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Protective effects of ethanolic extract of *Cuscuta reflexa* Roxb. against ethylene glycol-induced hyperoxaluria and urolithiasis in rats

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ABSTRACT

Introduction: Renal urolithiasis is a prevalent global health concern due to its high recurrence rate. *Cuscuta reflexa* (Roxb) has been traditionally used for its anti-inflammatory and antioxidant properties. This study investigated the antiurolithiatic potential of the ethanolic extract of *Cuscuta reflexa* (EECR) in an ethylene glycol (EG)-induced rat model of urolithiasis.
Methods: The alcoholic extract was subjected to phytochemical screening and quantification of bioactive compounds. Urolithiasis was induced using EG and followed by EECR treatment. Biochemical analysis of urine assessed calcium, oxalate, uric acid, and citrate levels. Serum was analyzed for urea, creatinine, and uric acid. Kidney tissues were examined histologically and assessed for oxidative stress markers and antioxidant enzyme activity.
Results: EECR at 400 mg/kg significantly ($P<0.001$) improved urinary parameters by reducing calcium, oxalate, and uric acid levels, while increasing citrate compared to the diseased group. Serum uric acid and calcium levels were also significantly reduced after administration of EECR ($P<0.05$). EECR enhanced catalase activity and decreased renal tissue calcium and oxalate ($P<0.05$). Histopathology confirmed reduced crystal deposition and restoration of normal renal architecture.
Conclusion: EECR demonstrates promising antiurolithiatic effects by reducing crystal formation, oxidative stress, and renal dysfunction, supporting its potential therapeutic application in urolithiasis management.

Implication for health policy/practice/research/medical education:
Cuscuta reflexa Roxb. extract may offer an affordable and accessible herbal alternative for urolithiasis prevention and management in clinical practice. Findings highlight the need for further clinical research and the possible inclusion of herbal therapy in medical curricula for urinary disorders.

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Introduction

Urolithiasis, characterized by calculus formation within the urinary tract, is one of the most prevalent benign urological conditions worldwide, imposing significant burdens on global healthcare systems (1,2). The incidence and prevalence of kidney stones have steadily increased, driven by factors such as dietary habits (particularly high sodium consumption), hereditary predisposition, and environmental conditions, including climatic variations. Projections indicate a considerable rise in populations at risk, from 40% in 2000 to an estimated 56% by 2050 and potentially up to 70% by 2095 (3). Pathophysiologically, alterations in urine composition, marked by increased urinary calcium excretion due to reduced tubular reabsorption and bicarbonate loss, elevated urinary pH, and decreased citrate concentrations, thus facilitating nucleation, crystal growth, and aggregation (4-7). Although traditionally considered non-fatal, urolithiasis poses significant health risks, as chronic kidney stone formation can lead to progressive renal damage and

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eventually chronic kidney disease (8,9).

Dietary factors significantly influence the risk of developing renal calculi. Excessive consumption of sodium, which is abundant in processed and fast foods, increases the excretion of calcium in urine, thus raising the risk of stone formation (10). This way, consuming a high protein, especially rich in animal proteins, is associated with a higher susceptibility to developing kidney stones, as they are associated with increasing calcium and decreasing citrate levels in urine (11).

Even with advances in medical therapy, urolithiasis is still a complicated condition to manage efficiently due to its complex pathophysiology as well as numerous etiological factors of this condition. Numerous herbal extracts containing flavonoids (12), polyphenols (13), and triterpenoids (14), which are utilized for the management of urolithiasis, possess an extensive, intricate, and peculiar range of action, with minimal adverse effects, encompassing diuretic, anti-inflammatory, analgesic, antioxidant, antispasmodic, and anti-calcifying properties. Various medicinal plants, traditionally used across different cultures to treat kidney stones, are now being scientifically investigated to validate their efficacy. Extracts of plants like *Aegle marmelos*, *Drymoglossum piloselloides,* and *Kalanchoe laciniata* have been studied for their inhibitory effects on calcium oxalate crystals, which are the primary components of kidney stones (15). Similarly, *Holarrhena antidysenterica* has proved effective in preventing stone formation by inhibiting calcium oxalate crystal aggregation and protecting renal epithelial cells (16). All these findings prove that herbal interventions are a safer and more effective alternative for present-day critical health problems.

Cuscuta reflexa Roxb., also known as Aftimoon or Kasoos in the Unani medicine system, is a member of the *Convolvulaceae* family and is a parasitic plant, with a long history of use in traditional medicine practices (17,18). It has been widely utilized globally for Unani treatment, especially in the Indian subcontinent, to treat numerous ailments, because of its antioxidant, anti-inflammatory, hepatoprotective, nephroprotective, and analgesic effects (19). Traditionally, this plant has been used to treat various ailments such as difficulties in urination, muscle pain, jaundice, and cough. It has also been used as a blood purifier, and its warm paste can be applied to relieve rheumatism and headaches. Besides this, the seeds of *C. reflexa* possess carminative and anthelmintic properties, making them useful in curing bilious disorders (20). *C. reflexa* has also been tested for its potential antihypertensive effects. The plant possesses positive inotropic and cardiotonic properties, thereby causing a reduction in blood pressure (21). The studies have revealed that the extracts of *C. reflexa* expedite the healing process in contact with frostbite injuries by mitigating inflammation and promoting tissue repair (22). *C. reflexa* (Aftimoon) has been widely used in Unani medicine as a safe and reliable herb with no reported adverse effects (23).

The present study evaluated the preventive effects of ethanolic extract of *Cuscuta reflexa* (EECR) in mitigating ethylene glycol (EG)-induced urolithiasis through the mitigation of kidney crystal formation. The study postulated the anti-urolithiasis activity by assessing key urinary parameters like urine pH, volume, serum indicators, histopathological analysis, etc., to evaluate the efficacy of EECR against urolithiasis. These evaluations investigated the antiurolithiatic potential of EECR by assessing its ability to restore kidney function and prevent calcium oxalate crystal formation, a key consequence of hyperoxaluria linked to urolithiasis.

Material and Methods

Chemicals and plant materials

Lumasiran (Oxlumo™), an FDA-approved drug, was utilized as the reference standard drug in the study for its prominent effect in the treatment of hyperoxaluria that has a close pathophysiological link to urolithiasis. EG (AR grade) was used to induce urolithiasis in the animal model, as it is a widely accepted method due to its ability to cause hyperoxaluria, a key contributor to urolithiasis. Biochemical diagnostic kits were employed for the analysis of urine and serum samples. Laboratory-grade reagents and chemicals were employed for phytochemical screening and quantitative assessment of the plant extract. All standard glassware, laboratory instruments, and equipment used in this study met the experimental requirements.

Whole plant of *C. reflexa* Roxb. was obtained locally from Kangra district, Himachal Pradesh, India, and authenticated by a botanist, Dr. K.C. Bhatt, Principal Scientist at the National Bureau of Plant Genetic Resources (ICAR) at New Delhi, India. A specimen was deposited there with the herbarium number AC-81/2022. Using the whole plant of *C. reflexa* ensures the extraction of a broad spectrum of bioactive compounds, enhancing therapeutic efficacy through synergistic action, which is crucial for managing multifactorial conditions like urolithiasis. This approach aligns with traditional practices and is pharmacologically relevant, as its phytochemicals are distributed throughout the plant due to its parasitic nature. Whole plant extracts offer a combination of flavonoids, phenolics, and triterpenoids that may act on multiple targets, improving efficacy while reducing side effects.

Animals, diet, and ethical statement

Male Wistar albino rats within a weight range of 200 and 250 g were kept in polypropylene cages with husk bedding filling under standard environmental conditions, with a temperature of 25 ± 2 °C and 55 ± 5% relative humidity. The rats consumed their standard pellet diet at their own pace while they received unlimited water access.

Upon arrival, all rats underwent a one-week

acclimatization before experimental groups were assigned. This study executed all procedures according to the Committee for the Control and Supervision of Experiments on Animals (CCSEA) and the Organization for Economic Co-operation and Development (OECD) guidelines. The Institutional Animal Ethical Committee granted ethical approval for the study under proposal No. CCSEA/LIPH/2023/35.

Preparation of plant extract

The plant material was cleaned, shaded, dried, and coarsely powdered using a mechanical grinder. A 100 g sample was initially defatted with petroleum ether (60-80 °C) and subsequently dried below 40 °C. The dried material was then subjected to extraction with 500 mL of 95% ethanol using a Soxhlet apparatus. The extract was filtered and concentrated by solvent evaporation under reduced pressure using a rotatory evaporator (ROLEX, Techno Scientific), yielding the crude ethanolic extract (EECR). The resulting extract was stored in a sealed container at 4°C until further use.

Qualitative analysis of phytochemicals

The qualitative phytochemical analysis of EECR was conducted using standard analytical procedures to detect the presence of major classes of bioactive compounds. These included alkaloids (identified by Mayer's and Dragendorff's tests), flavonoids (via the lead acetate and alkaline reagent tests), saponins (froth test), terpenoids (Salkowski test), glycosides (Keller-Kiliani test), tannins (Ferric chloride test), and polyphenols (Folin-Ciocalteu reagent). These methods are widely accepted for preliminary screening of phytochemicals in plant extracts (24,25).

Quantitative analysis of phytochemicals

Determination of total polyphenolic content

The total phenolic content determination for EECR by spectrophotometry relied on the Folin-Ciocalteu method, with gallic acid as the reference standard. A reaction mixture for testing was obtained through a combination of 0.5 mL plant extract solution with 3 mL demineralized water, followed by adding 0.25 mL Folin-Ciocalteu reagent after appropriate shaking. A mixture of 0.5 mL plant extract and 3 mL demineralized water received 0.25 mL Folin-Ciocalteu reagent before dark incubation for 5 minutes. Then, 1 mL sodium carbonate (Na_2CO_3) 7.5% solution was added to all tubes, followed by a 90-minute dark incubation at room temperature. The study included the use of reagent blanks for preparing all samples. The Shimadzu UV-1900i spectrophotometer measured absorbance using λ max = 760 nm against a reagent blank solution. The total phenolic compounds content in extracts showed an expression through milligrams of gallic acid equivalent (GAE) relative to 100 g sample weight (mg GA/100 g sample). The calculation of total phenolic compounds occurred through the application of the linear calibration curve. (26,27).

Determination of total flavonoid content

An aluminum chloride assay measured the flavonoid content of the EECR. The test tube received 0.5 mL of extract solution containing 2 mL of distilled water. 0.15 mL of 5% $NaNO_2$ solution was added to each test tube. A test solution of 0.15 mL 5% NaNO2 solution was added to each test tube. The test solution received 0.15 mL of 10% $AlCl_3$ solution following a 5-minute incubation period. The test tube received 1 mL of 1M NaOH solution, and the final volume was made up to 5 mL with distilled water. The Shimadzu UV-1900i spectrophotometer was used to evaluate the ensuing solution absorbance at λ max 510 nm after 10 minutes. Standard measurement of flavonoid content used Quercetin to determine total concentrations as quercetin equivalent per 100 g of sample (mg QE/100 g sample). The linear calibration curve's derived equation was used to determine the value (26).

Determination of total terpenoid content

The vanillin-H_2SO_4 assay, with minor modifications, served to determine the total terpenoid content found in EECR. The solution consisting of 2 g vanillin dissolved in 100 mL 5% H_2SO_4 comprised the 2% vanillin-H_2SO_4 reagent. One millilitre of 2% vanillin-H2SO4 reagent was mixed with 0.5 mL of methanol-extracted products. The ice bath agitation mixed all the components before the tubes were immersed at 60 °C for 20 minutes in a water bath. The absorbance was measured at λ max 608 nm through a Shimadzu UV-1900i spectrophotometer after the tubes reached 25 °C for 5 minutes. The analysis used standard compounds of Linalool to determine total terpenoid concentrations in the sample at 100 mg LU/100 g. The amount of total terpenoid content was calculated from the linear calibration curve equation (27).

Determination of diuretic activity

Determination of EECR's diuretic properties occurred through the protocol described by Lipschitz et al (28,29). The study involved 24 healthy Wistar albino male rats weighing 200–250 g that were randomly allocated into four groups comprising six animals in each set. The experimental animals received 18 hours of food restriction while receiving water, the only available fluid during this period. A single study group allocation divided the animals into multiple groups. 200 mg/kg and 400 mg/kg doses of EECR were administered to the animals belonging to the treatment group. The 200 mg/kg and 400 mg/kg doses of *C. reflexa* extract were selected based on prior studies demonstrating significant, dose-dependent therapeutic effects without toxicity. Acute toxicity evaluations confirmed safety up to 2000 mg/kg. These doses thus represent effective and safe ranges for pharmacological assessment.

- *Group I:* Normal control group received orally carboxymethyl cellulose (CMC) 0.5% w/v at a dose of 10 mL/kg.
- *Group 2:* The Positive control group received an oral administration of furosemide, serving as a reference diuretic drug, followed by a dose of 15 mg/kg.
- *Group 3:* Test group I followed by a single oral dose of EECR (200 mg/kg).
- *Group 4:* Test group II underwent a single oral administration of 400 mg/kg.

Following an administration duration of 1 hour, rats were placed into a separate metabolic cage for urine collection. The duration of the urine volume measurement lasted seven hours. The end of the tail base served for bladder drainage to obtain complete urine output. The electrolyte measurement of Na^+ and K^+ in rat urine proceeded through selective electrode analysis handled by AVL 9180 Electrolyte Analyzer, manufactured by Roche (30).

Antiurolithiatic studies

The study investigated the antiurolithiatic effects of EECR through testing on male Wistar albino rats (n=30) with EG-induced urolithiasis. This well-established method was adapted with minor modifications from previously reported protocols (31,32).

In this study, animals were randomly placed into various experimental groups. Urolithiasis was induced through daily administration of 0.75% v/v EG solution in drinking water ad libitum for 28 days. The first 14 days of EG administration were considered the induction phase, while the subsequent 14 days (Days 15–28) were designated as the treatment phase to assess the therapeutic potential of EECR.

Study design: Dosing and grouping

The experiment employed five groups of six animals each that were given different treatments as follows:

- *Group 1:* The normal control group received standard pellet food with free access to water for 28 days without any treatment.
- *Group 2:* The negative control group received 0.75% v/v EG through drinking water from day 1 to day 28 to induce urolithiasis.
- *Group 3:* The positive control group was administered the standard drug lumasiran with a dose of 3 mg/kg, s.c. on the 15th day of urolithiasis induction along with EG administration.
- *Group 4:* Treatment group I was involved in the daily administration of EECR with a dose of 200 mg/kg orally from day 15 to day 28; EG was also administered for lithiasis induction.
- *Group 5:* Treatment group II was involved in the daily administration of EECR with a dose of 400 mg/kg orally from day 15 to day 28; EG was also administered.

Determination of antiurolithiatic parameters

The antiurolithiatic potential of EECR was assessed using an EG-induced urolithiasis model, a well-established approach for inducing kidney stone formation in rats. EG (0.75% v/v) administration leads to hyperoxaluria, resulting in calcium oxalate crystal deposition in the renal system, causing structural and functional impairments.

To evaluate the antiurolithiatic effects, urine samples were collected on days 14 and 28 for the biochemical estimation of phosphate, oxalate, calcium, UA, urea, citrate, magnesium, and creatinine clearance, along with the measurement of urine pH and total urine volume. Additionally, serum biochemical parameters, including calcium, phosphate, uric acid, urea, magnesium, and creatinine, were analyzed at the end of the study to assess systemic metabolic alterations associated with urolithiasis.

On day 28, all animals were sacrificed, and both kidneys were harvested for further analysis. Histopathological examination was carried out to evaluate renal tissue changes, including crystal deposition, tubular damage, and inflammatory responses. Furthermore, kidney homogenates were analyzed for biochemical markers such as oxalate, calcium, uric acid, phosphate and catalase activity to assess oxidative stress and nephroprotective effects (31,32).

Estimation of biochemical parameters

Urine collection and analysis

Urine samples were drawn from all animals on the 14th and 28th days through metabolic cages. The total urine volume was measured, and a few drops of thymol were added as a preservative. Urinary parameters, including oxalate, calcium, phosphate, uric acid, urea, citrate, creatinine, and magnesium, were estimated using biochemical diagnostic kits as per the manufacturer's instructions (33). A properly calibrated digital pH meter was utilized to measure the pH of fresh urine samples.

Serum collection and analysis

Under light anesthesia, blood samples were taken via the retro-orbital plexus on day 28. The serum was separated for biochemical estimation after the samples were centrifuged for ten minutes at 4000 rpm to minimize cell damage. Urolithiasis-related markers such as calcium, phosphate, uric acid, urea, magnesium, and creatinine were quantified using biochemical kits according to the prescribed protocols (33).

Kidney histopathology analysis

After blood collection on the 28th day, all the animals of different groups were sacrificed, and both kidneys were harvested. In normal saline, at ice-cold temperature, organ specimens were cleaned to remove their extraneous contents. Each kidney was preserved in 10% v/v neutral formalin solution before paraffin embedding, followed by sectioning into 5 µm thin slices. Light microscopy

examination of hematoxylin and eosin (H&E)-stained sections evaluated crystal accumulation together with the extent of tissue damage and all other histological changes detected. The second kidney underwent a processing method that involved fine chopping followed by homogenization in Tris-HCl buffer with pH 7.4 to create a solution containing 20% tissue homogenate. A biochemical test used the centrifuged supernatant obtained from the 10-minute 2000 rpm spin of the homogenate. Analysis of lithiatic biomarkers, calcium, uric acid, oxalate, phosphate, and catalase was performed to determine the levels of the aforementioned nephric cell constituents (34,35).

Statistical analysis

The study analyzed data points by mean ± standard deviation (SD) through statistically significant analysis based on analysis of variance (ANOVA) between different experimental groups. The analysis of statistical significance used a *P* value below 0.005. A statistical method was employed to assess EECR's therapeutic impact on preventing and treating urolithiasis in rats induced by EG exposure.

Results

Extraction yield of EECR

The extract was successfully obtained with a percentage yield of 9.34 w/w and was further subjected to qualitative and quantitative analysis.

Qualitative analysis of phytochemicals

Phytochemical screening of EECR exposed the presence of alkaloids, steroids, terpenoids, tannins, glucosides, and polyphenols (Table 1).

Quantitative analysis of phytochemicals

Quantitative estimation of phytochemicals for EECR revealed the presence of various phytoconstituents, i.e. polyphenols (271±1.61 mg/100 g), flavonoids (186.30±1.05 mg/100 g), and terpenoids (123.30±2.57 mg/100 g).

Diuretic activity

Seven hours after administering furosemide (15 mg/kg), this standard reference drug showed a significant increase in urine output, with a diuretic index of 4.18. This was accompanied by a notable increase in sodium and potassium concentrations. In comparison, the EECR at doses of 200 mg/kg and 400 mg/kg produced diuretic indices of 1.58 and 1.80, respectively. EECR also significantly increased urine volume (2.24 mL at 200 mg/kg and 2.58 mL at 400 mg/kg), sodium concentrations (64.25 mEq/L and 64.95 mEq/L), and potassium concentrations (10.53 mEq/L and 10.76 mEq/L) at the same respective doses. These results indicate a dose-dependent diuretic effect of EECR, which, although lower than furosemide, demonstrates comparable activity and supports its potential as a natural diuretic agent (Figure 1).

Table 1. Phytochemical screening of ethanolic extract of *Cuscuta reflexa* (EECR; Whole plant)

Group of compounds	Observation EECR
Alkaloid	+
Flavonoid	+
Saponin	-
Steroid	+
Terpenoid	+
Glycoside	+
Tannin	+
Polyphenols	+

Note: + indicates the presence of phytochemicals, whereas the – sign indicates an absence

Effects on urine biochemical parameters

Following the induction of lithiatic condition with EG on the 14th day, the disease control group exhibited a marked decline in urine volume and altered urine biochemical markers, including calcium, oxalate, phosphate, UA, creatinine, and urea, as compared to the normal control group, with statistically significant increases in urinary parameter levels. After the 28th day, upon treatment with standard and test drugs (200 mg/kg and 400 mg/kg), the standard group demonstrated significant restoration of urine volume and near normalization of urinary biochemical markers. Treatment with the extract showed dose-dependent improvements, with the higher dose generally producing better outcomes than the lower dose. Furthermore, notable improvements in urinary pH (6.23 ± 0.16 and 6.37 ± 0.18), calcium (6.03 ± 0.20 mg/mL/24 h and 5.69 ± 0.25 mg/mL/24 h), oxalate (12.09 ± 0.39 mg/mL/24 h and 11.81 ± 0.41 mg/mL/24 h), citrate (9.43 ± 0.29 mg/mL/24 h and 9.64 ± 0.31 mg/mL/24 h) and magnesium concentrations (1.97 ± 0.14 mg/mL/24 h and 2.29 ± 0.19 mg/mL/24 h), towards normal levels, which indicates that EECR possesses potential as a dose-dependent therapeutic agent for diuretic and renal function enhancement (Figure 2).

Effects on serum biochemical parameters

On the fourteenth day of induction, the disease control group first showed markedly raised serum levels of creatinine, urea, phosphate, calcium, and uric acid, indicating the onset of a pathological state. By the 28th day after treatment, the standard drug effectively normalized the levels of calcium, phosphate, uric acid, and creatinine, and substantially reduced urea concentrations. The test drug treatment at different concentrations, followed by 200 and 400 mg/kg, resulted in a dose-dependent amelioration of these markers, while the higher dose of 400 mg/kg demonstrated greater efficacy. Specifically, calcium levels decreased to 15.52 mg/dL and 11.58 mg/dL in the 200 and 400 mg/kg groups, respectively. Phosphate

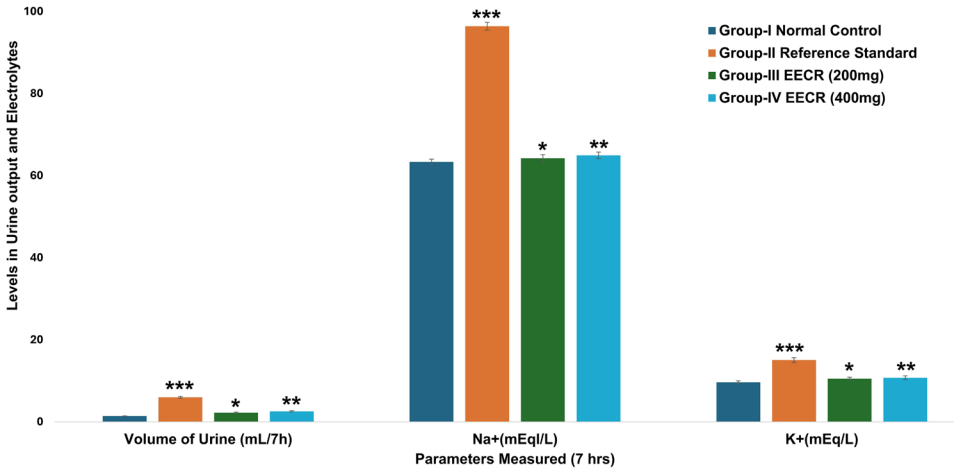

Figure 1. Graphical representation of the effect of ethanolic extract of *Cuscuta reflexa* (EECR) on diuresis and urinary parameters of experimental animals. Bar graph represents urine output volume (in mL) and sodium and potassium ion concentrations (mEq/L) in the urine of animals belonging to various experimental groups. Data show mean ± SD values (n=6/group). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ compared to the normal control.

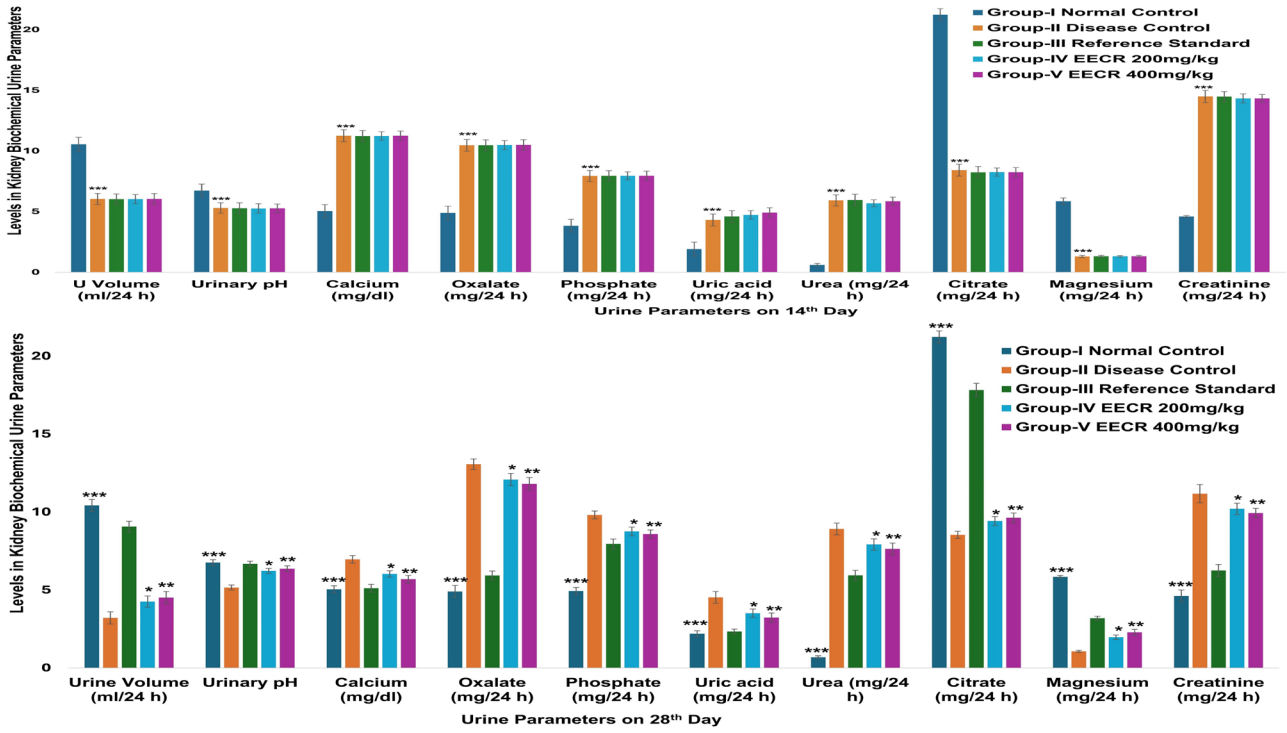

Figure 2. Effect of ethanolic extract of *Cuscuta reflexa* (EECR) on biochemical urine parameters of experimental animals. Data show mean ± SD values (n=6). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ compared to the normal control.

and uric acid levels comparably improved, with the higher dose yielding phosphate levels of 7.14 mg/dL and uric acid levels of 7.13 mg/dL. Creatinine levels also exhibited significant reductions, particularly with the 400 mg/kg dose reaching 1.69 mg/dL. These findings (Figure 3) suggest that EECR possesses therapeutic potential in mitigating serum biochemical disturbances associated with kidney dysfunction, with a clear dose-response relationship evident.

Effects on kidney homogenate parameters

The diseased group exhibited significantly increased levels of lithiatic biomarkers calcium, phosphate, uric acid, and oxalate, markedly reduced catalase activity in kidney homogenate, confirming the induction of renal dysfunction. Treatment with the standard drug significantly ameliorated the concentrations of these biomarkers and improved catalase activity. The extract-treated groups at doses of 200 and 400 mg/kg demonstrated

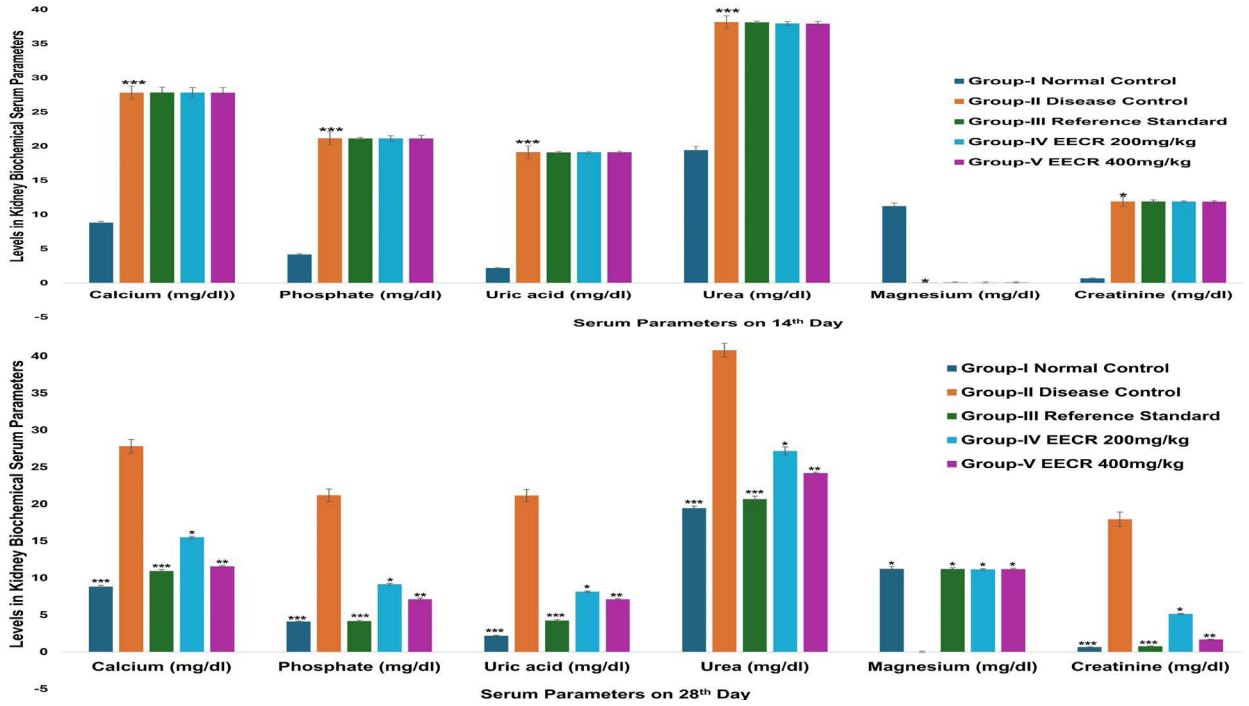

Figure 3. Effect of ethanolic extract of *Cuscuta reflexa* (EECR) on various serum parameters in experimental animals. Graphs illustrate the effect of EECR administration for 14 days (upper panel) and 28 days (lower panel) on key serum parameters. Data show mean ± SD values (n = 6). Comparisons were made between groups I, III, IV, and V relative to group II (disease control); ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ compared to the normal control.

dose-dependent marginally superior biochemical corrections; calcium levels decreased to 7.83 mg/gm tissue and phosphate to 5.09 mg/gm tissue at the higher dose. Uric acid and oxalate levels similarly improved, and catalase activity increased to 1.08 nmoles of H_2O_2 utilized/min/mg protein, approaching normal levels (Figure 4). The 400 mg/kg dose of EECR showed a stronger protective effect against kidney damage compared to 200 mg/kg dose ($P < 0.01$), indicating a clear dose-dependent response. Although its effectiveness was slightly lower than that of the standard drug ($P < 0.001$), the results suggest that EECR has promising potential for managing kidney-related conditions, including nephrolithiasis.

Histopathological evaluation of kidney tissues to assess EECR effect on EG-induced urolithiasis

The induction of renal urolithiasis by EG resulted in significant histological alterations. Microscopic examination of kidney histological sections revealed normal architecture in group I normal control (Figure 5A).

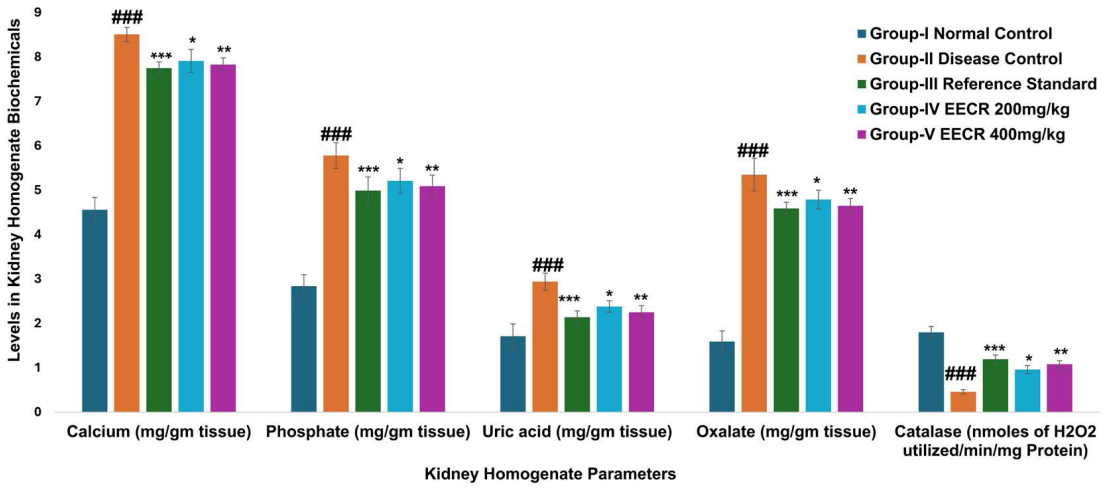

Figure 4. Effect of ethanolic extract of *Cuscuta reflexa* (EECR) on kidney homogenate parameters in experimental animals. Data are expressed as mean ± SD (n = 6). Group comparisons were made as follows: Groups III, IV, and V were compared with goup II (disease control); ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ versus disease control. Comparisons between group II (disease control) and group I (normal control) confirmed disease induction; ###$P < 0.001$, ##$P < 0.01$, and #$P < 0.05$ versus normal control.

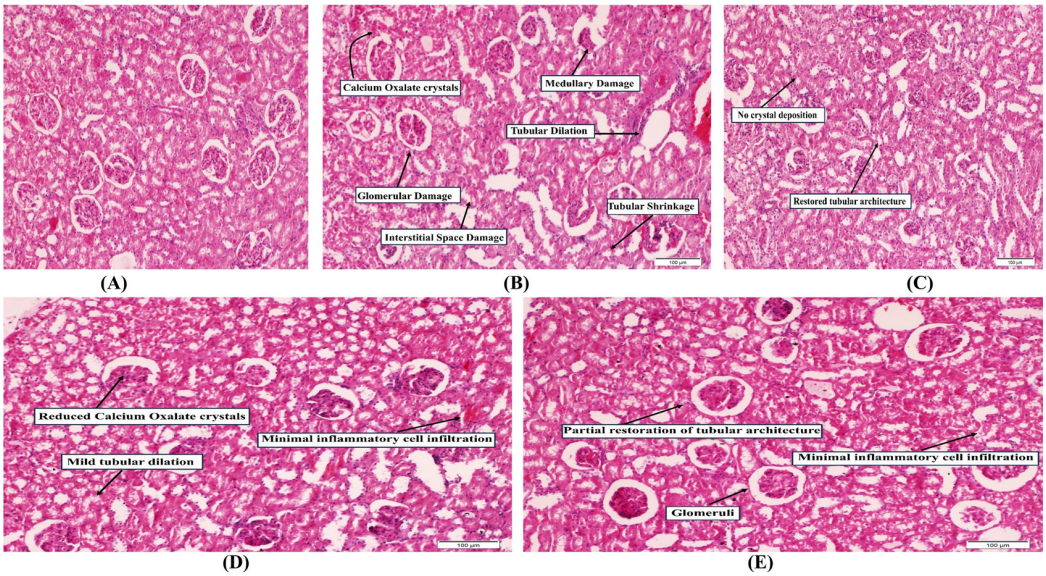

Figure 5. Effect of ethanolic extract of *Cuscuta reflexa* (EECR) on histopathology of the kidney excised from various experimental groups. Histopathological observations (100x) made from H&E-stained kidney sections collected from different groups of animals show the presence of calcium oxalate crystals in the negative control group. (A) Group I: Normal control, (B) Group II: Disease control (negative control), (C) Group III: Standard group (positive control) treated with lumasiran (3 mg/kg/S.C.), (D) Group IV: Treatment group I treated with EECR 200 mg/kg orally, and (E) Group V: Treatment group II treated with EECR 400 mg/kg orally.

In group II disease control (negative control), histological analysis demonstrated the existence of calcium oxalate crystals and serious harm to the medulla, glomeruli, tubules, and interstitial spaces. Along with noticeable histological alterations such as tubular dilatation and tubular shrinkage, interstitial mononuclear and inflammatory cell infiltration were also noted (Figure 5B). In group III standard group (positive control) treated with lumasiran, crystal depositions were not observed, and renal damage due to tubular atrophy was restored to normal levels (Figure 5C). In group IV treatment group-I (200 mg/kg/p.o.) and group V treatment group-II (400 mg/kg/p.o.) treated with EECR, microscopic examination indicated that major renal damage was ameliorated, accompanied by a reduced amount of calcium oxalate crystal deposition in the intratubular space and restoration of tubular abnormality compared to group II disease control, in a dose-dependent manner (Figure 5D and 5E).

Discussion

The rising global incidence of urolithiasis, driven by modern lifestyles, is marked by high recurrence despite conventional treatments, which often fail to prevent relapse. In contrast, medicinal plants rich in bioactive compounds show promise as effective, natural alternatives for preventing stone formation and supporting renal function (36-40).

The well-known model for urolithiasis by administering ethylene glycol was employed to examine the protective effects of EECR on the pathogenesis of kidney stone and assess it as a possible candidate for treatment against urolithiasis. EG undergoes hepatic metabolism to glycolic acid and oxalic acid, leading to hyperoxaluria, which plays a pivotal role in calcium oxalate crystal deposition within renal tubules. Additionally, glyoxylate, a byproduct of glycine and hydroxyproline catabolism, is normally converted to glycine by AGT in the liver. In AGT deficiency, glyoxylate accumulates and is oxidized to oxalate by cytosolic LDH. As oxalate cannot be metabolized, it is excreted in urine, where it combines with calcium to form crystals, leading to urolithiasis (41). This accumulation results in oxidative stress, renal inflammation, and progressive damage to kidney tissues, closely mimicking clinical nephrolithiasis (42).

In the present study, the quantitative and qualitative estimation of *C. reflexa* herbal extract exposed the existence of diverse compounds, including flavonoids, alkaloids, terpenoids, steroids, glycosides, polyphenols, and tannins (43).

The administration of EECR exhibited significant reno-protective potential, including raised urine output as well as pH, decreased calcium oxalate crystal deposition, reduced excretion of oxalate, calcium, and phosphate, and increased urinary magnesium excretion. The said potential may be attributed to the multiple functions of *C. reflexa*, recognized for its diuretic as well as nephroprotective activities (21,44). Furthermore, the antioxidant activity of EECR, attributed to its rich flavonoid and polyphenol content, likely contributes to oxidative stress reduction, improved kidney glutathione levels, and restoration of renal tissues following EG-induced nephrotoxicity (45). Additionally, the anti-inflammatory properties of

bioactive compounds present in Cuscuta reflexa may aid in protecting nephrons and alleviating renal inflammation, further supporting its therapeutic potential in managing urolithiasis (46).

The diuretic activity of an extract is divided into good (>1.50), moderate (1.00-1.50), mild (0.72-1.00), or nil (<0.72) based on diuretic index (47). The present investigation assessed the diuretic efficacy of EECR at 200 and 400 mg/kg doses, comparing it with furosemide and a normal group. Results revealed dose-dependent diuretic effects of EECR, with 400 mg/kg dose, that increased urine output (2.58 ± 0.14 mL) compared to the control (1.44 ± 0.03 mL), yielding a diuretic index of 1.80. However, this effect was less pronounced than that of furosemide (5.98 ± 0.21 mL, diuretic index 4.18). EECR at 400 mg/kg enhanced sodium excretion (64.95 ± 0.78 mEq/L) relative to the control (63.36 ± 0.68 mEq/L), but not to the extent of furosemide (96.42 ± 0.95 mEq/L). Potassium excretion also increased with EECR at 400 mg/kg (10.76 ± 0.46 mEq/L) compared to the control (9.65 ± 0.37 mEq/L), yet remained below furosemide levels (15.08 ± 0.59 mEq/L). The Na^+/K^+ ratio in EECR-treated groups (6.03 at 400 mg/kg) was lower than that of the control (6.56) and comparable to furosemide (6.39), suggesting moderate natriuretic selectivity. These observations indicate that EECR, while less potent than furosemide, may offer a more favourable electrolyte balance with reduced potassium loss, possibly operating through a mechanism akin to thiazide-like diuretics. Comparative analysis with prior research on herbal diuretics (48,49) corroborates the dose-dependent natriuretic and kaliuretic effects with milder potassium depletion, supporting its potential for safe long-term use. In summary, EECR demonstrates promise as a mild diuretic, with 400 mg/kg exhibiting more effectiveness compared with 200 mg/kg.

As per previous studies (50,51) on EG-induced urolithiasis, plant extracts rich in polyphenols and flavonoids ameliorated urinary parameters by mitigating the accumulation of lithogenic ions, particularly calcium and oxalate. In concordance with the said investigations, the administration of EECR at 400 mg/kg attenuated urinary calcium (5.69 ± 0.25 mg/dL) and oxalate (11.81 ± 0.41 mg/24 h) levels, albeit with less efficacy than lumasiran. Additionally, the studies (39) investigated the inhibitory action of citrate in the development of stone, stating that plant extracts elevate citrate levels to counteract calcium oxalate supersaturation. Correspondingly, EECR treatment enhanced urinary citrate concentrations (9.64 ± 0.31 mg/24 h) relative to the disease group (8.54 ± 0.23 mg/24 h, $P<0.005$), though this effect was less pronounced compared to the standard drug (17.83 ± 0.43 mg/24 h).

The observed diuretic effects in this study corroborate the findings of Ghelani et al (52). The authors established that increased urine output facilitates the reduction of stone-forming ion supersaturation. EECR at 400 mg/kg moderately augmented the volume of urine (4.52 ± 0.39 mL/24 h) compared to the disease group (3.21 ± 0.39 mL/24 h, $P<0.005$), although it did not reach the levels achieved by lumasiran (9.07 ± 0.35 mL/24 h, $P<0.001$). Furthermore, the observed improvements in urinary pH with EECR treatment (6.37 ± 0.18) suggest alkalizing properties, in alignment with the findings of (53) emphasized the importance of maintaining an alkaline urinary milieu for preventing calcium oxalate crystal aggregation.

Elevated magnesium levels play a critical role in preventing urolithiasis because magnesium binds with oxalate, which inhibits the development of calcium oxalate crystals. Present research demonstrated a significant increase in magnesium excretion (2.29 ± 0.19 mg/24 h) following 400 mg/kg EECR administration, in comparison to the disease group (1.06 ± 0.07 mg/24 h, $P<0.05$). This observation is congruent with previous reports published by Makasana et al (54) elucidating the role of magnesium-supplemented treatments in mitigating stone formation. However, lumasiran exhibited superior efficacy (3.19 ± 0.13 mg/24 h, $P<0.001$), indicating a more robust protective effect.

This study further elucidated the significance of restoring urinary creatinine and UA levels to mitigate renal damage associated with urolithiasis. While the disease group exhibited significantly elevated creatinine (11.18 ± 0.59 mg/24 h) and UA (4.53 ± 0.38 mg/24 h), EECR at 400 mg/kg reduced these levels to 9.94 ± 0.29 mg/24 h and 3.23 ± 0.29 mg/24 h, substantiating its protective effects and the outcomes synchronize with the results (55), which observed similar renal-protective effects of plant-based treatments in urolithiasis models.

Overall, the anti-urolithic effects of EECR are comparable to previously reported studies on herbal formulations, with its activity likely attributed to its polyphenolic content, alkalizing properties, and capacity to enhance citrate and magnesium excretion while reducing lithogenic ion concentrations. Although EECR demonstrated significant efficacy, particularly at 400 mg/kg, its effects were less pronounced than lumasiran, underscoring the necessity for further studies to optimize its dose and elucidate its mechanism of action. As per recent studies, the safety and therapeutic potential of ethanolic *C. reflexa* extract was well tolerated in rats up to 2000 mg/kg. It exhibits nephroprotective, anti-inflammatory, antioxidant, and anti-arthritic activities, with significant effects at 200 and 400 mg/kg doses (56). Traditional systems like Unani and Ayurveda also recognize its use in kidney disorders. Its phytochemicals—flavonoids, phenolics, and triterpenoids—contribute to free radical scavenging, anti-inflammatory, and diuretic actions, supporting its role in urolithiasis management. (56,57).

The findings of this investigation reveal that the ethanolic extract of the test compound (EECR) effectively ameliorates the pathological alterations in serum parameters associated with EG-induced urolithiasis,

corroborating and extending previous research. The disease group exhibited elevated serum calcium levels, which were markedly reduced by EECR administration at 400 mg/kg; observation aligns with the work of (58) and reported analogous reductions in calcium levels following treatment with polyphenol-rich plant extracts, attributing the effect to their calcium-binding capabilities. Serum phosphate, which was elevated to 21.19 ± 0.86 mg/dL in the disease group, decreased to 7.35 ± 0.14 mg/dL with EECR at 400 mg/kg. Similar findings were reported (59) and documented significant phosphate reduction in urolithiasis models treated with herbal formulations targeting renal phosphate transport. EECR administration (400 mg/kg) also led to a decrease in elevated UA levels from 21.16 ± 0.83 mg/dL to 7.43 ± 0.08 mg/dL, a trend that parallels the observations (60) elucidating the role of antioxidant-rich plant compounds in mitigating UA levels through the mitigation of oxidative stress and xanthine oxidase activity. The observed decrease in serum urea following EECR treatment is consistent with research (61), suggesting enhanced renal filtration due to the nephroprotective properties of herbal extracts. Serum magnesium, which was severely depleted in the disease group (0.02 ± 0.09 mg/dL), was restored to normal levels (11.26 ± 0.13 mg/dL) with EECR at 400 mg/kg. This finding aligns with the work (62) wherein the significance of magnesium in inhibiting calcium oxalate crystallization has been reported. Lastly, the reduction of creatinine levels following EECR treatment follows the outcomes reported (61), which revealed the efficacy of plant-based therapies in restoring renal function through the prevention of tubular damage. Collectively, these results highlight the potential of EECR as a promising anti-urolithic agent and elucidate its dose-dependent efficacy and nephroprotective mechanisms.

The present research demonstrated the efficacy of EECR in mitigating pathological changes in kidney homogenate parameters associated with EG-induced urolithiasis, aligning with and expanding upon findings from recent studies. Elevated tissue calcium levels in the disease group were significantly reduced by EECR at 400 mg/kg ($p<0.001$), consistent with the findings (63) and reported reductions in calcium deposition with polyphenol-rich herbal formulations due to their inhibitory effects on calcium crystallization. Similarly, EECR at 400 mg/kg reduced elevated phosphate levels, which highlighted the phosphate-lowering effects of plant-based antioxidants that reduce oxidative damage to renal tissues. The elevated UA levels observed in the disease group were also significantly reduced by EECR at 400 mg/kg, a trend similar to that reported (64), and it was found that plant-derived xanthine oxidase inhibitors effectively reduced UA accumulation in kidney tissues. Elevated oxalate levels were significantly lowered by EECR at 400 mg/kg, which demonstrated that phytochemicals can inhibit oxalate crystal aggregation and deposition in renal tissues. Furthermore, the improvement in catalase activity with EECR at 400 mg/kg compared to the negative control group was observed, and it was concluded that these findings collectively may affirm the potential of EECR as an anti-urolithic agent with mechanisms targeting lithogenic factors and oxidative damage, aligning with and expanding upon previous studies.

The histopathological evaluation of kidney tissues proved the therapeutic effects of EECR in protecting the tissues. The present study demonstrated clear differences in renal architecture across treatment groups. Group I (normal control) exhibited normal kidney histology, including intact glomeruli, tubules, and interstitial spaces. However, EG administration in group II (disease control) caused significant renal damage, as evidenced by extensive calcium oxalate crystal deposition, tubular dilatation, interstitial inflammation, and atrophy of renal tubules. These results are in line with previous research conducted (65) and show EG exposure led to similar histological damage due to oxidative stress and crystal-induced tubular obstruction.

Group III (standard group treated with lumasiran, 3 mg/kg/s.c.) showed significant amelioration of renal injury, with the absence of calcium oxalate crystals, and restoration of tubular architecture. These outcomes corroborate the findings (66) and demonstrate that lumasiran effectively reduced crystal deposition by targeting glyoxylate metabolism and decreasing oxalate production. Lumasiran, approved for hyperoxaluria, targets oxalate overproduction caused by AGT deficiency in primary hyperoxaluria type 1 (PH1), where excess glyoxylate is converted to oxalate by LDH. This leads to calcium oxalate crystal formation and urolithiasis. Similarly, the ethylene glycol-induced model in our study mimics this condition by elevating urinary oxalate. Thus, comparing the antiurolithiatic effects of *C. reflexa* extract with lumasiran under these pathophysiological conditions offers a scientifically relevant evaluation of therapeutic potential (67). Group IV (EECR 200 mg/kg) displayed a decrease in crystal deposition and moderate restoration of tubular structure, while group V (EECR 400 mg/kg) demonstrated further improvement with minimal calcium oxalate crystal accumulation, reduced interstitial inflammation, and near-complete recovery of tubular morphology. Previous studies also highlighted the nephroprotective potential of polyphenol-rich herbal extracts in reducing crystal deposition and oxidative damage (68).

The ameliorative potential of EECR seems to be due to its antioxidant and anti-inflammatory properties, which likely help in scavenging reactive oxygen species (ROS) and protecting renal tubular epithelial cells from oxidative stress-induced damage. As noted in earlier studies by (69), antioxidants reduce crystal aggregation and restore renal architecture by mitigating inflammation and tubular injury. In conclusion, histopathological findings

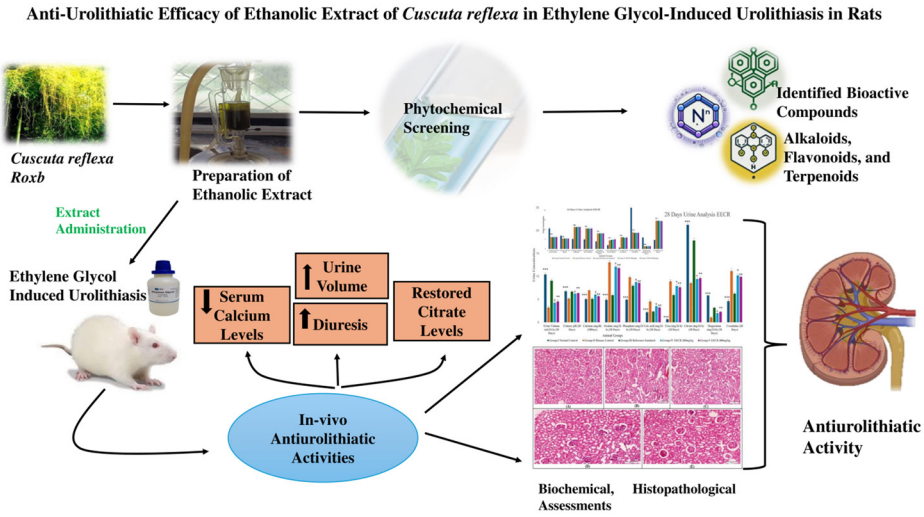

Figure 6. Schematic representation of the anti-urolithiatic efficacy of ethanolic extract of *Cuscuta reflexa* in ethylene glycol-induced urolithiasis in rats.

in the present study demonstrate that EECR significantly attenuates EG-induced renal damage, with the 400 mg/kg dose showing superior efficacy, thereby highlighting its effectiveness as a medicinal agent for urolithiasis, as shown in Figure 6. Future research should focus on elucidating its molecular mechanisms through gene expression studies, proteomics, and metabolomics. Identification of active constituents and validation via *in vitro* and *in vivo* models are essential. Advanced techniques like HPLC/UPLC, western blotting, and immunohistochemistry can aid in confirming biological targets. Standardization, safety evaluation, and clinical trials will be crucial for its development as an affordable, evidence-based herbal therapy.

Conclusion

The study highlights the therapeutic potential of *C. reflexa* ethanolic extract in managing EG-induced urolithiasis through its anti-urolithiatic, antioxidant, anti-inflammatory, and diuretic effects. EECR reduced calcium oxalate crystal deposition, modulated lithogenic factors, enhanced protective urinary components like citrate and magnesium, and restored renal function and architecture in a dose-dependent manner. Its efficacy depicts that it has potential as a natural alternative to standard therapies like lumasiran. Furthermore, EECR shows promise as an affordable therapeutic candidate. Isolation, standardization, and thorough preclinical and clinical evaluation might enable its development into a novel treatment, particularly for resource-limited settings. However, future research must validate its clinical efficacy, explore molecular mechanisms, and optimize dosing. Combining EECR with other treatments could also further enhance its potential in treating urolithiasis and related renal disorders.

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Conflict of interests

The authors declare no conflict of interest.

Data availability statement

All the data is available from the authors and shall be provided upon request.

Ethical considerations

The Institutional Animal Ethical Committee approved the reported protocol for this experiment under proposal No. CCSEA/LIPH/2023/35, and research was carried out following CCSEA and OECD Guidelines.

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