RESEARCH ARTICLE

Comprehensive Evaluation of Antilithiatic Potential: *In-vitro* and *In-vivo* Studies on Aqueous Extract and Fractions from *Punica granatum L*. Leaves

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ABSTRACT

The hydroalcoholic concentrate and its fractions of *Punica granatum L.* leaves were tested for antilithiatic activity. The evaluation includes *in-vitro* as well as *in-vivo* investigations. The extract and its fractions were tested *in-vitro* for their effects on urolithiasis-related processes such crystal formation, growth, & aggregation inhibition. *In-vivo* testing examined stone formation, growth, & dissolution in animal models with artificial urolithiasis after providing the extract and fractions. *In-vitro*, the hydroalcoholic extract & its components prevented crystal development, aggregation, and formation. These data imply that the extract & fractions may inhibit early stone growth, preventing urolithiasis. In animal models with artificial urolithiasis, the extract and fractions dramatically decreased stone load, with dichloromethane showing better antilithiatic effects. In addition, the therapy helped dissolve and pass stones. These findings demonstrate *P. granatum L.* leaves' powerful antilithiatic capabilities, supporting their use in urolithiasis.

Keywords: *Punica granatum L*. leaves, Urolithiasis, Stone formation, Traditional medicine, Aggregation

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INTRODUCTION

Urolithiasis, which causes urinary tract calculi or stones, can affect the bladder, kidneys, ureters, or urethra.[1] This disorder predominantly causes calcium oxalate stones and affects millions globally. Calcium phosphate, uric acids, & struvite stones can cause urinary tract infections. Diet, hydration consumption, and health affect stone composition.[2]

Depending on stone size and position, urolithiasis can cause strong renal colic (back or lateral pain), hematuria, higher urine urgency, and urinary tract infections.[3] Stone blockage can cause severe discomfort, urine retention, and kidney damage. For stone size, location, & composition, medical history, physical examination, an ultrasound, X-ray, or CT scan, and urine analysis are used to diagnose and guide therapy.[4,5] Conventional urolithiasis care involves pain alleviation and fluid intake to aid stone transit. Medical intervention may be needed to remove or fracture bigger stones.[6] However, traditional procedures may fail to remove certain stones, requiring additional treatment.[7] Pain and stone-prevention medications might cause gastrointestinal issues, sleepiness, constipation, and electrolyte abnormalities. Surgery includes ureteroscopy or percutaneous nephrolithotomy might cause bleeding, infection, or injury to adjacent tissues.[8, 9] Lithotripsy can cause discomfort, bruising, and tissue damage. Intervention healing may cause discomfort, pain, or urinary symptoms. Recurring stones may occur if metabolic abnormalities or nutrition are not addressed. Conventional management might cost money for procedures, medications, follow-up appointments, and job absences during recuperation.[10, 11]

Along with standard urolithiasis treatments, integrative and complementary therapies are considered. Some herbal treatments, such chancapiedra (Phyllanthusniruri), support kidney health and aid stone transit. Traditional Chinese acupuncture uses precise needle insertion to relieve pain, relax, and improve well-being during urolithiasis treatment. To restore balance, Ayurvedic therapies include nutrition, herbal medicines, lifestyle changes, and Panchakarma (detoxification). Homeopathy activates the body's natural healing processes using very diluted medicines. Vitamin B6, magnesium, & cranberry extract may also improve bladder health.[12,13]

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Herbal urolithiasis treatments have several benefits. They gain from years of Ayurvedic& Traditional Chinese Medicine knowledge and expertise. Herbal analgesics and diuretics may relieve urolithiasis symptoms like discomfort, pain, and urinary problems.[14, 15] Herbs that enhance kidney function, urine pH, and waste removal may improve urinary health. Other herbs may prevent urinary stones by changing urine composition or improving urinary health. Herbal medication is often considered as a natural alternative to pharmaceutical pharmaceuticals and associated with fewer side effects.[16, 17]

Pomegranate, or *Punica granatum*, is a Lythraceae fruit-bearing bush or small tree. Although native to the Middle East, its ruby-red arils and tangy, juicy aroma have made it a worldwide grown fruit.[18,19] Beyond their culinary purpose, pomegranates have long been used in traditional & alternative medicine. Pomegranates are appreciated for their flavor and health benefits in many cultures. Traditionally, pomegranate seeds, juice, & peel were used for therapeutic purposes.[20,21] These applications address many health disorders and issues. Pomegranates have been widely investigated for their medicinal benefits. Pomegranate juice and extracts contain antioxidant capabilities that may reduce oxidative stress associated with health issues.[22-25] This antioxidant property has contributed to its use as a medicinal treatment. Pomegranate may be antilithiatic and nephroprotective, according to emerging studies. These properties may prevent urinary stones (urolithiasis) & safeguard kidney function. Pomegranate has natural chemicals that may aid kidney health, although the processes and efficacy are still being studied.[26-31]

The planned study would investigate *Punica granatum's* therapeutic capabilities, focusing on urolithiasis & kidney protection. This study investigates pomegranate's ability to prevent and treat urinary stones based on traditional applications and new studies. This study helps us comprehend *P. granatum's* significance in traditional medicine & its prospective therapeutic uses by studying its features. It opens new opportunities for natural medicine research by revealing the wider use of pomegranate as a kidney stone treatment.

MATERIALS AND METHODS

Chemicals and Reagents

Celon laboratories presented a gift sample of ethylene glycol in Hyderabad. The compound quercetin was acquired *via* Sigma-Aldrich Chemicals Private Limited located in Bangalore. The study obtained all additional chemicals, solvents, & reagents through SD Fine Chem. Ltd. in Hyderabad.

Collection of *P. granatum L.* Leaves and Processing

Hyderabad, Telangana'sMedchal district provided *P. granatum L.* leaves from rural regions. Titupathi's qualified taxonomist verified the leaves' taxonomy. The leaves were rigorously cleaned after harvesting to eliminate pollutants. After washing, the leaves were air-dried under shady conditions until crisp and brittle. Blender crushed the dry leaves.[16]

Preparation of Extracts

First, 500 g of powdered *P. granatum L.* leaves were defatted in a soxhlet apparatus using petroleum ether. After being removed from the soxhlet, its contents were dried to remove any remaining moisture. The dry powder was then extracted with water in the soxhlet equipment for 72 hours. The extracted liquid was concentrated in a rotating evaporator at 40°C under decreased pressure. After calculating the extract yield, it was kept at 4°C in a dark condition. Dissolving the crude extract in distilled water & filtering it prepared it for analysis. After filtering, dichloromethane, ethyl acetate, and n-butanol were used to fractionally separate the solution. Each organic fraction following fractionation was concentrated to make dry extracts. All fractions were maintained in a dark refrigerator at 4°C after determining their yield percentage.[32, 33]

Phytochemical Analysis

A complete phytochemical study of *P. granatum L.* leaves extract organic fractions identifies and evaluates different bioactive components. This analytical method detects alkaloids, flavonoids, tannins, saponins glycosides, terpenoids, phenols, and various other phytochemicals.[34]

Quantification of total phenolic and flavonoid contents

Well-established colorimetric assays can measure TPC and TFC in *P. granatum L.* leaves extract as well as organic fractions. These assays are commonly used to estimate phenolic compound and flavonoid concentrations, which have bioactive potential and health benefits.[17]

Total phenolic content

To measure TPC in crude extract & organic fractions, a 100 µg/mL Gallic acid standard mixture is carefully produced. A measured extract or organic fraction volume is placed in a test tube. After this, the reagent Folin-Ciocalteu is added to the extract or organic part test tube and stirred well. A solution of sodium carbonate is added after 5 minutes of incubation, then the test tube is left for 30 minutes. This incubation time allows the extract's phenolic components to react with Folin-Ciocalteu and sodium carbonate. This reaction creates a blue complex. A UV-visible spectrophotometer measures the absorbing capacity of this blue-colored solution at 760 nm to quantify total phenolic content. The gallic acid standard solution with a known quantity is used to repeat this operation. The standard gallic acid solution data carefully creates a calibration curve. This calibration curve accurately matches gallic acid content to absorbance. The amount of phenolic compounds in the organic portion of the extraction is then determined using this consistent calibration curve. Gallic acid equivalents (mg GAE) per gram of sample are the final result.[20,26]

Total flavonoid content

Prepare an appropriate quercetin solution (e.g., 100 µg/mL) to measure TFC in *P. granatum L.* leaves crude extract plus organic fractions. An established amount of extraction or organic component is then placed in a test tube. Following

that, aluminum chloride was added to the extraction or organic component test tube and stirred well. The extract's flavonoid components react with aluminum chloride in the test tube for 30 minutes. This reaction creates a colorful complex. The wavelength of absorption of the colored solution is measured at 415 nm employing a UV-visible spectrophotometer to determine the total flavonoid concentration. Repeat the experiment with the known-concentration quercetin standard. The conventional quercetin solution data is used to create a calibration curve that links quercetin concentration to absorbance. The organic fraction's total flavonoid content is determined using the standard calibration curve. Quercetin alternatives (mg QE) per gram of material are reported.

In-vitro Antilithiatic Activity

Nucleation assay

The nucleation experiment measured the extracts' inhibitory effect on calcium oxalate crystal nucleation, a key kidney stone-forming process. A spectrophotometric test assessed inhibitory activity. The crystallization procedure began with the addition of 100 µL of 4 mM calcium chloride & 50 mM sodium oxalate solution to 0.5 mL of normal human urine. 0.5 mL of 0.05 mMTris buffer & 0.15 mMNaCl mixture at pH 6.5 were used to prepare the urine sample at 37°C. To correct volume, 1.5 mL of distilled water was added. At 0, 30, 60, 180, and 360 min, the induction period of crystals, which suggests when crystals attained a critical size and grew optically detectable, was compared to the negative control period without extract to determine nucleation rate. For positive control, Cystone was employed. Spectrophotometers measured OD at 620 nm. Formula used to calculate %inhibition.

$$Percentinhibition = \frac{1 - OD_{Extract}}{OD_{Control}} x\ 100$$

Crystal aggregation assessment

We used spectrophotometric assays with minor changes to assess urinary crystal aggregation. The main goal was to assess calcium oxalate crystal aggregation at 0, 30, 60, 180, and 360 minutes. We used 50 mM calcium chloride and sodium oxalate to make calcium oxalate monohydrate (COM) crystals to start crystallization. These solutions were thoroughly equilibrated, cooled to 37°C, and evaporated. After that, the COM crystals were dispersed in 0.5 mL of 0.05 mM tris buffer and 0.15 Mm NaCl at pH 6.5 to an ultimate concentration of 1-mg/mL. A spectrophotometer assessed absorbance at 620 nm, while the rate of agglomeration was calculated by comparing the extract/fractions' slope of turbidity to the control group.[23]

In-vivo Antilithiatic Activity

Acquisition and care of experimental animals

Male wistar albino rats weighing 150 to 250 grams were purchased through Vab Bioscience in Musheerabad, Hyderabad. The rats were habituated to lab settings for a week before trials. Animals were handled ethically during the investigation. Institutional Animal Ethical Committee (IAEC) registration number 1447/Po/Re/S/11/CPSCEA-51/A authorized the experimental procedure.

Experimental Design and Procedures

This study used male wistar albino rats with nephrolithiasis to test *P. granatum L.* leaves extract & its fractions for antilithiasis activities. The 42 animals in this experiment were carefully divided into seven groups of six rats. All animals had unlimited food during the trial.

Group A was the negative control & had a typical diet and free water. From day one to day 28th, the remaining groups were given ethylene glycol (0.75%) in the water they drank, which causes renal calculi.[35]

Group B, the disease control, had animals infected with lithiasis. Standard therapy was Cystone at 750 mg/kg from day 14 to day 28 in group C. Group D received 400 mg/kg crude extract from day 14 to 28. From day 14 to 28, group E received a dichloromethane fraction (400 mg/kg). Group F received 400 mg/kg ethyl acetate fraction from day 14 to 28. Group G received 400 mg/kg n-butanol fraction from day 14 to 28. This investigation examined how the extract/fractions affected renal calculi, revealing their antilithiatic potential.

Assessment of Serum and Urinary Parameters

Animal serum and urine tests were done on day 29. Blood samples were taken via the retro-orbital plexus & spun at 15000 rpm for 10 minutes in order to acquire serum samples under anesthesia. An automated clinical chemistry analyzer (Roche Cobas C501) measured serum uric acid, urea nitrogen, and creatinine. Rat urine samples were taken on the 14th& 28th days of the investigation. Automatic calcium, phosphate, & oxalate analysis was performed on these urine samples. The urine samples were examined using a 100X light microscope (Olympus DX 45, Japan). Histopathological examination of ethylene glycol-induced nephrolithiasis rats' left kidneys was performed by excising, weighing, fractionating, and fixing them in 10% neutral buffered formalin. Tissue specimens were dehydrated in ethanol, cleaned in xylene, and embedded in paraffin. Sections of 4 µm thick were cut and dyed with hematoxylin-eosin from embedded specimens. Calcium oxalate deposits and histological alterations were examined in kidney slices under a microscope.

Statistical Analysis

The experimental results (n = 3) were examined using one-way ANOVA and reported as mean ± SD. Statistical significance required a *p-value* below 0.005. For histopathological results, semi-qualitative analysis was used.

RESULTS AND DISCUSSION

P. granatum L. leaves are shiny, lance-shaped, and dark green to bright. They are essential to the pomegranate tree & may have medicinal properties. Polyphenols, tannins, flavonoids, and other phytochemicals are found in *P. granatum* leaves.[12] Many of pomegranate leaves' health advantages come from these chemicals. Pomegranate leaves contain antioxidants

Table 1: Results of total phenolic content and total flavonoid content

S. No.	*Extract/Fraction*	*TPC (mg EAG/gm)*	*TFC (mg QE/gm)*
1	Crude	41.34 ± 1.54	38.49 ± 1.27
2	Dichloromethane fraction	297.72 ± 5.32	204.67 ± 4.76
3	Ethyl acetate fraction	214.78 ± 3.63	129.86 ± 4.54
4	Butanol fraction	136.78 ± 4.12	78.56 ± 3.73

(The measurements were expressed as the mean ± standard deviation; n=3)

that neutralize free radicals, lowering oxidative stress and attendant health concerns. Pomegranate leaf phytochemicals may help treat inflammatory disorders. Pomegranate extract from leaves may help maintain healthy blood pressure & cholesterol, according to certain research. Pomegranate leaf extract may help regulate blood sugar and avoid diabetic problems. Pomegranate leaves are used in traditional medicine to cure illnesses due to their antibacterial characteristics. Pomegranate leaves may prevent kidney stones (urolithiasis) & preserve the kidneys.[22]

This study used the Soxhlet extraction method to extract dried pomegranate leaves aqueously, producing less than 26.89%. A comprehensive phytochemical examination of the extract and fractions showed promising results, which were supported by color changes. Flavonoids, alkaloids, & phenols dominated the extract and fractions.

Polyphenols—a wide set of secondary metabolites with health benefits—abound in the plant.[36] The Folin-Ciocalteu reagent assessed TPC as milligrams of gallic acid equivalents (mg GAE) per gram of material using the standard calibration curve y= 0.0059x + 0.2109, R2= 0.9974. Table 1 shows that the total phenol concentration in the extract varies from 41.34 ± 1.54 to 297.72 ± 5.32 mg EAG/g. Dichloromethane had the greatest polyphenol content (297.72 ± 5.32 mg EAG/g) among the fractions.

Flavonoids, polyphenolic chemicals present in many plants and thought to provide health advantages, were also examined. The aluminum chloride test measured total flavonoid content (TFC) in mg EQ per gram of material using the standard calibration curve y= 0.0124 x + 0.2109, R2 = 0.9958. Results indicate a total flavonoid concentration of 38.49 ± 1.27 to 204.67 ± 4.76 mg EQ/g in the extract. The dichloromethane fraction again has the greatest amount (204.67 ± 4.76 mg EQ/g) followed by the ethyl acetate fraction at 129.86 ± 4.54 mg EQ/g. Polyphenols and flavonoids are abundant in the plant, with dichloromethane showing good concentration potential.[37,33]

The nucleation assay showed that the extracts might prevent kidney stones by slowing calcium oxalate crystal formation. Table 2 shows extract %inhibition at different times. The dichloromethane fraction showed the strongest nucleation activity suppression, peaking at 30.92 ± 2.08% during 60 minutes of incubation. The result was 9.34% greater than the positive control, Cystone (21.58 ± 2.13%), at the same time point. At 360 min of incubation, the dichloromethane fraction maintained its inhibitory activity at 30.13 ± 1.34%, whereas Cystone reduced to 20.12 ± 1.57%. All extract/fractions showed an increase trend in inhibitory activities from 0 to 60 minutes, followed by a plateau or fall at 180 min. The ethyl acetate fraction showed the second-highest nucleation activity inhibition, with 24.53 ± 1.78% at 60 minutes and 23.78 ± 1.23% after 360 minutes. These data show that the extract/fractions include significant nucleation-preventing chemicals that can prevent kidney stones.[38] According to the nucleation assay, the

Table 2: Results of percent inhibition in the nucleation assay at different time points

S. No.	*Treatment*	*Percent inhibition at different time points (minutes)*				
		0	*30*	*60*	*180*	*360*
1	Negative control	-	-	-	-	-
2	Positive control	17.43 ± 1.28	19.58 ± 0.86	21.58 ± 2.13	20.98 ± 0.94	20.12 ± 1.57
3	Crude extract	11.12 ± 0.48	12.37 ± 0.76	13.16 ± 0.34	12.58 ± 0.72	12.17 ± 0.93
4	Dichloromethane fraction	26.78 ± 1.18	28.14 ± 1.53	30.92 ± 2.08	31.24 ± 1.32	30.13 ± 1.34
5	Ethyl acetate fraction	16.45 ± 1.49	17.78 ± 0.68	18.53 ± 1.78	19.08 ± 1.67	19.43 ± 1.23
6	Butanol fraction	13.67 ± 0.78	15.34 ± 0.94	15.78 ± 0.82	16.34 ± 1.39	16.89 ± 0.68

(The measurements were expressed as the mean ± standard deviation; n = 3)

Table 3: Results of percent inhibition in the aggregation assay at different time points

S. No.	*Treatment*	*Percent inhibition at different time points (minutes)*				
		0	*30*	*60*	*180*	*360*
1	Negative control	-	-	-	-	-
2	Positive control	34.74 ± 1.76	37.69 ± 0.74	41.32 ± 0.89	40.78 ± 1.17	40.25 ± 2.71
3	Crude extract	11.36 ± 0.59	12.74 ± 0.72	14.78 ± 0.96	12.78 ± 1.54	11.23 ± 0.28
4	Dichloromethane fraction	28.56 ± 1.25	34.23 ± 1.67	40.78 ± 2.03	43.18 ± 1.69	45.67 ± 1.13
5	Ethyl acetate fraction	20.15 ± 1.63	22.18 ± 1.73	25.38 ± 0.98	24.93 ± 1.18	24.54 ± 0.89
6	Butanol fraction	13.68 ± 0.68	15.58 ± 0.86	17.34 ± 0.59	16.87 ± 1.06	16.75 ± 1.03

(The measurements were expressed as the mean ± standard deviation; n = 3)

Table 4: Levels of serum biochemical parameters

Biochemical parameter	*Group*						
	A	*B*	*C*	*D*	*E*	*F*	*G*
	Plain control	*Disease control*	*Standard treatment*	*Crude extract*	*Dichloromethane fraction*	*Ethyl acetate fraction*	*Butanol fraction*
Blood urea nitrogen	23.78 ± 0.83	44.54 ± 0.58*	26.34 ± 0.65$^{\#}$	35.24 ± 1.86$^{\#\$}$	25.74 ± 0.72$^{\#}$	31.24 ± 0.83$^{\#\$}$	32.74 ± 0.86$^{\#\$}$
Creatinine	0.38 ± 0.07	1.06 ± 0.04*	0.63 ± 0.06$^{\#}$	0.86 ± 0.05$^{\#\$}$	0.58 ± 0.11$^{\#}$	0.74 ± 0.08$^{\#\$}$	0.79 ± 0.09$^{\#\$}$
Uric acid	1.65 ± 0.09	6.09 ± 0.16*	2.13 ± 0.08$^{\#}$	3.18 ± 0.23$^{\#\$}$	1.89 ± 0.23$^{\#}$	2.34 ± 0.16$^{\#\$}$	2.83 ± 0.17$^{\#\$}$

*Comparison with Plain control, $^{\#}$Comparison with disease control, $^{@}$Comparison with standard treatment, $^{\$}$Comparison with dichloromethane faction (The measurements were expressed as the mean ± standard deviation; n = 3)

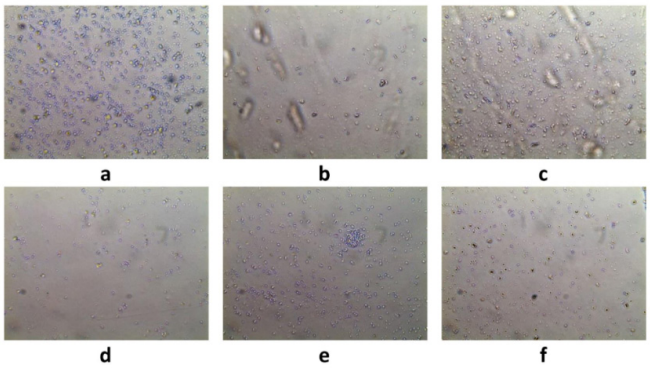
dichloromethane & ethyl acetate fractions may inhibit kidney stone development.

The aggregation assay revealed how the extract/fractions affected calcium oxalate crystal aggregation, which is important for understanding their capacity to avoid urinary stones.[19, 39] Table 3 compares extract/fraction aggregation inhibition effects. Dichloromethane inhibited aggregation activities the most compared to other components and Cystone. Cystone exhibited 34.74 ± 1.76% inhibitions at 0 minutes, while dichloromethane had 28.56 ± 1.25% inhibitions. After 360 minutes of incubation, dichloromethane fraction inhibition reached 45.67 ± 1.13%, 5.42% greater than cystone (40.25 ± 2.71%). Ethyl acetate & n-butanol inhibited aggregation by less than 30%, compared to cystone, the positive control. After 360 minutes at 37°C, the ethyl acetate & n-butanol fractions showed only 24.54 ± 0.89% & 16.75 ± 1.03% suppression of aggregation activities, respectively. The crude extract had the lowest inhibition rate, 11.23 ± 0.28%, following the same incubation time.

An inverse microscope at 10X resolution demonstrated nucleation and aggregation of CaOx crystals.[40] Figure 1 shows the extract/fractions' nucleation inhibition % and the negative and positive controls (Cystone). Crystals treated with extract/fractions and Cystone had diminished density, indicating CaOx crystal nucleation dissolution. Since they inhibit CaOx crystals from calcium chloride & sodium oxalate reactions, the extract/fractions may be antilithiatic. In Figure 2, microscopic pictures of extract/fractions, negative control, & positive control indicate aggregation inhibition. As shown by crystal size reduction, dichloromethane inhibited crystal aggregation best. This suggests the extracts might reduce stone size. The ethyl acetate component nucleated and aggregated better than the butanol fraction as well as crude extract.

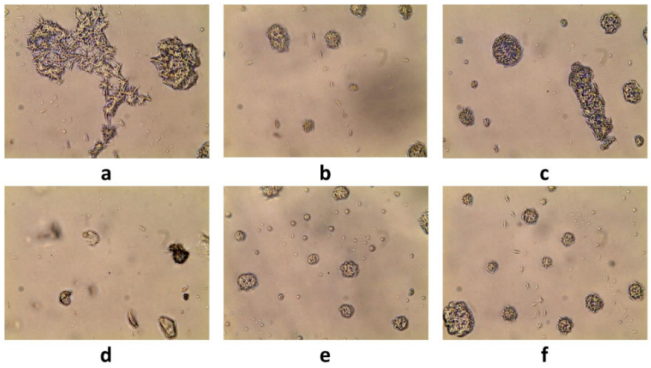
The sick group (B) showed a substantial rise ($p < 0.001$) in creatinine, urea nitrogen, & uric acid levels compared to the control group (I), indicating nephritic injury and impaired kidney function. However, animals administered the standard and varied extract/fraction test samples had significantly lower values of these parameters than the disease control animals. Additionally, Table 4 shows a substantial ($p < 0.01$) difference among groups given with different fractions. Dichloromethane fraction-treated groups had identical biochemical characteristics to those treated with the conventional medication.

In contrast to the control group, the sick group showed a substantial drop ($p < 0.001$) in urine production. In contrast, animals receiving the conventional therapy (Group C) had significantly higher urine production ($p < 0.001$) than the disease control group. A substantial rise ($p < 0.001$) in diuresis was seen in mice treated with the experimental extract/fractions when compared to the disease placebo group. The conventional medication and test samples greatly decreased lithogenic polyuria. This diluted urinary electrolytes, removing calcium and phosphorus and lowering saturation and precipitation. Thus, calculi were avoided. Both urine and serum



Note: A: Negative control; B: Positive control; C: Crude extract; D: Dichloromethane fraction; E: Ethyl acetate fraction; F: Butanol fraction

Figure 1: Inverted phase microscopic images of nucleation inhibition activity (10X magnification)



Note: A: Negative control; B: Positive control; C: Crude extract; D: Dichloromethane fraction; E: Ethyl acetate fraction; F: Butanol fraction

Figure 2: Inverted phase microscopic images of aggregation inhibition activity (10X magnification)

Table 5: Urine volume of different group of animals

Group	*Urine volume in mL*	
	14th day	*28th day*
A – Plain control	11.32 ± 1.12	12.17 ± 0.96
B- Disease control	4.78 ± 0.52*	4.13 ± 0.46*
C – Standard treatment	8.16 ± 0.72#	8.78 ± 0.54#
D – Crude extract	7.34 ± 0.54#@$	8.53 ± 0.28#@$
E – Dichloromethane fraction	8.89 ± 0.68#@	9.12 ± 0.67#@
F – Ethyl acetate fraction	7.78 ± 0.54#$	8.54 ± 0.48#$
G – Butanol fraction	6.89 ± 0.48#$	7.06 ± 0.76 #$

*Comparison with Plain control, #Comparison with disease control, @Comparison with standard treatment, $Comparison with dichloromethane faction

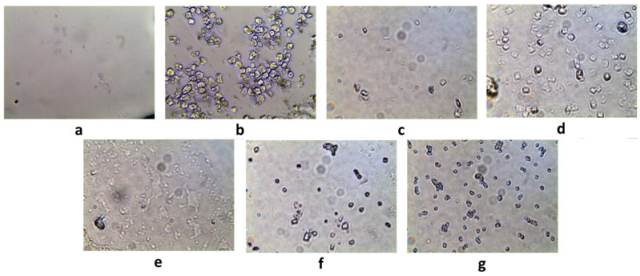
(The measurements were expressed as the mean ± standard deviation; n=3)

calcium excretion increased significantly in the therapy groups. Additionally, Table 5 shows a substantial ($p < 0.01$) difference among groups treated using various fractions.

On day 28, urine samples from separate groups were analyzed for pH, specific gravity, glucose, leukocytes, erythrocytes, bilirubin, urobilinogen, ketone bodies, protein, nitrite, phosphate, calcium, and oxalate. Table 6 shows the analysis's results.

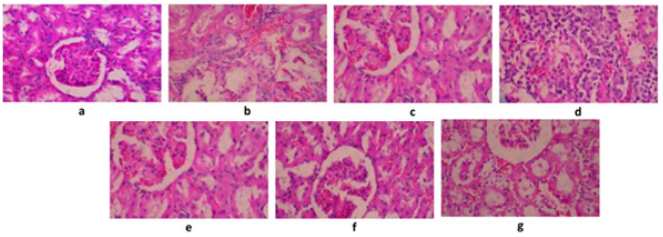
Urine samples in the negative group of controls did not crystallize. In the disease-induced category, urine samples had rectangular calcium oxalate crystals. Standard cystone treatment reduced or eliminated crystals. Crystal formation was significantly reduced in extract and fraction groups. Compared to the illness group, dichloromethane fraction prevented crystal formation the most (Figure 3).

Histopathology found no renal calculi or abnormalities in group A, the plain control group. Specifically, renal tubules



Note: A: Control; B: Disease control; C: Standard treatment; D: Crude extract; E: Dichloromethane fraction; F: Ethyl acetate fraction; G:Butanol fraction

Figure 3: Microscopic images of urine samples



Note: A: Control; B: Disease control; C: Standard treatment; D: Crude extract; E: Dichloromethane fraction; F: Ethyl acetate fraction; G:Butanol fraction

Figure 4: Histopathological findings of various groups

had calcium oxalate crystal deposits in group B, the disease control group. Also seen were epithelial desquamation, cellular inflammation, as well as blood vessel congestion.[28] Cystone-treated groups and test samples showed significant improvements in various anomalies, including calcium oxalate depositions (Figure 4). In lithiasis animals, the conventional medicine and testing samples (extract/fractions) prevented calcium oxalate supersaturation, reducing renal tubule deposition. These findings support the extract/fractions'

Table 6: Results of urine analysis

Parameter	Group						
	A	B	C	D	E	F	G
	Control	Disease control	Standard treatment	Crude extract	Dichloromethane fraction	Ethyl acetate fraction	Butanol fraction
pH	6.08 ± 0.23	8.66 ± 0.21*	6.54 ± 0.18#	7.65 ± 0.63	6.73 ± 0.86	7.12 ± 0.40	7.43 ± 0.10
Specific gravity	1.02 ± 0.001	1.04 ± 0.008	1.02 ± 0.001	1.06 ± 0.008	1.02 ± 0.008	1.07 ± .004	1.05 ± 0.003
Glucose	Ab	Ab	Ab	Ab	Ab	Ab	Ab
Erythrocytes	Ab	150 ± 44.7*	5 ± 2.24#	8.9 ± 1.61$	Ab	Ab	7.8 ± 1.32$
Leucocytes	Ab	41.67 ± 10.54*	8.33 ± 5.27#	19.5 ± 5.2@	10.89 ± 5.9@	20.32 ± 4.5$	18.68 ± 2.34$
Bilirubin	Ab	0.83 ± 0.32*	0.17 ± 0.12#	0.47 ± 0.10	0.18 ± 0.10	0.32 ± 0.20$	0.33 ± 0.16$
Urobilinogen	0.25 ± 0.15	1.0 ± 0.12*	0.4 ± 0.1#	0.7 ± 0.18	0.4 ± 0.18	0.63 ± 0.19	0.67 ± 0.15
Ketone	1.66 ± 1.05	22.5 ± 8.7*	1.66 ± 1.32#	2.98 ± 1.1	1.7 ± 1.1	2.33 ± 2.1$	3.01 ± 2.0$
Protein	Ab	20 ± 4.47*	3.33 ± 2.1#	8.3 ± 4.7$	Ab@	Ab@	7.66 ± 4.94$
Nitrite	Ab	0.91 ± 0.08*	0.75 ± 0.17#	0.76 ± 0.11	0.54 ± 0.16	0.66 ± 0.16	0.72 ± 0.20
Calcium	24.17 ± 0.31	40.17 ± 0.40*	25.32 ± 0.40#	32.43 ± 1.67	27.76 ± 1.21	30.5 ± 0.72	31.67 ± 0.57
Phosphate	0.56 ± 0.02	0.90 ± 0.03*	0.58 ± 0.04#	0.88 ± 0.06	0.61± 0.03	0.71 ± 0.03	0.74 ± 0.02
Oxalate	1.78 ± 0.06	5.96 ± 0.08*	1.85 ± 0.05#	2.76 ± 0.16	1.87 ± 0.09	2.26 ± 0.07	2.13 ± 0.06

(The measurements were expressed as the mean ± standard deviation; n=3)

antilithiatic efficacy in the ethylene glycol-induced urolithiatic model.[33]

In antilithiatic activity testing, the extract and its fractions improved blood creatinine, urea nitrogen, and uric acid levels.[15] These improvements showed reduced nephritic damage and improved kidney function. The extract and its fractions also increased urine production and improved urine composition, including pH, protein, and calculi-forming components including calcium, phosphate, and oxalate. Microscopic and histopathological tests confirmed that the extract and its components reduced calcium oxalate crystals, a usual urinary stone.[35]

The sick group had a higher urine pH (pH 8.66) than the control group. Animals given cystone and the test extract had decreasing urine pH. Urine glucose was missing in all groups, and ketone bodies, nitrites, and urobilinogen were not statistically different. Protein in lithiasis-induced mice (diseased group) suggested kidney damage.[25] Cystone and test extract-treated animals recovered quickly, whereas the sick group continued to have proteinuria, indicating serious nephritic injury. Dose-dependent improvement was shown in test extract and fraction groups.[36]

The calculi-induced group had higher calcium, phosphate, and oxalate levels, which create urinary stones. Cystone& the test extract and fractions decreased calculi-forming components in animals. In the disease control group, lithiasis dramatically raised serum and urine calcium levels. Higher urine calcium concentrations may form groups with charge-negative inhibitors. Oxalate levels in serum and urine increased with ethylene glycol-induced lithiasis. Treatment with standard and test extracts/fractions suppressed lithogen-induced hypercalciuria and hyperoxaluria.

Urolithic rats had elevated uric acid levels, which can affect calcium oxalate solubility and lithiasis medication efficacy. Each therapy reduced uric acid levels, lowering calculi formation.[16] Due to calculi blocking urine outflow, sick animals had lower glomerular filtration rates. Thus, blood urea, uric acid, and creatinine levels dropped. Animals treated with standard tests and extracts had lower urea, uric acid, and creatinine levels. These results showed a considerable difference between curative and preventative therapy.

CONCLUSION

The antilithiatic efficacy of the hydroalcoholic extract as well as its fractions from *P. granatum L.* leaves was evaluated *in-vitro* and *in-vivo*, and the findings were encouraging. The phytochemical analysis of organic fractions found bioactive chemicals that may be medicinal. *In-vitro*, the extract & fractions inhibited calcium oxalate crystal genesis and aggregation. This suggests they can prevent kidney stone formation by inhibiting crystal growth in the early stages. The extract & fractions were given to induced urolithiasis animal models for *in-vivo* testing. The treated groups had much less urinary stone development and growth than the control group. This shows *P. granatum L.* flower extracts may prevent and treat urolithiasis.

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Not applicable

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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