Scholars Academic Journal of Pharmacy
Abbreviated Key Title: Sch Acad J Pharm
ISSN 2347-9531 (Print) | ISSN 2320-4206 (Online)
Journal homepage: http://saspublisher.com/sajp/

OPEN ACCESS

Pharmaceutical Chemistry

Anti-Urolithiatic and *Invitro-Invivo* Anti-Oxidant Effects of Methanolic Extract of *Thunbergia laurifolia* on Ethylene Glycol-Induced Kidney Calculi in Rats

Amit Nayak[1], Madhu Garg[2], Saurabh Jain[3], Mohammed Azaz Khan[4*] Deepak Kumar Jain[5], Nushrat Khan[6]

[1]Lakshmi Narain College of Pharmacy, Bhopal, Madhya Pradesh, India
[2]Govt. Thakur Ranmat Singh College Rewa, Madhya Pradesh, India
[3]Pinnacle Biomedical Research Institute (PBRI), Bhopal, Madhya Pradesh, India
[4]Malhotra College of Pharmacy, Badwai, Bhopal, Madhya Pradesh, India
[5]Medicinal Chemistry Research Laboratory, SLT Institute of Pharmaceutical Sciences, Guru Ghasidas University, Bilaspur, Chhattisgarh, India
[6]School of Pharmacy Choksey Engineering College Masturi Road Lal Khadan Bilaspur, Chhattisgarh, India

***Corresponding author:** Mohammed Azaz Khan
| **Received:** 17.03.2019 | **Accepted:** 28.03.2019 | **Published:** 30.03.2019
DOI: 10.21276/sajp.2019.8.3.5

Abstract | **Original Research Article**

Oxidative stress acts as a vital mediator in the pathophysiology of urolithiasis. Renal calculi formation is one of the most general urological disorders. Urinary stone disease is a common disease which affects 10-12% of the population in developed countries. The objective of the present study is to investigate the antiurolithiatic and antioxidant activity of methanolic extract of *Thunbergia laurifolia* flowers on ethylene glycol-induced urolithiasis in Wistar albino male rats. Twelve male rats weighing 152-204 were used for this study. Urolithiasis was produced in Wistar albino rats by adding 0.75% v/v ethylene glycol (EG) to drinking water for 28 days. The methanolic extract of *Thunbergia laurifolia* flowers (TBL) was assessed for its preventive action in urolithiasis. In preventive treatment, the TBL given from 1st day to 28th day, various renal functional and injury markers such as urine volume, calcium, phosphate, uric acid, magnesium, urea and oxalate were evaluated using urine, serum and kidney homogenate. Antioxidant parameters such as DPPH radical scavenging activity, reducing power assay, lipid peroxidation, superoxide dismutase and Glutathione were also determined. The TBL treatment increased the urine output significantly compared to the control. The TBL treatment significantly reduced the urinary excretion of the calcium, phosphate, uric acid, magnesium, urea and oxalate and increased the excretion of citrate compared to EG control. The increased deposition of stone forming constituents in the kidneys of calculogenic rats were significantly lowered by treatment with TBL. It was also observed that alcoholic extract of TBL produced significant ($P < 0.001$) increased in lipid peroxidation (LPO), and decreased GSH, SOD. The results of the present study suggest that *Thunbergia laurifolia* has a strong antiurolithiatic and antioxidant activity.

Keywords: *Thunbergia laurifolia*, Antiurolithiatic, Antioxidant activity, DPPH radical scavenging activity, Reducing power assay, Lipid peroxidation.

INTRODUCTION

Urolithiasis (also called calculi or uroliths) or stone formation in the kidney causes a main impact on public health and economy worldwide since last two decades [1, 2]. It is also considered as the 3 most common problem of the urinary tract with an estimated lifetime risk of around 2%–5% in Asia, 8%–15% in America and Europe and 20% in the Middle East countries [3]. In spite of substantial progress in the study of physiological sign of urolithiasis, its exact mechanism is still not clearly unstated. The current proposed mechanism of stone formation involves urinary supersaturation, nucleation, precipitation, growth, aggregation of crystals and their retention in renal tubular epithelial cells with crystal matrix [1, 2]. These processes are modulated by a variety of urinary macromolecules which become incorporated in the growing crystals and eventually constitute organic component or matrix. Urolithiasis is a multifactorial disease and involves in modification of several biochemical pathways. Therefore, the present state insists a newer approach of therapy. Several current studies have highlighted the effectiveness of medicinal plants and natural compounds for treatment and management of urolithiasis [3-9]. Moreover, herbal remedies are known to contain numerous constituents, acting through multiple pathways such as antioxidant, analgesic, diuretic, pH neutralizing, etc., Several plants have been used to treat kidney stones including *Phyllanthus niruri*, *Zea mays*, *Agropyron repens* and *Herniaria hirsute* [10-14].Calcium oxalate urolithiasis

model has commonly been used to investigate the influence of urolithiasis on experimental model in rats. This model is induced by ethylene glycol (EG), a precursor to oxalate formation [15]. EG poisoning can lead to acute renal failure which is considered by a proximal tubular necrosis and an accumulation of calcium oxalate monohydrate crystals in the urine and kidney tissue. The prices mechanism is probably due to the calcium oxalate monohydrate's adherence to tubular cells primary hyperoxaluria and kidney stone formation [16]. Many studies indicated that the Cystone has a potent anti-lithiatic (prevents the formation of kidney stones) and lithotriptic (dissolves kidney stones) properties by declining urinary supersaturation or micropulverizes and diuretic that flushes out small kidney stones [17, 18]. *Thunbergia Laurifolia* Linn, commonly known as laurel clock vine or blue trumpet vine, is native to India [19], and in the Indomalaya ecozone, the species occurs from Indochina to Malaysia [20]. It is locally known as "kar tuau" in Malaysia and "Rang Chuet" in Thailand [21]. In Malaysia, juice from crushed leaves of *T. laurifolia* are taken for menorrhagia, placed into the ear for deafness and applied as a poultice for cuts and boils [22]. In Thailand, leaves are used as an antipyretic and for their detoxifying effects, e.g., as an antidote for poisons [23-25]. Several Thai herbal companies have started producing and exporting Rang Chuet tea [21]. The tea has been claimed to counteract the harmful effects of drugs, alcohol and cigarettes. Reported activities of *T. laurifolia* leaves include antioxidant, anti-inflammatory, anti-drug addiction, antidote, hepatoprotective, anti-diabetic activities, and wound healing effect [26]. We intended to explore the Anti-Urolithiatic of this plant. Its antiurolithiatic efficacy was not explored scientifically till now. Hence, the study was designed to evaluate the possible antiurolithiatic effect of methanolic extract of *Thunbergia laurifolia* flowers (TBL) in ethylene glycol (EG) induced lithiatic model in rat.

MATERIALS AND METHODS

Plant material

The flowers of *Thunbergia laurifolia* were collected from local area of Bhopal (M.P.) in the month of February, 2018.The sample was identified by senior Botanist Dr. Zia-Ul-Hassan, Professor and head of department of Botany, Safia College of Arts and Science, peer gate Bhopal. A herbarium of plants was submitted to the specimen library of Safia College of Arts and Science, peer gate Bhopal and The specimen voucher no. of *Thunbergia laurifolia* is 119/Bot/Saf/45.

Chemical reagents

Sodium carbonate, Ethylene Di amine, Acetic acid, Ammonia, Pyridine, Potassium dehydrogenate Phosphate, Ferric chloride, Chloroform, Ethanol were obtained from Merck India Ltd. (Mumbai, India). Nitro-blue Tetra zolium, Nutrient broth, Di potassium hydrogen phosphate, Agar- agar were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). All the chemicals used in this study were of analytical grade.

Animals

All ethical and handling guidelines were followed as set by Indian Legislation and approved by Institutional Animal Ethics Committee. All animals were procured and housed in animal house maintained under standard hygienic conditions. Animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal (Reg No. 1824/PO/ERe/S/15/CPCSEA). Protocol Approval Reference No. PBRI/IAEC/PN- 17.

Grouping of animals

Animals were housed in a group of five in separate cages under controlled conditions of temperature (22 ± 2°C). All animals were given standard diet (Golden feed, New Delhi) and water, *ad libitum*. The environment was also regulated at 25 ± 1 0C with 12/12 h (light/dark) cycle. Animals were further divided in four groups with six animals in each group. Group I: Control, Group II: Standard (Cystone at 750 mg/kg), Group III: Extract of *Thunbergia laurifolia* (200 mg/kg), Group IV: Extract of *Thunbergia laurifolia* (400 mg/kg).

Extraction of plant material

Cold maceration

Flowers of *Thunbergia laurifolia* were collected, washed and rinsed properly. They were dried in shade and powdered mechanically. About 500gm of the flowers powder was macerated with methanol and stored for 72 hours in ice cold condition for the extraction of phytochemicals. At the end of the third day extract was filtered using whatmann No. 1 filter paper to remove all un-extractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts [27].

Qualitative analysis of phytochemicals

The extracts prepared for the study were subjected to preliminary phytochemical screening by using different reagents for identifying the presence or absence of various phytoconstituents viz., carbohydrates, proteins, alkaloids, tannins, steroid, flavonoids and terpenoids in methanolic extracts of *Thunbergia laurifolia*. The above phytoconstituents were tested as per the standard method [28].

Quantification of secondary metabolites

Quantitative analysis is an important tool for the determination of quantity of phytoconstituents

present in plant extracts. For this TPC and TFC are determined. Methanolic flowers extracts of *Thunbergia laurifolia* plant material of subjected to estimate the presence of TPC and TFC by standard procedure.

Total phenolic content estimation

The amount of total phenolic in extracts was determined with the Folin Ciocalteu reagent. Concentration of (20-100 µg/ml) of gallic acid was prepared in methanol. Concentration of 100 µg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced in to test and mixed with 2 ml of a 10 fold dilute folin Ciocalteu reagent and 4 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and it was then Incubated at room temperature for 30 mins with intermittent shaking and the absorbance were taken at 765 nm against using methanol as blank. Total phenolic content was calculated by the standard regression curve of Gallic acid and the results were expressed as gallic acid equivalent (mg/g) [29].

Total flavonoid content estimation

Different concentration of rutin (20 to 100 µg/ml) was prepared in methanol. Test sample of near about same polarity (100 µg/ml) were prepared. An aliquot 0.5ml of diluted sample was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 min, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 5min, and then 2 mL of 4% NaOH solution was added to the mixture. The final volume was adjusted to 5ml with distilled water and allowed to stand for another 15 mins. Absorbance was determined at 510 nm against water as blank. Total Flavonoid content was calculated by the Standard regression curve of Rutin/ Quercetin [30].

Invitro antioxidant activity

DPPH radical scavenging activity

For DPPH assay, the method of Gulçin *et al.*[31] was adopted. A solution of 0.1mM DPPH (4mg/100ml) in methanol was prepared and 1 ml of this solution was mixed with 1 ml of different concentrations of the different extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Ascorbic acid was used as reference standard while methanol was used as control. Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of *Thunbergia laurifolia* extracts. The change in colour was measured at 517 nm wavelength using methanolic solution as a reference solution. This was related to the absorbance of the control without the plant extracts. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

Reducing power assay

A spectrophotometric method was used for the measurement of reducing power. For this 0.5 ml of each of the extracts was mixed with 0.5ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% potassium ferricyanide (10 mg/ml). The reaction mixture was incubated at 50 ºC for 20 min separately, and then rapidly cooled, mixed with 1.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (0.5ml) of the supernatant was diluted with distilled water (0.5ml) and then ferric chloride (0.5ml, 0.1%) was added and allowed to stand for 10 min. the absorbance was read spectrophotometrically at 700 nm. Ascorbic acid (AA) was used as standard for construction of calibration curve [32].

$$\text{Reducing Power (\%)} = (As / Ac) \times 100$$

Here, Ac is the absorbance of control (AA) and as is the absorbance of samples (extracts) or standards.

Acute oral toxicity

Acute toxicity study of the prepared flowers extracts of *Thunbergia laurifolia* was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423 [33] the animals were fasted for 4 h, but allowed free access to water throughout. As per the OECD recommendations, the starting dose level should be that which is most likely to produce mortality in some of the dosed animals; and when there is no information available on a substance to be tested in this regard; for animal welfare reasons, The dose level to be used as the starting dose is selected from one of three fixed levels 5, 300 and 2000 mg/kg body weight. Acute toxicity was determined as per reported method [34].

Ethylene glycol induced kidney stone

A. **Control group:** served as control group treated with normal Saline at 1ml/kg body weight and 0.75% ethylene Glycol for 28 days in Drinking Water.
B. **Standard treated group:** served as Standard group treated with cystone at 750 mg/kg body weight and 0.75% ethylene Glycol for 28 days in Drinking Water.
C. **200 mg/kg extract treated group:** Extract was dissolved in normal saline and was administrated by oral route at a dose of 200 mg/kg body weight each for 28 days and 0.75% ethylene Glycol for 28 days in Drinking Water
D. **400 mg/kg extract treated group:** Extract were dissolved in normal saline and was administrated by oral route at a dose of 400mg/kg body weight for 28 days and 0.75% ethylene Glycol for 28 days in Drinking Water.

Collection and analysis of urine

The 24-hour urine samples were collected on days 0, 7, 14 and 28, while each rat was kept in a metabolic cage. Urine oxalate was measured by atomic absorption. Each sample was prepared and the yielding color was read by spectroscopy at 422.7 nm wave length.

Serum analysis

At the end of the experiment (day 28), The blood was collected from the retro-orbital sinus under anaesthetic condition and serum was separated by centrifugation at 10,000rpm for 10 min and analyzed for BUN (Urea), creatinine and uric acid.

Kidney histopathology and homogenate analysis

All rats were killed by cervical dislocation. The kidneys were removed; isolated kidneys were cleaned off extraneous tissue and rinsed in ice-cold physiological saline and weighed. The right kidney was fixed in 10% neutral buffered formalin for histological processing and left kidney in 0.2 M phosphate buffer (W/V) for *In-vivo* antioxidant assays and biochemical estimation. The slides were examined under light microscope and CaOx deposits were determined. Aggregations of CaOx deposits (tubules containing CaOx deposits) were counted in 10 microscopic fields and expressed as mean ± standard error for each group [35, 36].

Biochemical estimation

Blood samples were centrifuged for 10 min at 3000 rpm to separate the serum. Creatinin, Urea (BUN) levels were estimated from the serum by using standard kits [37].

Invivo antioxidant activity

Lipid peroxidation (LPO) assay

LPO is an autocatalytic process, which is a common consequence cell death. This process causes peroxidative tissue damage in inflammation, cancer and toxicity of xenobiotics and aging. MDA is one of the end products in the LPO process. MDA is formed during oxidative degeneration as a product of free oxygen radicals, which is accepted as an indicator of LPO. This method described by *Okhawa et al.* [38], is as follows: The 10 % w/v tissues are homogenized in 0.15 M tris HCl buffer (pH 7.4) with a Teflon glass homogenizer. LPO in this homogenate is determined by measuring the amounts of MDA produced primarily. 0.2 ml tissue homogenate with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid and 1.5 ml of 8% TBA are added. The volume of the mixture is made up to 4 ml with distilled water and then heated at 95°C on a water bath for 60 minutes using glass balls as condenser. After incubation the tubes are cooled to room temperature and final volume was made to 5 ml in each tube. 5 ml of butanol: pyridine (15:1) mixture is added and the contents are vortexed thoroughly for 2 minutes. After centrifugation at 3000 rpm for 10 minutes, the upper organic layer is taken and its OD is taken at 532 nm against an appropriate blank without the sample (This absorbance will be of total MDA formed). The levels of lipid peroxides are expressed as n moles of TBARS/mg protein using an extinction coefficient of 1.56×105 mL/cm.

Superoxide dismutase (SOD) activity was determined using NBT method

The assay involves the production of superoxide from O_2 (using reduced β-nicotinamide adenine dinucleotide (NADH) as a reductant, and phenazine methosulphate (PMS) as a catalyst in the presence of an indicator, nitro blue tetrazolium (NBT), which turns blue when reduced by superoxide. The color change during the reaction was monitored spectrophotometrically in the visible range at 560 nm. When SOD enzyme is added to the reaction, (superoxide scavengers i.e., antioxidants) compete with NBT to react with superoxide. The percent inhibition of NBT reduction was used to quantify superoxide-scavenging. Prepared 10 % w/v tissue homogenate in 0.15 M Tris HCl or 0.1 M phosphate buffer. Centrifuged at 15000 rpm for 15 min at 4 °C. Supernatant (0.1 ml) was taken consider it as sample and 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052 M) + 0.1 ml phenazine methosulphate (186 μM) + 0.3 ml of 300 μM Nitroblutetrazolium + 0.2 ml NADH (750 μM) were added. Incubated at 30°C for 90 s .0.1 ml glacial acetic acid was added. Stirred with 4.0 ml nbutanol. Allowed to stand for 10 min, Centrifuged and separated butanol layer. OD at 560 nm was taken (taken butanol as blank) and concentration of SOD was expressed as units/g of liver tissue. Absorbance values were compared with a standard curve generated from known SOD [39,40].

One unit of SOD is the amount of enzyme that inhibit the rate of reaction by 50 %

Interpretation will be based on % inhibition is to SOD conc. Curve

$$\% \text{ Inhibition} = \frac{(\Delta \text{ Abs}_{\text{control}} - \Delta \text{ Abs}_{\text{ sample}}) \text{ x } 100}{\Delta \text{ Abs}_{\text{control}}}$$

*Control will consist of all reaction mixture except any sample, instead of sample take equal amount of buffer/water.

(1.5 unit/assay of the purified enzyme produced 80 % inhibition)

U/ml = % Inhibition x 3.75

U/gm tissue = % Inhibition x 3.75 x (1/gm tissue used)

Glutathione (GSH)

DTNB is reduced in presence of GSH to produce a yellow compound. The reduced chromogen is directly propoertional to GSH conc. And its absorbance can be measured at 412 nm. Prepared 10 % w/v tissue homogenate in 0.1 M phosphate buffer (pH 7.4). 0.2 ml of homogenateis taken and added with equal volume of 20% trichloroacetic acid (TCA) containing 1 mM EDTA (0.0430 gm in 100 ml D/W) to precipitate the tissue proteins. The mixture is allowed to stand for 15 min prior to centrifugation for 10 min at 2000 rpm. The supernatant (400 µl) is then transferred to a new set of test tubes and added with 1.8 ml of the Ellman's reagent (5,5-dithiobis-2- nitrobenzoic acid (0.1 mM) prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes are made up to the volume of 2 ml with distilled water. After completion of the total reaction, solutions are measured at 412 nm against blank (water). Absorbance values were compared with a standard curve generated from known GSH [41,42].

Calculation

1. **In Blood**

= A sample x 66.66 mg/dL

= A sample x 2.22 mmol/dL

2. **In Tissue**

= (A sample x 66.66)/ g tissue used (mg/ g tissue)

= (A sample x 2.22)/ g tissue used (mmol/ g tissue)

Histology of organ

During the collection of the tissue from body of mice for estimation of biochemical parameters, pieces of tissue were cut, washed and transferred in 10% formalin solution.

Dehydration of tissue pieces

The tissue pieces were removed from the 10% formalin solution and placed in absolute alcohol for dehydration. Three changes were given in absolute alcohol each for 15 minutes.

Preparation of tissues for embedding

After three changes in absolute alcohol, pieces were transferred in mixture of absolute alcohol+xylene (1:1) for 15-20 minutes, mixture were decanted off and pieces were put in xylene for 30 minutes and after 30 min. scraping of wax were added to the xylene up to saturation and kept for 24 hrs.

Paraffin infiltration and embedding

The matured sample of paraffin was filtered to remove any suspending particles and kept in molten state for 24 hrs at 62-64°C. The material was transferred directly in molten wax in the first infiltration pan for 45 minutes at 62°C in an oven. After the first embedding, tissue pieces were removed and laced in second infiltration and kept as such at controlled temperature.

Block preparation

The lid of coupling jar was applied on upper and side surface of lid. Filtered matured wax was poured in the lid up to 4/5 th of total height. The tissues were immediately removed from the infiltration and placed gently in to the lid. It was allowed to stand at room temperature till solidified. The lid was placed in tray containing water. It was kept as such till the block separated and floated in water. The block was cut and trimmed to remove excess wax.

Microtomy

The block was then cut in to ribbons like section with the help of microtome. The ribbon sections were transferred to a slide on which a fixative (Egg albumin solution) had been applied.

Staining of slide

The section on slide was de-waxed with xylol. Aqueous haematoxylin was used for staining. The sections were mounted with Canada balsam on the slides carefully with cover slip [42].

Biostatical interpretation

All data are presented in Mean ±SD. Data were analyzed by One Way ANOVA followed by Benfeffoni's test. $P<0.05$ was considered as level of significance (n=4).

RESULTS AND DISCUSSION

Phytochemical analysis of methanolic extract of *Thunbergia laurifolia* flowers showed the presence of carbohydrate, alkaloids, flavonoids, phenolics, tannin, saponins, and triterpenoids table 1.

Table-1: Result of phytochemical screening of *Thunbergia laurifolia*

Test	Methanolic extract
Test for carbohydrates	
Molish	+Ve
Fehling's	+Ve
Benedict's	-Ve
Test for protein and amino acid	
Biuret	+ Ve
Ninhydrin	+ Ve
Test for glycosides	
Borntrager's	-Ve
Keller-killani	-Ve
Test for alkaloids	
Mayer's	+Ve
Hager's	-Ve
Wagner's	+ Ve
Test for saponins	
Froth Test	+ Ve
Test for flavonoids	
Lead acetate	+ Ve
Alkaline reagent	+ Ve
Test for triterpenoids and steroids	
Salkowski's	+ Ve
Libermann-burchard's	-Ve
Test for tannin and phenolic compounds	
Ferric chloride	+Ve
Lead acetate	+ Ve
Gelatin	-Ve

Quantitative phytochemical assay was performed by calculating total phenolic content (TPC) and total flavonoid content (TFC). The TPC was calculated with respect to gallic acid (standard) and the TPC in methanolic extract was found to be 0.058 mg/g equivalent to gallic acid table 2& fig 1.

Table-2: Total phenolic content of extracts

S. No.	Methanolic Extract
1.	0.176
2.	0.178
3.	0.18
4.	0.181
5.	0.181
TPC	0.058 mg/gm equivalent to Gallic acid

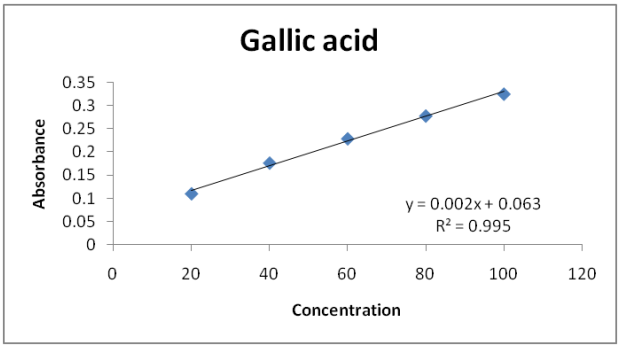

Fig-1: Graph of estimation of total phenolic content

TFC was then calculated with respect to rutin taken as standard. The TFC in hydroalcohoilc extract

was higher than that of the methanolic extract with concentration being 1.080 mg/g equivalent to rutin table 3& fig 2.

Table-3: Total flavonoid content of extracts

S. No.	Methanolic Extract
1.	1.179
2.	1.184
3.	1.166
4.	1.164
5.	1.168
TFC	1.080 mg/gm equivalent to Rutin

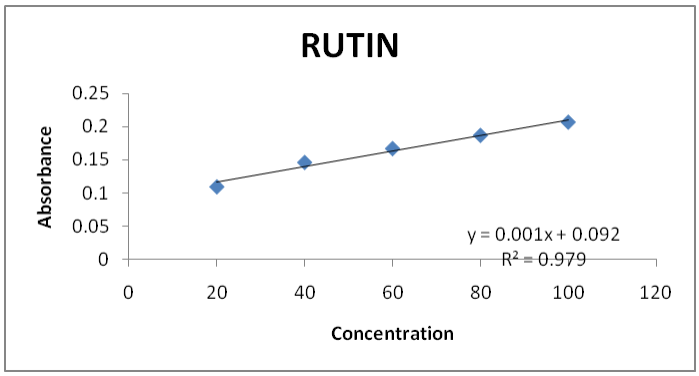

Fig-2: Graph of estimation of total flavanoid content

Antioxidant activity of the samples was calculated through DPPH assay and reducing power assay. % inhibition was calculated as an indicative of antioxidant potency. The higher the % inhibition the better the activity. Ascorbic acid was taken as standard in both the tests and the values were comparable with concentration ranging from 20µg/ml to 100µg/ml. A dose dependent activity with respect to concentration was observed. % inhibition was higher in the ascorbic acid where % inhibition ranged from 52.75% to 71.72 % while the values were lesser in methanolic extract ranging from 36.19% to 65.03% table 4.

Table-4: DPPH assay of ascorbic acid, methanolic extract

S. No.	Conc. (µg/ml)	Ascorbic acid (% Inhibition)	Methanolic Extract (% Inhibition)
1.	20	52.75	36.19
2.	40	56.36	42.11
3.	60	61.52	51.10
4.	80	68.97	56.25
5.	100	71.72	65.03

The reducing ability of the compound usually depends on the reductants, which have been exhibited antioxidative capacity by breaking the free radical chain, donating a hydrogen atom. Reducing power assay was calculated in extracts and the values indicated a better activity table 5& fig 3.

Table-5: Result of reducing power assay

S. No.	Ascorbic acid	Methanolic Extract
1.	0.987	0.533
2.	1.032	0.712
3.	1.145	0.716
4.	1.159	0.762
5.	1.201	0.800

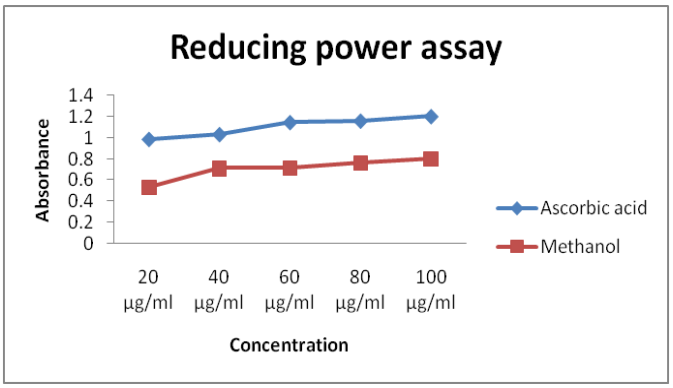

Fig-3 Reducing power assay

In the acute toxicity study, no signs of toxicity were found upto the dose of 2000 mg/kg body weight. Hence 1/10th and 1/5th doses i.e. 200 mg/kg and 400 mg/kg have been fixed as ED50 for present study. There was an increase in urinary oxalate concentration in calculi induced animals table 6. However, supplementation with TBL (200 and 400 mg/kg) significantly (P <0.05) inhibited these changes in urinary oxalate dose-independently in preventive regimen table 6.

Table-6: Urine oxalate concentration

Groups	0 Day	7 Day	14 Day	28 Day
Control	4.47±0.258	6.26±0.058	7.36±0.293	10.16±0.097
Standard	4.89±0.047	5.50±0.307	5.95±0.339	6.15±0.054
Ex (200 mg/kg)	5.60±0.212	6.22±0.078	7.63±0.136	8.66±0.044
Ex (400 mg/kg)	5.30±0.052	5.70±0.257	6.03±0.051	7.59±0.208

Renal stone induction caused impairment of renal functions of the untreated rats as evident from the makers of glomerular and tubular damage, i.e. elevated serum creatinine, uric acid and urea. These markers were significantly (P <0.05) reduced in the animals which were treated with TBL in a dose-dependent manner table 7.

Table 7: Effect of methanol extract of *Thunbergia laurifolia* and standard drug on serum biochemical parameters

Groups	Urea	Creatinine	Uric acid
Control	56.75±2.026	2.09±0.158	92.43±6.023
Standard	18.79±0.988	0.80±0.029	20.00±1.021
Ex (200 mg/kg)	45.68±2.012	1.97±0.255	60.12±2.345
Ex (400 mg/kg)	25.71±1.717	1.01±0.081	27.99±2.179

Ethylene glycol treatment significantly (P <0.05) increased the LPO levels and decreased the SOD and GSH levels in calculi-induced animals compared to the normal animals [Table 4, Group II].The treatment with TBL (200 and 400mg/kg) produced significant (P <0.05) reduction in LPO and improved the level of antioxidant enzymes like GSH. The elevated level of SOD was significantly (P <0.05) maintained by the treatment with TBL 400 in preventive treatment table 8.

Table-8: Effect of methanolic extract of *Thunbergia laurifolia* on antioxidant parameters of control and experimental animals

Groups	LPO	SOD	GSH
Control	11.11±0.255	24.72±4.759	0.95±0.061
Standard	4.72±0.255	44.90±5.608	3.42±0.123
Ex (200 mg/kg)	8.94±0.977	29.73±2.676	1.66±0.103
Ex (400 mg/kg)	6.33±0.764	42.72±14.596	2.51±0.110

Crystals were absent in urine of vehicle control animals, while in the lithogenic treatment-induced group, crystals were more numbers with larger sizes. Treatment with TBL powder clearly reduced the crystal number as well as the crystal size table 9 & fig 4.

Table-9: Number of calcium oxalate deposited in kidney (per 10 microscopic fields)

Groups	0 Day	7 Day	14 Day	28 Day
Control	0.56±0.013	0.31±0.013	0.19±0.013	0.15±0.018
Standard	0.62±0.013	0.56±0.022	0.53±0.022	0.52±0.024
Ex (200 mg/kg)	0.50±0.010	0.49±0.014	0.43±0.017	0.33±0.010
Ex (400 mg/kg)	0.64±0.013	0.55±0.013	0.51±0.013	0.41±0.013

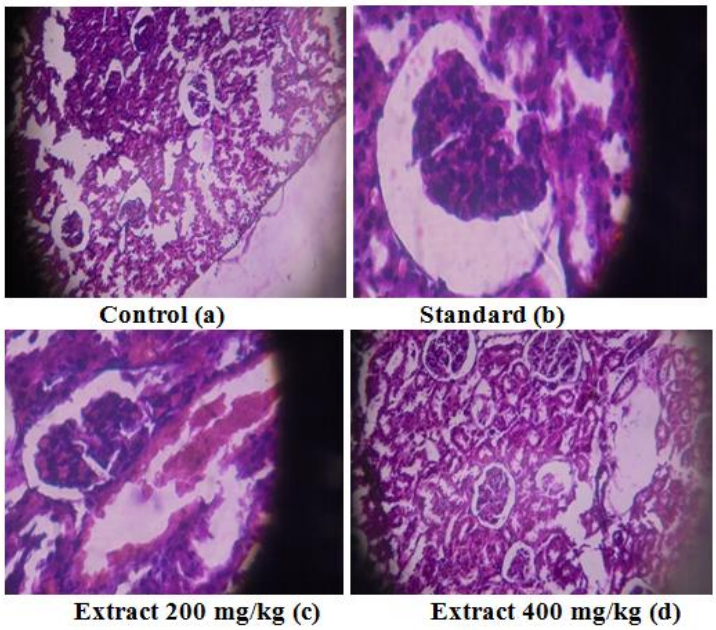

Fig-4: Light microscopic architecture and calcium oxalate deposits in the section of kidney Sections of (a) control, (b) cystone treated, (c) treatment with TBL at the dose of 200 mg/kg, (e) treatment with TBL at the dose of 400 mg/kg

CONCLUSION

The result indicates that administration of methanolic extract of *Thunbergia laurifolia* flowers to rats with the ethylene glycol- induced urolithiasis reduced and prevented the growth of urinary stones by diuresis, antioxidant activity and maintaining balance between stone promoters and inhibitors constituents. This study supporting folklore information regarding the antiurolithiatic activity of *Thunbergia laurifolia*. Further experimental and clinical studies are required to elucidate the chemical constituents of the extract and the mechanism that are responsible for the pharmacological activities.

Acknowledgment

The authors are very much thankful to Pinnacle Biomedical Research Institute (PBRI) Bhopal (M.P.) 462003, India for providing necessary facilities during this research work.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES

1. Aggarwal KP, Narula S, Kakkar M, Tandon C. Nephrolithiasis: Molecular mechanism of renal stone formation and the critical role played by modulators. Biomed Res Int. 2013;29:29-53
2. Panigrahi PN, Dey S, Jena SC. Urolithiasis: Critical analysis of mechanism of renal stone formation and use of medicinal plants as antiurolithiatic agents. Asian J Anim Vet Adv. 2016; 11:9-16.
3. Khan A, Bashir S, Khan SR, Gilani AH. Antiurolithic activity of Origanum vulgare is mediated through multiple pathways. BMC Complement Altern Med. 2011; 11:96-98.
4. Das M, Malipeddi H. Antiurolithiatic activity of ethanol leaf extract of Ipomoea eriocarpa against ethylene glycol-induced urolithiasis in male Wistar rats. Indian J Pharmacol. 2016; 48:270-274.
5. Jagannath N, Chikkannasetty SS, Govindadas D, Devasankaraiah G. Study of antiurolithiatic activity of Asparagus racemosus on albino rats. Indian J Pharmacol. 2012; 44:576-579.
6. Shah JG, Patel BG, Patel SB, Patel RK. Antiurolithiatic and antioxidant activity of Hordeum vulgare seeds on ethylene glycol-induced urolithiasis in rats. Indian J Pharmacol. 2012; 44:672-677.
7. Ahmadi M, Rad AK, Rajaei Z, Hadjzadeh MA, Mohammadian N, Tabasi NS. Alcea rosea root extract as a preventive and curative agent in

ethylene glycol-induced urolithiasis in rats. Indian J Pharmacol. 2012; 44:304-307.
8. Khan A, Khan SR, Gilani AH. Studies on the in vitro and in vivo antiurolithic activity of Holarrhena antidysenterica. Urol Res. 2012; 40:671-681.
9. Shirfule AL, Racharla V, Qadri SS, Khandare AL. Exploring antiurolithic effects of gokshuradi polyherbal Ayurvedic formulation in ethylene-glycol-induced urolithic rats. Evid Based Complement Alternat Med. 2013; 763-720.
10. Alexander H, Nestor S. Phyllanthus niruri inhibits calcium oxalate endocytosis by renal tubular cells: Its role in nephrolithiasis. Nephron. 1999; 81:393-397.
11. Freitas AM, Schor N, Boim MA. The effect of Phyllanthus niruri on urinary inhibitors of calcium oxalate crystallization and other factors associated with renal stone formation. BJU Int. 2002; 89:829-834.
12. Grases F, March JG, Ramis M, Costa-Bauz´a A. The influence of Zea mays on urinary risk factors for kidney stones in rats. Phytother Res. 1993; 7:146-149.
13. Grases F, Ramis M, Costa-Bauza A, March JG. Effect of Herniaria hirsuta and Agropyron repens on calcium oxalate urolithiasis risk in rats. J Ethnopharmacol. 1995; 45:211-214.
14. Yasui T, Fujita K, Sato M, Sugimoto M, Iguchi M, Nomura S. The effect of Takusya, a Kampou medicine, on renal stone formation and osteopontin expression in rat urolithiasis model. Urol Res. 1999; 27:194-199.
15. Bayir Y, Halici Z, Keles MS, Colak S, Cakir A, Kaya Y, Akçay F. Helichrysum plicatum DC. subsp. plicatum extract as a preventive agent in experimentally induced urolithiasis model. Journal of ethnopharmacology. 2011 Nov 18;138(2):408-14.
16. McMartin K. Are calcium oxalate crystals involved in the mechanism of acute renal failure in ethylene glycol poisoning?. Clinical Toxicology. 2009 Nov 1;47(9):859-69.
17. Karamakar D, Patki PS. Evaluation of efficacy and safety of a herbal formulation Cystone in the management of urolithiasis: Meta-analysis of 50 clinical studies. Int J Altern Med. 2010;8:1-8.
18. Kumaran MS, Patki PS. Evaluation of an Ayurvedic formulation (Cystone), in urolithiasis: A double blind, placebo-controlled study. European Journal of integrative medicine. 2011 Apr 1;3(1):23-8.
19. Starr F, Martz K, Loope LL. New plant records from East Maui for 1998. Bishop Mus Occ Pap.1999; 59(2):1-15.
20. Schönenberger J. Floral structure, development and diversity in Thunbergia (Acanthaceae). Botanical journal of the Linnean Society. 1999 May 1;130(1):1-36.
21. Chan EWC, Lim YY. Antioxidant activity of Thunbergia laurifolia tea. Jo Trop Forest Sci. 2006; 18(2):130-136.
22. Burkill IH. A dictionary of the economic products of the Malay Peninsula. Volume II (I–Z). Ministry of Agriculture and Cooperatives, Kuala Lumpur. 1966.
23. Usanawarong S, Thesiri T, Mahakunakorn P, Parasupattana S. Effect of Thunbergia laurifolia Linn. on detoxification of paraquat. Warasan Wichai Mo-Kho. 2000.
24. Ussanawarong S, Thesiri T. Effect of Thunbergia laurifolia Linn on detoxification of parathion in rat. Khon Kaen Uni Res J. 2001; 6:3-13.
25. Kanchanapoom T, Kasai R, Yamasaki K. Iridoid glucosides from Thunbergia laurifolia. Phytochemistry. 2002; 60(8):769-771.
26. Kwansang J, Itthipanichpong C, Limpanasithikul W. Evaluation of wound healing activity of Thunbergia laurifolia supercritical carbon dioxide extract in rats with second-degree burn wounds. J Adv Pharma Tech Res. 2015; 6 (3):103-107.
27. Sangeetha J, Vijayalakshmi K. Determination of bioactive components of ethyl acetate fraction of Punica granatum rind extract. Int J Pharm Sci Drug Res. 2011; 3(2): 116-122.
28. Kokate CK, Purohit AP, Gokhale SB. Pharmacognosy; 23 ed., Nirali prakashan. 2006;493-497.
29. Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissue using Folin-Ciocalteu reagent. Nature Protocol. 2007; 2(4): 875-877
30. Zhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 1999; 64: 555-559.
31. Gulçin I, Elias R, Gepdiremen A, Boyer L. Antioxidant activity of lignans from fringe tree (Chionanthus virginicus L.). Eur Food Res Technol. 2006; 223: 759-767.
32. Jain R, Jain SK. Total Phenolic Contents and Antioxidant Activities of Some Selected Anticancer Medicinal Plants from Chhattisgarh State, India. Pharmacologyonline. 2011; 2: 755-762.
33. Guideline Document on Acute oral Toxicity Testing, Series on Testing and Assessment No. 423. Paris: Organization for Economic Co-Operation and Development, OECD Environment, Health and Safety Publications. 1996. Available from: http//www.oecd.org/ehs.
34. Jonsson M, Jestoi M, Nathanail AV, Kokkonen UM, Anttila M, Koivisto P, Peltonen K. Application of OECD Guideline 423 in assessing the acute oral toxicity of moniliformin. Food Chem Toxicol. 2013; 53: 27-32.
35. Sriboonlue P, Suwantrai S, Prasongwatana V. An indirect method for urinary oxalate estimation. Clin Chim Acta. 1998; 273:59-68.

36. Khoei A, Hadjzadeh Z, Parizady M. Ethanolic extract of nigella sativa L seeds on ethylene glycol-induced kidney calculi in rats. Urol J. 2009; 4(2): 86-90.
37. Rajesh KG, Achyut NK, Geeta W, Murthy PS, Ramesh C, Vibha T. Nutritional and hypoglycemic effect of fruit pulp of Annona squamosa in normal healthy and alloxaninduced diabetic rabbits. Ann Nutr Metab. 2005; 49: 407-413.
38. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95(2):351-8.
39. Paoletti F, Aldinucci D, Mocali A, Caparrini A. A Sensitive Spectrophotometric Method for the Determination of Superoxide Dismutase Activity in Tissue Extracts, Analytical Biochemistr. 1986; 153: 536-541
40. Ellman GL. Tissue Sulfydryl Groups. Arch Biochem Biophy.1959; 82:70-77
41. Ferre S, Guix T, Prat G, Jane F, Cosa M. Is experimental catalepsy properly measured? Pharmacol Biochem Beh. 1990; 35: 753-757.
42. Slaoui M, Fiette L. Histopathology procedures: from tissue sampling to histopathological evaluation. InDrug Safety Evaluation 2011 (pp. 69-82). Humana Press.