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In Vitro evaluation of anti-urolithiatic activity of *Piper betle* L.

Research Article

Lekha Alwe[1], Sagar Gavas[1*]

1. Department of Zoology,
MES's The D.G.Ruparel College of Arts, Science and Commerce, Senapati Bapat Marg, Mahim, Mumbai. India.

Abstract

Urolithiasis or kidney stone is the most frequently occurring urinary disorders seen all over the world. They are saturated crystals formed due to the minerals present in the urine. It majorly comprises calcium oxalate, uric acid and phosphate crystals. The phosphate crystals, known as struvite, are formed due to the urinary tract infections. Although there are various synthetic medications, the use of traditional medicines/plants has been a promising method to treat kidney stones. To investigate the antiurolithiatic potential of traditional plant - *Piper betle*, the aqueous extract of *Piper betle* Linn. leaves was used. This extract was tested on two different crystals, first onto the raphides present in *Colocasia esculenta* leaf which is analogous to the calcium oxalate crystals. Second on the struvite crystals prepared from the crystallisation method. The study showed there was a considerable decrease in the amount of raphides present in *Colocasia esculenta* and the same effect was seen on struvite crystals, which reveals that the leaves of *Piper betle* have an effect in dissolving and/or minimising the size of both crystals, thus substantiating the traditional claim. It is concluded that the leaves of *Piper betle* have beneficial inhibitory effect on *in vitro* crystallisation of calcium oxalate and struvite crystals.

Keywords: Antiurolithiatic Property, *Piper betle,* FTIR, Traditional Medicines.

Introduction

Urolithiasis or kidney stone is one of the most frequently occurring urinary disorders seen in people of all ages throughout the world. It is a prevalent condition that is thought to affect about 12% of the population in India (1), with a recurrence rate of 70%-80% in males and 47%-60% in females (2). At present, in the global range, 4-15% of the human population suffers from urinary calculi (3). The stones are formed when the minerals present in the urine get saturated and crystallised. The crystalline forms of minerals such as salts of calcium and phosphate combine with the help of molecular interactions to form clusters, which gradually increase in size. These clusters are then retained and accumulated in the kidneys forming kidney stones (2,4). Several factors such as lifestyle switching, diet, metabolic disruptions, bacterial infections, hereditary factors and synthetic medications are responsible for the pathobiology of calculi formation in the kidneys and urinary tract (3). People suffering from urolithiasis often show symptoms such as blood in urine, abdominal pain, dark urine colour and a burning sensation while urinating.

The stones majorly comprise calcium oxalate, phosphate and uric acid crystals. Out of which the calcium oxalate (CaOx) crystal formation is more prevalent in people having high dietary fibres. It accounts for 80% of the total kidney stones (3). Oxalate usually enters the body through the diet including leafy green vegetables, fruits, cocoa, nuts, and seeds (5). Once entered into the body, it is cleaved into oxalic acid which eventually combines with the calcium ions to form calcium oxalate crystals (6). Along with high dietary fibre, inflammatory bowel disease is also responsible for the formation of calcium oxalate stones due to its high degree of calcium ion absorption into the body (6). Formulated drugs namely diuretics and antacids are primarily made of calcium ions that are in association with other active molecules. Consumption of these calcium-based drugs have a high rate of renal calculi formation.

The phosphate crystals, known as struvite ($MgNH_4PO_4.6H_2O$ - Ammonium Magnesium Phosphate Hexahydrate) is a bio-mineral (7,8) formed due to urinary tract infections (9). Struvites account for 10-20% of all renal calculi (10) and are found more frequently in women and persons older than 50 years (11). The condition arises due to a high intake of phosphate and magnesium-based food along with synthetic supplements (12). It is formed due to the presence of urolithic microorganisms containing urease enzymes (13,14). The enzyme leads to the fission of urea into ammonia molecules. Due to low water intake, ammonia splits into PO_4^{3-} and Mg^{2+} ions, thereby increasing the pH level of urine making it more alkaline (12,13,15). The raised pH level of urine reduces the solubility of magnesium ammonium phosphate and favours the precipitation of struvite crystals, thereby making the urine supersaturated which ultimately triggers the formation of struvite stones (13). The rapid growth of struvite stones leads to the formation of "staghorn-calculi", which is a very painful urological disorder intimidating

*** Corresponding Author:**
Sagar Gavas
Department of Zoology,
MES's The D.G.Ruparel College of Arts, Science and Commerce, Senapati Bapat Marg,
Mahim, Mumbai.
Email Id: sagarsgavas@gmail.com

human life, especially women (10). The presence of struvite staghorn calculi in the kidney is a warning signal for active treatment in most individuals (10).

Kidney stones can be treated using various interventional procedures such as extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS), or percutaneous nephrolithotomy (PNL) (16). It is an effective technique for generating smaller fragments of kidney stones. Since the techniques are very expensive, not everyone is benefited from these highly equipped technologies. Also, renal calculi are characterised by a high recurrence rate, therefore it requires preventive measures (11) that will shift the approach from using artificial and synthetic procedures and medications towards the utilisation of traditional knowledge in curing kidney stones.

India has a magnificent past of a traditional medical system and due to which the knowledge of traditional medicines has constantly been revolutionised over generations by tribal and rural groups (17). According to the WHO report, 60% of the world's population depends chiefly on traditional medicine whereas 80% of the population in developing countries largely uses traditional medicinal practices and herbal medicines as a primary base for the foundation of healthcare management (17). Plants are a good source of raw materials (1) having different combinations of phytoconstituents that serve as great potential markers for healing various ailments. Plant-based medicines have played a vital role throughout history with one promising effect of having the ability to repair the damage caused by any ailments in a natural way with minimum side effects (17). In Ayurveda, the extracts of different herbs/plants along with their vital parts are highly suggested in treating urinary stones and some of them have been assessed for their role as inhibitors of renal crystals (8). The potency of the medicinal plants was checked using *in vitro* and *in vivo* models of clinical trials. Data generated from these trials proved that the phytotherapeutic agents present in the plants could be a promising alternative therapy in the management of urolithiasis (1). Based on traditional knowledge, the pharmacological and phytochemical screening of medicinal plants can lead to the findings of new drugs and can help in enhancing human health through the production of formulated plant-based supplements.

In recent years, people have shown a revival interest in ethnomedicinal plants as a source of medicine because of their better cultural acceptability, better compatibility with the human body and lesser side effects. According to traditional knowledge and ayurvedic literature, *Piper betle* commonly known as betel vine is an evergreen, Malaysian-originated (18) perennial, shed-loving plant/ climber belonging to the family Piperaceae (19). It is an endemic species belonging to South and Southeast Asia (20). The plant is mostly found in areas where the climatic conditions are hot and moist (21). They have yellowish to green heart-shaped leaves with a pleasant odour and are sweet to pungent in taste (22). The morphological characteristics of *Piper betle* leaves are mentioned in Table 1 (21). They are widely known as *Paan* in India, which is second to tea and coffee based on daily utilisation (20,21). Different vernacular names are given for *Piper betle* in Indian languages, which are listed in Table 2 (22). The leaves of *Piper betle* are widely used for religious (23) purposes having the true essence of Indian rituals. In reference to Ayurveda, the Vedic name of betel leaf is *Saptasira* which can be used as an adjuvant with different medicines for voice, blood purification, laxative and appetiser (18).

As per Indian tradition, sweet betel leaves are served as an after-meal treat to boost up the digestion process due to its astringent taste (24). The significance of betel leaves is seen in different countries like Indonesia, Malaysia, Sri Lanka and Thailand. In accordance with traditional medicinal practices, *Piper betle* L leaves are utilised for vaginal douching in Indonesia, as a mouth freshener and mouthwash in Thailand and in Malaysia to treat various conditions related to teeth, joint pains, arthritis and headaches (25). The juice of betel leaves is used in the treatment of skin problems faced by people living in Sri Lanka (25). Additionally, the decoction of boiled leaves can be used as an effective tonic in treating cough and cold. The extracts (water, ethanol, methanol, acetone and chloroform) of the leaves have a wide range of properties such as - anticancer, antiulcer, antidiabetic, antifungal, antimalarial, analgesic, anti-inflammatory, antimicrobial, antiplatelet, immune modulatory activity, gastroprotective activity and wound healing activity (23,25). The effectiveness of *Piper betle* leaves is due to the presence of different chemicals along with micro and macronutrients that are listed in Table 3 and Table 4 (23).

Table 1: Morphological characters of *Piper betle* (21)

Characters	Description
Dimensions	Length of leaf: 8-16 cm Width of leaf: 6-12 cm
Colour and condition	Green to dark green and fresh leaves
Composition and venation	Simple and reticulate
Margin and apex	Entire and acuminate
Base	Broadly cordate leaves with a rounded base
Surface and Texture	Thick lamina with smooth and glabrous surface
Petiole	Long petiole 1.5 to 4.5 cm long
Leaf Base	Stipulate leaves
Phyllotaxis	Alternately arranged leaves

Table 2: Vernacular Names of *Piper betle* (21)

Indian languages	Synonym of *Piper betle*
Ayurvedic	*Taambula, Naagvallari, Naagini, Taambulvalli, Saptashiraa, Bhujangalataa.*
Unani	*Paan, Tambool*
Sidha/Tamil	*Vetrilai Nagavalli, Kammaaruvetritai*
Hindi, Bengal, Gujraji, Urdu	*Paan*
Konkani	*Phodi paan*
Malayalam	*Vettila*
Marathi	*Vidyache paan*
Telugu	*Tamalapaka*

Table 3: Elemental Composition of *Piper betle* (23)

Sr. No.	Constituents	Approximate Composition
1	Water	85-90%
2	Protein	3-3.5%
3	Fat	0.4-1.0%
4	Minerals	2.3-3.3%
5	Fibres	2.30%
6	Chlorophyll	0.01-0.25%
7	Carbohydrate	0.5-6.10%
8	Energy	44 kcal/100g
9	Essential Oil	0.08 - 0.2%
10	Iodine	3.4 µg/100g
11	Iron	0.005-0.007%
12	Calcium	0.2-0.5%
13	Potassium	1.1-4.6%
14	Nicotinic acid	0.63-0.89 mg/100g
15	Vitamin C	0.005-0.01%
16	Vitamin A	1.9-2.9 mg/100g
17	Thiamine	13-70 µg/100g
18	Riboflavin	1.9-30
19	Tannin	0.1-1.3%
20	Nitrogen	2.0-7.0%
21	Phosphorus	0.05-0.6%

Table 4: Chemical constituents of *Piper betle* (23)

Component	Percentage of Components (%)
Chavibetol	53.1
Caryophyllene	3.71
Chavibetol acetate	15.5
Allylpyrocatechol Diacetate	0.71
Chavibetol methyl ether	0.48
Camphene	0.48
f-Pinene	0.21
Eugenol	0.32
u-Limonene	0.14
a-Pinene	0.21
1,8-Cineol	0.04
Saprobe	0.11
Allylpyrocatechol Monoacetate	0.23

1.1 Taxonomic Classification of *Piper betle* (22)

- Kingdom: Plantae
- Division: Magnoliophyta
- Order: Piperales
- Family: Piperaceae
- Subfamily: Piperoideae
- Genus: Piper
- Species: *Piper betle* Linn

Fig.1: Habitat of *Piper betle* Linn (21)

A preliminary study reported that the extracts of *Piper betle* leaves contain large numbers of bioactive molecules (23). The presence of active phytoconstituents has made the leaves to be a source of precursors for various traditional as well as modern drug formulations (21). Optimisation of extraction techniques during phytochemical screening is very important in order to retain medicinal ingredients for further processing in pharmaceutical industries (20). *Piper betle* leaves have many healing and curing properties for an ailment but there is no mention of its antiurolithiatic potential in the literature. Hence, to investigate the antiurolithiatic potential of the traditional plant - *Piper betle*, the present study demonstrated the use of the aqueous extract of *Piper betle* leaves on *in vitro* crystallisation of calcium oxalate and struvite crystals. Further, phytochemical screening was done for the determination of active functional groups that were analysed using different solvent extracts of *Piper betle* leaves. Characterisation of *Piper betle* leaves was done by using Fourier Transform Infrared Spectroscopy (FTIR). Since humans consume these leaves daily, their antiurolithiatic properties would be a good preventive option available. The main aim of the study is to investigate the potential of *Piper betel* in preventing or treating kidney stone and evaluate the in vitro effect on kidney stones dissolution. The demonstration of the aqueous extract of *Piper betle* ability to dissolve kidney stones highlights its potential usefulness as a natural remedy for kidney stone dissolution. This finding suggest that *Piper betle* could serve as non-invasive and potentially cost effective alternative to traditional medical interventions for kidney stone treatment.

Materials and Methods

Plant Material, Drugs and Chemicals - (2)

The leaves of *Piper betle* were collected from the local market of Dadar in September 2022. Cystone tablets (Himalaya Drug Company) were purchased from a nearby medical store. The highest-grade commercially available chemicals were utilised for the experiment.

Preparation of Aqueous Extract of *Piper betle* (2)

The collected leaves were washed to remove foreign particles and exposed to shade drying. The dried leaves were pulverized into fine powder by using a mortar pestle. The powder was immersed in distilled water (5g in 50 mL = 10%) and kept for a few hours. After that the solution was filtered by Whatman Filter Paper No. 1. Similarly, a series of concentrations (0.5%, 1%, 2%, 4%, 6% and 8%) of aqueous leaf extract was prepared. The filtered extract was then used for further analysis.

Preparation of Aqueous Extract of Standard Drug (Cystone)

The cystone tablets were crushed into a fine powder using a mortar pestle. A series of concentrations (0.5%, 1%, 2%, 4%, 6%, 8% and 10%) of cystone powder was prepared by aqueous extraction with distilled water.

Experimental Procedure

For calcium oxalate crystals

Freehand sections of petiole and leaves of *Colocasia esculenta* showing raphides were taken on a glass slide and treated with the *Piper betle* leaves and standard drug (cystone) extracts (17).

For struvite crystals (26)

In a 250 mL beaker, 100 mL of 0.11 M $MgCl_2$ and 0.11 M $NH_4H_2PO_4$ solutions were combined to form the crystals. To keep the pH at the desired level of 9.00, 1.0 N KOH solution was added dropwise from a burette. The solution was continuously stirred for 90 minutes at a low speed to maintain the homogeneity of the solution. After 90 minutes, the solution was filtered using Whatman Filter Paper No. 1 to obtain the crystals. Thereafter, the crystals were washed with distilled water to get rid of impurities. The wet crystals were air-dried for 48 h, and the resulting dry crystals were used for the experiment.

The outcome of the extracts [*Piper betle* leaves and standard drug (cystone)] on raphides (calcium oxalate) and struvite crystals were observed by comparing the images taken before and after the treatment.

Phytochemical Screening (22)

The powdered leaves are subjected to successive extraction by maceration by using water, water + acetone, 90% methanol and 80% ethanol solutions. The filtrate extracts were further used for preliminary phytochemicals with a chemical test listed below in Table 5 (27).

FTIR Analysis

Samples were put into sample containers, and the FTIR spectrophotometer was equipped with ATR attachments. The detector used is called DTGS (deuterated triglycine sulphate). Measurements are made using wave numbers between 1000-4000 cm^{-1}. With the aid of the software OPUS 7.2.139.1.24, the FTIR spectrum is displayed. The spectrum is further analysed to determine the different molecular bonds and functional groups present in the sample (28).

Table 5: Qualitative analysis of phytoconstituents (27)

Test	Procedure	Observations (Indicating
Detection of Alkaloids		
Picric acid test	Few mL filtrate + 3-4	An orange colour
Detection of Carbohydrates		
Barfoed's test	1mL filtrate + 1mL	A red precipitate
Benedict's test	0.5mL filtrate + 0.5mL Benedict's reagent +	Green/yellow/red colour
Detection of amino acids		
Ninhydrin test	2mL filtrate + 2 drops of Ninhydrin solution	A purple-coloured solution
Xanthoproetic	Plant extract + Few	A yellow-coloured
Detection of Glycosides		
Keller - Killani's test	1mL filtrate + 1.5mL glacial acetic acid + 1 drop of 5% ferric	A blue-coloured solution (in the acetic acid
Detection of flavonoids		
Conc.H_2SO_4	Plant extract + conc.	An orange colour
Ferric chloride test	Extract aqueous solution + few drops of	A green precipitate
Lead acetate	1mL plant extract + few	A yellow
Pew's test	Few mL aqueous extract solution + 0.1gm	A red colour {flavanols}
Detection of Phenolic Compounds		
Ferric chloride test	Extract aqueous solution + few drops of	Dark green/bluish-black colour
Detection of Tannins		
Braymer's test	1mL filtrate + 3mL distilled water + 3 drops	Blue-green colour
10% NaOH test	0.4mL plant extract + 4mL 10% NaOH +	Formation of emulsion
Detection of Saponins		
Foam test	0.5gm plant extract +	Persistent foam for
Detection of Steroids		
Salkowski's test	Filtrate + few drops of conc. H_2SO_4	Golden yellow layer
Detection of Coumarin		
NaOH test	Plant extract + 10%	A yellow colour

Results

In Vitro Crystallisation of Calcium Oxalate and Struvite Crystals

The present investigation was performed to test the antiurolithiatic potential of the traditional plant - *Piper betle*, for which a series of concentrations of aqueous extract of *Piper betle* leaves (0.5%, 1%, 2%, 4%, 6%, 8% and 10%) were prepared and were compared with the standard drug (cystone). Sections of *Colocasia esculenta* leaf and petiole showing raphides (17) along with struvite crystals prepared from the crystallization method were utilised for the study. It was observed that with a 0.5% concentration of aqueous leaf extract, there was no considerable decrease in the number of raphides (calcium oxalate) and struvite crystals. The concentrations of 1% and 2% showed a slight change in the quantity of both crystals. A

moderate change in the size and number was seen in raphides and struvite crystals treated with 4% and 6% aqueous leaf extract of *Piper betle*. The highest degree of change was seen with concentrations of 8% and 10%, by dissolving and/or minimising the size of each crystal. Similar effects were seen using the standard drug (cystone). The results are shown in Table 6, Table 7, Table 8 and Table 9.

Table 6: *Colocasia esculenta* leaf section showing raphides before and after treatment with the leaf extract

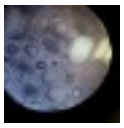
Leaf extract	0.5%	1%	2%	4%	6%	8%	10%
Before Treatment							
After Treatment							

Table 7: *Colocasia esculenta* leaf section showing raphides before and after treatment with the standard drug extract

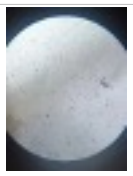
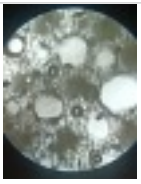
Std. drug extract conc.	0.5%	1%	2%	4%	6%	8%	10%
Before Treatment							
After Treatment							

Table 8: Struvite crystals before and after treatment with the leaf extract.

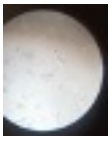
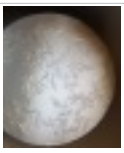
Leaf extract conc.	0.5%	1%	2%	4%	6%	8%	10%
Before Treatment							
After Treatment							

Table 9: Struvite crystals before and after treatment with the standard drug extract.

Std. drug extract conc.	0.5%	1%	2%	4%	6%	8%	10%
Before Treatment							
After Treatment							

Phytoconstituents Present in the Extracts of *Piper betle* Leaves -

In the present study, phytochemical screening of *Piper betle* leaves was done by preparing the extracts, using solvents such as water, water + acetone, 90% methanol and 80% ethanol. These extracts were analysed for the presence of alkaloids, carbohydrates, amino acids, glycosides, steroids, tannins, flavonoids, phenolic compounds, saponins and coumarins using standard protocols. The phytochemical screening showed the presence of alkaloids, carbohydrates, amino acids, flavonoids, phenolic compounds and coumarin, which are shown in Table 10.

Table 10: Data showing the presence of phytoconstituents in the *Piper betle* leaves extracts.

Sr. No.	Phytoconstituents	Water extract	Water + Acetone extract	90% Methanol extract	80% Ethanol extract
1	Alkaloids	-	+	-	-
2	Carbohydrates	+	+	+	+
3	Amino acids	+	+	+	+
4	Glycosides	-	-	-	-
5	Flavonoids	+	+	+	+
6	Phenolic compounds	+	+	+	+
7	Tannins	-	-	-	-
8	Saponins	-	-	-	-
9	Steroids	-	-	-	-
10	Coumarin	-	-	+	+

(+) indicates presence and (−) indicates absence of phytoconstituents.

Characterization of *Piper betle* Leaves

The FTIR analysis was carried out to examine the chemical bondings which eventually provided the information about the molecular structure of the compound (29). Two samples (leaves powder and standard drug powder) were provided for FTIR analysis and were compared for similarities in terms of chemical bondings and active functional groups. The FTIR spectra are shown in Fig. 2 and Fig. 3.

In Fig. 2 – FTIR spectrum of *Piper betle* leaves powder

A broad band between the wavelengths 3305 to 3385 cm^{-1} indicates the presence of OH group of alcohol along with hydrogen bonding at wavelength 3385 cm^{-1}. Thus, the leaf powder shows the presence of hydroxychavicol molecules, thereby giving a positive test for phenolic compounds. The peak at 2925 cm^{-1} is due to the presence of saturated aliphatic compounds especially having a C-H asymmetrical stretch of alkane. The band at 1740 cm^{-1} shows different compounds having ester linkages out of which the leaf powder has coumarin molecules that fall around the same band of the spectra i.e., 1745 cm^{-1}. The peak at 1608 cm^{-1} indicates C=C-C aromatic ring stretch. The peak at 1414 cm^{-1} shows nitrogen group thus confirming the presence of amino acids in the given leaf powder. The band at 1105 cm^{-1} and 1043 cm^{-1} indicates several aromatic C-H linkages with in-plane bend structures. The lowest peak at 723 cm^{-1} indicates aromatic C-H 1,2-disubstitution (ortho) molecules. The chemical bondings and the active functional groups are summarised in Table 11.

In Fig. 3 – FTIR spectrum of standard drug (cystone) powder

The spectrum generated by IR spectroscopy for the standard drug (cystone) powder shows similar peaks and broad bands to that of the *Piper betle* leaf powder. Thereby, it confirms the presence of phenolic compounds, amino acids, alkaloids, and flavonoids in the standard drug. Since the phytoconstituents in *Piper betle* leaves and the standard drug (cystone) are nearly the same in nature, the antiurolithiatic potential of its extracts shows similar effects on raphides (calcium oxalate) and struvite crystals seen in [Table 6, Table 7, Table 8 and Table 9].

Fig 2: FTIR spectrum of *Piper betle* leaves powder

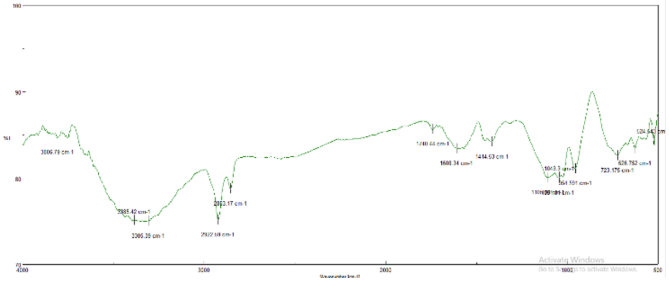


Fig 3: FTIR spectrum of standard drug (cystone) powder

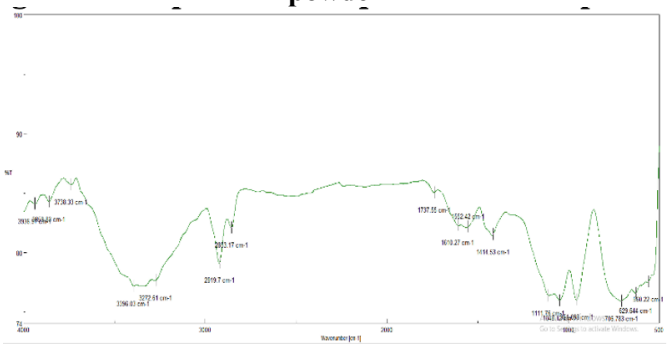


Table 11: Wavelength Numbers and Functional Groups of *Piper betle* leaves

Sr. No.	Wavelengths (cm^{-1})	Bonds	Functional groups
1	3385	O-H stretch, H-bonded	Alcohol, Hydroxyl
2	2925	C-H asymmetrical stretch	Alkane
3	1740	-COO stretch	Ester
4	1608	C=C-C aromatic ring stretch	Aryl
5	1414	N-H stretch	Amino
6	1105 & 1043	aromatic C-H linkages	Aryl
7	723	aromatic C-H 1,2-disubstitution (ortho)	Aryl

Discussion

Renal stones are reportedly affecting humankind for a long time and have been one of the causes of renal failure (30). The reappearance of kidney stones in the human body is of crucial concern in the therapeutic management of urolithiasis (2). Advancement in drug therapy has met the needs of the population's healthcare. There are many vital areas in medical sciences such as infectious diseases, arthritis, liver diseases and cancer where the use of ordinary and synthetic medications are devoid of satisfactory treatments (1). Also, the surgical methods being expensive and unaffordable to the common man, the use of natural drugs is considered to be the next alternative step (30). The use of plant resources in the formulation of new medicines has become a crucial part of research and development. Plants have effective factors that are responsible for the development of novel and very impactful drugs along with therapeutic markers against kidney stones. The formation of kidney stones is an intricate process that involves a series of biological episodes that are ideally triggered by genetic factors together with dietary factors and changes in lifestyle patterns (3). Saturation of these dietary factors along with minerals in the body leads to the formation of basic crystals majorly made of calcium oxalate salts, which have the potential to form kidney stones. These crystals formed are aggregations of calcium oxalate molecules due to the chemical forces between its atoms.

In the present study, raphides of *Colocasia esculenta* showed a similar composition to that of a kidney stone made of calcium oxalate crystals. A series of concentrations of aqueous extract of *Piper betle* leaves were prepared and tested on the raphides of *Colocasia esculenta*. Increased concentrations of leaf aqueous extract showed considerable effects in minimising the size of raphides. This indicates the antiurolithiatic property of *Piper betle* against the raphides. With the help of the crystallisation method, struvite also known as phosphate crystals was prepared which resembled the stones formed during the urinary tract infections. Urease enzyme, a virulence factor, is responsible for the formation of struvite crystals. In this process, the urea splits into ammonia in the presence of urease enzymes. Hydrolysis of ammonia increases the urine pH levels which ultimately increases the solubility of polyvalent cations in the urine thereby precipitating struvite crystals leading towards the progression of infectious kidney stones. To claim the antiurolithiatic property of *Piper betle* against the struvite crystals, a series of concentrations of aqueous extract of Piper *betle* leaves were prepared. As compared to the calcium oxalate crystals, similar effects were seen in the size of struvite crystals that were tested with different concentrations of aqueous extract of *Piper betle* leaves.

One possible mechanism of the antiurolithiatic activity of *Piper betle* could be the acidic nature of bioactive molecules such as flavonoids and phenolics, having the ability to neutralize the basic nature of calcium oxalate and struvite crystals, thus preventing its accumulation in the body. In contrast to allopathic medicine which targets one of the facets of the pathophysiology of urolithiasis, plant-based drugs have proven to be effective at multiple stages in the prevention of the formation of renal calculi (31). Screening of medicinal plants for their phytoconstituents is very essential in identifying new sources of therapeutic and industrial importance. In the present investigation, water, water + acetone, methanol and ethanol extracts of *Piper betle* leaves were tested for the presence of alkaloids, flavonoids, amino acids, carbohydrates, glycosides, phenolic compounds, tannins, saponins and coumarin. The results indicated that alkaloids, amino acids, carbohydrates, flavonoids, phenolic compounds and coumarin were present in the leaves of *Piper betle* taken under study. Water + acetone, methanolic and ethanolic extracts of the plant leaves showed the presence of maximum phytocomponents and therefore, can be used for the isolation of pure compounds for novel drug discoveries. The *Piper betle* leaves contain flavonoids and phenolic compounds that might serve as an impactful precursor in the formulation of plant-based drugs against urolithiasis. Further verification of active functional groups of *Piper betle* leaves was done using the FTIR technique. The spectrum generated revealed the presence of hydroxyl groups along with aromatic molecules, saturated aliphatic compounds and amino acids, thereby giving a positive confirmation for its phytoconstituents screening using different solvent extracts. A similar spectrum was established for the standard drug (cystone) which showed the presence of similar functional groups, thus showing similar effects on the calcium oxalate and struvite crystals. Hence these leaves can be a good substitution factor against synthetic drugs like cystone.

The use of various plants in the treatment of kidney stones has been recorded under a broad term called ethnobotanical studies. The knowledge of traditional medicine serves as a promising method in treating various diseases and disorders including urolithiasis. Still, it requires further detailed research to understand the cause and the mechanism of these medicines to generate the best efficient and safe lithotriptic agents. Also, the present study utilizes plant cells as test subjects thus providing an absolute replacement for animal models (17).

Conclusion

Urolithiasis is a major health ailment associated with pain and inflammation as a consequence of the recurrence of kidney stones caused due to the supersaturation of urine (32). The ayurvedic approach is a multifaceted remedial treatment that occurs due to modifications of lifestyle through diet; internal medicines and Basti therapy are highly effective in the management of renal stones (6). The present study revealed that the aqueous extract of *Piper betle* leaves has a beneficial inhibitory effect on *in vitro* crystallisation of calcium oxalate and struvite crystals. Screening of phytochemicals along with the characterisation of *Piper betle* leaves through FTIR

indicated that the leaves have a complex structure with active ingredients having the potential to dissolve and/ or minimise/neutralise the crystals made of calcium oxalate and struvite containing basic properties. The findings of the present study in association with the previous research studies claim that *Piper betle* leaves are promising commercial medicinal in having different therapeutic activities thereby, substantiating its traditional claim.

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