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ANTILITHIATIC ACTIVITY OF ETHANOLIC LEAVES EXTRACT OF *DALBERGIA SISSOO* LINN. ETHYLENE GLYCOL INDUCED LITHIASIS IN RATS

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Keywords:

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ABSTRACT: Aim of the Study: The aim of this study was to investigate the antilithiatic activity of ethanolic leaves extract of *Dalbergia sissoo* Linn. ethylene glycol induced in rats. **Materials and Methods:** All the chemicals were analytical grade. Cystone was obtained from from Himalaya Health Care Ltd. The ethanolic extract of plant *Dalbergia sissoo* Linn. leaves were given with tween 80 as vehicle administered orally. All chemicals were used of analytical grade available; ethylene glycol was obtained from Merck Ltd., Mumbai, India. Male Albino rats of wistar strain weighing between 150-200 g were selected for the antiurolithiatic activity. Animals were housed in polypropylene cages with the filter tops and maintained at 25 ± 2 ºC, relative humidity 55 ± 10% under controlled conditions of 12-h light: 12-h dark cycle. **Results:** In the present *in-vivo* study DSEE (at 100 and 200 mg/kg) exhibited a dose dependent significant anti-lithiatic activity on treatment. The extract dose of 100 mg/kg also caused reduction of calcium, oxalates, phosphorus and creatinine in blood serum level the results were found statistically significant. The antilithiatic effect of ethanol extract at was found significant than the reference standard. **Conclusion:** The ethanolic extract of *Dalbergia sissoo* linn. has protective effect against ethylene glycol, induced in rats.

INTRODUCTION: Around 1500 BC, Ayurveda's fundamental and applied principles got organised and enunciated. Ayurveda traces its origins to the Vedas, Atharvaveda in particular, and is connected to Hindu religion. Atharvaveda (one of the four most ancient books of Indian knowledge, wisdom and culture) contains 114 hymns or formulations for the treatment of diseases. Ayurveda originated in and developed from these hymns. In this sense, Ayurveda is considered by some to have divine origin. Indian medicine has a long history, and is one of the oldest organised systems of medicine.

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Its earliest concepts are set out in the sacred writings called the Vedas, especially in the metrical passages of the Atharvaveda, which may possibly date as far back as the 2nd millennium BC.

According to a later writer, the system of medicine was received by Dhanvantari from Brahma, and Dhanvantari was defined as the god of medicine. In later times his status was gradually reduced, until he was credited with having been an earthly king. The Sushruta Samhita of Sushruta appeared during the 1st millennium BC on the work of the surgeon Sushruta - write: "The main vehicle of the transmission of knowledge during that period was by oral method. The language used was Sanskrit - the Vedic language of that period (2000-500 BC). The most authentic compilation of his teachings and work is presently available in a treatise called Sushruta Samhita". This contains 184 chapters and description of 1,120 illnesses, 700 medicinal plants,

64 preparations from mineral sources and 57 preparations based on animal sources [1]. Medicinal plants have been the part and parcel of human society to combat diseases since the dawn of human society to combat diseases since the dawn of human civilization [2].

Introduction to Urolithiasis: All over the world especially in developing countries, approximately 80% of population continues to use traditional medicine in primary medical problems. In the past decade, therefore, research has been focused on scientific evaluation of traditional drugs of plant origin [3]. Also called Nephrolithiasis or kidney stones, Urolithiasis is the presence of calculi in the urinary tract. The male-to-female incidence ratio is 4: 1. Eighty percent of calculi are composed of calcium (either oxalate or phosphate), with others composed of struvite, uric acid, or cystine. Approximately 1 million Americans develop a kidney stone each year and an estimated 12% of the population forms a stone some time during their life [4, 20].

Annual incidences of kidney stones are about 0.1-0.4% of the population and lifetime prevalence's in the USA and Europe range between 8 and 15%. Kidney stones occur more frequently with increasing age and among men. Within ten years, the disease usually recurs in more than 50% of patients. Nowadays, about 85% of all kidney stones contain calcium salts (calcium oxalate and/or calcium phosphate) as their main crystalline components. Because human urine is commonly supersaturated with respect to calcium salts as well as to uric acid, crystalluria is very common, *i.e*. healthy people excrete up to ten millions of microcrystals every day.

Introduction to Urinary Tract: The urinary tract system consists of the kidneys, ureters, bladder and urethra. The kidneys are two bean-shaped organs located below the ribs toward the middle of the back. The kidneys remove extra water and wastes from the blood, converting it to urine. They also keep a stable balance of salts and other substances in the blood. Narrow tubes called ureters carry urine from the kidneys to the bladder, a triangle-shaped chamber in the lower abdomen. Like a balloon, the bladder's elastic walls stretch and expand to store urine. They flatten together when urine is emptied through the urethra to outside the body. A kidney stone is a hard mass developed from crystals that separate from the urine and build up on the inner surfaces of the kidney. Normally, urine contains chemicals that prevent the crystals from forming. These inhibitors do not seem to work for everyone, however, so some people form stones. If the crystals remain tiny enough, they will travel through the urinary tract and pass out of the body in the urine without being noticed.

(From Greek nephros, "kidney") refers to the condition of having calculi in the urinary tract (which also includes the kidneys), which may form or pass into the urinary bladder [5]. Stone formation is the one of the oldest and most wide spread diseases known to man. Urinary calculi have been found in the tombs of Egyptian mummies dating back to 4000 BC and in the graves of North American Indians from 1500-1000 BC. Calcium-containing stones especially calcium oxalate monohydrate (Whewellite), calcium oxalate de-hydrate (Weddellite) and basic calcium phosphate (Apatite) are the most commonly occurring ones to an extent of 75-90% followed by magnesium ammonium phosphate (Struvite) to an extent of 10-15%, uric acid 3-10% and cystine 0.5-1%. In most of the cases the commonly occurring stones are calcium oxalate or magnesium ammonium phosphate type [6].

Natural Products Used in the Treatments of Urolithiasis: A large number of indigenous drugs have been used for this purpose in our country since ancient times. Sushruta (1000 B.C.) has very systemically described this disease and the medicines for its cure. Amongst the medicinal plants used in urolithiasis are 'patharphor' (*Didymocarpus pedicellata*), several Bergenia species, 'Kulath-ki-dal,' three species of Tribulus (T. *systoides, T. terrestris* and *T. alatus*), 'manjit' (*Rubia cordifolia* and *Rubia tinctorum*), 'varuna' (*Crataeva nurvala*) and 'imli' (*Tamarindus indica*) [7], *Costus spiralis* [8], *Raphanus sativus* [9], *Moringa oleifera* [10] and *Crataeva adansonii* [11].

Plant Profile: *Dalbergia sissoo* is the state tree of Punjab state (India) and the provincial tree of Punjab province (Pakistan). It is found growing along river banks below 900 m (3,000 ft) elevation, but can range naturally up to 1,300 m (4,300 ft). It

can withstand average annual rainfall up to 2,000 mm (79 in) and droughts of 3-4 months. It prefers soils from pure sand and gravel to rich alluvium of river banks. Shisham can grow in slightly saline soils. Seedlings are intolerant of shade [2].

FIG. 1: LEAVES OF *DALBERGIA SISSOO* LINN. [19]

Scientific Classification:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Fabales
Family : Fabaceae
Subfamily : Faboideae
Genus : Dalbergia
Species : *D. sissoo*

Geographical Distribution: Exotic range: Afghanistan, Bangladesh, Bhutan, India, Malaysia, Pakistan Native range: Cameroon, Cyprus, Ethiopia, Ghana, Indonesia, Iraq, Israel, Kenya, Mauritius, Nigeria, Sudan, Tanzania, Thailand, Togo, US, Zimbabwe [12].

Tradional Uses: Ayurveda describes the bark and wood as bitter, hot and acrid used as aphrodisiac, abortifacient, expectorant, antihelmintic, antipyretic and diseases of the blood, leucoderma, dyspepsia and dysentery. The wood is good for diseases of the eye, and of the nose, used in scabies and syphilis. A decoction of the leaves are given in the acute stage of gonorrhea. The whole plant has long been employed in ancient Yunani preparations [13, 14]. Ayurvedics has also prescribed the leaf juice for eye ailments Yunana use the wood for blood disorders, burning sensations, eye and nose disorders, scabies, scalding urine, stomach problems, and syphilis. The sissoo plant is a folk remedy for excoriations, gonorrhea and skin ailments [15].

Chemical Constituents: The plant is having the isoflavones irisolidone, biochanin-A, muningin, tectorigenin, prunetin, genestein, sissotrin and prunetin-4-Ogalactoside. The flavone norarto-carpotin and F3-amyrin, F3- sitosterol and stigmasterol were isolated and identified from the green branches of aerial parts of *D. sissoo* [16].

Pharmacological Activity:

Ayurveda: Leaf juice for eye ailments, considering the wood and bark as abortifacient, anthelmintic, antipyretic, aphrodisiac, expectorant, and refrigerant. The wood and bark for anal disorders, blood diseases, burning sensations, dysentery, dyspepsia, leucoderma, and skin ailments.

MATERIALS AND METHODS:

Collection of Plant: The leaves of *Dalbergia sissoo* Linn. (Family: Fabaceaee) were collected during the month of December 2015 from Jhansi District, Uttar Pradesh, India.

Collection and Authentification:

Identification and Authentication: The identi-fication and authentication of the plant was done by Assistant professor Department of Botany, Bundelkhand University, Jhansi, Uttar Pradesh, India. A voucher specimen of the plant was kept in the herbarium of Department of Botany, Bundelkhand University, Jhansi with accession number of the specimen is BU/Bot/Spe/Phar/01-2016/01, on date 05/01/2016.

Preparation of Extraction: The air-dried in shade plant leaves of *Dalbergia sissoo* (250 g) was coarsely powdered using grinder and continuous extracted in a Soxhlet apparatus at 50 ºC with 750 ml of petroleum ether than 750 ml of ethanol. The extract was filtered through a paper filter (Whatman, no.1) and evaporated under reduced pressure by the rotary evaporator.

Extractive Value: Extracts were prepared with various solvents. Percentages of the extractive values were calculated with reference to air-dried drug are given in **Table 1.**

TABLE 1: EXTRACTIVE VALUE OF *DALBERGIA SISSOO* LINN.

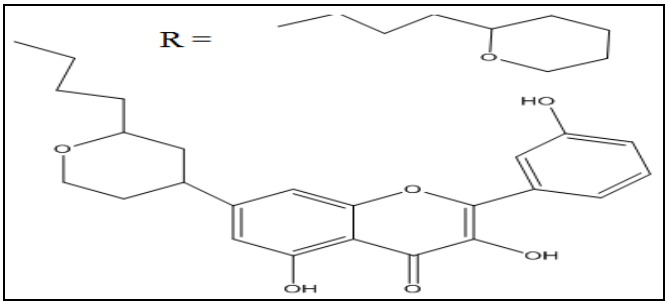
S. no.	Types of solvents	% w/w
1	Petroleum ether (60-80 °C)	3.342
2	Ethanol 100%	8.198

Extraction of Plant Materials: The leaves of the plant were extracted with ethanol and then ethanolic extract of leaves extract of *Dalbergia sissoo* Linn. was subjected for phytochemical screening for the detection of various plant constituents it is found that steroids and flavonoids compounds are present as major active principle.

Preliminary Phytochemical Studies: Phyto-chemical tests were done in plant extracts for the detection of presence of different chemical constituents such as; alkaloids, glycosides, steroids, flavonoids, essential oils, carbohydrates, proteins, tannins and other substances which are responsible for the biological activity. So the chemical tests are performed in the ethanolic extract (EE) of *Dalbergia sissoo* Linn. for the detection of different chemical constituents: The leaves of the plant were extracted with ethanol and then ethanolic extract of leaves extract of *Dalbergia sissoo* Linn. was subjected for phytochemical screening for the detection of various plant constituents it is found that Steroids and flavonoids compounds are present as major active principle.

The best solvent system for TLC of *Dalbergia sissoo* Linn. is toluene: ethyl acetate: formic acid (few drops) TLC of *Dalbergia sissoo* Linn. shows the presence of eight compounds with different R_f values in Different colour using the 0.5% vanillin in dil. H_2SO_4 detecting reagent which suggests that the presence of eight compounds in the extract. For the better isolation & identification of the different components of ethanolic extract of *Dalbergia sissoo* Linn., the HPTLC of ethanolic extract of *Dalbergia sissoo* Linn, was carried out at PG Tech Pvt. Ltd. Indore.

The report of HPTLC indicates the presence of 9 spots with different R_f values which shows to occupy the different area with different R_f values. It shows that the compounds contain about 9 constituents. The Column Chromatography of isolated compounds from the ethanolic extract of *Dalbergia sissoo* Linn. were carried out with *n*-hexane (100) to toluene: ethyl acetate (98:5) and stationary phase silica gel. One fraction was collected D_1 on performing the TLC of fraction D_1 were showing one spots respectively. Since fraction D_1 showing only one spot so it was further investigated for ^{1}H-NMR, C-NMR MASS, FTIR and Elemental analysis (CHNO) & structure was proposed. From comparison of IR, NMR and MASS spectra of standard with isolated compound, it indicated that this compound may be flavanoid derivative having Quercetin like structure (R= $C_9H_{18}O$) as a proposed structure. The molecular formula $C_{24}H_{26}O_6$ and molecular mass of isolated compound.



Chemical Formula: $C_{24}H_{26}O_6$ and Exact Mass: 410.17

FIG. 2: FLAVANOID DERIVATIVE HAVING QUERCETIN LIKE STRUCTURE (R= $C_9H_{18}O$)

Chemical (Drugs and Solutions) and Apparatus: All the chemicals were analytical grade. Cystone was obtained from from Himalaya Health Care Ltd. The ethanolic extract of plant *Dalbergia sissoo* Linn. leaves were given with tween 80 as vehicle administered orally. All chemicals were used of analytical grade available; ethylene glycol was obtained from Merck Ltd., Mumbai, India. The dose of 100 mg/kg, 200 mg/kg of ethanolic extract was selected for the test. All the doses was given orally after making suspension in vehicle *i.e.* 1% tween 80 and the standard drug *i.e*. Cystone was given orally (750 mg/kg) in vehicle [17].

Animal Selection:

Animals: Male Albino rats of wistar strain weighing between 150-200 gm were selected for the antiurolithiatic activity. Animals were housed in polypropylene cages with the filter tops and maintained at 25 ± 2 ºC, relative humidity 55 ± 10% under controlled conditions of 12-h light: 12-h dark cycle. The animals were fed up with commercial rat chow and were given water *ad libitum*. All protocols of the study was approved by the Institutional Animal Ethical Committee with reference number Bu/Pharm/IAEC/a/17/01/. The IAEC is approved by committee for the purpose of control and supervision of experiments on animals (CPCSEA) with registration number 716/02/a/CPCSEA.

Animal Groups: Thirty rats were divided in to 5 groups comprising six animals per groups.

Group 1: Normal, *ad libitum* access to regular food and drinking water administered.

Group 2, 3, 4, 5 *ad libitum* acess to regular food and *ad libitum* access to drinking water containing 0.75% (v/v) ethylene glycol (EG) in order to promote hyperoxaluria and CaOx deposition in the kidneys.

Group 2: Ethylene glycol 0.75% (v/v)

Group 3: Standard drug (Cystone 750 mg/kg).

Group 4: Ethanolic extract of *Dalbergia sissoo* leaves (100mg/kg).

Group 5: Ethanolic extract of *Dalbergia sissoo* leaves (200 mg/kg).

The rats were house in cages and divided into 5 groups of six animals each. Group 1 serve as control and received regular rat food and drinking water *ad libitum.* Ethylene glycol (0.75%) in drinking water was fed to group 2 to 5 for induction of renal calculi till 28th day. Group 2 receive only Ethylene glycol 0.75% (v/v), Group 3 receive standard antilithiatic drug, cystone (750 mg/kg) from 15th day till 28th day [15]. Group 4 to 5 received as curative regimen, group 4 received ethanolic extract 100 mg/kg, group 5 received ethanolic extract 200 mg/kg and group 15th day till 28th day.

Statistical Analysis: The results were expressed as mean ± standard error mean (SEM). The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test and P<0.05 was considered significant.

RESULTS AND DISCUSSION: In the present *in -vivo* study, renal stone inducing treatment to male Albino wistar rats resulted in hyperoxaluria. There was an increased oxalate, calcium, phosphate, creatinine and uric acid excretion. However, supplementation with DSEE significantly prevented these changes in urinary oxalate, calcium, phosphate, creatinine and uric acid excretion dose-dependently. Ethanolic extract of *Dalbergia sissoo* Linn. leaves was subjected to antilithiatic activity in rats where ethylene glycol (0.75% (v/v) used as the causing lithiasis agent.

A marked rise in calcium, oxalates, phosphorus and creatinine in blood serum level observed in lithiatic compare standard to different curative doses of rats. Ethanolic extract of *Dalbergia sissoo* (at 100 and 200 mg/kg) exhibited a dose dependent significant antilithiatic activity on treatment. The extract dose of 100 mg/kg also caused reduction of calcium, oxalates, phosphorus and creatinine in blood serum level the results were found statistically significant. The antilithiatic effect of ethanol extract at was found significant than the reference standard.

Assessment of Antiurolithiatic Activity:

Serum Analysis: The blood was collected from the retro-orbital sinus under anaesthetic condition and serum was separated by centrifugation at 10,000 g for 10 min and analyzed for creatinine, uric acid and urea nitrogen. The creatinine kit (Reckon Diagnostics Pvt. Ltd., India) and uric acid diagnostic kit (Span Diagnostics Ltd., India) were used to estimate ser-um creatinine and uric acid levels respectively 2.7. Kidney histopathology and homogenate analysis.

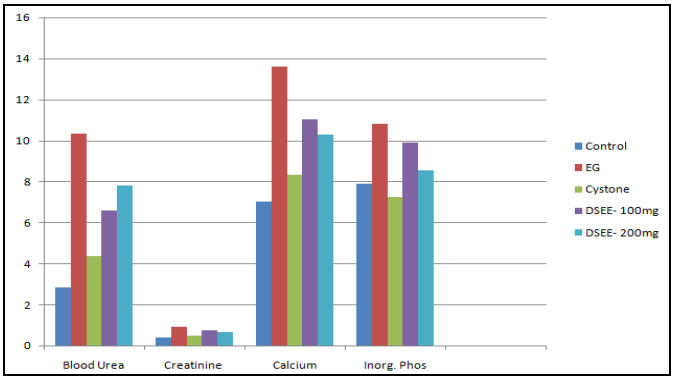


FIG. 3: GRAPHICAL REPRESENTATION OF ANTI-LITHIATIC ACTIVITY OF *DALBERGIA SISSOO* LINN. n= 6, values are expressed as Mean ± SEM *P*< 0.05 when compared with control group

TABLE 2: SERUM BIOCHEMICAL DATA

Parameter Unit (mg/dl)	Group 1 Control	Group 2 (EG)	Group 3 Cystone	Curative regimen (DSEE)	
				Group 4 DSEE- 100mg	Group 5 DSEE- 200mg
Blood Urea	2.83±0.30	10.34±0.20	4.37±0.33	6.59±0.27	7.72±0.61
Creatinine	0.38±0.01	0.91±0.02	0.48±0.02	0.73±0.02	0.66±0.02
Calcium	7.05±0.4	13.62±0.21	8.36±0.21	11.05±0.23	10.32±0.52
Inorganic Phosphorus	7.92±0.17	10.83±0.24	7.27±41	9.92±0.13	8.59±0.27

Kidney Histopathology and Homogenate Analysis: The abdomen was cut open to remove both kidneys from each animal. Isolated kidneys were cleaned off extraneous tissue and rinsed in ice-cold physiological saline. The right kidney was fixed in 10% neutral buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 lm and stained with H and E (Haematoxylin and Eosin) for histo-pathological examination. The slides were examined under light microscope to study light microscopic architecture of the kidney and calcium oxalate deposits. The left kidney was finely minced and 20% homogenate was prepared in Tris-HCl buffer (0.02 mol/l, pH 7.4). Total kidney homogenate was used for assaying tissue calcium, oxalate [15] and lipid peroxidation activity [18].

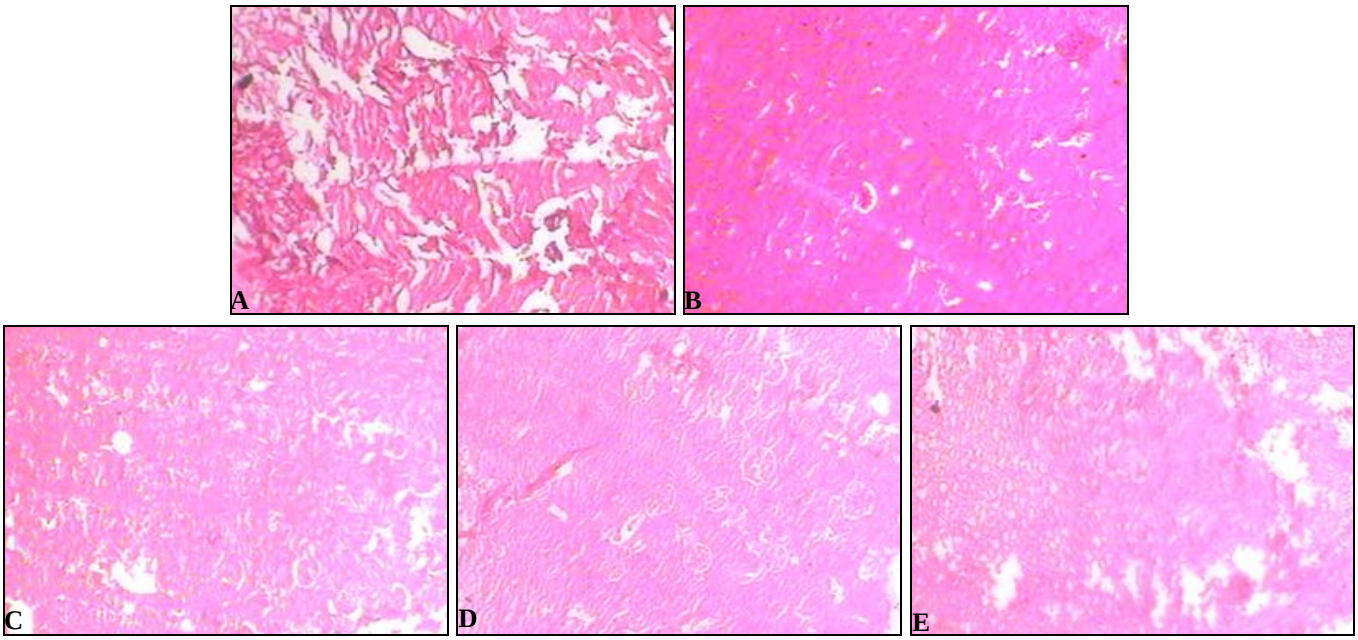


FIG. 4: MICROSCOPIC ARCHITECTURE AND CALCIUM OXALATE DEPOSITS IN THE KIDNEY SECTION. KIDNEY SECTIONS OF (A) VEHICLE CONTROL (B) UROLITHIC (C) CYSTONE TREATED (D) TREATMENT WITH DSEE AT THE DOSE OF 100 mg/kg (E) TREATMENT WITH DSEE AT THE DOSE OF 200 mg/kg

CONCLUSION: As traditional medicines are usually taken by the oral route, same route of administration was used for evaluation of protective effect of the DSEE against ethylene glycol induced urolithiasis in rats. Male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans and earlier studies shown that the amount of stone deposition in female rats was significantly less.

The result showed the nephroprotective effect of leaves extract of *Dalbergia sissoo* Linn. in ethylene glycol induced urolithiatic model. Earlier study reported a high antioxidant and anti-inflammatory capacity of leaves extract of *Dalbergia sissoo* Linn. Therefore, DSEE may prevent statistically significant calcium oxalate crystal deposition in the kidney by preventing hyperoxaluria-induced peroxidative damage to the renal tubular membrane surface (lipid peroxidation), which in turn can prevent statistically significant calcium oxalate crystal attachment and subsequent development of kidney stones. Urinary chemistry is one of the important factors in determining the type of crystal formed and the nature of macromolecules included on the surface of the crystals. Hence, the study of the urinary chemistry related to the calculi forming minerals will provide a good indication of the extent of stone formation.

Microscopic examination of kidney sections derived from ethylene glycol induced urolithic rats showed polymorphic irregular crystal deposits inside the tubules which causes dilation of the proximal tubules along with interstitial inflammation that might be attributed to oxalate. Co-treatment with the DSEE decreased the number and size of calcium oxalate deposits in different parts. Results indicate that administration of leaves extract of *Dalbergia sissoo* Linn. reduced and

prevented statistically significant the growth of urinary stones. It also seems that the preventive effect is more effective than its treatment effect Therefore; the leaves extract of *Dalbergia sissoo* Linn. is helpful to prevent the recurrence of the disease as it showed its effect on early stages of stone development.

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CONFLICT OF INTEREST: We declare that we have no conflict of interest.

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