithic rats. A reduction in oxalate content in urine samples of t-CA treated rats was observed. This could be attributed to the inhibitory activity of t-CA treatment on oxalate formation.

Furthermore, urinary phosphorus excretion was increased in EG-induced urolithic rats. It has been
reported that increased urinary phosphorus excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces CaC$_2$O$_4$ deposition. Treatment of t-CA lowered the excretion of phosphorus and reduced the risk of stone formation. Moreover, urine samples from urolithic group showed increased urinary uric acid excretion. Uric acid interferes with CaC$_2$O$_4$ solubility and reduces the inhibitory activity of glycosaminoglycans on adherent crystals. Treatment with t-CA remarkably lowered the uric acid content in urine samples of t-CA treated rats.

It is well characterized that high urinary calcium concentration leads to an increase in urinary saturation of calcium salts and reduction in urinary inhibitory activity by the way of complex formation with negatively charged inhibitors such as citrate. The present study showed a marked increase in urinary calcium excretion and urinary calcium levels in urolithic rats. Treatment strategies aimed at reducing urinary calcium levels showed reduced stone recurrence rates. Interestingly, treatment with t-CA efficiently prevented lithogen-induced hypercalciuria thereby inhibiting calcium stone formation better than cystone.

In addition, urinary magnesium levels were diminished in EG-induced urolithic rats, whereas treatment with t-CA restored the magnesium levels in urine and thus, reduced the growth of CaC$_2$O$_4$ crystals. These results are in harmony with the previous studies which reported that the low magnesium levels may pose a threat of stone formation as the supersaturation of CaC$_2$O$_4$ is reduced by the magnesium complexes with oxalate and thereby reduces the growth and nucleation rate of CaC$_2$O$_4$ crystals.

Calcium and oxalate levels were markedly increased in the renal tissue of EG-treated rats due to the expression of intracellular CaC$_2$O$_4$ crystal. The t-CA treatment suppressed the increase in intracellular CaC$_2$O$_4$ level as clearly seen in the histopathological study. However, these elevations of calcium and oxalate were attenuated by treatment with the t-CA and, thereby decreased CaC$_2$O$_4$ crystal-induced cell-injury. t-CA significantly prevented all these effects of lithogenic treatment. Thus, confirming in vivo antiurolithic effect.

Moreover, renal function was assessed by measuring serum creatinine, and uric acid. The glomerular filtration rate (GFR) decreases due to the obstruction of the urine outflow by stones in urolithiasis. Therefore, the waste products particularly creatinine, and uric acid accumulate in the blood. In conclusion, the administration of EG caused nephrotoxicity which was characterized by marked elevation of serum creatinine and uric acid levels. Treatment with t-CA significantly decreased the nephrotoxicity indicated by improved GFR and thereby decreased the levels of serum creatinine, and uric acid. The elevated level of LPO causes renal damage by reacting with polyunsaturated fatty acids in the cell membrane of the kidney. The LPO levels were increased in the kidney homogenate of EG-treated rats. The t-CA treatment successfully prevented an increase in LPO in experimental rats confirming its antioxidant potential.

In the present study, we examined the effect of t-CA on urinary pH. It is reported that a decrease in urinary pH is associated with increased CaC$_2$O$_4$ production. Obtained data showed that urine pH was significantly decreased in EG-treated rats and thus, increased CaC$_2$O$_4$ production. These results are in harmony with previous studies. The treatment with t-CA increased the urinary pH leading to promote secondary nucleation of CaC$_2$O$_4$ by precipitation of calcium phosphate and confirmed its antiurolithic effect.

Histopathological examination of the kidney sections of EG-induced urolithic rats showed polymorphic irregular and needle-shaped crystal deposits inside the tubules causing tubular dilatation along with interstitial inflammation, renal tubular damage and cell necrosis. Treatment with t-CA restored these changes and also prevented the damages to the tubules. Several studies have reported that cell damage and cell detachment resulted from the basement membrane due to crystal formation and released degradation products which further promote nucleation of crystals. In the present study, a microscopic examination of urine exhibited CaC$_2$O$_4$ crystal deposition in EG-treated rats. Administration of t-CA to EG-exposed rats, prevented supersaturation of CaC$_2$O$_4$ as confirmed by fewer numbers of crystal deposits in renal tubules.

**Conclusion**

The present study, for the first time, shows the beneficial effect of t-CA in prevention as well as the management of urolithiasis in experimental rats. The present study showed that t-CA significantly augmented serum creatinine, uric acid as well as urine oxalate, calcium, magnesium, and phosphate in EG-induced urolithiasis after 28 days. Further, t-CA also protected renal tissue damage and reduced the level of CaC$_2$O$_4$. Our findings demonstrated antiurolithic effect conferred
by t-CA is mediated through a combination of CaC2O4 crystal inhibitory, diuretic, antioxidant, and hypermagnesemia activity. Future study should focus on the possible role of t-CA in treatment and prevention of urolithiasis using different animal models and clinical set up.

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Conflict of interest
Authors declare no conflict of interests.

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