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EVALUATION OF ANTIUROLITHIATIC ACTIVITY OF GREWIA HIRSUTA L. AGAINST ETHYLENE GLYCOL–AMMONIUM CHLORIDE-INDUCED UROLITHIASIS IN ALBINO WISTAR RATS

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ABSTRACT
This study aimed to evaluate the antiurolithiatic activity of Grewia hirsuta L. in an ethylene glycol–ammonium chloride-induced urolithiasis model in albino Wistar rats. The ethylene glycol–ammonium chloride-induced urolithiasis model was established in albino Wistar rats by administering ethylene glycol (0.75%) and ammonium chloride (2%) in drinking water for 28 days. Concurrently, rats were treated with Grewia hirsuta L. extract orally at doses of 200 and 400 mg/kg body weight for the same duration. Various parameters including urinary pH, urine volume, and levels of calcium, oxalate, and phosphate were assessed. Treatment with Grewia hirsuta L. extract significantly reduced urinary calcium, oxalate, and phosphate levels in a dose-dependent manner compared to the urolithiasis-induced control group. Additionally, the extract restored urinary pH and increased urine volume, indicating its potential to inhibit stone formation and promote stone expulsion. In conclusion, Grewia hirsuta L. demonstrates significant antiurolithiatic activity in an ethylene glycol–ammonium chloride-induced urolithiasis model, potentially mediated through its ability to modulate urinary parameters and mitigate renal tissue damage. These findings support the therapeutic potential of Grewia hirsuta L. as a natural remedy for urolithiasis and warrant further investigation into its mechanism of action and clinical efficacy.

KEYWORDS: Grewia hirsuta L., Urolithiasis, Ethylene glycol–ammonium chloride, Herbal medicine.

INTRODUCTION
Urolithiasis, the formation of urinary calculi within the urinary tract, presents a significant global health burden with its prevalence steadily increasing over the years. Recurrence rates of urolithiasis are high, and conventional treatment options often come with adverse effects and limitations, underscoring the urgent need for effective alternative therapies. In recent years, there has been a growing interest in exploring natural products derived from medicinal plants as potential remedies for urolithiasis due to their perceived safety and therapeutic efficacy.[1-2]

Grewia hirsuta L., a member of the Malvaceae family, is a medicinal plant traditionally used in various regions of India and Africa for its therapeutic properties. Grewia hirsuta L. has been documented in traditional medicine for its diuretic, anti-inflammatory, and antioxidant activities.[3] Despite its traditional use, scientific investigations into the antiurolithiatic potential of Grewia hirsuta L. are relatively scarce.

Animal models of urolithiasis, induced by substances such as ethylene glycol and ammonium chloride, provide valuable platforms for studying the pathophysiology of urinary stone formation and evaluating the efficacy of potential therapeutic agents.[4] Ethylene glycol promotes the deposition of calcium oxalate crystals, a predominant component of urinary stones, while ammonium chloride acidifies urine, creating a conducive environment for stone formation. Utilizing these models allows for the assessment of the preventive and therapeutic effects of herbal extracts against urolithiasis.

Urolithiasis, characterized by the formation of urinary calculi within the urinary tract, is a prevalent and burdensome condition affecting millions worldwide.[5] The recurrence rates of urolithiasis remain high, prompting the search for effective therapeutic interventions with minimal side effects. Natural products derived from medicinal plants have emerged as promising candidates due to their perceived safety and potential efficacy.[6]

Grewia hirsuta L., a member of the Malvaceae family, is traditionally used in various regions of India and Africa for its medicinal properties.[7] Several pharmacological studies have highlighted the diverse therapeutic potential of Grewia hirsuta L., including its diuretic, anti-inflammatory, and antioxidant activities.[8]

Experimental models of urolithiasis play a crucial role in evaluating the antiurolithiatic potential of medicinal plants. The ethylene glycol–ammonium chloride-induced urolithiasis model in albino Wistar rats is a well-established model that closely mimics the pathophysiological processes observed in humans.[9] Ethylene glycol promotes the formation of calcium oxalate crystals, while ammonium chloride induces acidic urine, creating a conducive environment for stone formation.

Studies investigating the antiurolithiatic activity of medicinal plants have provided valuable insights. Herbal extracts rich in bioactive compounds such as flavonoids, polyphenols, and saponins have demonstrated promising results in preventing stone formation and promoting stone expulsion.[10] Additionally, these extracts have been shown to modulate urinary parameters, including pH, volume, and composition, thereby reducing the risk of stone recurrence.

In the context of Grewia hirsuta L., limited scientific evidence exists regarding its antiurolithiatic potential. However, given its traditional use and pharmacological properties, Grewia hirsuta L. represents a promising candidate for further investigation in the management of urolithiasis.[11]

This study seeks to investigate the antiurolithiatic activity of Grewia hirsuta L. in an ethylene glycol–ammonium chloride-induced urolithiasis model in albino Wistar rats. By examining various urinary parameters and conducting histopathological analyses of renal tissues, we aim to elucidate the mechanisms underlying the protective effects of Grewia hirsuta L. against stone formation and renal damage.

The findings of this study hold promise for the development of novel therapeutic interventions for urolithiasis, potentially offering safer and more efficacious alternatives to conventional treatments. Furthermore, understanding the pharmacological mechanisms of Grewia hirsuta L. in mitigating urolithiasis may facilitate its integration into clinical practice, addressing an unmet need in urological care.

MATERIAL AND METHODS

Collection and Authentication of plant material

Plant *Grewia hirsuta* was purchased and collected from Vindhya Herbals Sanjeevani Ayurveda, Bhopal, (M.P) India. The identification and authentication of plant was done was identified by Botanist Dr. Saba Naaz, Professor and Head of Department of Botany, Saifia College of Arts and Science, peergate Bhopal. Collected plant material washed under running tap water and kept in shade for drying. Dried plant materials were then powdered using blender and further observed for colour, odour, and texture then placed in packed labelled air tight container for further use.

Plant extraction

In present study, plant material was extracted by continuous hot percolation method using Soxhlet apparatus. Powdered material of *Grewia hirsuta* was placed in thimble of soxhlet apparatus. Soxhlation was performed at 60°C using petroleum ether as non-polar solvent. Exhausted plant material (marc) was dried and afterward re-extracted with methanol solvent. For each solvent, soxhlation was continued till no visual colour change was observed in siphon tube and completion of extraction was confirmed by absence of any residual solvent, when evaporated. Obtained extracts was evaporated using rotary vacuum evaporator (Buchi type) at 40°C. Dried extract was weighed and percentage yield for each extract was determined using formula:

$$\% \text{ Yield} = \frac{\text{Weight of extract}}{\text{Weight of Plant Materialused}} \times 100$$

Qualitative phytochemical estimation of extracts

In order to identify various phyto-constituents namely alkaloids, terpenoids, glycosides, steroids, triterpenoids, flavonoids, carbohydrates, saponins and tannins in different extracts, phytochemical screening was performed using standard procedures.[12-13]

Tests for carbohydrates

A. Molisch test: To 1ml of test sample, 2-3 drops of α-naphthol was added. Conc. sulphuric acid was added along the side of the test tube. The appearance of purple ring at the junction of two liquids was observed, which confirms the presence of carbohydrates in the test samples.

B. Fehling's test: To 1 ml of test sample, similar quantity of Fehling's solution A and B was added and heated on a water bath for few minutes. The development of brick red precipitate was observed.

C. Benedict's test: To 1 ml of test sample, equal quantity of Benedict's reagent was added, boiled. Development of red colour precipitate confirmed the presence of carbohydrates.

Test for alkaloids

All the test extracts were first treated with dil. hydrochloric acid separately and filtered. The filtrate of all the test extracts were exposed to following tests.

A. Mayer's test: 1 ml of the test extract filtrate was treated with 1ml of Mayer`s reagent and formation of cream precipitate was observed.

B. Hager's test: 1 ml of the test extract filtrate was treated with 1mL of Hager`s reagent and formation of yellow precipitate was observed.

C. Wagner's test: 1ml of the test extract filtrate was added to 1ml of Wagner's reagent and formation of reddish-brown precipitate was observed.

Tests for terpenoids

A. Salkowski test: Chloroform solution of test sample was treated with equal amount of conc. sulphuric acid. The presence of steroid components in the test extracts was observed by the appearance of red color in chloroform layer and green fluorescence in acid layer.

B. Libermann - Burchard test: To 2ml of test sample, chloroform was added followed with 2-3 drops of acetic anhydride and conc. sulphuric acid. The test solution was observed for colour change from red to blue and then finally to bluish green colour which confirms the presence of steroids in the test extracts.

Test for flavonoids

A. Shinoda test: The test extract when treated with magnesium turnings and 2-3 drops of conc. hydrochloric acid, pink colour appeared was noted.

B. Alkaline reagent test: 1mL of test sample was dissolved in dilute sodium hydroxide solution, yellow colour appeared was noted.

Tests for tannins and phenolic compounds

A. 5% $FeCl_3$ solution: To 2-3 ml of extract, few drops of 5% $FeCl_3$ solution was added, deep blue-black colour was observed.

B. 10% lead acetate solution: To 2-3 ml of extract, few drops of 10% lead acetate solution was added, white precipitate was observed.

C. Gelatin test: After dissolving some quantity of extract in distilled water, 2 ml of 1% gelatin solution containing 10% sodium chloride was added which led to the formation of white precipitate indicating presence of phenolic compounds.

Tests for Saponins

A. Foam test*:* 1 ml of test sample was dissolved in 20 ml of distilled water and shaked for 15min in a graduated cylinder. Formation of persistent foam around 1cm layer was observed.

Test for proteins and amino acids

A. Ninhydrin test: 3 ml of the test solution was heated with 3 drops of 5% ninhydrin solution, placed a test-tube in a boiling water bath for 10 min and then the colour change was observed.

B. Biuret test: Test sample was treated with same volume of 1% copper sulphate and 4% sodium hydroxide solution and appearance of violet or pink colour was observed.

Test for glycoside

A. Legal test: 1 ml of sodium nitroprusside was added in 1 ml of pyridine solution containing test sample and colour change was observed.

B. Keller killani test: To 2 ml of extract, 3 ml of glacial acetic acid and 1 drop of 5% ferric chloride were added. This solution was carefully transferred to the surface of 2 ml concentrated H_2SO_4 and the observation was noted down.

Test for fats and lipids

A. Spot test: One drop of extract was placed on filter paper and solvent was allowed to evaporate. An oily stain on filter paper indicates the presence of fixed oil.

***In-vitro* anti urolithiasis (Nucleation assay)**

Preparation of synthetic urine

We chose the classical model for the study of oxalate crystallization because of its simplicity and satisfactory reproducibility. This model includes the study of crystallization without inhibitor and with it, in order to assess the inhibiting capacity of any chemical species used. Two solutions of following composition were mixed: A: $Na_2C_2O_4$ (2 m mol/1) and B: Ca Cl_2 $2H_2O$ (10 m mol/1). The two solutions were prepared stock NaCl 9 g to obtain the ionic force like the Indoor environments. The formation and growth of the COM crystals of oxalate from artificial urine at different concentration was the object of our investigation. Artificial urine is prepared by mixing and stirring two equal volumes of 50 ml of solutions A and B at constant temperature (37°C) in capped vessels to give final artificial urine. Mixture agitation was maintained to prevent elimination.

Simulation of the sedimentary crystal formation

The crystal size development was monitored in sample drops every five minutes by polarized microscope. A drop of sample was put on hemacytometer counting chamber and observed sample under microscope at time after 30 min. Calculated number of crystals and catches of sight with a camera. A series of experiments corresponding to the physiological concentrations of 25, 50, 75, and 100% of plants extracts were used. The follow-up of the crystal size development by microscope was carried out at time after 30 min of formation of crystals and catches of sight with a camera. Calculated the percentage of Inhibition (I %) was based on the formula

$$I\% = [(TSI - TAI) / TSI] * 100$$

TSI- represents the number of calcium oxalate monohydrate crystals without inhibitor.

TAI- represents the number of calcium oxalate monohydrate crystals after addition of inhibitor.

Pharmacological study (*In vivo* studies)

Animals were selected randomly from animal house of MCP, Bhopal, India and further divided into various treatment groups randomly and kept in propylene cage with sterile husk as bedding. Relative humidity of 30.7 % at 22±2 °C and 12:12 light and dark cycle was maintained in the animal house and fed with standard pellets (Golden Feeds, New Delhi, India) and water was available *ad libitum*. Institutional Animal Ethics Committee (IAEC), Bhopal has approved all animal experiments with CPCSEA Reg. No.-1824/PO/ERe/S/15.

Animals used

- Strain - *Albino Wistar* rats
- Age - 5-6 weeks
- Sex - either sex
- Body weight - 200±20 g

Acute oral toxicity

Acute toxicity was performed as per OECD 423 guidelines and three albino rats per step were selected. Two to four steps may be necessary to allow for making a judgment on the acute toxicity of the test substance depending on the mortality and/or the morbidity status of the animals. The test extracts were administered in fixed doses as shown in Figure 4.1, using oral cannula and signs of toxicity and mortality was observed, if any (OECD 423, 2001).

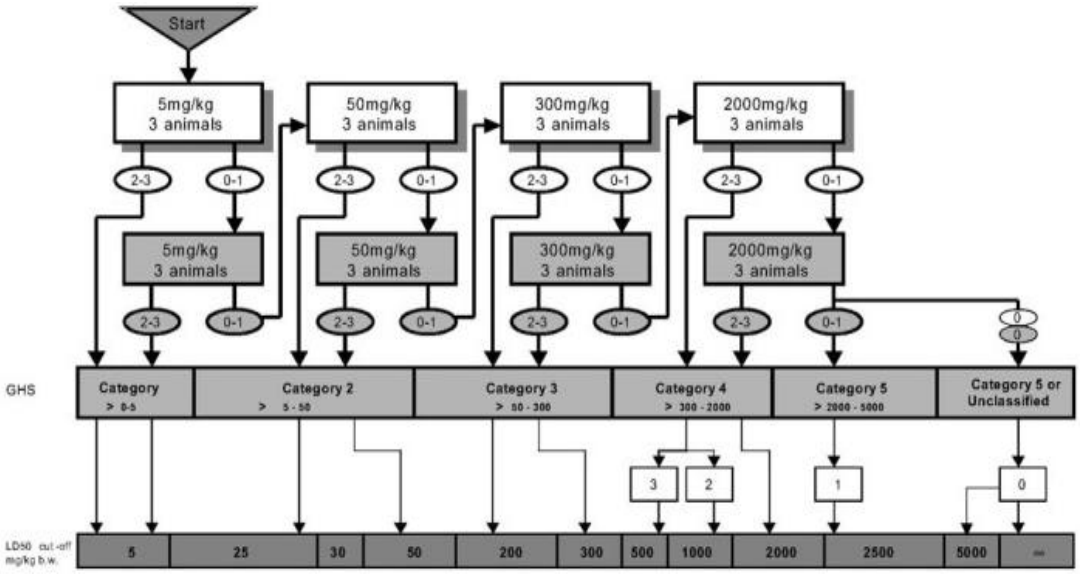

Figure 4.1: Flowchart for Acute Toxicity Study (As per OECD 423 Guidelines).

Table 1: Treatment groups.

Group No.	Treatment	No of animals per group
Group 1	Control	Six
Group 2	EG + NH_4Cl Control	Six
Group 3	GHME200	Six
Group 4	GHME400	Six
Group 5	Cystone750	Six

***In-vivo* urolithiasis activity**

Ethylene glycol + ammonium chloride induced

To study the effect of test material on CaC_2O_4 urolithiasis, group I rats, serving as vehicle treated control, received vehicle once in 24 h, whereas, the untreated rats (group II) received stone inducing treatment for 21 days, comprised of 0.75% (w/v) Ethylene Glycol with 1% (w/v) ammonium chloride for 5 days, following this the water supply will be switched to 0.75% EG alone in water, along with vehicle treatment. Other treatment group will be given test sample/ standard antiurolithic drug dissolved/suspended in vehicle once in 24 h and simultaneously received stone inducing treatment similar to the untreated groups.

Analysis Parameters (For *in-vivo* model)

The weight and activity will be regularly monitored to assess their overall health so that any animal looking lethargic or excessively losing weight could be excluded from the study. Water intake will be also determined simultaneously. Volume and pH will be determined, for last 24 h urine sample.

Blood will be collected through cardiac puncture/ retro orbital puncture from animals under ether anaesthesia for serum separation in order to assess serum creatinine and BUN. Animals will be sacrificed and both the kidneys will be excised, rinsed in ice cold physiological saline and weighed.

The right kidney will be fixed in 10% neutral buffered formalin, processed in series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5µm and stained with Haematoxylin & Eosin for examination under polarized light or by Pizzolato's method; which selectively stains CaC_2O_4 (Pizzolato, 1971), for examination under light microscope. To count the number of crystalline deposits, a sagittal section of each renal specimen will be divided into 8 equal sized regions by four virtual lines according to the method of Tsai et al. (2008). A field of 100× will be then randomly selected from each region and CaC_2O_4deposits will be counted. The total number of CaC_2O_4 deposits in each specimen will be reported as average of the eight readings.

The left kidney will be worked for estimation of enzymes involved in oxidative stress (LPO, SOD, GSH, and Catalase)

Kidney homogenate analysis

The abdomen will be cut open to remove both kidneys from each animal. Isolated kidneys will be cleaned off extraneous tissue and preserved in 10% neutral formalin. The kidneys will be dried at 80°C in a hot air oven. A sample of 100 mg of the dried kidney will be boiled in 10 ml of 1N hydrochloric acid for 30 min and homogenized. The homogenate will be centrifuged at 2000×g for 10 min and the supernatant will be separated. The calcium, phosphate and oxalate content in kidney homogenate will be determined.

Urea nitrogen

Estimation of blood urea nitrogen was estimated by Berthelot method **(Fawcett and Scott, 1960)** using the commercially available kit (Kamineni Life Sciences Pvt. Ltd. Hyderabad, India). 1000 µl of working reagent-I containing urease reagent, and a mixture of salicylate, hypochlorite and nitroprusside was added to 10 µl of serum, 10 µl of standard urea (40 mg/dl) and 10 µl of purified water to prepare test, standard and blank, respectively. All the test tubes were mixed well and incubated at 37 °C for 5 min. Then 1000 µl of reagent-II containing alkaline buffer, was added to all the test tubes, which were incubated at 37 °C for 5 min. Urease catalyses the conversion of urea to ammonia and carbon dioxide. The ammonia thus released reacts with a mixture of salicylate, hypochlorite and nitroprusside to yield indophenol, a blue-green coloured compound. The intensity of the colour produced is directly proportional to the concentration of urea in the sample and is measured spectrophotometrically at 578 nm.[12]

The blood urea was calculated using the following formula

Blood urea nitrogen (mg/dl) = Serum urea X 0.467.

Estimation of calcium oxalate

Calcium has numerous functions within the body, not only as a structure factor in bones and teeth but also in normal neuromuscular function and the clotting of blood. Estimation of calcium was estimated by O-Cresolphthaleincomplexone (OCPC) method **(Moorehead and Briggs, 1974)** using the commercially available kit (Transasia Biomedicals Ltd. baddi). Reagent 1 containing 2-Amino-2-methyl-1-propanol and reagent 2 containing OCPC, 8-hydroxy Quinoline and Hcl, add in equal volume and allow to stand at room temperature. Then take absorbance at 578 nm.[13]

Calcium (mg/dl) = Abs. of Test/Abs of standard×conc. of standard (mg/dl)

Estimation of phosphate

Estimation of phosphate was estimated by Berthelot method **(Daly, J. A., & Ertingshausen, 1972)** using the commercially available kit (vital Diagnostics Pvt. Ltd. Thane, India). Molybdate reagent using as a working reagent for estimation of phosphate present in urea. And take absorbance at 340 nm.[14]

Estimation of urea

Estimation of phosphate was estimated by Berthelot method **(Gutmann, I., & Bergmeyer, 1974)** using the commercially available kit (Transasia Biomedicals Ltd. baddi), India). Reagent 1 and aqua 4 added equally and attain the temperature 15-30^o C. reagent and serum were added in appropriate volume then take absorbance at 340nm.[15]

Biostatical interpretation

Data was calculated as Mean ± SD/SEM. One-way ANOVA was used to interpret the results followed by Bonferroni's test. $P<0.001$ was considered as level of significance.

RESULTS

Extraction

The whole plant of *Grewia hirsuta* was extracted by soxhlation extraction method and the percentage yield calculated by the formula Yield (%) = Weight of the residue obtained/ Weight of the plant material taken×100 was found to be 2.56 % (petroleum ether), and 4.85 % (methanol).

Table 2: Percentage yield of whole plant extracts of *Grewia hirsute.*

Plant name	Percentage yield (%)	
	Pet. ether	Methanol
Grewia hirsuta	2.56	4.85

Qualitative phytochemical screening

Qualitative phytochemical testing of extracts was done to study the presence or absence of various phytochemical constituents using standard tests **(**Kokate *et al.,* 1993). Phytochemical testing of petroleum ether extract of *Grewia hirsuta* (GHPE), methanolic extract of *Grewia hirsuta* (GHME) was performed. Results showed presence of various phytoconstituents in extracts. Phytochemical estimation of GHPE showed the presence of alkaloids and fatty acids. The results of qualitative phytochemical estimation are shown in the table.

Table 3: Phytochemical screening of pet. ether extract of *Grewia hirsuta* (GH).

S. No.	Experiment	Result
		Pet ether extract ER
1. Alkaloids		
1.1	Mayer's reagent test	+ve
1.2	Wagner's reagent test	+ve
1.3	Hager's reagent test	+ve
2. Carbohydrates		
2.1	Molish's test	-ve
2.2	Barfoed's test	-ve
3. Test for Reducing Sugar's		
3.1	Fehling's test	+ve
3.2	Benedict's test	+ve
4. Flavonoids		
4.1	Alkaline reagent test	-ve
4.2	Shinoda test	-ve
4.3	Lead acetate test	-ve
5. Glycoside		
5.1	Borntrager test	-ve
5.2	Legal's test	-ve
5.3	Killer- Killiani test	-ve
6. Tannin and Phenolic compound		
6.1	Ferric chloride test	-ve
6.2	Lead Acetate test	-ve
6.3	Dilute Iodine solution	-ve
7. Saponin		
7.1	Foam Test	-ve
8. Test for Proteins and amino acid		
8.1	Ninhydrin test	-ve
9. Test for Triterpenoids and Steroids		
9.1	Salwonski Test	-ve
9.2	Libberman and Burchard's test	-ve

+ve: Present **–ve**: Absent

Table 4: Phytochemical screening of methanolic extract of *Grewia hirsuta* (GH).

S. No.	Experiment	Result
		Methanolic extract ER
1. Alkaloids		
1.1	Mayer's reagent test	+ve
1.2	Wagner's reagent test	+ve
1.3	Hager's reagent test	+ve
2. Carbohydrates		
2.1	Molish's test	+ve
2.2	Barfoed's test	+ve
3. Test for Reducing Sugar's		
3.1	Fehling's test	-ve
3.2	Benedict's test	-ve
4. Flavonoids		
4.1	Alkaline reagent test	+ve
4.2	Shinoda test	+ve
4.3	Lead acetate test	+ve
5. Glycosides		
5.1	Borntrager test	+ve
5.2	Legal's test	+ve
5.3	Killer- Killiani test	+ve
6. Tannin and Phenolic compound		
6.1	Ferric chloride test	+ve
6.2	Lead Acetate test	+ve

6.3	Dilute Iodine solution	+ve
7. Saponin		
7.1	Foam Test	+ve
8. Test for Proteins and amino acid		
8.1	Ninhydrin test	+ve
***9.* Test for Triterpenoids and Steroids**		
9.1	Salwonski Test	-ve
9.2	Libberman and Burchard's test	-ve

+ve: Present **–ve**: Absent

***In-vitro* anti urolithiasis (Nucleation assay)**

A large number of people in this world are suffering from urinary stone problem. Calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) containing stones are commonly found in kidney.

Herbal extracts may contain substances that inhibit the growth of CaOx crystals. This property of plants may be important in preventing kidney stone formation; CaOx crystals induced by urinary macromolecules are less tightly bound to epithelial cell surfaces, which are then excreted with urine (Wessan *et al*., 1998). The extract may also contain substances that inhibit CaOx crystal aggregation; the agglomeration of particles is a critical step in urinary stone formation, as larger crystals are less likely to pass spontaneously in the urinary tract (Kok and Khan, 1994). If the extract keeps CaOx particles dispersed in solution, they are more easily eliminated.

Table 5: Effect of different concentrations of methanolic extract of *Grewia hirsuta L.* on COM crystal nucleation.

S. No.	Sample	COM/mm3	% Inhibition
1.	Control	362.5	-
2.	25 %	162.5	55.17
3.	50 %	125	65.52
4.	75 %	100	72.41
5.	100 %	62.5	82.76

The formation and growth of the calcium oxalate monohydrate crystals from artificial urine at different concentrations were studied. Stone formation was the due to supersaturation of urine with some urinary salts such as calcium oxalate. The number of calcium oxalate monohydrate crystals was found to be maximum in control. Different percentages of plant extract were tested in order to assess the inhibiting potential of plant extract for oxalate crystallization. In the presence of different percentages of plant extract, the size (length and the width) of the crystals were reduced. It was observed that the plant used in this study inhibited the crystal development with maximum number of crystals found at 25 % physiological extract concentration while at 100 % physiological concentration of extract, the crystal formation was found minimal. Results show that the decrease in number of crystal as well as % inhibition of the formation of calcium oxalate monohydrate crystals was directly proportional to the increase in percentage of plant extract, with minimum inhibition of 55.17% at 25 % physiological concentrations of *Grewia hirsuta L.* extract in methanol while maximum inhibition of 82.76% at 100 % physiological concentrations of *Grewia hirsuta L.* extract in methanol.

Acute oral toxicity

Toxicity testing of new compounds is significant for drug development process. The toxicity of the substances can be determined on experimental animals by analyzing accidental exposures to an *in vivo* substance. Acute oral toxicity study of methanolic extract of *Grewia hirsuta L.* according to OECD-423 guidelines in mice.

Table 6: Acute oral toxicity study of methanol extract of *Grewia hirsuta L.* according to OECD-423 guidelines.

S. No.	Dose	Leathality
1.	5 mg/kg	0/3
2.	50 mg/kg	0/3
3.	300 mg/kg	0/3
4.	2000 mg/kg	0/3

To determine the safety of *Grewia hirsuta L.*, toxicological evaluation was carried out in experimental animals. The selected extracts showed neither visible sign of toxicity nor mortality. The results clearly indicated non-toxicity of the extracts at a dose of 2000 mg/kg. No abnormality in the general behavior of the test animals either in the short term or long term, was observed. There was no body weight loss during the observation period. All the animals were found to be normal and there were no gross behavioral changes till the end of the observation period. Thus, there is no mortality and all the extracts tested are considered safe and nontoxic. Hence from this, 1/10th and 1/5th of MTD (maximum tolerated dose) was selected and the effective doses were fixed as 200 and 400 mg/kg for future pharmacological studies.

***In vivo* Anti-urolithiatic activity**

Urolithiasis or kidney stone formation is a complex process which take place due to supersaturation of stone forming constitute like calcium, oxalate, uric acid, urea, phosphate in urine and an imbalance of inhibitors like glycosaminoglycans, magnesium (Basavaraj *et al*.,2007). Calcium, struvite, uric acid, cystine stones and others are the five main types of kidney stones but calcium oxalate crystals are most commonly found kidney stones in humans. Some of the treatments for treating kidney stones are Extracorporial Shock Wave Lithotripsy (ESWL), Ureteroscopy (URS) or Percutaneous

nephrolithotomy (PNL) (Coll *et al*., 2002). However, the success rate of treatment is poor; they have many short comings and side effects and reoccurrence is quite common. Hence, herbal medicine has gained much popularity. They are more effective, have less side effects and reduce recurrence rate of stone formation; thus, search for antilithiatic drug from natural sources has assumed greater importance and is promising. Herbal medicines have many phytoconstituents which may exert their beneficial effect alone or in combination in kidney stone treatment. Plant extracts contain phytochemicals that inhibit stone formation by inhibiting synthesis and agglomeration of crystals.

Ethylene Glycol and Ammonium chloride induced urolithiasis (EG+NH_4Cl Model)

In the present study, methanol extracts of the whole plant of *Grewia hirsuta L*. was assessed for its antiurolithiatic activity in ethylene glycol and ammonium chloride induced urolithiasis in rats and the results obtained was recorded.

Kidney analysis

The kidney parameters of the rats were assessed by evaluating the calcium, phosphorus and oxalate concentrations in the kidney homogenate. The kidney parameters exhibited a similar result as the urine parameters. For kidney, EG control gave the value of oxalate, calcium and phosphate as 6.65±0.339, 5.68±0.397, 5.12±0.483 respectively. Negative control showed decrease in value as 1.23±0.191, 1.97±0.296, 1.48±0.190 of oxalate, calcium and phosphate respectively. GHME 200 showed increase in value of oxalate as 2.73±0.463, whereas GHME 400 was not found to be effective as it gave lowest increase level of oxalate, calcium and phosphate as 2.07±0.489, 3.22±0.319, 2.57±0.294 respectively.

Urine analysis

The administration of ethylene glycol supplemented with ammonium chloride resulted in hyperoxyluria in rats. The administration of ethylene glycol in the rat's system leads to ethylene glycol toxicity. The toxicity results from the metabolic degradation of ethylene glycol into toxic metabolites. This leads to acidosis which further causes precipitation of oxalates as calcium oxalate crystals. The urine was collected for 24 h and analysed for the presence of crystalline components such as calcium, phosphorus and oxalate. The concentration of urine phosphorus, calcium, and oxalate present in Group I–V is shown in Table. For urine, EG+NH_4Cl control gave the value of oxalate, calcium and phosphate as 4.25±0.266, 5.37±0.333, 7.02±0.494. Negative control showed decrease in value as 0.31±0.060, 1.13±0.079, 2.52±0.356 of oxalate, calcium and phosphate respectively. GHME 200 showed increase in value of all three constituents as 3.12±0.512, 3.38±0.417, 4.78±0.118 and GHME 400 showed decrease in all the three values as 1.07±0.339, 1.95±0.383 and 3.53±0.378.

Table 6: Effect of *Grewia hirsuta L.* whole plant Extract on Urinary and Kidney Parameters in Ethylene Glycol Induced Wistar Rats.

Group No.	Treatment	In kidney			In urine		
		Oxalate	Calcium	Phosphate	Oxalate	Calcium	Phosphate
I	Control	1.23±0.191	1.97±0.296	1.48±0.190	0.31±0.060	1.13±0.079	2.52±0.356
II	EG+NH_4Cl Control	6.65±0.339	5.68±0.397	5.12±0.483	4.25±0.266	5.37±0.333	7.02±0.494
III	GHME 200	2.73±0.463	3.97±0.501	3.13±0.557	2.68±0.556	3.12±0.271	4.53±0.147
IV	GHME 400	2.07±0.489	3.22±0.319	2.57±0.294	1.07±0.339	1.95±0.383	3.53±0.378
V	Cystone 750	1.43±0.378	3.10±0.477	2.10±0.607	0.73±0.163	1.55±0.259	3.18±0.331

Serum analysis

The renal function of the rats was evaluated at the end of the study by assessing the serum calcium, phosphorus and oxalate levels. In urolithiasis, glomerular filtration rate (GFR) is reduced due to hindrance in the urinary flow in the urinary system. Due to this, waste products mainly nitrogenous compounds such as, urea and creatinine gets hoarded in the blood (Betanabhatala *et al*., 2009). Ethylene glycol and ammonium chloride administration led to the elevation of serum calcium, phosphorus and oxalate levels in the stone induced animals which indicated the precipitation and nucleation of calcium stones (Table 5.24). In case of serum, EG+NH_4Cl control gave the value of oxalate, calcium and phosphate as 57.03±3.232, 4.47±0.301, 2.22±0.354 respectively. Negative control showed decrease in value as 25.60±1.307, 1.04±0.040, 0.59±0.069 of oxalate, calcium and phosphate respectively. GHME 400 found effective as it increases the value of oxalate, calcium and phosphate i.e., 51.32±4.772, 3.05±0.521, 1.09±0.052 respectively.

Table 7: Effect of *Grewia hirsuta L* whole plant Extract on Serum Parameters in Ethylene Glycol Induced Wistar Rats.

Group No.	Treatment	In Serum		
		Oxalate	Calcium	Phosphate
I	Control	25.60±1.307	1.04±0.040	0.59±0.069
II	EG+NH_4Cl Control	57.03±3.232	4.47±0.301	2.22±0.354
III	GHME 200	48.42±4.642	2.73±0.314	1.03±0.060

IV	GHME 400	51.32±4.772	3.05±0.521	1.09±0.052
V	Cystone 750	36.27±2.495	1.60±0.374	0.73±0.087

CONCLUSION

The current study was able to show the antiurolithiatic effect of whole plant extract of *Grewia hirsuta L* in ethylene glycol ammonium chloride-induced urolithiasis model. Therefore, GHME may reduce and inhibits the growth of urinary stones.

Therefore, it is useful to prevent the recurrence of urolithiasis as it proved its effect on the early stages of stone development. The mechanism causing this effect is still unspecified, but is possibly related to increased diuresis and lowering of urinary concentrations of stone-forming components. However, it requires more investigation to clarify the exact mechanism of this action.

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Conflict of interest

No authors declared Conflict of Interest.

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