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Original Article

Curative Effect of Extractive Phytoconstituents of *Psidium Guajava* Leaves on Ethylene Glycol Induced Urolithiasis in Experimental Animals

Hemant K. Nagar [a,*], Harinarayan S. Chandel [a], Pawan Rathore[a], Amit K. Srivastava[b], Girjesh Vishwakarma[b], Rajnish Srivastava[c], Mahendra S. Ranawat[d]

[a]*Department of Pharmacology, Truba Institute of Pharmacy, Bhopal, Madhya Pradesh, India.*

[b]*Department of Pharmacology, Sapience Bio-analytical Research Lab, Bhopal, Madhya Pradesh, India.*

[c]*Moradabad Educational Trust Group of Institutions Faculty of Pharmacy, Moradabad, Uttar Pradesh, India.*

[d]*Bhupal Nobles' College of Pharmacy, Udaipur, Rajasthan, India.*

Corresponding Author. Tel : +91 9993633755, *E-mail address:*hemant_nagar81@yahoo.co.in

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ABSTRACT

The aim of this study was to investigate the curative effect of extractive phytoconstituents of *Psidium guajava* leaves on ethylene glycol induced urolithiasis in experimental animals. Urolithiasis was induced in Wistar albino rats by 0.75% Ethylene glycol (v/v) in drinking water for 28 days and preventive effect of ethanolic extract of *Psidium guajava* leaves (EEPG) was evaluated. The animals were divided into six group's i.e. normal control, negative control, positive control (Cystone, 750 mg/kg body weight, p.o.) and test (EEPG, 100, 150, and 200 mg/kg body weight, p.o.). All extracts and standard drug were given once daily by oral route from 15th to 28th day. Evaluation parameters such as body weight, urinary volume, urine and serum calcium, oxalate, creatinine, urea and uric acid were evaluated. Kidneys from each animal were removed on the 28th day for histopathological examination. EEPG prevents the reduction in body weight and significantly increased the urine volume. EEPG treatment significantly ($P < 0.05$) decreased the elevated urinary and serum creatinine, urea, uric acid, calcium and oxalate level. Histopathological examination of EEPG treated showed decreased degenerative changes in the kidney tissue like epithelial cell dissociation, interstitial inflammation within the renal tissue and proximal tubules dilation. Extractive phytoconstituents of *Psidium guajava* leaves was found to show potential anti-urolithiatic activity.

1. INTRODUCTION

Urolithiasis is a recurrent painful disease, affecting 1-5% of the global population with a higher incidence between 20 and 50 years of age, whereas about 10-12% prevalence is in India [1, 2]. Epidemiological studies stated that urolithiasis is more common and prevalent in men than in women [3]. It is clinically characterized by calculus formation at any region in the urinary tract, leading to urolithiasis. Symptoms include dysuria, burning and painful micturition, pain in the pelvic and lumbo-sacral region and the presence of small stone crystals in the urine [4]. Urolithiasis is a disease of multiple etiologies and it also strongly associated with environmental-nutritional factors (decreased urinary volume, animal protein rich diet, etc.) and metabolic disturbances (hyperuricosuria, hypercalciuria, and deficiency of stone-inhibiting factors like citrate, magnesium and glycosaminoglycans) [5, 6].

Various pharmacological and surgical treatment options (thiazide, potassium alkali therapy, nonsteroidal anti-inflammatory drugs, alpha-1 blockers, intracorporeal lithotripsy, laser lithotripsy and shock wave lithotripsy) are available for the management of urolithiasis [7, 8]. But majority of drugs and surgical strategies produce several adverse effects (haematuria, haemorrhage,

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tubular necrosis and consequent fibrosis of the kidney) and also may alter normal biochemical homeostasis of the body on chronic use [9,10].

Out of many strategies, herbal medication is promising as an alternative treatment for the safe and effective treatment of urolithiasis, due to availability, affordability, lesser adverse effects and proved effectiveness [11,12]. Many natural herbs have been pharmacologically reported to possess potent anti-urolithiatic activity [13].

Psidium guajava (Family: Myrtaceae) is a branched tropical tree, growing about 8-10 m in height. It is commonly known as guava and distributed throughout the tropics, commercially cultivated in almost all states of India [14]. Leaves are 5-12 cm in length and 3-5 cm wide, simple, opposite, oblong, ovate with prominent parallel veins, arranged in pairs, with relatively hairy underneath [15].

In Ayurvedic and Unani systems of medicines, it is an extensively used medicinal plant. In addition, *Psidium guajava* leaves were traditionally used for the treatment of spasmodic pain, inflammation, and diuretic properties [12,16]. Although all parts of this plant is possessed valuable medicinal activities, but leaves are extensively studied in recent years to formulate the herbal medicines. Phytochemical studies of *Psidium guajava* leaves can be ascribed for its important bioactive phytoconstituents such as alkaloids, flavonoids, glycosides, triterpenoids, tannins and phenolic compounds [17].

An indigenous drug having lesser side effects is the major area of the present research, looking for a better and safe formulation for the management of urolithiasis. Leaves of *Psidium guajava* are reported to possess important pharmacological activities such as antiinflammatory, antimicrobial and antioxidant activity [18-22]. Therefore, in the light of phytochemical and ethnopharmacological facts of plant, aim of the present study was to investigate the curative effect of extractive phytoconstituents of *Psidium guajava* leaves on Ethylene glycol induced Urolithiasis in experimental animals.

2. EXPERIMENTAL

Materials and Method

Cystone tablets were procured from Himalaya Health Care, India, Ethylene glycol was purchased from Hi-Media Pvt. Ltd., Mumbai, India. All the other reagents, solvents and chemicals used in the study were of analytical grade and procured from S.D. Fine Chemicals (Mumbai, India) Diagnostic kits for various biochemical studies were obtained from Span diagnostics, India.

Plant Material

Fresh leaves of *Psidium guajava* for the present study were collected in the month of January, locally from Bhopal, (M.P.) India. It was identified and authenticated by Dr. Zia Ul Hasan, Head of Department, Department of Botany, Safia Science College, Bhopal, (M.P.) India, and a specimen voucher (493/Bot/Safia/14), deposited in the Herbarium of the Department of Pharmacognosy, Truba Institute of Pharmacy, Bhopal, (M.P.), India, for future reference.

Extraction

The leaves of *Psidium guajava* were shade dried for 2 weeks, pulverized to coarse powder, and then passed through sieve no. 20 to maintain uniformity. Coarsely dried powder of the leaves was first defatted with petroleum ether (60-80ºC) for 72 h to remove fatty materials and then extracted with ethanol (95%) using soxhlet apparatus for 36 h, obtained green color extract was collected, filtered through Whatman filter paper (No. 42), concentrated in vacuum under reduced pressure using a rotary flash evaporator and the dried crude extract was stored in airtight container at 4ºC for further study. The yield of the extract was 8.64%.

Phytochemical Screening

EEPG was subjected to various phytochemical screening tests for the identification of the phytoconstituents presents in *Psidium guajava* leaves using standard procedures [23].

Animals

The experiment was carried out on Healthy male Wistar albino rats, weighing between 150-200 g. Animals were provided from the authorized animal house of Truba Institute of Pharmacy, Bhopal (M.P.). The animals were acclimatized to the standard laboratory conditions in cross ventilated animal house at temperature 25±2°C relative humidity 44-56% and light and dark cycles of 12:12 hours, fed with standard pellet diet and water *ad libitum* during experiment. The experiment was approved by the institutional animal ethics committee (IAEC) as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (Approval No. 1196/a/08/CPCSEA).

Preparation of Test Formulation of Extract

A suspension formulation of EEPG was prepared in 0.5% carboxy methyl cellulose solution with distilled water and stored at 2-8ºC for further studies.

Acute Oral Toxicity Study

The acute oral toxicity study was evaluated as per Organization for Economic Cooperation and Development (OECD) guidelines no. 425 [24] on Wistar albino rats, weighing between 150-155g. Before the experiment, rats were fasted overnight with water *ad libitum*. Three animals were selected which receives a dose of 2000 mg/kg. All three animals were received a single dose of 2000 mg/kg body weight of EEPG by oral gavage. Animals were observed individually for any sign of toxicity, behavioral changes, and mortality after dosing, with special attention given during the first 4 hours, and thereafter for 24 hours, for a total period of 7 days.

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In-vivo Anti-urolithiatic Activity

Ethylene glycol-induced urolithiasis in rats [25, 26]

Ethylene glycol-induced urolithiasis model was commonly used to evaluate the antiurolithiatic activity in experimental animals. Thirty-six male Wistar albino rats were randomly divided into six groups, consisting of six animals in each group. Group I served as control and received normal food and drinking water *ad libitum* for 28 days. Group II, served as negative control group received only 0.75% Ethylene glycol (EG) in drinking water for 28 days. Group III served as positive control group received Ethylene glycol (EG) 0.75% (v/v) in drinking water, for induction of renal calculi for 28 days and standard antiurolithiatic drug, Cystone (750 mg/kg body weight, p.o.) from the 15th day till 28 days. Groups IV, V, and VI served as test groups and received Ethylene glycol 0.75% (v/v) in drinking water, for induction of renal calculi for 28 days and EEPG 100, 150, and 200 mg/kg body weight respectively from 15th to 28th day. All extracts and standard drug were given once daily by oral route.

Evaluation of Anti-urolithiatic Activity

Change in body weight

Body weight of each animal from all the groups was measured every 7th day to assess the percentage change in body weight during overall study period.

Analysis of urine

At the end of the study, all the animals from each group were kept in individual metabolic cages and 24 h urine samples were collected. Animals had free access to drinking water during the urine collection period. After 24 h urine collection the volume of urine and pH was measured and one drop of 0.1 N hydrochloric acid was added to the collected urine samples before being stored at 4°C to inhibit the microbial growth during storage. The urine samples were analyzed for calcium, oxalate, creatinine, urea and uric acid content using the Span diagnostic kit in clinical auto-bioanalyzer.

Analysis of serum

At the end of the study, after urine collection, blood sample was collected from all the animals by retro-orbital puncture under mild anesthesia. Serum was separated by centrifugation at 3000 rpm for 15 minutes and analyzed for calcium, creatinine, urea and uric acid content using the Span diagnostic kit in clinical auto-bioanalyzer.

Histopathological study of kidney

At the end of the study, all rats were anesthetized and abdomen was cut open to isolate both the kidneys from each animal. Isolated kidneys were fixed in 10% neutral buffered formalin. Histological sections (about 5µm thickness) were prepared by microtomy and stained with hematoxylin-eosin (H&E) dye for histological examination. Histological slides were examined under a light microscope at 10X magnification.

Statistical analysis

The results are expressed as mean ± Standard error of mean (SEM). The statistical significance was analyzed using One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test by employing statistical software, Graph Pad, In Stat 3. Differences between groups were considered significant at $P<0.05$ levels.

3. RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical investigation of ethanolic extract revealed the nature of phytochemicals present in *Psidium guajava* leaves. EEPG was found to contain alkaloids, glycosides, flavonoids, tannins, triterpenoids, polyphenols, carbohydrates and proteins.

Acute Oral Toxicity Study

The acute oral toxicity study of EEPG was determined according to the OECD (Organization for Economic Corporation and Development) guidelines no. 425. No sign of any toxicity, no change in sleep and behavior pattern and mortality observed throughout the study period. Administered dose was found tolerable (as no death found).

Change in Body Weight

Body weight of animals was recorded every seventh day during overall 28 days of the study period. During the overall study period of 28 days, the body weight of normal control animals was increased a bit [Table 1, group I], but at the end of study on 28th day it decreased for the untreated calculi induced animals receiving 0.75% (v/v) Ethylene glycol for 28 days [Table 1, group II]. Supplementation with EEPG (100, 150, and 200 mg/kg, body weight, p.o.) inhibits the marked change in body weight of test animals, in comparison to calculi induced animals showing its curative effect [Table 1, group IV-VI].

Table 1. Change in body weight

Groups	Body Weight (g)					% Change in Body Weight
	Initial	Day 7	Day 14	Day 21	Day 28	
I	156.83 ± 2.97	157.83 ± 3.17	156.66 ± 2.75	157.83 ± 2.81	158.16 ± 3.00	–
II	176.5 ± 2.75	175.16 ± 2.60	165 ± 2.08	159.83 ± 1.85	155.5 ± 1.62	11.89
III	172.16 ± 4.18	171.0 ± 4.12	167.16 ± 4.24	165.0 ± 4.12	165.5 ± 4.38	3.86
IV	175.83 ± 3.54	174.0 ± 3.59	170.66 ± 3.75	163.83 ± 4.02	160 ± 3.93	9.04
V	176.33 ± 2.56	172.83 ± 2.44	171.0 ± 2.55	164.16 ± 2.56	161 ± 2.56	8.69
VI	179.33 ± 1.45	177.0 ± 1.52	172.83 ± 1.64	170.5 ± 1.92	169.83 ± 2.11	5.29

All values are represented as mean ± SEM, n = 6 animals in each group.

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Analysis of Urine

Urinary volume remained constant in normal control group throughout the experiment [Table 2, group I], while calculi induced animals showed decreased urine volume in comparison to normal control group animals on the 28th day [Table 2, group II]. While in the positive control and test groups, the urine out-put was somewhat higher than that of the calculi induced animals [Table 2, group III and group IV-VI]. In the present study, EEPG at the dose of 100 and 200 mg/kg, body weight, p.o., significantly (P<0.05) increased the urine volume demonstrating its diuretic activity.

Table 2. Urine analysis

Group	Volume of urine (ml)
I	2.85 ± 0.03
II	2.17 ± 0.04a***
III	3.83 ± 0.02a***, b***
IV	2.63 ± 0.04a*, b***, c***
V	2.83 ± 0.03b***, c***
VI	3.09 ± 0.10a*, b***, c***

All values are represented as mean ± SEM, n = 6 animals in each group, Data were analyzed by one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test, a- Significant difference as compared to normal control group (group-I), b- Significant difference as compared to negative control group (group-II), c- Significant difference as compared to standard group (group-III) and *P<0.05, **P<0.01, ***P<0.001

Table 3. Effect of ethanolic extract of *Psidium guajava* on urine parameters

Group	Calcium (mg/day)	Oxalate (mg/day)	Creatinine (mg/day/ml)	Urea (mg/day/ml)	Uric acid (mg/day/ml)
I	2.88±0.10	3.5 ±0.13	1.15 ± 0.12	36.41 ± 0.76	2.26 ± 0.15
II	7.61 ± 0.25a***	10.21 ± 0.31a***	4.1 ± 0.33a***	55.38 ± 0.90a***	5.33 ± 0.23a***
III	3.4 ± 0.23b***	6.4 ± 0.17a***, b***	2.31 ± 0.16a**, b***	30.4 ± 1.05a***, b***	3.76 ± 0.22a***, b***
IV	7.55 ± 0.31a***, c***	9.58 ± 0.13a***, c***	3.73 ± 0.22a***, c***	48.45 ± 0.74a***, b***, c***	4.68 ± 0.17a***, c**
V	6.28 ± 0.12a***, b**, c***	8.23 ± 0.08a***, b***, c***	3.36 ± 0.13a***, c**	40.26 ± 0.44a*, b***, c***	4.5 ± 0.13a***, b*
VI	4.66 ± 0.19a***, b***, c**	7.13 ± 0.15a***, b***	2.03 ± 0.10a*, b***	32.78 ± 0.57a*, b***	3.4 ± 0.12a***, b***

All values are represented as mean ± SEM, n = 6 animals in each group, Data were analyzed by one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test, a–Significant difference as compared to normal control group (group-I), b–Significant difference as compared to negative control group (group-II), c–Significant difference as compared to standard group (group-III) and *P<0.05, **P<0.01, ***P<0.001

In the present study, urinary calcium oxalate, creatinine, urea and uric acid excretion was significantly (P<0.001) increased in calculi induced animals [Table 3, group II], indicating distinct renal damage by the chronic administration of Ethylene glycol 0.75% (v/v) for 28 days which was evident from the results of biochemical analysis of urine. However, supplementation with EEPG at 100, 150, and 200 mg/kg, body weight, p.o. and Cystone 750 mg/kg, body weight, p.o. significantly (P < 0.05 and P < 0.001) inhibited these changes in positive control and test groups animals [Table 3, group III and group IV-VI] as compared to calculi induced animals [Table 3, group II].

Analysis of Serum

In the present study, calculi induced by chronic administration of 0.75% (v/v) ethylene glycol for 28 days resulted in impairment of renal functions of the calculi induced animals as evident from markers of glomerular and tubular damage, i.e., significantly (p<0.001) increased serum calcium, creatinine, urea and uric acid levels in rats [Table 4, group II]. However, treatment with EEPG (100, 150, and 200 mg/kg, body weight, p.o.) significantly (p<0.05) decreased the elevated levels of calcium and creatinine in serum as compared to the calculi induced animals [Table 4, group IV-VI]. Serum urea and uric acid level was significantly (p<0.001) increased in calculi induced animals, indicating marked renal and glomerular damage [Table 4, group II]. Supplementation with Cystone significantly and EEPG (group IV, V and VI) dose dependently lowered the elevated serum level of urea and uric acid as compared to group II [Table 4, group III and group IV-VI].

Table 4. Effect of ethanolic extract of *Psidium guajava* on serum parameters

Group	Calcium (mg/day)	Creatinine (mg/dl)	Urea (mg/dl)	Uric acid (mg/dl)
I	8.63 ± 0.14	1.11 ± 0.15	35.31 ± 0.90	1.16 ± 0.10
II	13.4 ± 0.29 a***	2.83 ± 0.17 a***	70.5 ± 0.52 a***	3.2 ± 0.16 a***
III	9.15 ± 0.29 b***	1.46 ± 0.06 b***	53.15 ± 0.34 b***	1.4 ± 0.08 b***
IV	13.83 ± 0.28 a***, c***	2.68 ± 0.13 a***, c***	62.31 ± 0.80 a***, c***	2.05 ± 0.16 a***, b***, c*
V	11.83 ± 0.42 a***, b*, c***	2.46 ± 0.16 a***, c***	57.78 ± 0.82 a***, b*, c***	2.18 ± 0.12 a***, b***, c**
VI	10.1 ± 0.33 a*, b***	2.13 ± 0.14 a***, b*, c*	55.18 ± 0.60 a*, b***	2.21 ± 0.13 a***, b***, c**

All values are represented as mean ± SEM, n = 6 animals in each group, Data were analyzed by one-way ANOVA, followed by Tukey-Kramer Multiple Comparisons Test, a–Significant difference as compared to normal control group (group-I), b–Significant difference as compared to negative control group (group-II), c–Significant difference as compared to standard group (group-III) and *P<0.05, **P<0.01, ***P<0.001.

Histopathological Study of Kidney

Figure 1 represents the histopathological examination of the kidney. Histopathological examination was done with the help of Trinocular microscope. Histopathological section of normal control animal's kidney revealed no abnormalities like proximal tubules dilation and interstitial inflammation within the renal

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tissue [Figure 1a]. On the other hand, section of the calculi induced rat kidney, represented, marked interstitial inflammation within the renal tissue along with proximal tubules dilation, deposition of intratubular and interstitial crystal inside the tubules was a characteristic sign of calculi formation on chronic administration of 0.75% Ethylene glycol (v/v) for 28 days [Figure 1b]. However, the number of intratubular and interstitial crystals inside the tubules of EEPG treated rats [Figure 1d-f] and standard drug Cystone treated rats [Figure 1c] reduced in comparison to calculi induced group. EEPG treatment also reduced the degenerative changes in the kidney tissue like epithelial cell dissociation, interstitial infiltration of the inflammatory cells, proximal tubules dilation [Figure 1d-f].

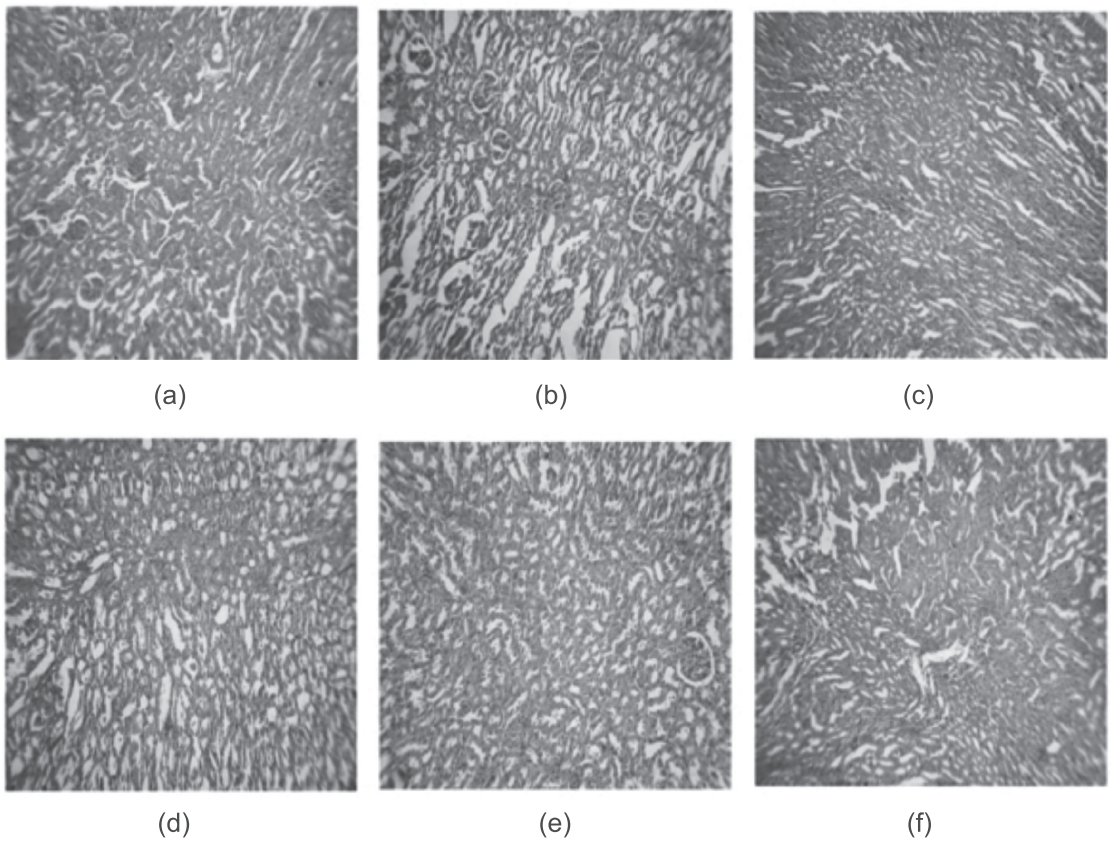

Fig. 1. Histopathological examination of kidney, Sections of (a) normal control (group-I), (b) negative control (group-II), (c) standard, Cystone treated (group-III), (d) treatment with EEPG at the dose of 100 mg/kg (group-IV), (e) treatment with EEPG at the dose of 150 mg/kg (group-V), (f) treatment with EEPG at the dose of 200 mg/kg (group-VI)

Since urolithiasis is a recurrent, painful urinary stone disease involving the major organs of the urinary system, in recent years out of many treatment strategies for treatment of urolithiasis, herbal medications have proven their role to find out efficient therapy for urinary stones [27, 28]. The World Health Organization also encourages the importance of herbal drugs for the cure of several disorders due to their availability, affordability and lesser side effects [29]. In the present study an attempt was made to evaluate the effect of extractive phytoconstituents of *Psidium guajava* on Ethylene glycol induced Urolithiasis in experimental animals. Phytochemical screening revealed that EEPG contains alkaloids, glycosides, flavonoids, tannins, triterpenoids, polyphenols, carbohydrates and proteins. Male rats were selected; on the basis of previous studies for the induction of urolithiasis because the urinary system of male rats is similar to that of humans [30]. The results of the present study indicated that administration of 0.75% Ethylene glycol (v/v) to the animals for 28 days administration, form renal calculi composed mainly of calcium, oxalate and uric acid stones [31].

During the overall study period of 28 days, normal control animals were remained active and gained the weight, while untreated calculi induced animals receiving 0.75% (v/v) Ethylene glycol consistently lost their weight over 28 days. Supplementation with EEPG (100, 150, and 200 mg/kg, body weight, p.o.) inhibits the marked change in body weight of test group animals, in comparison to calculi induced animals showing its protective effect.

Chronic administration of 0.75% Ethylene glycol (v/v) facilitates the increased risk of urolithiasis by elevating urinary levels of stone forming constituents (calcium, oxalate, and uric acid) and provides a favorable environment for the nucleation and stone growth. Ethylene glycol increase the action of oxalate synthesizing liver enzyme glycolate oxidase this enzyme further increases oxalate production by means of increasing substrate

availability. An increase in urine output in EEPG treated animals was observed, which dilutes the concentration of urinary waste products and electrolytes. As a result, calcium, creatinine, urea and uric acid were flushed out via urine, decreasing the chance of precipitation, formation and growth of urinary stone. Up to 80-90% of stones in the urinary system originated from the general components of urine such as calcium and oxalate, which causes the hypercalciuria and hyperoxaluria respectively during stone formation [32]. However, EEPG lowered the level of oxalate as well as facilitates the excretion of calcium, creatinine, *urea and uric acid via urine*, which is valuable in the prevention of calculi formation.

In urolithiasis, urinary supersaturation with respect to stone-forming constituent's e.g., calcium and oxalate are usually considered to be one of the etiological factors. During this process glomerular filtration rate and outflow of urine get decreased due to the presence of calculi in the urinary system as a result of waste products, majorly nitrogenous materials such as creatinine, urea and uric acid get accumulated in blood. These nitrogenous substances have been reported to cause renal and tubular damage by inducing lipid peroxidation in cell membrane [33]. Blood urea and uric acid level are considered as excellent markers of equilibrium in the nitrogen metabolism.

In the present study marked renal damage was seen in calculi induced animals, as indicated by the significant increase in serum calcium, creatinine, urea and uric acid level on treatment with 0.75% (v/v) Ethylene glycol for 28 days, whereas serum urea and uric acid levels were found to be reduced in a dose dependent manner in the EEPG treated group, indicating a protective effect of EEPG. Increased serum creatinine level in the calculi induced animals is a sign of renal impairment due to hyperoxaluria. Supplementation of EEPG reduced the serum creatinine level significantly.

Histopathological examination of kidney sections of calculi induced animals showed interstitial inflammation within the renal tissue, proximal tubules dilation. On the other hand, treatment with EEPG decreases the interstitial inflammation in different parts of the renal tissue and proximal tubules dilation. EEPG showed its protective effect via reducing the degenerative changes in the kidney tissue like epithelial cell dissociation, interstitial infiltration of the inflammatory cells, proximal tubules dilation.

Probable mechanism of action *Psidium guajava* may be due to its important phytoconstituents present in leaves such as flavanoids, triterpenoids and polyphenols, which were confirmed in phytochemical study. Previous studies reported that flavonoids and polyphenolic compounds exert their curative effect in urolithiasis via reducing the spasmodic pain, inflammation, increasing the output of urine and antioxidant effect [34, 11]. Moreover, anti-inflammatory, diuretic and antioxidant activity [12, 35, 19] of *Psidium guajava* probably contributes to its antiurolithiatic activity. All these extractive phytoconstituents and previous pharmacological activities may be responsible for the antiurolithiatic activity of EEPG.

4. CONCLUSION

In conclusion, the results indicate that co-administration of EEPG reduced and prevented the growth of urinary stones. The probable mechanism could be due to its extractive phytoconstituents possessing diuretic, anti-inflammatory, and antioxidant property, and decreasing the concentration of urinary stone-forming substances. Finally, our results showed that the anti-urolithiatic activity of the *Psidium guajava* was a result of the presence of the rich amount of bioactive phytoconstituents in leaves. These findings suggest the potential for use of *Psidium guajava* leaves in the treatment of urolithiasis confirming their traditional use in urinary disorder. Further studies are required to elucidate the isolated phytoconstituents of the extract and to understand their mechanism of action.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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