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Protective potential of *Quercus leucotrichophora* A. Camus seeds in chemically induced urolithiasis in rats

Research Article

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Abstract

Background: *Quercus Leucotrichophora* A. Camus (QL) seeds are used in traditional medicine to treat various urinary and renal diseases. It is used as a diuretic, antioxidant, and nephroprotective agent. In the present study, we investigated the in vitro and in vivo antiurolithiatic activity of methanolic extract of *Quercus Leucotrichophora* A. Camus (MEQL) Seeds. Methods: An *in vitro* assay was used to determine whether MEQL prevents the formation of calcium oxalate (CaOx) or promotes CaOx dissolution. In in vivo studies, the effects of oral administration of Methanolic extract of test drug were studied on calcium oxalate urolithiasis in male Wistar rats. Male Wistar rats were divided into six groups of 6 animals each. Animals of Normal control received regular drinking water ad libitum throughout the study. whereas in other groups nephrolithiasis was induced by providing drinking water containing 0.75% ethylene glycol and 1% ammonium chloride for 7 days. Test groups were treated with 250 mg/kg, 375 mg/kg, and 500 mg/kg of the test drug and standard control with Cystone (750 mg/kg) for 21 days. Urine was examined for the presence of crystals on the 8th and 22nd days. On the 22nd day, 24 h urine, serum was collected and various biochemical parameters were estimated in urine, serum, and kidney homogenate along with histology of the kidney. Results: In the in vitro experiments, MEQL exhibited a concentration-dependent inhibitory activity on nucleation and aggregation. The urine volume increased significantly in all test groups. Test groups showed a significant reduction in the number of CaOx crystals in urine. Levels of urinary calcium, phosphorus, and sodium; Serum levels of calcium, phosphorus, creatinine, and urea decreased significantly in standard and test groups. Histopathology shows significant changes. From the above results, it can be concluded that the methanolic extract of *Quercus leucotrichophora* seeds has a significant inhibitory effect on calcium oxalate urolithiasis due to their diuretic and antioxidant activity. Conclusion: These results indicate that MEQL showed significant activity in urolithiasis which might be due to diuretic and antioxidant action.

Keywords: Antiurolithiatic, Calcium oxalate, *Quercus leucotrichophora,* Ethylene glycol, Antioxidant.

Introduction

Urolithiasis (kidney stone disease) is a common and often devastating condition with varying causes and pathogenesis. Symptoms of kidney stones can range from completely inconspicuous to extremely painful with microscopic or overt haematuria, ureteral obstruction, and urinary tract infection. They are also associated with symptoms such as malaise, cloudy or foul-smelling urine, more frequent urination, burning with urination, chills, fever, nausea, and vomiting. It is a multifactorial disease that results from the combined influence of epidemiological, biochemical, and genetic risk factors. Urolithiasis mainly affects people of productive age, and men are more frequently affected by this disease than women. (1, 2) The complex process of kidney stone formation is the result of a series of physicochemical processes such as supersaturation, nucleation, growth, aggregation, and retention in the renal tubules. (3) Stone formation occurs due to disturbances in urine flow rate, uric acid excretion, and urine pH. Increased bone resorption, hypercalciuria, hypocitraturia, hypomagnesemia, and hyperphosphaturia are risk factors for calcium oxalate kidney stones. (4, 1) The main risk factor for urolithiasis is the supersaturation of urine with mineral salts. The type of stones formed correlates with the degree of supersaturation of urine. Therefore, a reduction in supersaturation is useful in preventing the recurrence of stones. (5)

Quercus leucotrichophora, commonly known as banj oak or ban tree, belongs to the Fagaceae family. (6) Several species of the Quercus genus have strong medicinal and therapeutic effects. In the Himalayan region, the ban tree is the main source of fuel and fodder. The leaves, seeds, and bark of QL are used in human and veterinary medicine to cure a variety of

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diseases. In northern Pakistan, seeds and fruit powder are used to cure urinary tract infections. (7, 8)

It has shown pharmacological effects such as diuretic activity (8), antiasthmatic (9), antimicrobial activity (10), and antiarthritic potential (11). In traditional Indian medicine, different parts of the plant are used to treat various human ailments, such as toothache and hemorrhoids in Jammu, Kashmir, and Ladakh (India); the leaves are used as astringents and to treat diarrhea, and the gum resin is used to treat stomach pain (12). Tribal people in

Uttarakhand's Tehri Garhwal district makes a paste from the gum resin of the plant and uses it to treat gonorrhea, asthma, hemorrhage, diarrhea, and dysentery (13). The seeds of the plant are used to cure urinary tract diseases (14). *Quercus leucotrichophora* A. Camus has also been used to treat dysentery and diarrhea at home. (15) The seeds of *Quercus leucotrichophora* are highly revered for their nutritional value and medicinal properties. The seeds of the plant are used as an astringent, antioxidant, and diuretic in the treatment of urinary tract disorders. (8)

The present study aims to substantiate the traditional use of *Quercus leucotrichophora* in renal diseases using various biochemical parameters. The study investigates the anti-urolithiasis activity of seed extracts of *Quercus leucotrichophora* using in vitro and in vivo models.

Material and methods

Drug and chemicals

Ethylene glycol was purchased from Chem Think Lab, Ankleshwar, Gujarat, India. Cystone was purchased from Himalaya Health Care Pvt. Ltd. Various kits for biochemical determination of urine and serum were purchased from Lab Care Diagnostics Pvt Ltd, Gujarat, India. All other chemicals and reagents used were of analytical grade and were purchased from approved chemical suppliers.

Plant material

Seeds of *Quercus leucotrichophora* were obtained from Chail Chowk, Mandi, Himachal Pradesh, and authenticated from DR. B. R. Patel, associate professor of botany, Patidar Gin Science College, Bardoli, Dis Surat.

Preparation of extract

The dry seeds were ground in a mortar and pestle and sieved through sieve No. 14. Before extraction, the powdered seeds were kept in an airtight container. The dried powder of QL seeds (100 g) was then extracted in a Soxhlet extractor at 60-65°C for 5 hours with methanol (250 ml). The extract was filtered while still hot and concentrated in a rotating vacuum evaporator. The dried extract samples were stored in an airtight container in a refrigerator at low temperature until use. (8)

Experimental animals

Experiments were conducted in accordance with the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA). The experimental protocol of the study was approved by the Ethical Committee of the Institution for Animals (CPCSEA/SNLPCP/IAEC/04/02/140). The male albino rats of Wistar strain weighing 200-250 g were obtained from Jay Research Foundation, Vapi, Gujarat. The rats were maintained at a temperature of 25 ± 1°C and relative humidity of 50 ± 55%. The rats were fed with standard chow and water ad libitum. Animals were acclimatized in institutional animal shelters and exposed to a 12-day night cycle.

Evaluation of the in vitro antiurolithiatic activity

The classical model for the study of oxalate crystallization was chosen for its simplicity and satisfactory reproducibility, depending on the method, crystallization without inhibitors and with them to evaluate the inhibitory capacity of the test material. The anti-urolithic activity of the plant extracts was evaluated in vitro by inhibition of calcium oxalate nucleation, aggregation, and growth in the presence of inhibitors (standard drug and extracts) and the absence of inhibitors. A UV/Vis spectrophotometer (Shimadzu UV-1700) was used to measure turbidity changes in each assay. Five different concentrations (100µg/ml, 200µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml) of the plant extracts were tested in each assay.

Nucleation assay

Calcium chloride (4 mmol/L) and sodium oxalate (50 mmol/L) solutions were prepared in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 and 37°C. Calcium chloride solution (950µl) was mixed with 100 µl of different extracts at different concentrations (100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml)). Crystallization was initiated by adding 950 µl sodium oxalate solution at 37°C. The nucleation rate was determined by comparing the induction time of crystals (the time of appearance of crystals that have reached a critical size and are thus optically detectable) in the presence of the plant extract and the control without extract. Absorbance (optical density) was measured at 620 nm after 10, 20, 30, 60, and 90 minutes. The development of the crystals was probably due to the associated reaction: (16)

$$CaCl_2 + Na_2C_2O_4 \rightarrow CaC_2O_4 + 2NaCl$$

$$\% \text{ Inhibition of nucleation} = \frac{\text{Absorbance of control - Absorbance of test}}{\text{Absorbance of control}} \times 100$$

Aggregation assay

Calcium oxalate monohydrate crystals (COM) were prepared by mixing calcium chloride and sodium oxalate at 50 mmol/l. Both solutions were brought to 60°C in a water shower for 1 hour and then cooled to 37°C overnight. Crystals were obtained by centrifugation and then dissolved at 37°C. CaOx crystals were used at the final concentration of 0.8 mg/ml supported with Tris 0.05 mol/l and NaCl 0.15 mol/l at pH 6.5. Assays were performed separately in the

absence and presence of the plant (100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml) after stopping the mixture. Cystone tablets were used as the standard drug solution. Percent aggregation inhibition was calculated by comparing turbidity in the presence of the extract to that of the control using the following formula: (17)

$$\% \text{ aggregation (Ir)} = 1 - \text{Turbidity sample} / \text{Turbidity control} \times 100$$

Evaluation of the in vivo antiurolithiatic activity

Screening of the antiurolithiatic potential of Methanolic Extract of Quercus leucotrichophora was performed on rats using an ethylene glycol (EG) and ammonium chloride (NH_4Cl) induced model of urolithiasis. Forty-two male Wistar rats were divided into seven groups (n = 6).

- Group I: Normal control group received regular drinking water ad libitum throughout the study.
- Group II: The model control group received Ethylene glycol 0.75% (V/V) and Ammonium chloride 1% (W/V) for 7 days (p.o)
- Group III: Standard control group received Cystone (750 mg/kg) from the 8th to the 21st day (p.o.)
- Group IV: MEQL (250mg/kg) from 8th to 21st day (p.o.)
- Group V: MEQL (375mg/kg) from 8th to 21st day (p.o.)
- Group VI: MEQL (500mg/kg) from 8th to 21st day (p.o.)
- Groups II-VII, received EG and AC in their drinking water ad libitium for 7 days, respectively, to induce urolithiasis and generate CaOx deposition into the kidneys. The body weight of all the animals was noted on the 0, 8th, and 22nd days of the experiment. All the animals were sacrificed at the end of the experiments. (18)

Analysis of urine Samples

All animals were housed in individual metabolic cages, and a 24-hour urine sample was collected on day 21. A 24-hour urine sample was collected on day 21. A 24-hour urine sample was collected on the 21st day. Body weight (19), water intake, volume, urine pH (20), and the number of CaOx crystals in the urine were determined.

Urine was acidified with a drop of concentrated HCl and stored at –20 ºC for the determination of calcium, oxalate, magnesium, phosphate, and uric acid using commercially available kits. Oxalate and citrate were determined by the method described previously. (21, 22)

Collection and Analysis of Serum

After the experimental period, blood was collected from the retroorbital region under mild ether anesthesia, and the animals were sacrificed under high-dose anesthetics. The serum was separated by centrifugation at 10,000 g for 10 minutes and analyzed for calcium, creatinine, urea, urea, and blood urea nitrogen (BUN) using commercially available diagnostic kits, while oxalate was measured. (23)

Kidney Histopathology and homogenate analysis

The abdomen was cut open and opened, and both kidneys were removed from each animal. The detached kidneys were freed from nonessential tissue, calibrated, and washed with super-cold saline. The left kidney was fixed with 10% v/v neutral formalin and, after removal, sectioned on a flat surface and sent to the Histology Administration (Khandwala Pathology Lab, Surat) for hematoxylin and eosin staining.

The same histological specimens were examined microscopically for the presence of glomerular congestion, tubular impressions, peritubular congestion, epithelial adhesions, vascular congestion, interstitial edema, and inflammatory cells.

The right kidney was finely minced, and a 20% homogenate was prepared in Tris-HCl buffer (pH 7.4). The absolute kidney homogenate was used for the assay of tissue calcium and oxalate, lipid peroxidase (LOP), reduced glutathione (GSH), and SOD (24, 25, 26).

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant. The data obtained were analysed by one-way analysis ANOVA followed by Dunnett's test for multiple comparisons using Graph Pad Prism version 8.0. (27).

Results

Results of the qualitative analysis of the extract revealed the presence of tannins, saponins, phenols, flavonoids, Terpenoids, Steroids, and Amino acids.

In vitro studies

Table 1: Inhibitory effects of MEQL on nucleation and aggregation of calcium oxalate

Concentration (µg/ml)	Percentage Inhibition of Nucleation	Percentage Inhibition of Aggregation
MEQL		
100	31.67	49.34
200	46.35	55.95
300	54.43	60.41
400	68.28	67.25
500	73.62	75.96
Cystone		
100	52.70	58.12
200	58.18	65.25
300	64.70	72.95
400	68.43	76.37
500	75.90	77.96

In Vivo studies

Table 2: Effects of MEQL on Body weight and water intake

Groups	Body weight			water intake (mL)		
	Day 0	Day 8	Day 22	Day 0	Day 8	Day 22
Group-I Normal Control	250.5 ± 1.33	253 ± 0.85	251.6 ± 1.60	25.51 ± 0.77	22.25 ± 1.10	24.5 ± 0.42
Group-II Model Control (EG+NH_4Cl)	252.3 ± 1.05	203.8 ± 1.19###	208.5 ± 1.70***	23.83 ± 0.94	14.41 ± 1.01###	13.16 ± 0.94***
Group-III Standard (Cystone 750mg)	253.1 ± 0.60	212.6 ± 1.83###	243.5 ± 0.76***	23.8 ± 0.84	17.0 ± 0.75###	24.16 ± 1.07***
Group -IV MEQL 250 mg	251.6 ± 1.05	205.5 ± 1.33###	217.6 ± 0.88***	23.91 ± 0.66	17.16 ± 0.54###	18.16 ± 0.60***
Group-V MEQL 375 mg	254.5 ± 1.47	204.3 ± 1.68###	230 ± 0.57***	23.5 ± 0.76	17.5 ± 0.5###	19 ± 0.36***
Group-VI MEQL 500 mg	251.5 ± 0.76	206.1 ± 2.35###	240.3 ± 1.40***	22.41 ± 0.69	19.11 ± 0.63###	20.58 ± 0.66***

The values are expressed as mean ± SEM (n=6); Statistical analysis by ANOVA, followed by Dunnett's Multiple Comparison tests. ***p<0.001, ** p<0.01, *p<0.05 # Day 8 compared with Day 0 * Day 22 compared with Day 8.

Table 3: Effects of MEQL on Urine volume, Urine pH, and No. of urine CaOx crystal

Groups	Urine volume			Urine pH		No. of urine Caox crystal	
	Day 0	Day 8	Day 22	Day 8	Day 22	Day 8	Day 22
Group-I Normal Control	4.96 ± 0.01	4.97 ± 0.07	4.93 ± 0.01	6.73 ± 0.01	6.49 ± 0.11	1.79 ± 0.01	1.51 ± 0.00
Group-II Model Control (EG+NH_4Cl)	4.86 ± 0.07	3.91 ± 0.11###	3.80 ± 0.16***	5.38 ± 0.08###	5.51 ± 0.06***	10.13 ± 0.01###	8 ± 0.36***
Group-III Standard (Cystone 750mg)	4.76 ± 0.16	3.45 ± 0.21###	5.22 ± 0.16***	5.45 ± 0.01###	7.09 ± 0.09***	10.22 ± 0.00###	4.91 ± 0.27***
Group –IV MEQL 250 mg	4.36 ± 0.25	3.44 ± 0.35###	4.11 ± 0.05***	5.60 ± 0.03###	6.35 ± 0.10*	10.21 ± 0.00###	6.91 ± 0.27*
Group-V MEQL 375 mg	4.24 ± 0.24	3.64 ± 0.21###	4.27 ± 0.09***	5.36 ± 0.01###	6.95 ± 0.14**	10.26 ± 0.01###	6.50 ± 0.22**
Group-VI MEQL 500 mg	4.71 ± 0.19	3.90 ± 0.10###	5.04 ± 0.03***	5.43 ± 0.05###	7.21 ± 0.08***	10.19 ± 0.00###	5.75 ± 0.31***

The values are expressed as mean ± SEM (n=6); Statistical analysis by ANOVA, followed by Dunnett's Multiple Comparison tests. ***p<0.001, ** p<0.01, *p<0.05 # Day 8 compared with Day 0 * Day 22 compared with Day 8.

Administration of ethylene glycol and ammonium chloride (EG 0.75% v/v with AC 1% w/v in drinking water) significantly decreased the body weight of model control rats. These changes were significantly ($p < 0.001$) prevented in CYSTONE (750 mg/kg) and MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) treated groups compared to model control rats. A significant ($p < 0.001$) decrease in 24-hour urine volume (ml) was observed in the model control rats, while MEQL showed a significant ($p < 0.001$) improvement in urine output compared to the model control animals. In addition, a significant ($p < 0.001$) decrease in urine pH was observed in the model control group, which was a significant ($p < 0.001$) increase in the MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) and Cystone (750 mg/kg) treated group.

A significantly higher number ($P < 0.001$) of crystals was found in the urine of rats in the model control group than in the urine of the MEQL- and Cystone-treated groups, which had fewer and smaller crystals.

Table 4: Effect of MEQL on Urine Parameters

Groups	Calcium (mg/24 h)	Phosphorus (mg/24 h)	Creatinine (mg/24 h)	Sodium (mEq/dl)	Oxalates (mg/24 h)	Uric acid (mg/24 h)	Magnesium (mg/24 h)
Group-I Normal Control	4.03 ± 0.12	4.91 ± 0.41	0.74 ± 0.01	81.87 ± 1.79	4.41 ± 0.27	2.43 ± 0.21	3.25 ± 0.07
Group-II Model Control	6.32 ± 0.37###	7.58 ± 0.37###	0.59 ± 0.00###	168.8 ± 1.24###	10.5 ± 0.76###	4.76 ± 0.24###	1.21 ± 0.00###
Group-III Standard (Cystone 750mg)	4.62 ± 0.25**	5.08 ± 0.34***	0.71 ± 0.01***	101.8 ± 1.74***	6.58 ± 0.32***	2.50 ± 0.42***	2.51 ± 0.11***
Group-IV MEQL 250 mg	5.19 ± 0.12*	5.75 ± 0.30**	0.67 ± 0.02**	77.83 ± 1.90***	7.83 ± 0.47***	3.33 ± 0.21**	2.00 ± 0.01***
Group-V MEQL375	5.05 ± 0.30**	5.58 ± 0.37**	0.71 ± 0.00***	130.0 ± 2.17***	6.83 ± 0.30***	3.0 ± 0.25***	2.15 ±0.07***
Group-VI MEQL500	4.72 ± 0.17***	5.25 ± 0.26***	0.75 ± 0.00***	141.5 ±1.23***	5.08 ± 0.22***	2.66 ± 0.21***	2.63 ± 0.04***

The values are expressed as mean ± SEM (n=6); Statistical analysis by ANOVA, followed by Dunnett's Multiple Comparison tests. ***p<0.001, ** p<0.01, *p<0.05 Compared with the Model control group. ### p<0.001 when compared to the normal control group.

In the present study, treatment with kidney stones in male Wistar rats resulted in hyperoxaluria. Oxalate, calcium, sodium, phosphate, and uric acid excretion were increased in the model control group. However, supplementation with MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) significantly and dose-dependently prevented these changes in urinary oxalate, calcium, sodium phosphate, and uric acid excretion in the groups IV, V, and VI.

Urinary excretion of magnesium and creatinine was significantly decreased in the model control group compared with the normal control group ($P < 0.001$). Supplementation with MEQL essentially prevented these changes and returned them to near-normal values.

Serum Analysis

Table 5: Effect of MEQL on Serum Parameters

Groups	Calcium (mg/dl)	Phosphorus (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Oxalate (mg/dl)	Uric acid (mg/dl)	BUN (mg/dl)
Group-I Normal	8.81 ± 0.54	3.83 ± 0.32	0.71 ± 0.01	42.6 ± 0.76	0.760 ± 0.00	2.55 ± 0.01	43.3 ± 0.80
Group-II Model Control	12.36 ± 0.79###	6.17 ± 0.29###	1.34 ± 0.05###	54.5 ± 0.90###	2.08 ± 0.08###	6.44 ± 0.01###	73.3 ± 0.88###
Group-III Standard (Cystone 750mg)	9.25 ± 0.19***	4.63 ± 0.2***	0.74 ± 0.01***	43.5 ± 1.23 ***	0.62 ± 0.00***	3.29 ± 0.09***	44.0 ± 0.36***
Group-IV MEQL 250mg	10.26 ± 0.06**	5.16 ± 0.16 *	1.05 ± 0.14*	50.2 ± 1.21*	0.96 ± 0.01***	4.77 ± 0.02***	55.8 ± 0.53***
Group-V MEQL 375mg	9.94 ± 0.08**	5.12 ± 0.17*	0.89 ± 0.01***	47.2 ± 0.77***	0.82 ± 0.00***	4.01 ± 0.04***	54.0 ± 0.57***
Group-VI MEQL 500mg	9.32 ± 0.21***	4.72 ± 0.13***	0.73 ± 0.04***	45.5 ± 0.98***	0.78 ± 0.00***	3.36 ± 0.04***	45.8 ± 0.40***

The values are expressed as mean ± SEM (n=6); Statistical analysis by ANOVA, followed by Dunnett's Multiple Comparison tests. ***p<0.001, ** p<0.01, *p<0.05 Compared with the Model control group. ### p<0.001 when compared to the normal control group.

Renal stone induction caused impairment of the renal function of the untreated rats as was evident from the markers of glomerular and tubular damage: elevated serum creatinine, urea, uric acid, and BUN, which were dose-dependently prevented in the animals receiving a simultaneous treatment with MEQL at the dose of 250 mg/kg, 375 mg/kg, and 500 mg/kg.

Kidney homogenate analysis

Kidney stone induction caused a significant increase ($p < 0.001$) in lipid peroxidation of renal tissues of the model control group, which was prevented in a dose-dependent manner in animals receiving 250, 375, and 500 mg/kg

MEQL. On the contrary, antioxidant enzymes GSH and SOD decreased significantly ($p < 0.001$) in the model control group compared to the untreated rats. However, they increased significantly ($p < 0.001$) after MEQL treatment.

The deposition of calcium and oxalates in kidney tissue was increased in the model control group. However, 250, 375, and 500 mg/kg doses of methanolic extract significantly ($p < 0.001$) reduced the increase of calcium and oxalates in kidney tissues, while 500 mg/kg of methanolic extract significantly ($p < 0.001$) reduced the increase of calcium and oxalates in kidneys.

Table 6: Effect of MEQL on Kidney parameters in ethylene glycol and ammonium chloride-induced urolithiasis

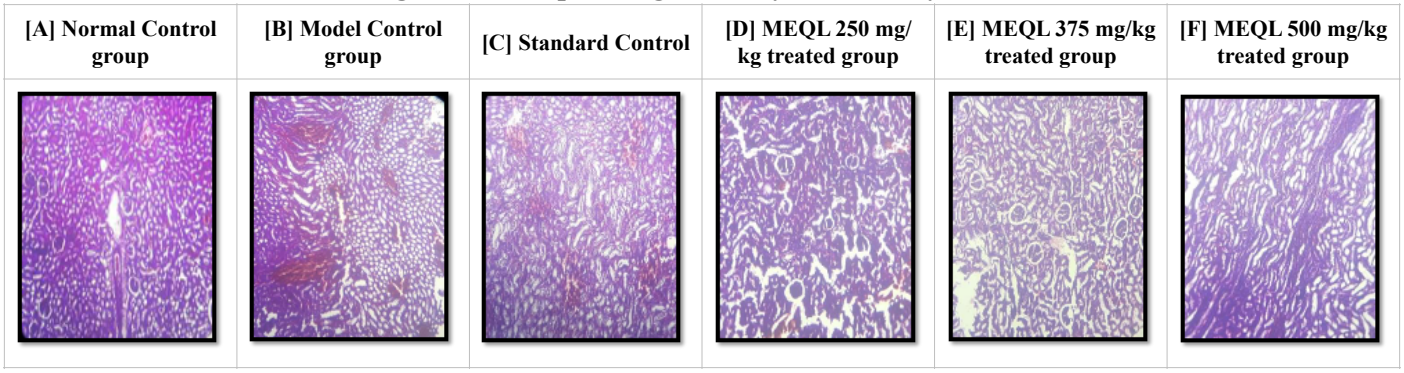
Groups	Calcium (mg/gm)	Oxalate (mg/gm)	GSH	SOD	LOP
Group-I Normal Control	3.22 ± 0.04	1.53 ± 0.02	62.72 ± 0.00	8.17 ± 0.00	9.51 ± 0.42
Group-II Model Control (EG+NH_4Cl)	5.30 ± 0.10###	5.42 ± 0.08###	43.77 ± 0.02###	4.23 ± 0.02###	18.24 ± 0.59###
Group-III Standard (Cystone 750mg)	3.66 ± 0.11***	3.51 ± 0.18***	63.84 ± 0.01***	7.79 ± 0.01***	9.14 ± 0.33***
Group-IV MEQL 250 mg	4.91 ± 0.02**	4.88 ± 0.02*	53.17 ± 0.77***	4.82 ± 0.22*	12.32 ± 0.11***
Group-V MEQL 375 mg	4.15 ± 0.03***	4.63 ± 0.15***	59.40 ± 0.51***	5.19 ± 0.18***	9.48 ± 0.14***
Group-VI MEQL500 mg	3.70 ± 0.06***	4.17 ± 0.17***	68.94 ± 0.17***	7.19 ± 0.08***	9.24 ± 0.04***

The values are expressed as mean ± SEM (n=6); Statistical analysis by ANOVA, followed by Dunnett's Multiple Comparison tests. ***p<0.001, ** p<0.01, *p<0.05 Compared with the Model control group. ### p<0.001 when compared to the normal control group.

Kidney histopathology

The microscopy of the histological section of the kidney revealed the presence of calcium oxalate crystals mostly in renal tubules in the cortex region. The Normal control rats did not show any calcium oxalate crystals in the kidney section. However, repeated administration of cystone 750 mg/kg and MEQL at dosages of 250, 375, and 500 mg/kg for 14 days substantially reduced the amount of calcium oxalate deposits and other anomalies in the renal tubules in a dose-dependent manner.

Figure 1: Histopathological analysis of kidney section



Discussion

Urolithiasis is a urinary stone disease in which calcifications occur in the kidney, bladder, or urethra. The formation of kidney stones has been confirmed by the imbalance of promoter and inhibitor in the kidney and urinary tract, which leads to the graduation phase of crystal nucleation, aggregation, and finally fluid retention. It occurs in both men and women, but the risk is generally higher in men (28, 29, 30)

The EG-induced urolithiasis model has generally been used as an experimental model and may be moderately inconsistent. However, AC-induced rats lead to metabolic acidosis. Therefore, in this study, a high rate of CaOx deposition in the kidney was used with a combination of 0.75% ethylene glycol (EG; V/V) and 1% ammonium chloride (AC; W/V). (31)

Administration of EG and ammonium chloride resulted in crystalluria, hyperoxaluria, and low urine output, which could be the result of renal dysfunction. It is known that EG causes necrosis of proximal tubule cells due to the formation of several metabolites (glycolaldehyde, glyoxylate, glycolate, and oxalate) and the accumulation of large calcium oxalate monohydrate crystals (COM) in the lumen of the renal tubules. The impairment of renal function was evident from the significant increase in serum urea and creatinine levels

and the decrease in creatinine clearance in the positive control group. It also increased urinary calcium and phosphate excretion.

Male rats were selected to induce urolithiasis because the urinary system of male rats is similar to that of humans and previous studies have shown that stone deposition was significantly lower in female rats. (24)

The seeds of *Quercus leucotrichophora* are highly venerated for their nutritional values and medicinal properties. The seeds of the plant are used as an astringent, Antioxidant, and diuretic in the treatment of urinary disorders. (7)

In the present study, we evaluated the anti-urolithic activity of *Quercus leucotrichophora* using a methanolic extract of seeds of *Quercus leucotrichophora* A. Camus (MEQL) both in vitro and in vivo and compared it with the activity of a standard treatment (Cystone).

Kidney stone formation is a complex process involving a series of biological events most likely triggered by genetic susceptibility along with nutritional factors and lifestyle changes. Nucleation is the most critical step in the process of stone formation, which begins with the combination of stone salts in solution into loose clusters that can increase in size with the addition of new components. (32)

Aggregation of crystals is the process by which numerous crystals come together in solution and combine to form large crystal agglomerates. Aggregation is a critical factor in crystal retention because large crystal agglomerates clog the renal tubules, promoting stone formation.

In the nucleation test of the different extracts of Quercus leucotrichophora, the highest percentage of nucleation inhibition of MEQL was obtained at a concentration of 500µg/ml (73.62%). It was found that the methanolic extracts (100µg/ml to 500µg/ml) had a significant percentage inhibition of nucleation compared to the standard Cystone (100µg/ml to 500µg/ml).

In the aggregation assay, the highest percentage of aggregation inhibition of MEQL was obtained at a concentration of 500µg/ml (75.96%). It was found that the methanolic extracts (100µg/ml to 500µg/ml) had a significant percentage inhibition of aggregation compared to the standard Cystone (100µg/ml to 500µg/ml). (33)

Methanolic extract (100µg/ml to 500µg/ml) of *Quercus leucotrichophora* A. Camus showed a dose-dependent inhibition on CaOx nucleation and aggregation.

Changes in body weight and food and water intake serve as initial and helpful indicators of a disease state. A gradual increase in aversion to food intake was observed in all EG-treated groups. It is reported that after 7 days of administration of EG 0.75% with AC 1%, the animals became ill, appeared lethargic, drank less water, stopped eating, and lost body weight. [34]

In the present study, it was found that the body weight of the animals decreased on day 8 in all groups compared with the body weight on day 1. After treatment with the test extract MEQL at the doses of 250 mg/kg, 375 mg/kg, and 500 mg/kg on day 22, the body weight increased compared to day 8. The MEQL at a dose of 500 mg/kg showed greater significance on body weight.

It is reported that after a 7-day course of EG, 0.75% with AC 1%, animals became ill, appeared lethargic, drank less water, and their urine volume and water intake decreased. After 7 days of treatment with plant extracts from the 8th to the 22nd, the animals' urine volume and water consumption reversed. Treatment with MEQL (250 mg/kg, 375 mg/kg, 500 mg/kg) increased urine volume and water intake. The test substance MEQL 500 mg/kg showed a more pronounced effect. (17)

After treatment with MEQL, which increased urine volume, it eventually decreased the saturation of oxalate and calcium ions, preventing the formation of crystals of calcium oxalate in the kidney. Thus, the diuretic effect of MEQL played a role in flushing out the excess ions and helped in the mechanical excretion of the stone. (35, 7)

The PH value of urine is important in kidney stone formation because acidic urine promotes crystal retention by adhesion, leading to stone formation in the distal region of the kidney. When urine pH is low, the solubility of calcium oxalate stones in urine decreases, which promotes stone formation. The increase in pH may be responsible for the dissolution of the complexes of calcium and oxalate, which contributes to their significant anti-urolithiasis activity. In EG and AC-induced rats, urine pH was significantly decreased compared to the normal control group, and a significant increase was observed after treatment with MEQL (250 mg/kg, 375 mg/kg, 500 mg/kg). The test drug MEQL 500 mg/kg showed a more significant pH of about (7.21 ± 0.08, $p < 0.001$). (18)

After induction of crystalluria, the number of calcium oxalate crystals increased significantly in all groups, when compared with Normal control on the 8th day, but on the 22nd day after treatment with the test drug (250 mg/kg, 375 mg/kg, 500 mg/kg) it decreased significantly when compared with on 8th day and with model control on 8th and 22nd day. The dose of 500mg/kg of MEQL was found more significant in CaOx crystal about (5.75 ± 0.31, $p < 0.001$). (36)

The increased urinary calcium promotes the nucleation, and precipitation, of calcium oxalate from urine and subsequent crystal growth. An increase in urinary phosphate excretion was observed in Model control (Lithiatic) when compared to the normal. Elevated urinary phosphate excretion along with oxalate-induced stress appears to provide a suitable environment for stone formation. (37)

In the present study, EG and AC-treated group significantly increased the excretion of Calcium, phosphate, sodium, and Oxalates in urine and decreased creatinine in urine. The increased urinary calcium promotes the nucleation, and precipitation, of calcium oxalate from urine and subsequent crystal growth. Elevated urinary phosphate excretion along with oxalate-induced stress appears to provide a suitable environment for stone formation. Treatment with

MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) showed a significant reduction in urinary calcium, phosphorus, sodium, Oxalates, and creatinine level was significantly increased. MEQL with a dose of 500 mg/ kg showed a more significant balance in calcium, phosphorus, sodium, Oxalates, and creatinine. (32)

In the present investigation, uric acid excretion was increased in urolithic rats. Uric acid interferes with calcium oxalate solubility and reduces the inhibitory activity of glycosaminoglycans. Treatment with MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) lowered the excretion of uric acid and reduced the risk of stone formation.

Normal urine contains many calculi inhibitors and magnesium is one such inhibitor. Low levels of magnesium are observed in stone-forming rats. The magnesium level returns to a normal level after the drug treatment. Magnesium forms a complex with oxalate and reduces the supersaturation of calcium oxalate and as a consequence reduces the growth and nucleation rate of calcium oxalate crystals. Magnesium inhibits oxalate absorption and excretion and thus prevents its supersaturation. Treatment with MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) showed a significant increase in urinary magnesium levels. MEQL with a dose of 500 mg/kg showed a more significant level of magnesium. (38)

The result showed that the Methanolic extract of *Quercus leucotrichophora* reduced supersaturation, which reduced the risk of stone formation.

Induction of EG and AC results in increased serum calcium, phosphorus, and oxalate levels. Treatment with MEQL restored serum calcium, phosphorus levels, and oxalates thus reducing the risk of stone formation.

In urolithiasis, the glomerular filtration rate (GFR) decreases due to the obstruction of the outflow of urine by stones in the urinary system. Due to this, waste products, particularly nitrogenous substances such as urea, BUN, creatinine, and uric acid accumulate in the blood**.** In the present study, administration of EG and AC showed a significant elevation in serum creatinine, BUN, and urea was observed in the model control group compared with a normal control group, which indicates glomerular and tubular damage. Treatment with MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) prevented the elevation of creatinine, BUN, and urea serum levels. Which test drug MEQL 500 mg/kg showed a more significant effect in the elevation of creatinine, BUN, and urea in serum levels. (17)

The present study also showed that there were increased calcium and oxalate levels in kidney homogenate by ethylene glycol and ammonium chloride treatment in model control group. Treatment with MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) lowered the calcium and oxalate levels as compared to the model control group. The MEQL with a dose of 500 mg/kg showed a more significant decrease in calcium and oxalate levels in kidney homogenate.

The development of tissue injury is probably dependent on the balance between the generation of reactive oxygen species (ROS) and the antioxidant defence mechanism of the tissue. Reduced tissue antioxidant enzymes may be followed by increasing free radical generation in the early and late stages of nephrolithiasis, putting the renal tissue under oxidative stress. The oxidative stress will increase due to cell structural injury and inflammation. Oxidative damage is reflected by increased levels of markers of oxidative injury by higher LOP and decreased antioxidant enzyme activity such SOD and GSH. Treatment with MEQL (250 mg/kg, 375 mg/kg, and 500 mg/kg) significantly increased SOD and GSH activity and decreased LOP levels. (39)

Microscopic examination of the kidney sections of ethylene glycol and ammonium chloride-induced urolithiatic rats showed polymorphic irregular crystal deposits inside the tubules causing dilation of the proximal tubules and interstitial inflammation that may be attributed to oxalate. Treatment with the MEQL decreased the number and size of calcium oxalate deposits in different parts of the renal tubules and also prevented damage to the tubules and calyxes. (24)

Conclusion

Antiurolithiatic activity of MEQL seeds is mediated, possibly due to the prevention of urinary supersaturation, inhibition of mineralization of stone-forming constituents, and normalization of the cellular function by neutralizing the effect of ROS, which could have caused oxidative stress in renal tubules. The study suggests that flavonoids, steroids, terpenoids, and phenolic compounds of MEQL are therapeutically effective for the treatment of calcium oxalate stones, exhibiting effects through a combination of a diuretic and antioxidant action, which could be responsible for its antilithiatic activity. Further studies are necessary to determine the mechanism of action of MEQL in the treatment of urolithiasis.

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References

1. Siddiquia WA , Shahzada M, Shabbirb A , Ahmadc A. Evaluation of anti-urolithiatic and diuretic activities of watermelon (*Citrullus lanatus*) using in vivo and in vitro experiments. Biomedicine & Pharmacotherapy 2018; 97:1212-1221.
2. Makasana A, Ranpariya V, Desai D, Mendpara J, Parekh V. Evaluation for the anti-urolithiatic activity of *Launaea procumbens* against ethylene glycol induced renal calculi in rats. Toxicol Reports. 2014; 1:46–52.
3. Zaki S, Jahan N, Kalim M, Islam G, In vitro antilithiatic activity of the hydro-alcoholic extract of *Cinnamomum zeylanicum* Blume bark on calcium oxalate crystallization, Journal of Integrative Medicine. 2019; 273–281.
4. Ahmeda S, Hasana MM, Khan H, Mahmood ZA, Patel S. The mechanistic insight of polyphenols in

calcium oxalate urolithiasis mitigation. BiomedPharmacother. 2018;106: 1292-1299.

5. Hewagama SP, Hewawasam RP. Antiurolithiatic Potential of Three Sri Lankan Medicinal Plants by the Inhibition of Nucleation, Growth, and Aggregation of Calcium Oxalate Crystals *in Vitro*. ScientificWorldJournal. 2022 12; 2022:8657249, 1-13.
6. Kumari P, Joshi GC, Tewari LM. Diversity and status of ethno-medicinal plants of Almora district in Uttarakhand, India. International Journal of Biodiversity and Conservation 2011; 3(7):298-326.
7. Singla S, Kumari C, Soni A, Goyal S. The In-Vivo Diuretic activity of methanolic extract of *Quercus leucotrichophora* A. Camus seeds. Phytomedicine Plus. 2022; ; 2, 4: 100346,1-7.
8. Akhtar N, Rashid A, Murad W, Bergmeier E. Diversity and use of ethno-medicinal plants in the region of Swat, North Pakistan. J Ethnobiol Ethnomed. 2013; 9:25, 1-13.
9. Saleem A, Najda A, Mubeen A*, Akhtar MF,Bukhari SA, Zeb A*. HPLC-DAD analysis of *Quercus leucotrichophora* extract and appraisal of its antiasthmatic potential via modulation of aquaporins, inflammatory, and oxidative stress biomarkers in Albino mice. Biomed Pharmacother. 2022; 155:1-10.
10. Semwal P, Painuli S, Badoni H, Bacheti RK. Screening of phytoconstituents and antibacterial activity of leaves and bark of *Quercus leucotrichophora* A. Camus from Uttarakhand Himalaya. Clin Phytosci. 2018; 4, 30, 1-6.
11. Saleem A, Hameed I, Akhtar MF, Rahman MF, Ashraf GM. Appraisal of Anti-Arthritic Potential of *Quercus Leucotrichophora* Methanolic Extract in Complete Freuns Adjuvant Induced Arthritic Rats; a Mechanistic Study. 2022, 1-27
12. Gairola S, Sharma S, Bedi YS. A cross-cultural analysis of Jammu & Kashmir and Ladakh India medicinal plant use. J Ethnopharmacol. 2014; 155 (2):925-86.
13. Chauhan PS, Bisht S, Ahmad S. Traditional, and ethnobotanical uses of medicinal trees in district Tehri Garhwal Western Himalayas. Ann Geriatr Educ Med Sci 2016;3 (1): 14-19.
14. Rawat VS. Medicinal plants and sustainable livelihood in Pauri district of Garhwal Himalaya, Uttarakhand, India. International Journal of Bioassays. 2016, 5:4589-4592.
15. Joshi AK, Juyal D. Traditional and ethnobotanical uses of *Quercus leucotrichophora* A. Camus (*Quercus oblongata* D. Don) in Kumaun and Garhwal regions of Uttarakhand, India : A review. 2017. 5(5), 6–8.
16. Heirangkhongjam MD, Ngaseppam IS, "*Rhus chinensis Mill*: A medicinal plant with promising inhibition of calcium oxalate crystallization, an in-vitro study", Journal of Herbal Medicine, 2021, 1-25.
17. Venkatachalam S, Singanallur RR, Bavaniamma V. In vitro evaluation of *Rotula aquatica* Lour. For antiurolithiatic activity. Journal of pharmacy research, 2013; 6(3) 378-382.
18. Kumara BN, Waduda A, Jahana N, Ghulamuddin S , Bano H , Makbul SA, *et al* Antilithiatic effect of *Peucedanum grande C. B. Clarke* in chemically induced urolithiasis in rats. J Ethnopharmacol. 2016; 194:1122-1129.
19. Sikarwar I, Dey YN, Wanjari MM, SharmaA, Gaidhani SN, Jadhav AD. *Chenopodium album* Linn. Leaves prevent ethylene glycol-induced urolithiasis in rats. J Ethnopharmacol. 2017; 195:275-282.
20. Choudhary SS, Panigrahi PN, Dhara SK, Sahoo M, Dan A, Thakur N, *et al*. *Cucumis callosus* (Rottl.) Cogn. Fruit extract ameliorates calcium oxalate urolithiasis in ethylene glycol induced hyperoxaluric Rat model. Heliyon. 2023; 1-13.
21. Ahmed OM, Ebaid H, El-Nahass S, Ragab M, Alhazza IM. Nephroprotective Effect of *Pleurotus ostreatus* and *Agaricus bisporus* Extracts and Carvedilol on Ethylene Glycol-Induced Urolithiasis: Roles of NF-κB, p53, Bcl-2, Bax, and Bak. Biomolecules. 2020, 10(9), 1317. 1-37.
22. Marhoume FZ, Aboufatima R, Zaid Y, Limami Y, Duval RE , Laadraoui J, Belbachir A , Chait A , Bagri A . Antioxidant and Polyphenol-Rich Ethanolic Extract of *Rubia tinctorum* L. Prevents Urolithiasis in an Ethylene Glycol Experimental Model in Rats. Molecules. 2021; 26(4):1005, 1-17.
23. Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. J Pharmacol Pharmacother, 2010, 87-93. 1(2):87-93.
24. Karadi RV, Gadge NB, Alagawadi KR, Savadi RV. Effect of *Moringa oleifera* Lam. root-wood on ethylene glycol induced urolithiasis in rats. J Ethnopharmacol. 2006; 105(1-2):306-11.
25. Panigrahi PN, Dey S, Sahoo M, Choudhary SS, Mahajan S. Alteration in Oxidative/nitrosative imbalance, histochemical expression of osteopontin and antiurolithiatic efficacy of *Xanthium strumarium* (L.) in ethylene glycol induced urolithiasis. Biomed Pharmacother. 2016; 84: 1524-1532.
26. Ahmed A, Wadud A, Jahan N, Bilal A, Hajera S. Efficacy of *Adiantum capillus* veneris Linn in chemically induced urolithiasis in rats. Journal of Ethnopharmacology 146 (2013) 411–416.
27. Divakar K, Pawar AT, Chandrasekhar SB, Dighe SB, Divakar G. Protective effect of the hydro-alcoholic extract of *Rubia cordifolia* roots against ethylene glycol induced urolithiasis in rats. Food Chem Toxicol. 2010; 48(4):1013-8.
28. İlhan M, Ergene B, Süntar I, Özbilgin S, Saltan Çitoğlu G, Demirel MA, *et al.* Preclinical evaluation of Antiurolithiatic activity *ofviburnum opulusl*. on sodium oxalate-induced urolithiasis rat model, Evidence-Based Complementary and Alternative Medicine. 2014 (2014) 1–10.
29. Ghale-Salimi MY, Eidi M, Ghaemi N, Khavari-Nejad RA. Antiurolithiatic effect of the taraxasterol on ethylene glycol induced kidney calculi in male rats. Urolithiasis. 2018; 46(5):419-428.

30. Sairi NH, Muhammad N, Pauzi AN, Bakar MF, Talip BHJ, Abdullah N, *et al*. The evaluation of Antiurolithiathic Properties of *Ananas nanus, Aquilaria malaccensis, Curcuma xanthorrhiza*, *pandanus atrocarpus* and *Garcinia Mangostana*. Materials Today: Proceedings. 2019;19:1145–50.
31. Mammate N, El Oumari FE, Imtara H , Belchkar S ,Lahrichi A, Alqahtani AS, *et al*. Antioxidant and Anti-Urolithiatic Activity of Aqueous and Ethanolic Extracts from *Saussurea costus* (Falc) Lispich Using Scanning Electron Microscopy. Life (Basel). 2022; 12(7):1026, 1-7.
32. Nambururu SL, Dodoala S, Koganti B, Prasad KVSRG. Antiurolithiatic activity of *Phaseolus vulggaris* seeds agains ethylene glycol- induced renal calculi in wistar rats. International jornal of green pharmacy, 2017, 11 (4), 281-289.
33. Agawane SB, Gupta VS, Kulkarni MJ, Bhattacharya AK, Koratkar SS, Rao VK. Patho-physiological evaluation of *Duranta erecta* for the treatment of urolithiasis. J Ayurveda Integr Med. 2019; 10(1):4-11.
34. Jamshed A, Jabeen Q. Pharmacological Evaluation of *Mentha piperita* Against Urolithiasis: An In Vitro and In Vivo Study. Dose Response. 2022; 20(1):1-15.
35. Korah MC, Rahman J, Rajeswari R, Sherief H, Lalitha V,Sengottavelu S , *et al.* Evaluation of diuretic efficacy and antiurolithiatic potential of ethanolic leaf extract of *Annona squamosa* Linn. in experimental animal models. Indian J Pharmacol. 2020; 52(3):196-202.
36. Saleem U, Ahmad N, Shah MA, Anwar F, Ahmad B. Anti-urolithiatic activity of *Salvia hispanica* L. seeds in ethylene glycol induced urolithiasis rat's model. An Acad Bras Cienc. 2020; 92(4): 1-13.
37. Madhuri GD, Devi AM, Srija JS, Narendra D. Evaluation of Antiurolithiatic Activity of *Solidago virgaurea* Against Ethylene Glycol Induced Renal Calculi in Rats. Int. J. Adv. Pharm. Biotech., 2019, 05(04), 05–11.
38. Patel VB, Acharya NN. Effect of *Macrotyloma uniflorum* in ethylene glycol induced urolithiasis in rats. Heliyon; 2020; e04253, 2020, 1-7.
39. Salamaa AAA, El-Kassabyb MI, Hassan A. Anti-urolithiatic activity of *Solanum nigrum* hydroalcoholic extract in ethylene glycol-induced urolithiasis in rats. Egypt Pharmaceut J, 2019, 18:311–319.

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