International Journal of Phytopharmacology
Research Article
www.onlineijp.com
e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

PHYTOCHEMICAL SCREENING & PHARMACOLOGICAL INVESTIGATION OF THUNBERGIA ERECTA FOR UROLITHIASIS

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ABSTRACT

Medicinal plants play an important role in our natural wealth. They serve as an important therapeutic agent as well as valuable raw material for manufacturing numerous traditional medicines and also acts as the lead for modern medicines. The present study was designed to investigate the phytochemical screening and characterization of active component from plant Thunbergia erecta. The percentage yield of extraction is very important in phytochemical extraction in order to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. Urolithiasis is a common medical condition affecting people all across the globe, though the rate of incidence and prevalence may vary. The last five decades have witnessed a gradual increase in renal stone incidence rate in industrialized countries. The greater incidence rates in those parts of the world might be attributed to changing eating habits. An established therapy is lacking for the reduction of urinary oxalate excretion in calcium oxalate stone patients and also its relapses. Herbal therapies of preventing kidney stones have gained importance in the last decade. Recent studies have highlighted the potential of several medicinal herbs and natural compounds for the treatment of nephrolithiasis. Numerous scientific literatures are available suggesting that herbal therapies are effective and at the same time potentially safer alternative to other forms of management. There are varieties of herbal formulations available commercially for the treatment of urolithiasis, although the scientific data showing their advantage over others is missing in most of the cases. Moreover, various phytotherapeutic agents have also been proposed as useful alternative or complementary therapies for the management of urolithiasis, in part due to their anti-oxidative effects.

Key words: Urolithiasis, Medicinal Plant, Kidney Stone, Thunbergia erecta.

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Access this article online

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Received:25.09.2023 **Revised**:12.10.2023 **Accepted**:26.10.2023

INTRODUCTION

Medicinal plants provide useful tools for treating various diseases. The practices of these traditional medicines are based on old beliefs and observations, which predate the development and spread of modern medicine (Herlach, F.*et al.*, 1996) Today, people have developed interest in herbal drugs. This interest is developed as that they are safe, inexpensive and without any adverse or side effects (Kwoh, C. K. *et al.*, 1996) Medicinal plants are more popular among the people seeking herbal remedies and healthy approach (Sukanya, S. L. *et al*., 2009) The world's 1.42 billion people use traditional medicines for treating various disorders (Fabricant, D. S. *et al.*, 2001)

The process of standardization can be achieved through stepwise photochemical studies and pharmacognostic studies. These studies are helpful in the standardization and identification of the plant material. Correct quality assurance and identification of the starting materials is an essential to ensure reproducible quality of herbal medicine. It will contribute to its safety and efficacy (Farnsworth, N. *et al*., 1985)

Many of the herbs that have been used against various disorders are not properly investigated with the findings correlated with pharmacological and photochemical studies. Medicinal plants include those

plants that are used directly or indirectly in the extraction of the drug for the treatment of disorders. Scientists are trying to explore the precious assets of medicinal plants for the people suffering from ailments. In the world 30% of the pharmaceutical preparations are manufactured from plants (Doharey, V. *et al.*, 2011)

The recent resurgence of plant remedies results from several factors like effectiveness of plant medicines without side effects compared to modern medicines. In present time, there is a need for basic scientific investigations done on the medicinal plants. This is evident due to the increase in number of reports by various investigators that supports the claims of medicinal plants (Kumar, S., *et al.* 2011)

Urolithiasis

Urolithiasis refers to the formation of stones in the urinary system, i.e. in the kidney, ureter, and urinary bladder or in the urethra. 'Urolithiasis' is the combination of two words i.e. ouron [urine] and lithos [stone]. Urolithiasis affects the urinary tract badly. It is a major source of morbidity. Stone formation is a very painful urologic disorder that occurs in approximately 12% of the global population. Its re-occurrence rate in males is much more i.e. 70-81% than in female's i.e 47-60% (Saha, P., *et al.*, 2011)

It is reported that 10% of the population living in the urban area are suffering from the problem of stone formation. The prevalence of renal calculi is less in the southern part (Selvaraj, N. *et al*., 2011) Men are affected more than females and the rate of occurrence is three times higher in men as compared to females. The reason behind this is the enhancing capacity of testosterone and inhibiting capacity of estrogen in stone formation (Kadam, D. *et al.*, 2011) It has been found that the formation of urinary calculi dates back not only to 4000 B.C in the tombs of Egyptian mummies also in graves of North American Indians from 1500 to1000 B.C (Khan, A. U., *et al.*, 1979) Stone formation is also documented in the early Sanskrit documents during 3000 and 2000 B.C **(**Pareta, S. *et al.*, 2011) Due to the high rate of reoccurrence as well as multifactorial etiology this disorder is considered as a medical challenge. The imbalance between inhibitors and promoters results in the formation of stones (Devi, V. K., *et al*., 1993)

Urolithiasis has also known as uroliths or calculi; the most common prevalent urinary disorder. It involves the formation of stones in any part of urinary tract by the successive physiochemical events of aggregation supersaturation, nucleation and retention (Divakar, K., *et al.*, 2010 & Bouanani, S., *et al.*, 2010). It is a multifactorial disorder that results from multifactorial etiopathogenesis like metabolic, epidemiological, biochemical, nutritional, socio-economic, drug-induced and genetical risk factors. (Stefano, S., *et al.*, 2003 & Alessandra, A. N., *et al.*, 2003)

The key pathological event in the formation of stones includes the super-saturation of urine. This also includes elements like calcium oxalate, struvite, uric acid that contribute to the formation of stone in the urinary system (Osborne, C. A., *et al.*, 2009). 10-12% of the population living in the urban area are affected globally. The cases of this disease have been boosting up in the last five decades (Worcester, E. M., *et al.*, 2010) . The reported reoccurrence rate was recorded as 40% in 3 years, 74% in 10 years, and 98% in 25 years **(**Lathadevi, H. *et al.*, 2005) Age and gender are the two important factors on which the disorder urolithiasis depends. On a clinical basis, children are rarely affected by this disorder **[31].** Urolithiasis often leads to life-threatening complications like chronic renal failure, pyelonephritis**.**

MATERIAL AND METHOD

Selection and Collection of Plant

The plant material was selected on the basis of ethano – botanical survey. Various considerations were involved in the plant selection especially for its anti-urolithiasis activity. The leaves of Thunbergia erecta was collected in the month of July 2023.

Authentication of Plant

The identification and authentication of plant was done by Dr. Saba Naaz, Botanist, from the Department of Botany, Saifia College of Science and Bhopal. A voucher specimen number **195/Saif. /Sci./Clg/Bpl.** was kept in Department of Botany, Saifia College of Science, Bhopal for future reference.

Solvent Extraction

Cold maceration method

The extraction of the plant materials was carried out using cold maceration method. The leaves of were collected, washed and rinsed properly. Extraction with different organic solvents like petroleum ether and methanol were performed and allow standing for 4-5days each. Filteration of extract was done using whatman no.1 filter paper as it removes all unextractable matter, including cellular materials and other constituents that are insoluble in the extraction solvent. Extract was transferred to beaker and evaporated & amp; excessive moisture was removed and extract was collected in air tight container. Each extract was dissolved by Dimethyl sulfoxide [DMSO] and sterilized using 0.22 µm syringe filters [Axiva, Scichem Biotech] for further use.

The yield of the extract is calculated by using the following formula.

Yield [%] = Weight of the residue obtained × 100
Weight of the plant material taken

Phytochemical Investigation

Qualitative Test

To determine the presence and absence of phytoconstituents a detailed qualitative phytochemical analysis was planned. The analytical responses of these tests were confirmed by color intensity or the precipitate formation.

In vitro Anti Urolithiasis Activity

Nucleation Assay

Preparation of synthetic urine:

We have chosen the oldest model to study oxalate crystallization because of its simplicity and satisfactory reproduction. This model involves the crystallization study without the inhibitor as well as with it, in order to test the inhibitory power of any type of chemical used. Two solutions of the following formulations were combined: A: Na2C2O4 [2 m mol / 1] and B: Ca Cl2 2H2O [10 m mol / 1]. These two solutions were prepared in stock NaCl 9 g to obtain ionic energy as the properties of the House. The formation and growth of COM oxalate crystals from artificial urine in different concentrations became our research object. Artificial urine is prepared by mixing and stirring two equal volumes of 50 ml of solutions A and B at a constant temperature [37 ° C] in closed vessels to provide final artificial urine. The mixture was kept to prevent dissipation.

Simulation of the sedimentary crystal formation

Crystal size growth was observed with samples dropping every five minutes with a separate microscope. A drop of sample was placed in the hemacytometer calculator and the sample was detected under a microscope at a time after 30 min. a calculated number of crystals and eye contact with the camera. A series of experiments involving the body of 25, 50, 75, and 100% of plants extracted were used. Tracking of crystal size growth with a microscope was done on time after 30 minutes of crystalline formation and camera capture. Calculated the percentage of Inhibition [I %] was based on the formula:

$$I\% = [[TSI - TAI] / TSI] * 100$$

TSI- represents the number of calcium oxalate monohydrate crystals without inhibitor.

TAI- represents the number of calcium oxalate monohydrate crystals after addition of inhibitor.

RESULT & DISCUSSION

Collection of Plant Material

The leaves of Thunbergia erecta was collected in the month of July 2019 and was dried in shade at room temperature. The dried leaves of Thunbergia erecta was course powdered and used for the preparation of the extract **Table 2**.

Percentage Yield

The percentage yield of extraction is very important in phytochemical extraction in order to evaluate the standard extraction efficiency for a particular plant, different parts of same plant or different solvents used. The yield of extracts obtained from the leaves of the Thunbergia erecta using petroleum ether and methanol as solvents are depicted in **Table 3**.

The plant material was extracted by cold maceration and the percentage yield calculated by the formula Yield [%] = Weight of the residue obtained/Weight of the plant material taken×100 was found to be% [petroleum ether] and % [methanol].

Formula of Percentage yield = $\frac{\text{Actual yield}}{} \times 100$

Percentage yield of Petroleum extract = $\frac{2.913}{100} \times 100 = 1.456\ \%$

Percentage yield of Methanolic extract = $\frac{7.887}{100} \times 100 = 3.983\%$.

Phytochemical Analysis

Qualitative Analysis

Qualitative phytochemical testing of extracts was done to study the presence or absence of various phytochemical constituents using standard tests [139]. Phytochemical testing of petroleum ether extract and methanolic extract of Thunbergia erecta were performed. Results showed presence of various phytoconstituents in extracts. The results of qualitative phytochemical estimation are shown in the **Table 4**.

The results of qualitative phytochemical analysis of the crude powder of leaves of T.erecta are shown in **Table no. 4**. Methanolic extracts of sample of T.erecta showed the presence of carbohydrates, alkaloids, flavonoids, glycosides, saponins, tannins and phenolic compounds; but in petroleum ether extracts all phytoconstituents are absent.

Quantitative Phytochemical Screening

Preliminary phytochemical testing of crude extracts confirmed the presence of Phenolics and flavonoids in plant material. To estimate their amount total phenolic [TPC] and total flavonoid content [TFC] assays were performed.

Total Phenolic Contents [TPC]

Total phenolic content of all the extracts was determined by Folin –Ciocalteu's method using gallic acid as standard. Results were expressed as mg of gallic acid equivalent weight [GAE].

The Folin–Ciocalteu reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid. This mixture causes oxidation of phenols and is reduced to blue color solution of tungsten and molybdenum which is measured by spectrophotometer

having absorption maxima at 750 nm. The blue coloration is proportional to the total quantity of Phenolics present.

The total phenolic contents of T. erecta extracts were calculated with a regression equation based on a standard curve using Gallic acid [20-100µg/ml] as standard [**Table 6**]. The methanolic extract of Thunbergia erecta showed 174.66 mg/g equivalent of Gallic acid [**Table 7**].

Phenolics are the most important secondary metabolites present in plants [141]. They contribute to the antioxidant activity of plants due to their redox properties, act as hydrogen donators, reducing agents, and oxygen scavengers. This leads to prevention of various diseases associated with oxidative stress such as cardiovascular, neurodegenerative diseases and cancer**.**

Table 1: Plant part selected for the present study

S. No.	Plant	Family	Parts used
01	*Thunbergia erecta*	*Acanthaceae*	Leaves

Table 2: Collection of Thunbergia erecta.

S.No.	Plant Name	Plant Part Used
1.	***Thunbergia erecta***	Leaves

Table 3: Yield of crude extracts of Thunbergia erecta leaves extract.

S.No.	Solvent	% Yield
1.	Pet. Ether	1.456
2.	Methanol	3.983

Table 4: Phytochemical evaluations of different extracts of Thunbergia erecta.

S. No.	Experiment	Results	
		Pet Ether Extract	Methanolic extract
1.	Test for Carbohydrates		
	Molisch's Test	-ve	+ve
	Fehling's Test	-ve	+ve
	Benedict's Test	-ve	+ve
2.	Test for Protein & Amino acids		
	Biuret's Test	-ve	-ve
	Ninhydrin Test	-ve	-ve
3.	Test for Glycosides		
	BorntragerTest	-ve	+ve
	Killer killaniTest	-ve	+ve
4.	Test for Alkaloids		
	Mayer's Test	-ve	+ve
	Hager's Test	-ve	+ve
	Wagner's Test	-ve	+ve
5.	Test for Saponins		
	Froth Test	-ve	+ve
6.	Test for Flavonoids		
	Lead acetate	-ve	+ve
	Alkaline reagent test	-ve	+ve
7.	Test for Triterpenoids and Steroids		
	Libermann-Burchard Test	-ve	-ve
	Salkowski Test	-ve	-ve
8.	Test for Tannin and Phenolic Compounds		
	Ferric Chloride Test	-ve	+ve
	Gelatin Test	-ve	+ve
	Lead Acetate Test	-ve	+ve

Table 5: In vitro antiurolithic activity of extract.

S. No.	Sample	COM/mm3	% Inhibition
1	Control	325	-
2	25	225	30.77
3	50	187.5	42.31
4	75	137.5	57.69
5	100	112.5	65.38

Table 6: In vitro antiurolithic activity of standard.

S. No.	Sample	COM/mm3	% Inhibition
1	Control	325	-
2	25	187.5	42.31
3	50	150	53.85
4	75	100	69.23
5	100	62.5	80.77

Table 7: Acute oral toxicity of extract of selected plants (in albino wistar rats.

S.No.	Dose	No. of animal	Mortality*
			Methanolic
	5 mg/kg	Three	0/3
	50 mg/kg	Three	0/3
	300 mg/kg	Three	0/3
	2000 mg/kg	Three	0/3
	5 mg/kg	Three	0/3
	50 mg/kg	Three	0/3
	300 mg/kg	Three	0/3
	2000 mg/kg	Three	0/3

Table 8: Standard Curve of Gallic acid

S.No.	Concentration[µg/ml]	Absorbance
1.	20	0.1098
2.	40	0.1763
3.	60	0.229
4.	80	0.2783
5.	100	0.3258

In vitro Anti urolithiasis activity

A large number of people in this world are suffering from urinary stone problem. Calcium oxalate monohydrate [COM] and calcium oxalate dihydrate [COD] containing stones are commonly found in kidney. Herbal extracts may contain substances that inhibit the growth of CaOx crystals. This property of plants may be important in preventing kidney stone formation; CaOx crystals induced by urinary macromolecules are less tightly bound to epithelial cell surfaces, which are then excreted with urine.

The formation and growth of the calcium oxalate monohydrate crystals from artificial urine at different concentrations were studied.Stone formation was the due to supersaturation of urine with some urinary salts such as calcium oxalate. The number of calcium oxalate monohydrate crystals was found to be maximum in control. Different percentages of plant extract were tested in order to assess the inhibiting potential of plant extract for oxalate crystallization. In the presence of different percentages of plant extract, the size [length and the width] of the crystals were reduced.

It was observed that the plant used in this study inhibited the crystal development with maximum number of crystals found at 25% physiological extract concentration while at 100% physiological concentration of extract, the crystal formation was found minimal. Results show that the decrease in number of crystal as well as % inhibition of the formation of calcium oxalate monohydrate crystals was directly proportional to the increase in percentage of plant extract. Hence the calcium oxalate monohydrate showed minimum inhibition of 30.77% at 25 % physiological concentration of T. erecta methanolic extract, while maximum inhibition of 65.38% at 100 % physiological concentrations of T. erecta methanolic extract.

Acute Oral Toxicity

Toxicity testing of new compounds is significant for drug development process. The toxicity of the substances can be determined on experimental animals by analyzing accidental exposures to an in vivo substance. Acute oral toxicity study of methanolic extract of Thunbergia erecta according to OECD-423 guidelines in mice.To ascertain safety of any component its acute oral toxicity is an important parameter to assess. Toxicity was assessed at 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg. As per OECD 423 guideline up to the dose of 2000 mg/kg safety was assessed. All two extracts were found to be safe at all selected doses. As all two extracts were safe up to the dose of 2000 mg/kg and no mortality was observed [**Table 12**], dose was selected accordingly.$1/10^{th}$ and $1/5^{th}$ of 2000 mg/kg was selected as dose for present investigation. Doses selected were 200 mg/kg and 400 mg/kg.

CONCLUSION

In the study, we have done the qualitative phytochemical anaylsis of extract. Qualitative phytochemical testing of extracts was done to study the presence or absence of various phytochemical constituents using standard tests. Plants produce primary and secondary metabolites with divergent functions. In phytochemical tests, aqueous and organic extracts are prepared from those plant samples that are the reservoir of secondary metabolites, such as leaves, stems, roots, or bark. The plant extracts are then analyzed for the presence of secondary metabolites like alkaloids, terpenes, and flavonoids. The phytochemical category includes compounds recognized as essential nutrients, which are naturally contained in plants and are required for normal physiological functions, so must be obtained from the diet in humans. Here, phytochemical testing of petroleum ether extract of *Thunbergia erecta* and methanolic extract of *Thunbergia erecta* was performed. Results showed presence of various phytoconstituents in methanolic extract. Phytochemical estimation of *Thunbergia erecta* showed the presence of alkaloids and carbohydrates, glysoside, saponin, flavonoids and phenol.

To know the chemical properties of selected *Thunbergia erecta*, preliminary phytochemical analysis, spectroscopic and chromatographic analysis such as UV-Vis, TLC was carried out. In addition, HPTLC and IR were studied. To know the biological potentials of *Thunbergia erecta*, invivoantiurolithic and histopathological and acute oral toxicity activities were also carried out.

Kidney stone disease is a multi-factorial disorder resulting from the combined influence of epidemiological, biochemical and genetic risk factors**.** Urolithiasis is considered as the third most common affliction of the urinary tract. It refers to the solid non-metallic minerals in the urinary tract. It is a complex process that is a consequence of an imbalance between promoters and inhibitors in the kidney. Since urolithiasis is a multifactorial disease it has a very complicated and highly uncertain etiology. The formation of kidney stones involves several phytochemical events beginning with crystal nucleation, aggregation and end with retention within the urinary tract. Among the several types of kidney stones, the most common are calcium oxalate stones representing up to 80% of the analyzed stones. Calcium-containing stones may be in the form of pure calcium oxalate [50%] or calcium phosphate [5%] and a mixture of both [45%] followed by magnesium phosphate [15-20%], uric 5 acids [10%] and cystine [1%]**.** It is estimated that at least 10% of the population in the industrialized part of the world is afflicted by urinary tract diseases and among these kidney stones are common with an annual incidence of 0.5-1.9%. About 12% of the population of India is expected to have urinary stones and out of that 50% of cases encounter loss of one or both 2 kidneys with or without renal damage up to some extent.

The Antiurolithias is assay was performed to investigate the formation and growth of the COM crystals of oxalate from artificial urine at different concentration. It was observed that the plant used in this study inhibited the crystal development with maximum number of crystals found at 25% physiological extract concentration while at 100% physiological concentration of extract, the crystal formation was found minimal. Results show that the decrease in number of crystal as well as % inhibition of the formation of calcium oxalate monohydrate crystals was directly proportional to the increase in percentage of plant extract. Hence the calcium oxalate monohydrate showed minimum inhibition of 30.77% at 25 % physiological concentration of *T. erecta methanolic extract*, while maximum inhibition of 65.38% at 100 % physiological concentrations of *T. erecta methanolic* extract as depicted in table.

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