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ORIGINAL RESEARCH

# Sub-Acute Toxicity Evaluation and Antiurolithiatic Activity of *Tribulus terrestris* Ethanol Extract in Rats

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**Background:** *Tribulus terrestris* (*T. terrestris*) is traditionally used in the management of urinary disorders; however, evidence regarding its safety upon repeated administration and its antiurolithiatic efficacy remains limited. This study aimed to evaluate both the sub-acute toxicity and antiurolithiatic potential of the ethanol extract of *T. terrestris* (EETT) in experimental rats.

**Methods:** Sub-acute toxicity was assessed in Wistar rats (n = 30; 5 males and 5 females per group) following 28-day repeated oral administration of EETT (500 and 1000 mg/kg). Antiurolithiatic activity was evaluated in an ethylene glycol/ammonium chloride-induced nephrolithiasis model (n = 36; 6 rats per group), where EETT (150, 300, and 450 mg/kg, intraperitoneally) was administered and compared with potassium citrate. Hematological, biochemical, and histopathological parameters were analyzed.

**Results:** EETT exhibited no mortality or treatment-related toxicity, with no significant alterations in body weight, relative organ weights, hematological indices, or serum biochemical parameters. Histological examination confirmed normal tissue architecture in treated animals. In the nephrolithiasis model, EETT significantly ($P < 0.05$) reduced elevated serum creatinine, urea, calcium, and potassium levels in a dose-dependent manner. Histopathological findings revealed decreased calcium oxalate crystal deposition and preservation of renal structure, particularly at higher doses, with effects comparable to potassium citrate.

**Conclusion:** The findings demonstrate that EETT possesses a favorable safety profile alongside significant antiurolithiatic activity. This dual evidence supports its potential as a therapeutic candidate for nephrolithiasis and warrants further pharmacological and clinical investigation.

**Plain Language Summary:** Kidney stones can cause severe pain and may affect long-term kidney health. Many people use herbal remedies such as *Tribulus terrestris*, but clear scientific evidence about its safety and effectiveness is still limited.

In this study, we tested an ethanol extract of this plant in rats to evaluate both safety and its ability to reduce kidney stone formation. Repeated use for 28 days did not produce harmful effects at the tested doses. In addition, the extract reduced stone formation and helped maintain normal kidney structure and function.

These findings suggest that *Tribulus terrestris* may be a potential candidate option for supporting kidney health. However, further studies in humans are required before it can be recommended for clinical use.

**Keywords:** *Tribulus terrestris*, sub-acute toxicity, nephrolithiasis, renal protection, rats

## Introduction

Kidney disorders represent a major global health burden and contribute substantially to morbidity and mortality worldwide. Acute kidney injury (AKI), chronic kidney disease (CKD), nephrolithiasis, and recurrent urinary tract infections share interconnected pathological mechanisms, including oxidative stress, inflammation, and progressive nephron loss.[1–3] Among these conditions, nephrolithiasis remains a significant clinical challenge due to its high prevalence, frequent recurrence, and association with progressive renal impairment, despite advances in preventive and therapeutic strategies.[2–5]

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Although current pharmacological interventions have improved the management of kidney stone disease, many agents are associated with adverse effects, particularly with long-term use, which can limit adherence and clinical applicability.[6,7] In addition, patients often require repeated interventional procedures, such as extracorporeal shock wave lithotripsy, ureteroscopy, or percutaneous nephrolithotomy, followed by prolonged preventive therapy. Consequently, there is increasing interest in identifying complementary and alternative agents that may provide effective renal protection with an improved safety profile.[8,9] Importantly, natural products should not be assumed to be inherently safe without rigorous toxicological evaluation.[10,11]

Available toxicological data on *Tribulus terrestris (T. terrestris)* suggest a generally favorable safety profile. Acute exposure studies have reported no mortality at relatively high doses, indicating a broad margin of safety.[12] Likewise, limited sub-acute investigations have not demonstrated significant alterations in hematological, biochemical, or histopathological parameters following repeated administration.[13,14] However, recent reviews emphasize that, despite these findings, the safety profile of *T. terrestris* remains incompletely characterized, particularly under conditions of repeated administration or high-dose exposure.[15,16] In addition, variability in extract composition and occasional clinical reports of renal and hepatic adverse effects highlight the need for cautious interpretation and underscore the importance of standardized preparations and further systematic evaluation.[17]

*T. terrestris* is a medicinal plant widely used in traditional systems of medicine for the management of urinary tract and renal disorders. It has been reported to possess diuretic, antiurolithiatic, antioxidant, and antimicrobial activities, highlighting its potential therapeutic relevance in nephrolithiasis and associated complications.[15,18,19] However, comprehensive preclinical evidence regarding repeated-dose safety and its association with antiurolithiatic activity remains limited.[20]

Therefore, the present study aimed to evaluate the sub-acute toxicity profile of the ethanol extract of *T. terrestris* in rats, with particular emphasis on hematological, biochemical, and histopathological parameters, and to investigate its antiurolithiatic activity using an ethylene glycol/ammonium chloride-induced nephrolithiasis model.

# Materials and Methods

## Plant Material

Aerial and subterranean parts of *T. terrestris* (leaves, stems, roots, and fruits) were collected from wild populations in Taiz and Ibb governorates, Yemen (altitude 1800–2000 m above sea level), during May–June 2023 (Figure 1).

The plant material was taxonomically identified by Prof. Dr. Hasan M. Hasan, a taxonomist at the Department of Botany, Faculty of Science, Sana'a University, Yemen. A voucher specimen (No. 721) was deposited at the Yemeni National Herbarium, Sana'a University.

## Chemicals and Reagents

All chemicals and reagents were of analytical grade. Ethanol and dimethyl sulfoxide (DMSO) were obtained from (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) via a local distributor. Ethylene glycol, ammonium chloride, and potassium citrate were purchased from (HiMedia Laboratories, Mumbai, India) through a local authorized supplier.

## Preparation of Extract for Administration

The powdered plant material of *T. terrestris* was subjected to sequential solvent extraction using solvents of increasing polarity. The comprehensive extraction procedure and phytochemical characterization of the ethanol extract (EETT) were established in our previous work.[21] In the present study, the same standardized protocol was strictly followed using the identical extract batch to ensure absolute experimental consistency. The phytochemical profile was previously validated through standard qualitative screening methods, including tests for saponins (foam test), alkaloids (Dragendorff's and Mayer's tests), flavonoids (aluminum chloride and ammonia tests), anthraquinones (Borntrager's test), tannins (ferric chloride test), terpenoids (Salkowski test), and resins (acetic anhydride–sulfuric acid test).[22–24] For clarity and to provide a complete pharmacological context, the qualitative composition of this standardized extract is summarized in Table 1.

**Figure 1** Photograph of *Tribulus terrestris* in its natural habitat. The photograph illustrates the typical growth pattern and leaf morphology of *Tribulus terrestris* in its natural habitat. It was taken at the same geographic location where the study specimens were collected, serving documentation purposes.

The dried extract was dissolved in 4% DMSO in normal saline, filtered through a 0.45 µm membrane, and used as the dosing solution. The same vehicle was administered to the control group.

## Experimental Animals

Adult Wistar rats (180 ± 20 g) were obtained from the animal facility of the University of Science and Technology, Sana'a, Yemen. Animals were housed under controlled environmental conditions (23 ± 2 °C, 12 h light/dark cycle) with free access to standard diet and water. Rats were acclimatized for two weeks prior to experimentation. All experimental procedures were conducted in accordance with ARRIVE guidelines and approved by the institutional ethics committee (Approval No. EAC/UST233).

The determination of EETT doses was informed by a prior study conducted by Chaudhary et al[25] and subsequently refined through a preliminary pilot experiment in our laboratory to verify safety and tolerability.

**Table 1** Qualitative Phytochemical Profile of EETT (Data Adapted from Previous Study)[21]

| Phytochemical Constituent | Test Performed | Result |
|---|---|---|
| Saponins | Frothing test | + |
| Flavonoids | Alkaline reagent test | + |
| Tannins | Ferric chloride test | + |
| Alkaloids | Dragendorff's test | + |
| Anthraquinones | Bornträger's test | + |
| Terpenoids | Salkowski test | – |
| Resins | Acetic anhydride–sulfuric acid test | – |

**Note**: (+) indicates the presence of the phytochemical constituent; (−) indicates its absence.

## Sub-Acute Toxicity Study

The 28-day repeated-dose toxicity study was conducted in accordance with OECD Guideline 407.[9] Thirty Wistar rats (both sexes) were randomly allocated into three groups (n = 10; 5 males and 5 females per group).

- Group I: Control (vehicle)
- Group II: EETT (500 mg/kg/day, oral)
- Group III: EETT (1000 mg/kg/day, oral)

Animals were observed daily for mortality and clinical signs of toxicity. General health status and body condition were monitored throughout the study period. Quantitative body weight measurements were recorded at baseline and at the end of the experimental period to assess overall changes during treatment.

On day 29, animals were anesthetized and blood samples were collected via cardiac puncture into EDTA tubes for hematological analysis and gel tubes for serum biochemical evaluation. Hematological parameters were assessed using a complete blood count (CBC). Biochemical parameters included ALT, AST, total bilirubin, cholesterol, albumin, blood urea nitrogen (BUN), urea, and creatinine. Following blood collection, kidneys, liver, and heart were excised and weighed. Relative organ weight (ROW) was calculated as:[9]

$$\text{ROW} = \text{organ weight (g)} / \text{body weight at sacrifice (g)}.$$

Organs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E) for liver and heart, and periodic acid–Schiff (PAS) for kidneys. Histological evaluation was performed by a blinded pathologist.[26] Histological alterations were evaluated using a semi-quantitative scoring system based on lesion severity (0 = no lesion, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe), as commonly applied in toxicological pathology studies.[27]

Satellite groups were not included in the present study, as the experimental design was focused on evaluating sub-acute toxicity and antiurolithiatic efficacy following repeated administration. Safety assessment was based on hematological, biochemical, and histopathological parameters at the end of the treatment period.

## Antiurolithiatic Activity (Nephrolithiasis Model)

Thirty-six adult male Wistar rats were divided into six groups (n = 6 per group) and treated for 28 days. Group I (control) received standard diet and water, and vehicle used for extract dissolution. Group II received 0.75% ethylene glycol and 1% ammonium chloride in drinking water to induce nephrolithiasis.[28] Groups III–V received the lithogenic regimen plus EETT at 150, 300, or 450 mg/kg intraperitoneally (IP), respectively.[25] Group VI received potassium citrate (200 mg/kg,

IP) as a standard reference. At the end of treatment, animals were anesthetized and blood was collected for serum creatinine, urea, calcium, and potassium measurement. Kidneys were harvested and processed for histopathological evaluation. Crystal deposition was assessed semi-quantitatively at ×100 magnification using the scale: 0 (none), + (1–5), ++ (6–10), +++ (>11 deposits per field).[29]

## Statistical Analysis

Data were analyzed using GraphPad Prism (version 8.0.2). Results are expressed as mean ± SEM. Group comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was set at $p < 0.05$.

# Results

## Extract Yield and Phytochemical Screening

Ethanol extraction of *Tribulus terrestris* (EETT) yielded 5.7% (w/w) of dried crude extract. Phytochemical screening confirmed the presence of saponins, alkaloids, anthraquinones, flavonoids, and tannins (Table 1).

## Sub-Acute Toxicity Study

No mortality or treatment-related clinical signs of toxicity were observed throughout the 28-day period. General appearance, behavior, and physiological functions remained comparable between control and treated groups.

### Body Weight and Relative Organ Weight

Body weight gain did not differ significantly between control and treated groups in both sexes, indicating the absence of treatment-related effects on growth (Table 2). Relative organ weights of the liver, kidneys, and heart did not differ significantly among groups, indicating absence of hypertrophy or atrophy (Table 2).

### Hematological Parameters

No statistically significant differences ($p > 0.05$) were observed in hematological parameters between groups, and all values remained within normal physiological ranges (Table 3).

### Serum Biochemical Analysis

Serum biochemical analysis showed no significant changes in ALT, AST, total bilirubin, albumin, cholesterol, creatinine, BUN, or urea in treated rats compared with controls, with no observable treatment-related toxicity within the tested dose range (Table 4).

**Table 2** Impact of EETT on Rats' Body Weight and Relative Organ Weight

| | | Control | | EETT-500 mg/kg | | EETT-1000 mg/kg | |
|---|---|---|---|---|---|---|---|
| | | Male | Female | Male | Female | Male | Female |
| Body Weight | Day 1 | 171.5 ± 4.4 | 172.8 ± 3.81 | 170.3 ± 4.82 | 173 ± 3.17 | 178 ± 1.73 | 167.8 ± 1.70 |
| | Day 28 | 202.8 ± 1.6 | 197.3 ± 3.42 | 195.3 ± 3.90 | 207.6 ± 4.92 | 208.8 ± 5.79 | 195.5 ± 2.53 |
| Relative Organ Weight | R. Kidney | 0.366 ± 0.01 | 0.350 ± 0.007 | 0.366 ± 0.016 | 0.344 ± 0.014 | 0.366 ± 0.015 | 0.368 ± 0.016 |
| | L. Kidney | 0.356 ± 0.01 | 0.338 ± 0.008 | 0.350 ± 0.016 | 0.329 ± 0.012 | 0.348 ± 0.017 | 0.357 ± 0.014 |
| | Liver | 4.03 ± 0.12 | 3.80 ± 0.186 | 4.04 ± 0.144 | 3.36 ± 0.167 | 3.65 ± 0.055 | 3.98 ± 0.162 |
| | Heart | 0.391 ± 0.005 | 0.411 ± 0.021 | 0.403 ± 0.012 | 0.380 ±0.002 | 0.420 ± 0.020 | 0.403 ± 0.017 |

**Notes**: Data expressed as mean ± SEM; n = 10 (5 male and 5 female).
**Abbreviation**: EETT, Ethanol extract of *Tribulus terrestris*.

**Table 3** Effect of EETT on Hematologic Parameters in Rats

| | Control | | EETT-500 mg/kg | | EETT-1000 mg/kg | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| Hb (g/dl) | 14.03 ± 0.20 | 14.4 ± 0.40 | 14.9 ± 0.56* | 15.1 ± 0.16 | 14.45 ± 0.29 | 14.1 ± 0.19 |
| PVC (%) | 40.58 ± 2.15 | 45.55 ± 0.76 | 47.25 ± 1.16 | 45.3 ± 0.69 | 43.08 ± 1.42 | 43.1 ± 1.42 |
| RBC (10^12/µL) | 6.47 ± 0.37 | 7.17 ± 0.17 | 7.18 ± 0.32 | 7.2 ± 0.09 | 6.42 ± 0.14 | 6.70 ± 0.27 |
| MCV (fl) | 62.85 ± 0.73 | 61.16 ± 1.26 | 63.35 ± 0.91 | 63.12 ± 0.30 | 62.68 ± 1.66 | 64.98 ± 1.11 |
| MCH (pg) | 21.13 ± 0.59 | 21.3 ± 0.47 | 21.73 ± 0.62 | 20.98 ± 0.17 | 21.68 ± 0.68 | 21.68 ± 0.70 |
| MCHC (g/dl) | 33.63 ± 0.63 | 32.5 ± 0.77 | 34.25 ± 0.45 | 33.38± 0.26 | 33.5 ± 0.39 | 32.53 ± 0.63 |
| WBC (x10^9/L) | 6.35 ± 0.21 | 4.62 ± 0.13 | 7.22 ± 0.54 | 5.14 ± 0.39 | 6.14 ± 0.80 | 5.04 ± 0.41 |
| Platelets (10^9/µL) | 511.6 ± 26.66 | 519.3 ± 19.53 | 574.5 ± 46.21 | 537.8 ± 27.41 | 588 ± 49.62 | 571.5 ± 59.08 |
| Neutrophils (%) | 51.8 ± 2.56 | 42.45 ± 1.25 | 50 ± 2.69 | 41.08 ± 1.09 | 49.15 ± 2.09 | 38.96 ± 1.1 |
| Eosinophils (%) | 2.225 ± 0.43 | 2.36 ± 0.25 | 2.36 ± 0.21 | 2.24 ± 0.77 | 2.77 ± 0.25 | 2.28 ± 0.32 |
| Lymphocytes (%) | 39.63 ± 1.29 | 45.7 ± 1.89 | 35.9 ± 1.59 | 47.3 ± 1.30 | 39.13 ± 1.43 | 45.15 ± 1.18 |
| Monocytes (%) | 0.96 ± 0.34 | 0.93 ± 0.47 | 1.43 ± 0.53 | 0.55 ± 0.38 | 1.12 ± 0.57 | 0.91 ± 0.43 |
| RDW-CV % | 16.45 ± 0.47 | 16.05 ± 1 | 16.05 ± 0.47 | 15.7 ± 0.27 | 15.5 ± 0.35 | 16.38 ± 0.33 |

**Notes**: Data expressed as mean ± SEM, * Significantly different from the control at $p < 0.05$, n = 10 (5 male and 5 female).
**Abbreviation**: EETT, Ethanol extract of *Tribulus terrestris*.

**Table 4** Effect of EETT on Serum Parameters in Rats

| | Control | | EETT-500 mg/kg | | EETT-1000 mg/kg | |
|---|---|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** | **Male** | **Female** |
| Total bilirubin (mg/dl | 0.5 + 0.07 | 0.56 ± 0.09 | 0.55 ± 0.06 | 0.48 ± 0.12 | 0.24 ± 0.05 | 0.475 ± 0.1 |
| Cholesterol (mg/dl) | 479.9 ± 45 | 488.2 ± 29 | 435.4 ± 22 | 451.3 ± 28 | 328.8 ± 35 | 440.6 ± 26 |
| ALT (U/L) | 36.2 ± 3 | 28.75 ± 3.9 | 25.25 ± 3.9 | 28.4 ± 3.4 | 25.6 ± 3.7 | 22.25 ± 1.25 |
| AST (U/L) | 119 ± 5.7 | 103.6 ± 5.8 | 101.2 ± 4.9 | 98.8 ± 3.5 | 95.8 ± 3.4 | 100.4 ± 3.14 |
| BUN (mg/dl) | 1.47 ± 0.22 | 1.61 ± 0.11 | 0.96 ± 0.12 | 0.98 ± 0.16 | 1.028 ± 0.15 | 0.878 ± 0.02 |
| Creatinine (mg/dl) | 0.76 ± 0.09 | 0.8 ± 0.03 | 0.56 ± 0.04 | 0.76 ± 0.05 | 0.72 ± 0.02 | 0.676 ± 0.02 |
| Albumin (mg/dl) | 457.5 ± 10.26 | 468.2 ± 21.22 | 453.6 ± 17.74 | 483.6 ± 10.11 | 395.3 ± 21.79 | 464 ± 21.79 |

**Notes**: Data expressed as mean ± SEM; n = 10 (5 male and 5 female).
**Abbreviations**: EETT, Ethanol extract of *Tribulus terrestris*; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BUN, Blood urea nitrogen.

## Histopathological Analysis

Histological examination of kidneys, heart, and liver revealed preserved tissue architecture in all treatment groups. No evidence of tubular degeneration, interstitial inflammation, necrosis, or fibrosis was observed, with no observable pathological alterations across examined organs (Figures 2–4).

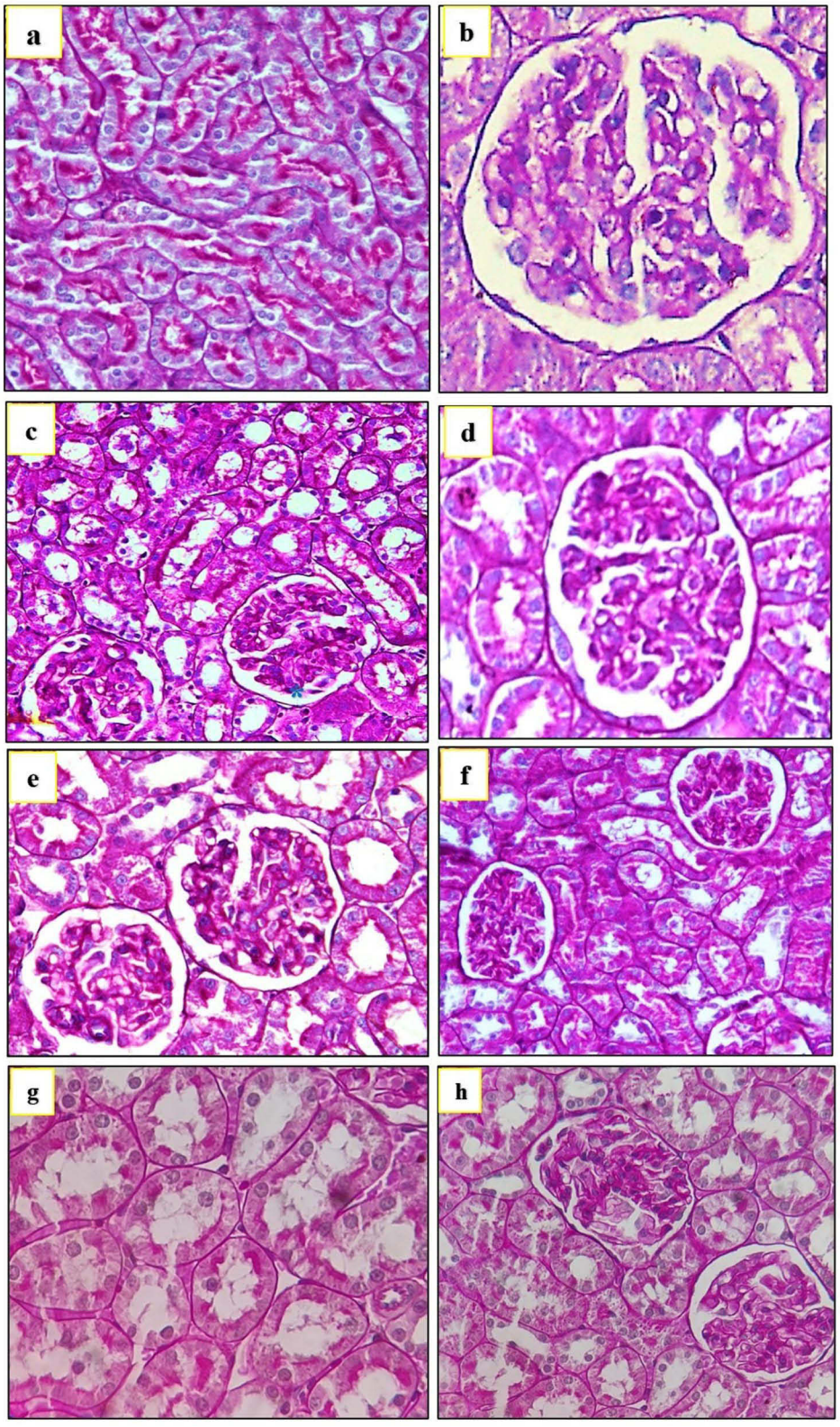


**Figure 2** Representative PAS-stained photomicrographs of kidney sections. (**a** and **b**) (normal control): Normal renal architecture showing intact glomeruli and well-preserved proximal tubules with clearly defined brush borders and basement membranes. (**c** and **d**) (EETT 500 mg/kg): Renal sections exhibiting preserved cortical architecture with normal glomeruli and tubular structures, without evidence of degenerative or pathological alterations. (**e**–**h**) (EETT 1000 mg/kg): Representative renal sections from different microscopic fields demonstrating maintained renal morphology, including intact proximal tubules and normal renal corpuscles with well-defined Bowman's capsule, Bowman's space, and glomeruli. Images were captured at 200× and 400× magnification.


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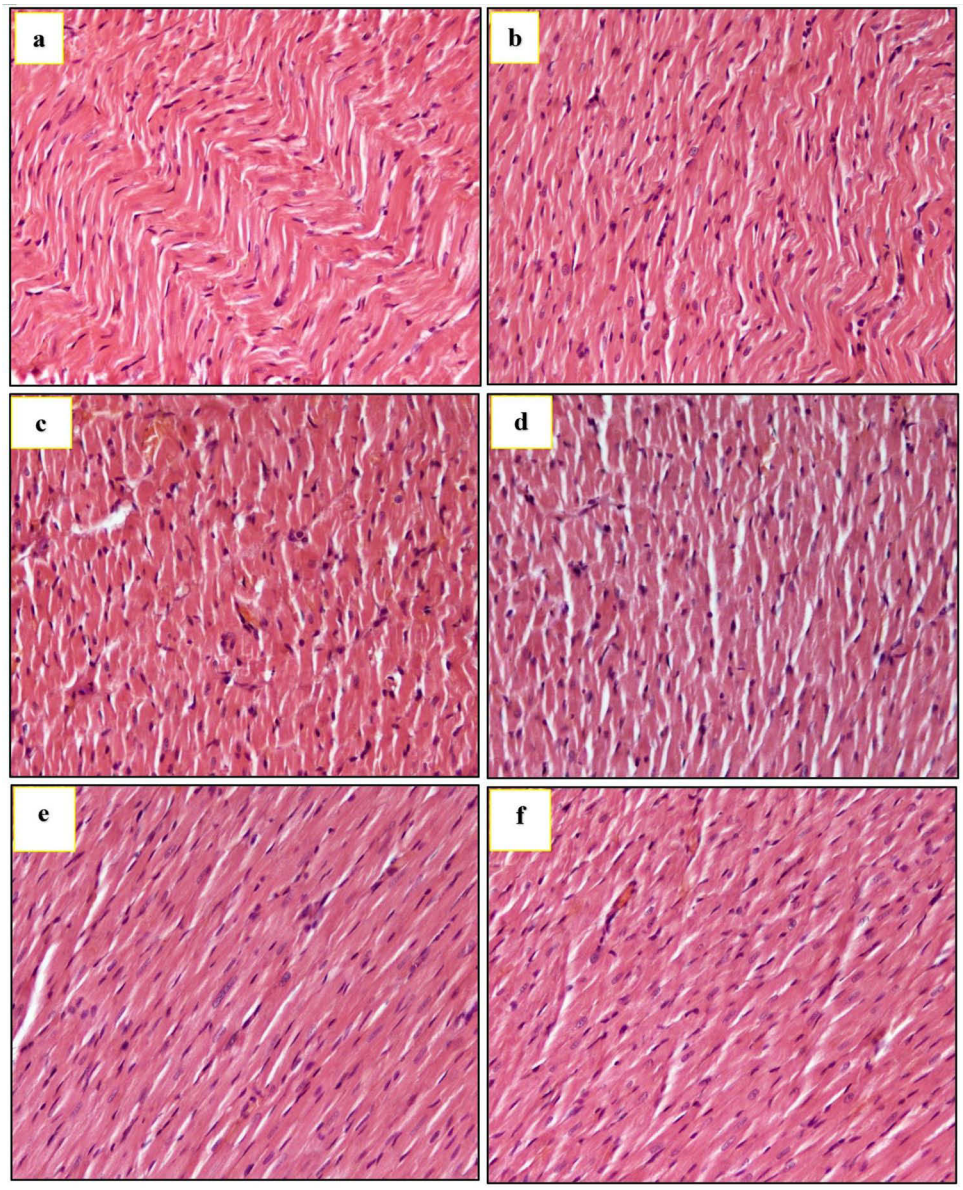


**Figure 3** Representative hematoxylin and eosin (H&E)-stained photomicrographs of cardiac tissue. (**a** and **b**) (Normal control): Myocardial sections showing normal cardiac architecture with regularly arranged muscle fibers, eosinophilic cytoplasm, centrally located oval nuclei, and minimal interstitial connective tissue. (**c** and **d**) (EETT 500 mg/kg): Cardiac sections showing preserved myocardial architecture with intact muscle fibers and normal nuclear morphology, without evidence of inflammation, necrosis, or structural alterations. (**e** and **f**) (EETT 1000 mg/kg): Sections demonstrating well-organized myocardial fibers and maintained tissue architecture, comparable to the control group, with no detectable pathological changes.

## Antiurolithiatic Activity

Administration of ethylene glycol and ammonium chloride (EG/AC) induced nephrolithiasis, as reflected by significant elevations in serum creatinine, urea, calcium, and potassium levels compared to control. Treatment with EETT produced dose-dependent improvement in all parameters, with the highest dose (450 mg/kg) restoring values close to normal. These effects were comparable to potassium citrate.

### Effect on Serum Creatinine and Urea

The EG/AC group showed a marked increase in serum creatinine and urea, indicating impaired renal function. EETT at 300 and 450 mg/kg significantly reduced these markers, whereas the 150 mg/kg dose showed limited improvement. Potassium citrate produced a similar protective effect (Figures 5 and 6, Table 5).

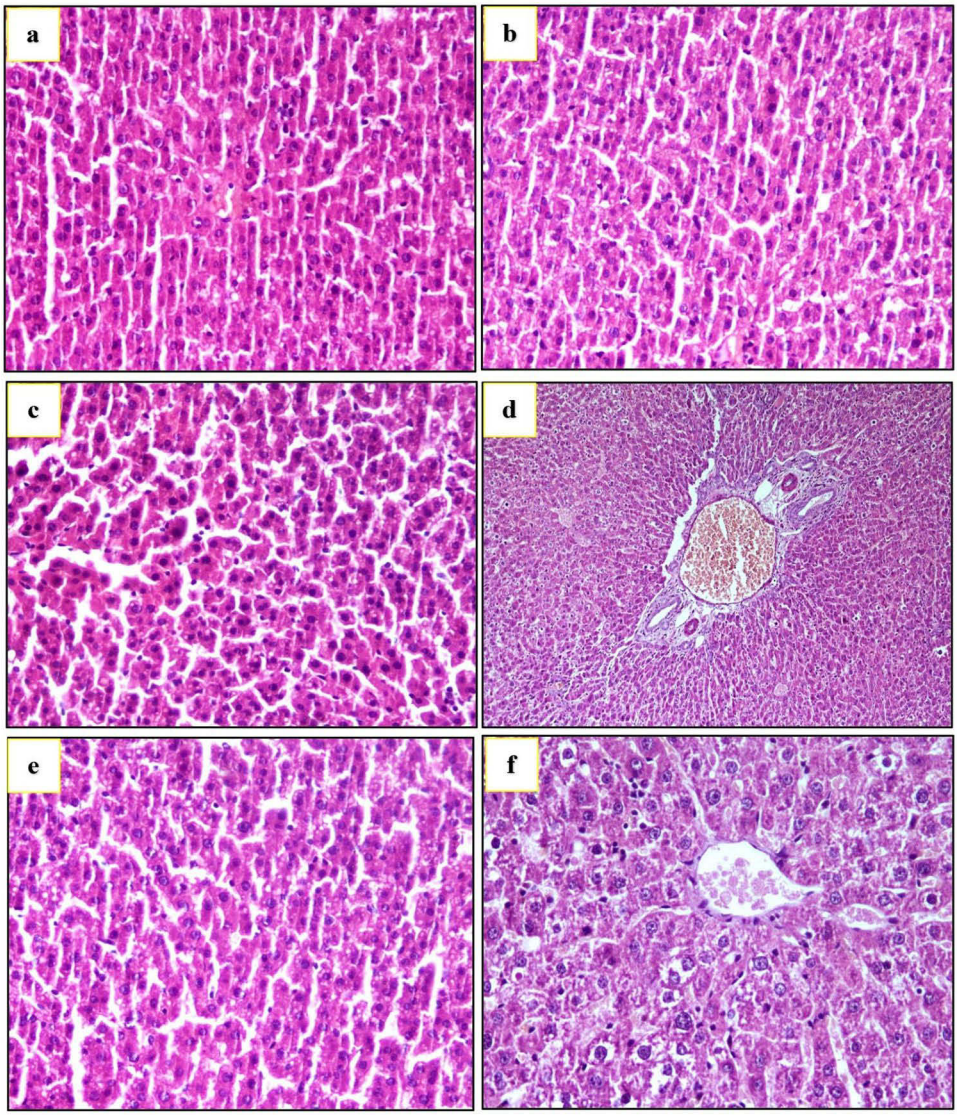


**Figure 4** Representative hematoxylin and eosin (H&E)-stained photomicrographs of liver tissue. (**a** and **b**) (Normal control): Liver sections showing typical hepatic architecture with radially arranged hepatic cords surrounding the central vein, polygonal hepatocytes with eosinophilic cytoplasm, and well-defined sinusoids. (**c**) (EETT 500 mg/kg): Liver sections showing largely preserved hepatic architecture with minimal cytoplasmic vacuolation of hepatocytes and slight disorganization of hepatic cords, without evidence of structural damage. (**d**) (Low magnification): Overview of the hepatic lobule demonstrating preserved lobular organization with mild central vein congestion and minimal periportal cellular presence. (**e** and **f**) (EETT 1000 mg/kg): Liver sections showing preserved hepatic architecture with mild cellular alterations, including limited cytoplasmic vacuolation and slight variation in nuclear morphology, without evidence of necrosis, fibrosis, or significant inflammatory infiltration. Magnification: (a–c, e–f) ×400; (**d**) ×100; scale bar = 100 μm.

## Effect on Serum Calcium and Potassium

EG/AC administration significantly elevated serum calcium and potassium. EETT at 300 and 450 mg/kg significantly reduced serum calcium levels toward normal values, while potassium was significantly improved only at 450 mg/kg. Potassium citrate effectively corrected both electrolyte disturbances (Figures 7 and 8, Table 5).

## Histopathological Evaluation of Kidneys

Kidneys from the EG/AC group demonstrated extensive calcium oxalate crystal deposition, tubular dilatation, epithelial degeneration, and disruption of normal architecture. Treatment with EETT reduced crystal deposition in a dose-dependent manner. At 450 mg/kg, EETT resulted in marked improvement in renal histology, with minimal or absent crystal deposits, showing effects comparable to the potassium citrate group (Figures 9 and 10, Table 6).


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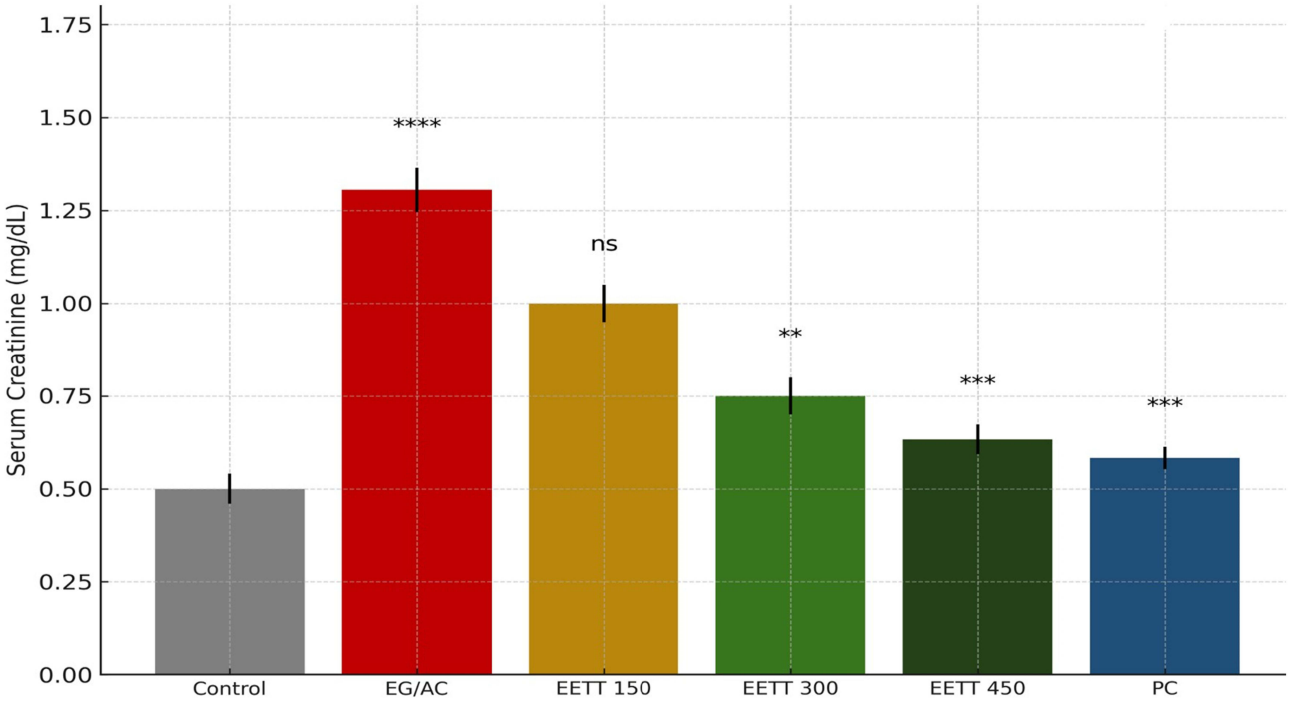


**Figure 5** Effect of EETT on serum creatinine levels in rats. Data are expressed as mean ± SEM (n = 6). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ ns: not significant. EG/AC group was compared to the control group to confirm disease induction, while all other groups were compared to the EG/AC group.
**Abbreviations**: EG/AC, ethylene glycol + ammonium chloride; EETT, ethanol extract of *Tribulus terrestris*; PC, potassium citrate.

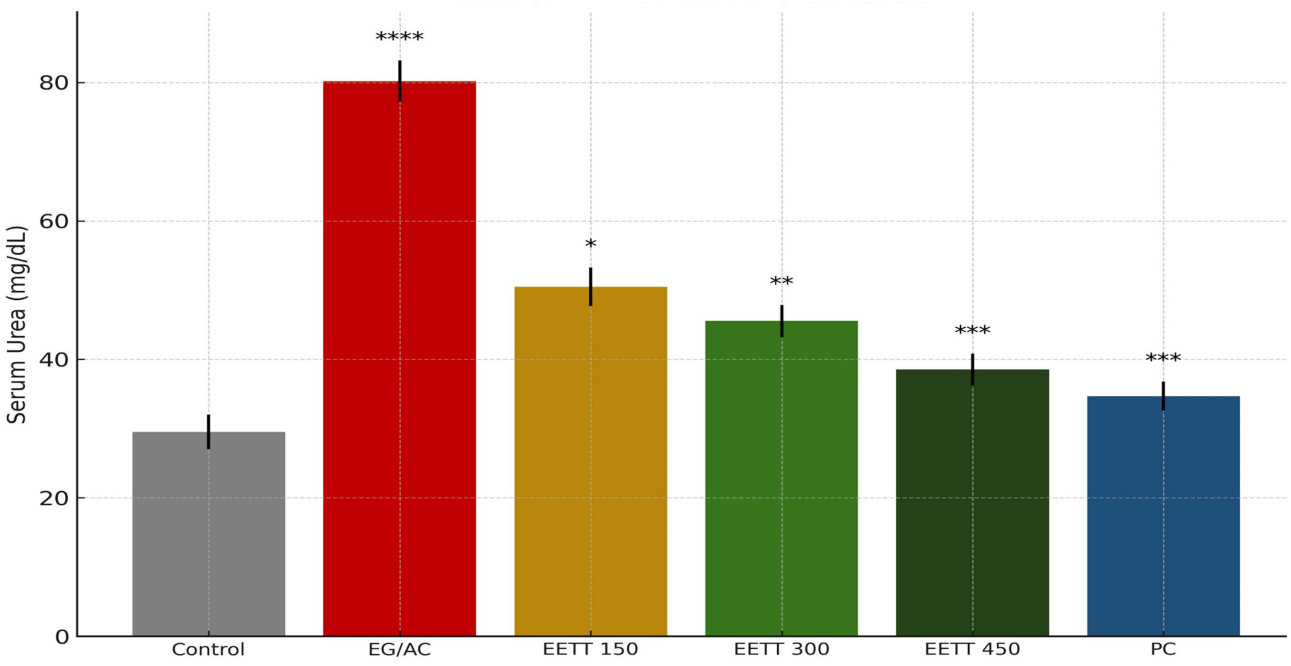


**Figure 6** Effect of EETT on serum urea levels in rats. Data are expressed as mean ± SEM (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. EG/AC group was compared to the control group to confirm disease induction, while all other groups were compared to the EG/AC group.
**Abbreviations**: EG/AC, ethylene glycol + ammonium chloride; EETT, ethanol extract of *Tribulus terrestris*; PC, potassium citrate.

**Table 5** Effect of EETT on Serum Biochemical Parameters in Rats with EG/AC-Induced Nephrolithiasis

| Group | Creatinine (Mean ± SEM) | Urea (Mean ± SEM) | Calcium (Mean ± SEM) | Potassium (Mean ± SEM) |
|---|---|---|---|---|
| Control | 0.500 ± 0.093 | 29.500 ± 3.603 | 9.233 ± 0.244 | 7.117 ± 0.240 |
| EG/AC | 1.305 ± 0.152**** | 80.17 ± 5.41**** | 11.73 ± 0.088**** | 8.98 ± 0.464** |
| EG/AC-EETT-150 | 1.000 ± 0.151ns | 50.500 ± 11.42* | 11.18 ± 0.345 ns | 8.70 ± 0.316 ns |
| EG/AC-EETT-300 | 0.750 ± 0.062** | 45.500 ± 7.084** | 9.98 ± 0.208*** | 7.817 ± 0.373 ns |
| EG/AC-EETT-450 | 0.633 ± 0.061*** | 38.500 ± 5.20*** | 9.75 ± 0.240**** | 7.467 ± 0.263* |
| EG/AC-PC-200 | 0.583 ± 0.06*** | 34.670 ± 5.52*** | 9.53 ± 0.272**** | 7.15 ± 0.371** |

**Notes**: Data are expressed as mean ± SEM (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. EG/AC group was compared to the control group to confirm disease induction, while all other groups were compared to the EG/AC group.
**Abbreviations**: ns, not significant; EG/AC, ethylene glycol + ammonium chloride; EETT, ethanol extract of *Tribulus terrestris*; PC, potassium citrate.

## Discussion

The present study aimed to contribute to addressing existing gaps in the literature, particularly the limited data on repeated-dose safety and the scarcity of studies that simultaneously evaluate antiurolithiatic efficacy within a unified experimental framework. The current work demonstrates that the ethanol extract of *Tribulus terrestris* (EETT) exhibits a favorable safety profile alongside significant antiurolithiatic activity in an experimental model. Phytochemical screening confirmed the presence of saponins, flavonoids, tannins, and alkaloids, which are reported in the literature to possess antioxidant and crystal-aggregation inhibitory properties.[30]

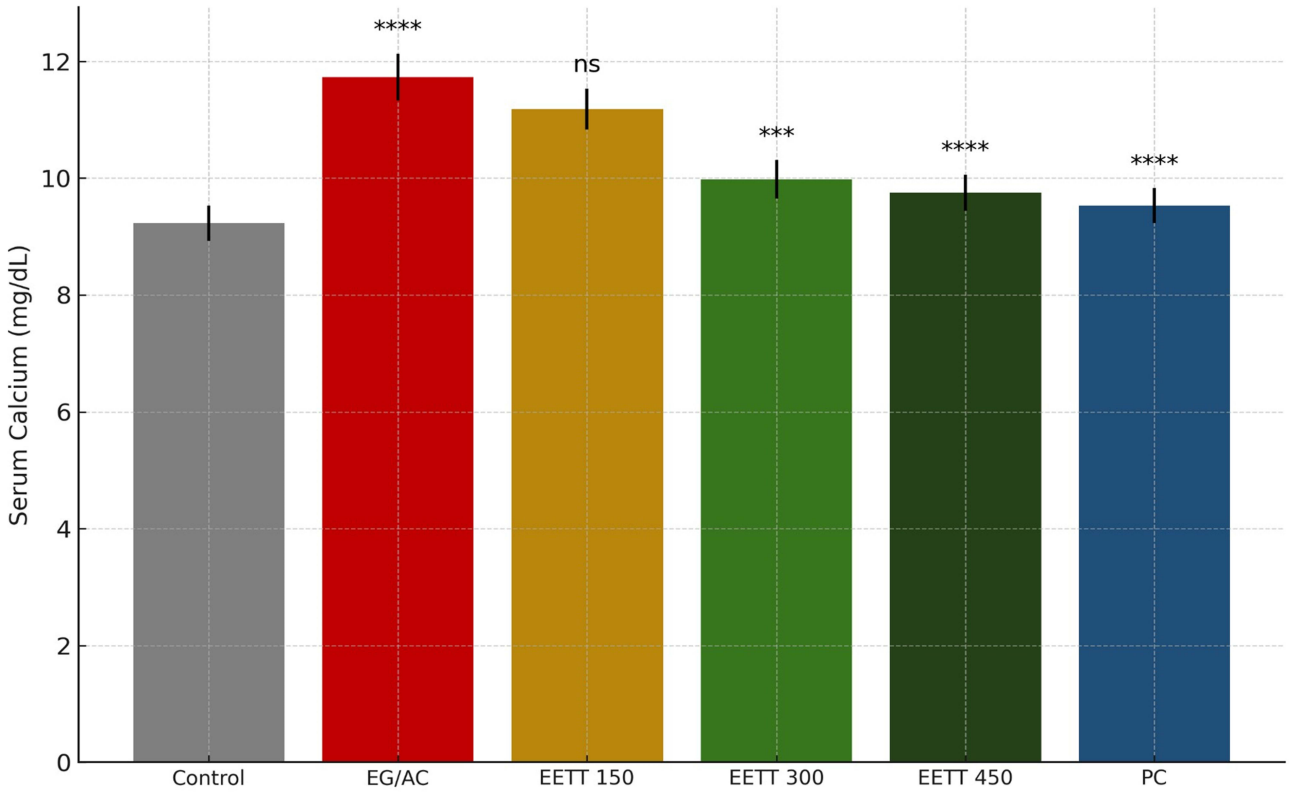


**Figure 7** Effect of EETT on serum calcium levels in rats. Data are expressed as mean ± SEM (n = 6). *** $p < 0.001$, **** $p < 0.0001$ ns: not significant. EG/AC group was compared to the control group to confirm disease induction, while all other groups were compared to the EG/AC group.
**Abbreviations**: EG/AC, ethylene glycol + ammonium chloride; EETT, ethanol extract of *Tribulus terrestris*; PC, potassium citrate.


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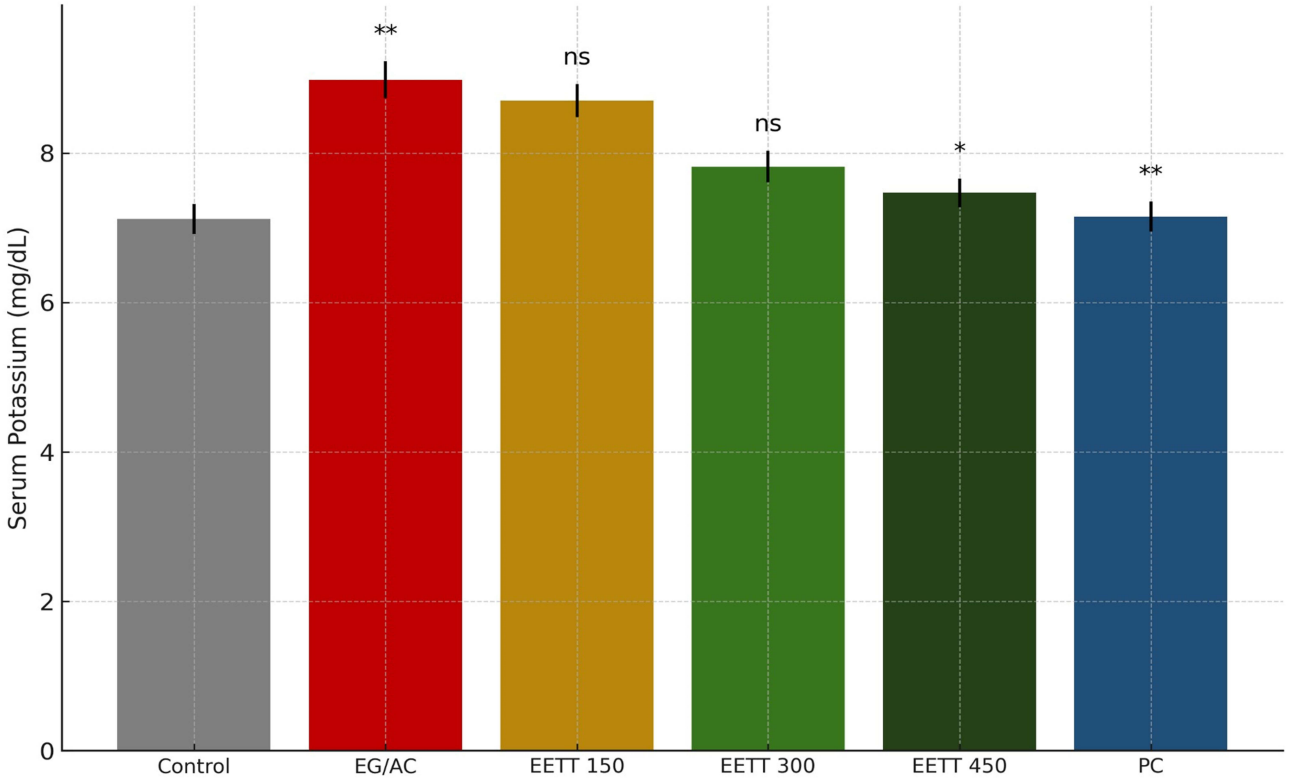


**Figure 8** Effect of EETT on serum potassium levels in rats. Data are expressed as mean ± SEM (n = 6). * $p < 0.05$, ** $p < 0.01$, ns: not significant. EG/AC group was compared to the control group to confirm disease induction, while all other groups were compared to the EG/AC group.
**Abbreviations**: EG/AC, ethylene glycol + ammonium chloride; EETT, ethanol extract of *Tribulus terrestris*; PC, potassium citrate.

In the 28-day repeated-dose toxicity assessment, EETT did not produce mortality or observable clinical signs of toxicity at doses up to 1000 mg/kg. Furthermore, no significant alterations were detected in body weight, relative organ weights, hematological indices, or serum biochemical parameters, indicating the absence of systemic toxicity under the tested conditions.[13,14,31] Histopathological examination further supported these findings, revealing preserved architecture of the kidney, liver, and heart without detectable pathological alterations.[32–34] These results collectively indicate a favorable sub-acute safety profile of the extract within the tested dose range.

With respect to efficacy, EETT significantly ameliorated ethylene glycol/ammonium chloride (EG/AC)-induced nephrolithiasis. This was evidenced by a dose-dependent reduction in serum creatinine, urea, calcium, and potassium levels, along with a marked decrease in renal calcium oxalate crystal deposition. These findings suggest that EETT may exert its antiurolithiatic effects through multiple mechanisms, primarily the inhibition of crystal aggregation and the preservation of renal structural integrity, possibly mediated by antioxidant and anti-inflammatory actions.[25] Notably, the higher dose (450 mg/kg) demonstrated an effect comparable to the standard treatment, potassium citrate, highlighting its potential therapeutic relevance.

It should be noted that different routes of administration were employed for toxicity (oral) and efficacy (intraperitoneal) assessments. The intraperitoneal route was selected in the nephrolithiasis model to ensure controlled systemic exposure and to minimize variability in absorption. However, this difference should be considered when interpreting dose translation to clinical settings.

The findings of this study are consistent with previous studies evaluating the antiurolithiatic and safety profile of *T. terrestris*. For example, earlier investigation using an aqueous extract reported significant reduction in renal calculi formation alongside improvements in biochemical markers, with a reported no observed adverse effect level (NOAEL) of

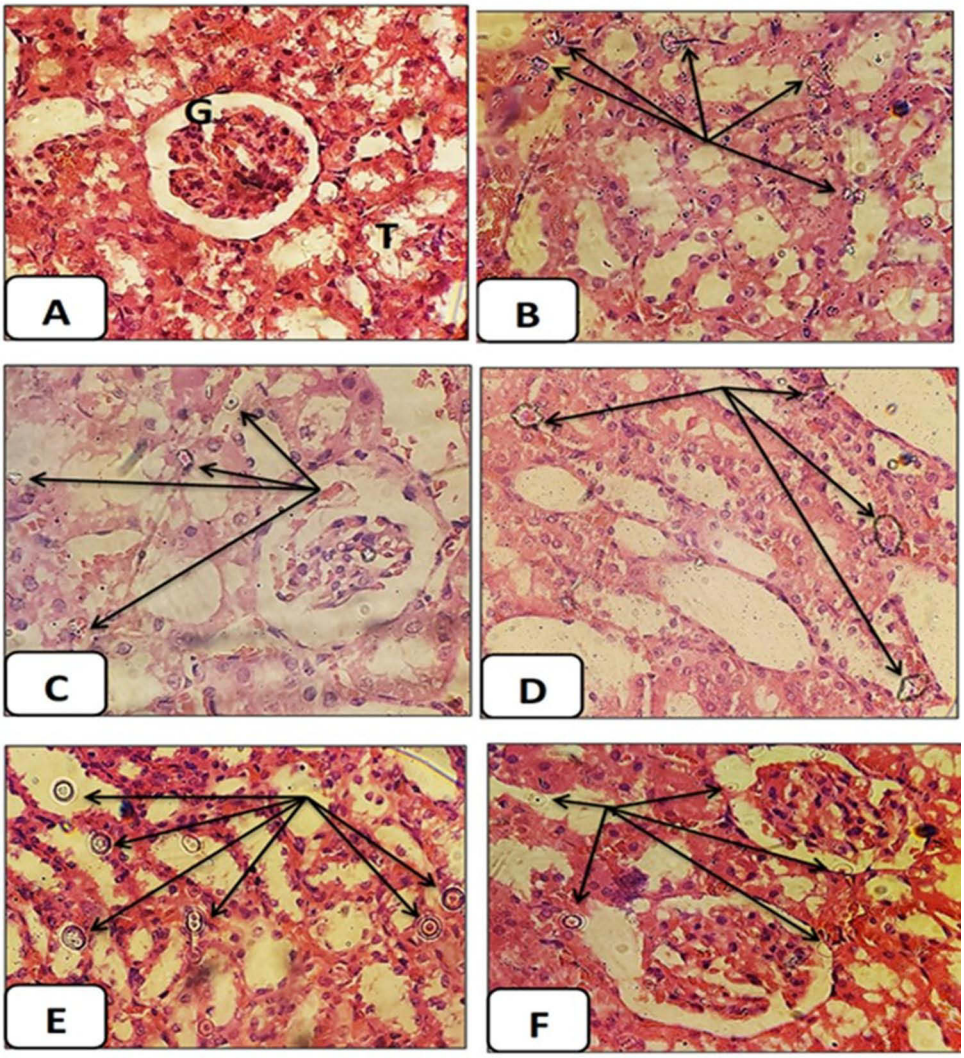


**Figure 9** Representative histological sections of rat kidneys from the EG/AC-induced nephrolithiasis group. (**A**) Normal renal architecture in the negative control group, showing intact glomerulus (G) and renal tubules (T) with no pathological changes. (**B–F**) Renal sections from the EG/AC group showing extensive crystal deposition (arrows) within renal tubules and interstitial spaces, tubular dilatation, and partial disorganization of renal tissue.

750 mg/kg following repeated oral administration.[20] In addition, sub-acute toxicity studies of various *T. terrestris* preparations have consistently reported an absence of mortality at doses up to 1500 mg/kg.[14]

From a translational perspective, dose extrapolation indicates that the highest effective dose corresponds to a relatively elevated human equivalent dose. However, such discrepancies are anticipated due to interspecies differences in pharmacokinetics, metabolism, and routes of administration. Clinical evidence suggests that *T. terrestris* is generally well tolerated at commonly used doses.[35,36] Nevertheless, reports of adverse effects at excessive intake levels underscore the importance of standardized extract formulations and the need for rigorous clinical evaluation.[17]

Collectively, the integration of safety and efficacy data in the present study provides a coherent pharmacological profile of EETT. These findings indicate that its antiurolithiatic activity is achieved within a non-toxic dose range, supporting its potential for further pharmacological development. Future investigations should focus on the isolation of active constituents and elucidation of the underlying molecular mechanisms to better define its therapeutic applicability.


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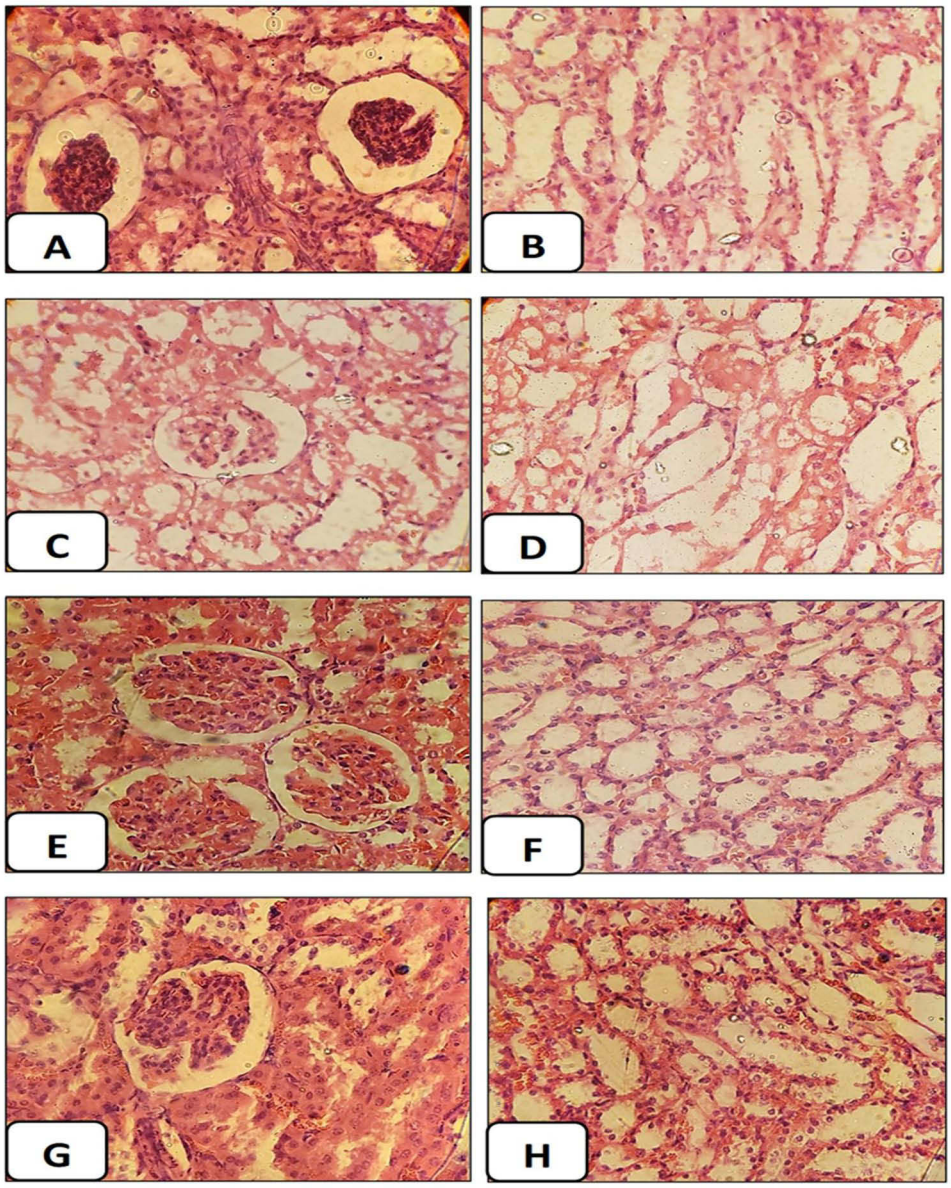


**Figure 10** Representative histological sections of rat kidneys from treated groups in the EG/AC-induced nephrolithiasis model. (H&E). (**A** and **B**) Renal sections from rats treated with low-dose EETT (150 mg/kg) showing persistent crystal deposits within renal tubules. (**C** and **D**) Renal sections from the intermediate-dose EETT group (300 mg/kg) showing reduced crystal deposition with partial preservation of tubular structure. (**E** and **F**) Renal sections from the high-dose EETT group (450 mg/kg) showing largely preserved renal architecture with minimal or no visible crystal deposits. (**G–H**) Renal sections from the potassium citrate–treated group showing normal glomeruli and renal tubules without observable pathological alterations. Sections stained with hematoxylin and eosin (H&E); magnification ×400.

## Limitations of the Study

There are some limitations that should be considered. A formal acute toxicity assessment, including determination of the $LD_{50}$ of EETT, was not carried out in the present investigation. In addition, urinary biochemical parameters were not assessed, which could have provided further insight into the mechanisms underlying stone inhibition. Furthermore, the specific bioactive constituents responsible for the observed effects were not isolated or quantified. Therefore, additional studies focusing on detailed phytochemical characterization and mechanistic evaluation are warranted.

**Table 6** Histopathological Scoring of Renal Injury in Different Experimental Groups

| Group | Presences of Crystals | Interpretation |
|---|---|---|
| Control | 0 | No visible crystals |
| EG/AC | +++ | >11 deposits/field (abundant) |
| EG/AC-EETT-150 | ++ | 6–10 deposits/field (moderate) |
| EG/AC-EETT-300 | + | 1–5 deposits/field (mild) |
| EG/AC-EETT-450 | 0 | No visible crystals |
| EG/AC-PC-200 | 0 | No visible crystals |

**Note**: Crystal deposition was evaluated microscopically at ×100 magnification based on the number of intratubular deposits per field. Scoring was assigned as follows: 0 = no deposits; + = 1–5 deposits/field; ++ = 6–10 deposits/field; +++ = >11 deposits/field.
**Abbreviations**: EG/AC, ethylene glycol + ammonium chloride; EETT, ethanol extract of *Tribulus terrestris*; PC, potassium citrate.

## Conclusion

The findings of this study indicate that the ethanol extract of *Tribulus terrestris* (EETT) exhibits a favorable safety profile, with no observable treatment-related systemic toxicity following 28-day repeated oral administration at doses up to 1000 mg/kg. In addition to its safety, EETT demonstrated significant antiurolithiatic and renoprotective effects, as reflected by the reduction in renal crystal deposition and the improvement of key biochemical markers of kidney function in a dose-dependent manner.

Notably, the observed effects were comparable to those of the standard treatment, potassium citrate, highlighting the potential of EETT as a promising natural candidate for the management of urolithiasis. These results are consistent with previous reports on the therapeutic properties of *T. terrestris* and further support its traditional use.

Overall, this study provides important preclinical evidence supporting both the safety and efficacy of EETT. However, further studies are warranted to identify the active constituents and to elucidate the underlying mechanisms of action. Future investigations, including well-designed clinical studies, will be essential to confirm its therapeutic potential in humans.

## Abbreviations

AKI, Acute Kidney Injury; CKD, chronic kidney disease; EETT, Ethanol Extract of *Tribulus terrestris*; EG/AC, ethylene glycol + ammonium chloride; PC, Potassium Citrate; *T. terrestris, Tribulus terrestris*.

## Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Ethics Approval

All animal experiments were conducted in accordance with institutional guidelines and were approved by the Ethical Committee of the University of Science and Technology prior to the commencement of the experiments (Approval No. EAC/UST233). In compliance with the International Guide to Laboratory Animal Care and Use.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest in this work.

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