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Anti-Urolithiasis Activity of Selected Plant Extracts by Titrimetry Method and Aggregation Assay

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

A major global health concern with a high recurrence rate is urolithiasis. Various in vivo and in vitro techniques have been effective in assessing the anti urolithiatic capacity of therapeutic plants. Renal stone production can be studied using in vitro models, whereas the pathological implications of urolithiasis are declared using in vivo models. Therefore, preventative management can be considerably and successfully evaluated using in vitro models, whereas urolithiasis treatment can be directed by using in vivo models. This study explains the benefits, drawbacks, and uses of both models, with a focus on the contribution of in vitro research to the assessment of preventive care. We done Physicochemical Tests for *Phyllanthus acidus* (fruits) *Tinospora cordifolia* (leaves) and *Psidium gujava* (fruits and leaves) and also Preliminary Phytochemical Investigation of *Phyllanthus acidus* (fruits), *Tinospora cordifolia* (leaves) and *Psidium gujava* (fruits and leaves).

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Keywords: Kidney stones; aggregation; cystone; spectroscopy; calcium oxalate crystals.

1. INTRODUCTION

One of the major kidney illnesses that necessitates a well-targeted therapeutic approach is urolithiasis. For the treatment of lithiaisis, several medications are available, such as diuretics and stone inhibitors; however, clinical examination of these medications has revealed a prevalence of relapses, adverse effects, and drug interactions. This has served as the justification for the creation of novel antilitiatic medications, and the hunt for novel molecules has expanded to include herbal medications that provide improved protection and a lower risk of relapse [1,2]. Plant-based medications are becoming more and more well-liked and are being researched for a variety of illnesses, including lithiasis. This has served as the justification for the creation of novel antilitiatic medications, and the hunt for novel molecules has expanded to include herbal medications that provide improved protection and a lower risk of relapse [3,4]. Plant-based medications are becoming more and more well-liked and are being researched for a variety of illnesses, including lithiasis.[5,6] "Particularly calcium oxalate dihydrate (Weddellite), calcium oxalate monohydrate (Whewellite), and basic calcium phosphate (Apatite) are the calcium-containing stones that most often occurring ones, comprising 75–90%, then magnesium ammonium phosphate (Struvite), which makes up 10-15%, uric acid, which makes up 3–10%, and cystine, which makes up 0.5–1%. Most often occurring stones are of the calcium oxalate or magnesium ammonium phosphate kind" [7-10].

"Urolithiasis, formation of kidney stone presence of one or more calculi in any location within the urinary tract, is one of the oldest and wide spread diseases known to man. It is a serious, debilitating problem in all societies throughout the world, affecting approximately 12% of the population, and men are three times more prone than women. It is more prevalent between the ages of 20 and 40 in both sexes. Etiology is multifactorial and is strongly related to dietary lifestyle habits or practices. Increased rates of hypertension and obesity, also contribute to an increase in stone formation" [11,12].

2. CLASSIFICATION OF UROLITHIASIS

Urolithiasis Can be Classified as:

1. Calcium Oxalate
2. Uric Acid
3. Struvite
4. Cystine

1. Calcium Oxalate

- The most common type of kidney stone which is created when calcium combines with oxalate in the urine.
- Inadequate calcium and fluid intake, as well other conditions, may contribute to their formation.

2. Uric Acid

- This is another common type of kidney stone.
- Foods such as organ meats and shellfish have high concentrations of a natural chemical compound known as purines.
- High purine intake leads to a higher production of monosodium urate, which, under the right conditions, may form stones in the kidneys.
- The formation of these types of stones tends to run in families.

3. Struvite

- These stones are less common and are caused by infections in the upper urinary tract.
- More common in women, struvite stones form as a result of certain types of urinary tract infections.
- These stones tend to grow quickly and become large, sometimes occupying the entire kidney.
- Left untreated, they can cause frequent and sometimes severe urinary tract infections and loss of kidney function.

4. Crystaine

- "Cystine stones are caused by a hereditary genetic disorder called cystinuria that can lead to excessive amounts of the amino acid

cystine collecting in the urine. This can result in the formation of stones in the kidneys, bladder, and ureters, which transport urine from the kidneys to the bladder" [11].

3. SIGNS AND SYMPTOMS OF UROLITHIASIS

Urolithiasis is formation of kidney stones which are solid mass made up of tiny crystals. One or more stones can be in the kidney or ureter at the same time. If you ever have severe pain in your belly or one side of your back that comes and goes suddenly, you may be passing a kidney stone.

- Feeling pain in your lower back or side of your body.
- Having nausea and/or vomiting with the pain.
- Seeing blood in your urine.
- Feeling pain when urinating.
- Being unable to urinate.
- Feeling the need to urinate more often.
- Fever or chills.
- Having urine that smells bad or looks cloudy.

3.1 Pathogenesis of Urolithiasis

Urinary stone formation is a result of different mechanisms. Whereas exceeding supersaturation (i.e., free stone formation) is the cause of uric acid or cystine calculi, infection stones result from bacterial metabolism. The formation of the most common fraction, the calcium-containing calculi, is more complex and, surprisingly, is not yet completely understood. Recent evidence suggests that both free and fixed stone formation is possible.

- **Inhibitors of stone formation:**

"Stones can form when there is a deficiency of substances that normally prevent crystallization in the urine, such as Citrate, Magnesium, Nephrocalcin and Uropontin. (that inhibit the nucleation, growth and aggregation of calcium-containing crystals)" [11].

- **Supersaturation of urine:**

"When the urine becomes supersaturated with one or more calculogenic (crystal- forming) substances, a seed crystal may form through the process of nucleation" [11].

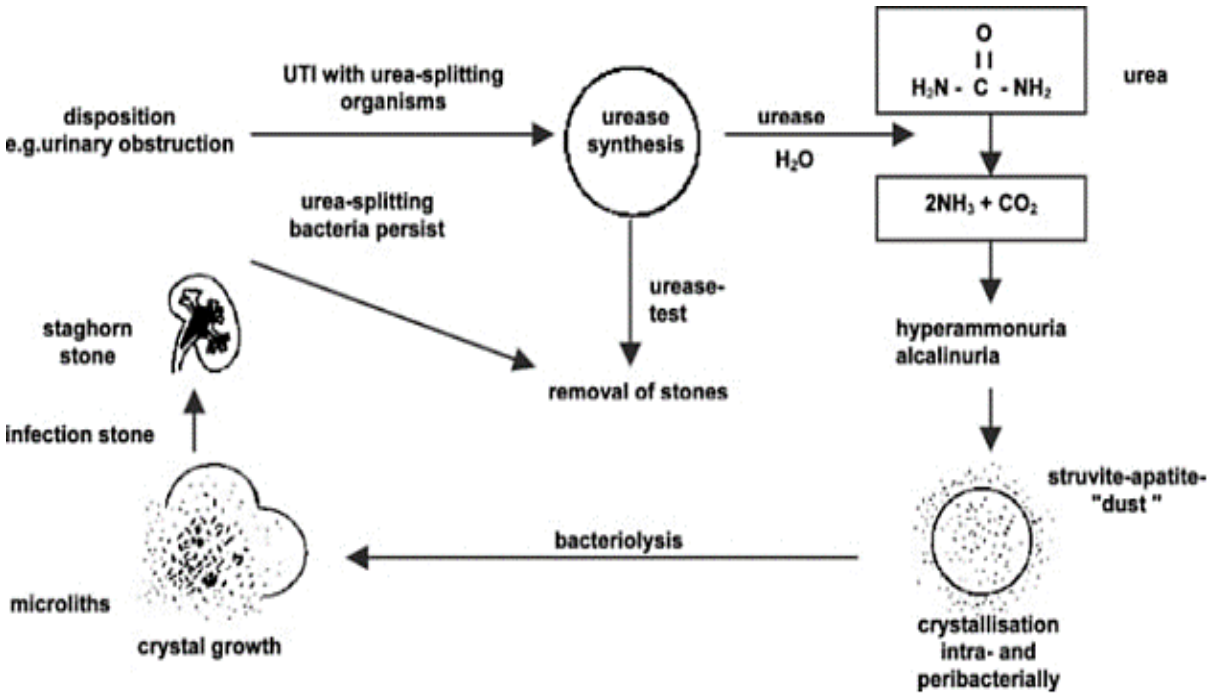

Fig. 1. Supersaturation of urine

PLANT PROFILE

Phyllanthus acidus

Plant Introduction:

Botanical name : *Phyllanthus acidus*
Family : Phyllanthaceae. Indian Name : Usiri, Holphali, Leyoir.
Habitat : It is small deciduous tree found in moist tropical and subtropical coastal woodlands and disturbed sites.
Parts Used : Fruit.
Phytochemical : Alkaloids, flavonoids, tannins, glycosides, lignin, terpenes, sterols. constituents

Botanical Classification:

Phyllanthus acidus belongs to the family of “Phyllanthaceae.”

Classification:

Kingdom : Plantae
Division : Tracheophyta
Class : Magnoliopsida
Order : Malpighiales
Family : Phyllanthaceae
Genus : Phyllanthus
Species :*P. acidus*

Tinospora cordifolia

Plant Introduction:

Botanical name : *Tinospora mcordifolia*
Family : Menispermaceae.
Indian Name : Guduchi, Giloy.
Habitat : Distributed throughtout tropical regions of India that are located 1200meters above from sea level of kumaon to assam. Native to India, Myanmar and Srilanka.
Parts Used : Leaves.
Phytochemical : Alkaloids, steroids, glycosides, tannins, flavonoids constituents

Botanical Classification:

Tinospora cordifolia belongs to the family of “Menispermaceae.”

Classification:

Kingdom : Plantae
Division : Tracheophyta
Class : Magnoliopsida
Order : Ranunculales
Family : Menispermaceae
Genus : Tinospora
Species : *T. cordifolia*

Psidium guajava

Plant Introduction:

Botanical name : *Psidium guajava*
Family : Myrtaceae.
Indian Name : Amrudh, Amarood, Peyara.
Habitat : Guava is successfully grown under tropical and subtropical climate. The quality of fruit is better in ares having distinct winters. Guava tolerates drought, protective irrigation facilities are required.
Parts Used : Fruits and Leaves.
Phytochemical Constituents : The fruit contains Saponins, Oleanolic acid, Lyxopyranoside, Araboyranoside, and Flavonoids.

Botanical Classification:

Psidium guajava belongs to the family of “Myrtaceae.”

Classification:

Kingdom : Plantae
Division : Mangnoliophyta
Class : Magnoliopsida
Order : Myrtales
Family : Myrtaceae
Genus : Psidium L
Species : *P.guajava*

3.2 Plants and Plant Products with Antiurolithiatic Activity

In order to dissolve urinary calculi in the kidney and bladder, the commercialized composite herbal formulations Cystone (Himalaya Drug Company, India), Calcuri (Charak Pharmaceuticals, Bombay, India), and Chandraprabha bati (Baidyanath, India) have been used extensively in clinical settings. Some of the herbal plants with Urolithiatic activity are *Phyllanthus acidus, Tinospora cordifolia, Psidium gujava, Cyperus rotundus.*

3.3 Phytochemical Tests

3.3.1 Flavonoids

Shinoda test: A piece of metallic magnesium was added to 1ml of extract and add 2 drops of HCl and heat the test tube in water bath. The occurance of orange, red /violet precipitate indicates presence of flavonoids.To 1ml aqueous extract add 1ml 10% lead acetate results in yellow precipitate indicates presence of flavonoids.

3.3.2 Saponins

Foam test: Take 3ml of each extract and add 2ml of distilled water in test tube to dilute it. Now shake the mixture vigorously. The formation (or) occurance of foam results test /The formation of foam indicates saponins.

3.3.3 Tannins

Take 2ml of each extract in each test tubes and boil them for 2mins and allow the test tubes to cool. After cooling add 3 drops of ferric chloride solution to each extract. The colour changes to dark blue results presence of tannins.

4. METHODS AND METHODOLOGY

4.1 Preparation of Extracts

The selected plant materials were separately extracted successively with selected solvent with increase order of polarity using suitable extraction process and preliminary phytochemical study was performed on various liquid extracts.

The fresh plant materials of *Phyllanthus acidus, Tinospora cordifolia, Psidium gujava* were collected from young matured plants and authenticated. After authentication, the plant materials were collected in bulk, washed under running tap water to remove adhering dirt followed by rinsing with distilled water. The plant materials were then shade dried and separately pulverized in a mechanical grinder to obtain coarse powder.

- **Preparation of Extracts**

The dried powdered plants materials (500 g each) were separately successively and extracted with methanol and aqueous using a soxhlet extractor. The period of extraction was fixed at 5 h for every solvent at every stage of the extraction process. After completion of extraction, the extractive value was determined with respect to the dried plant material. After the filtrate has obtained, it was then transferred into a weighed petri plates. The obtained extracts were concentrated to dryness by keeping filtrate for complete evaporation of solvent. The extractive value in percentage was calculated by using following formula and recorded.

Extractive value (%) = Weight of dried extract/Weight of plant material X 100

4.2 Methods

4.2.1 Titrimetry methods

Step 1:

"Preparation of experimental kidney stones (Calcium oxalate stones) by homogenous precipitation 1.47gm of calcium chloride dihydrate was dissolved in 100ml distilled water and 1.34gm of sodium oxalate was dissolved in 100 ml of 2N H2SO4. Both were mixed equally in a beaker to precipitate out calcium oxalate with stirring. Equimolar solution of calcium chloride dehydrate (AR) in distilled water and Disodium hydrogen phosphate (AR) in 10 ml of (2N H2SO4), was allowed to react in sufficient quantity of distilled water in a beaker. The resulting precipitate was calcium phosphate. Both precipitates freed from traces of H2SO4 by ammonia solution. Washed the precipitates with distilled water and dried at 600C for 4 hours"[11].

Step 2:

Preparation of semi-permeable membrane from farm eggs "The semi - permeable membrane of eggs lies in between the outer calcified shell and the inner contents like albumin & yolk. Apex of eggs was punctured by a glass rod in order to squeeze out the entire content. Empty eggs were washed thoroughly with distilled water and placed in a beaker consisting 2 M HCl for an overnight, which caused complete decalcification. Further, washed with distilled water, placed it in ammonia solution for for neutralization of acid traces in the moistened condition for a while & rinsed it with distilled water. Stored in refrigerator at a pH of 7-7.4" [11].

Step-3:

Estimation of Calcium oxalate by Titrimetry:

"The dissolution percentage of calcium oxalate was evaluated by taking exactly 1 mg of calcium oxalate and 10,20,30,40 mg of the extract, packed it together in semipermeable membrane of egg. This was allowed to suspend in a conical flask containing 100 ml of 0.1M Tris buffer. First group served as blank containing only1 mg of calcium oxalate. The second group served as positive control containing 1 mg of calcium oxalate and along with the 10,20,30,40 mg of standard drug, i.e. cystone. The 3rd and 4th groups along with 1 mg of calcium oxalate containing, aqueous and methanolic extracts. The conical flasks of all groups were kept in an incubator preheated to 370C for 2 h. Remove the contents of semipermeable membranes from each group into separate test tubes, add 2 ml of 1N sulphuric acid to each test tube and titrated with 0.9494N KMnO4 till a light pink colour end point obtained. The amount of remaining undissolved calcium oxalate is substracted from the total quantity used in the experiment in the beginning to know the total quantity of dissolved calcium oxalate by various solvent extracts. Each ml of 0.9494N KMnO4 equivalent to 0.1898mg of Calcium oxalate" [11].

4.3 Aggregation Assay

"The rate of aggregation of the CaOx crystals was determined by the method of Hess et al. with slight modifications. The COM crystals were prepared by mixing both the solutions of calcium chloride and sodium oxalate at 50 mmol/L. Both solutions were then equilibrated in a bath for 1 h at 60°C. The solutions were then cooled to 37°C and then evaporated. The COM crystals were then dis- solved with Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 to a final concentration of 1 mg/mL. The absorbance at 620 nm was recorded

at 30, 60, 90, 180 and 360 min. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract with that obtained in the control. The percentage inhibition was calculated as (1-Si/Sc)/100, where S_1 is the slope of the plot in the presence of inhibitor (ex- tract) and Sc the slope of the control plot (with no inhibitor)" [11].

5. RESULTS AND DISCUSSION

"Kidney stone function is a complex process that results from a succession of several physico-chemical events including supersaturation, nucleation, growth, aggregation and retention within renal tubules. Thus if supersaturation or later steps in crystallization can be prevented, then lithiasis should be avoided. Indeed, several measures are usually taken to reduce supersaturation, e.g. increasing fluid intake and medical therapy. In India, as in many less developed areas, phytotherapy is a common method of primary health care because pharmaceutical products are expensive and the 'folk' pharmacopoeia provides apparently effective remedies for many diseases".[7] Furthermore, in developing nations, traditional medicine is necessary to treat urolithiasis, especially to reduce the cost burden that the general population bears when utilizing regular dose forms.[13,14] These results could be considered positives because the herb extracts inhibits crystallization and prevents stone formation [11,15].

5.1 Results of Preliminary Phytochemical Investigation of *Phyllanthus acidus, Tinospora cordifolia* and *Psidium gujava*

On preliminary phytochemicals analysis of *Phyllanthus acidus* (Usiri) fruits, *Tinospora cordifolia* (Giloy) leaves and *Psidium gujava* (Guava) fruits, leaves have shown the presence of flavonoids, saponins and tannins. All the results are shown in the table.

5.2 *In-Vitro* Anti-Urolithiasis Activity by Titrimerty Method and Aggregation Assay

In-vitro anti-urolithiasis activity of *Phyllanthus acidus, Tinospora cordifolia* and *Psidium gujava* Methanolic and Aqueous extract by Titrimerty method.

In-vitro anti-urolithiasis activity of *Phyllanthus acidus, Tinospora cordifolia* and *Psidium gujava* Methanolic and Aqueous extract by Aggregation assay.

In-vitro anti-urolithiasis activity of *Phyllanthus acidus, Tinospora cordifolia* and *Psidium gujava* Methanolic and Aqueous extract by Spectroscopy.

Table 1. Phytochemical studies of *phyllanthus acidus, tinospora cordifolia* and *psidium gujava*

Test	Flavonoids	Alkaloids	Glycosides	Tannins	Saponins	Phenolics
Phyllanthus acidus(Fruit)	+	-	-	+	+	-
Tinospora cordifolia(Leaf)	+	-	-	+	+	-
Psidium gujava(Fruit)	+	-	-	+	+	-
Psidium gujava(Leaf)	+	-	-	+	+	-

Table 2. The titrimetric value of the sample extract

Sample	Titrimetry value
Methanolic fruit extract of *Phyllanthus acidus*	0.2
Methanolic leaf extract of *Tinospora cordifolia*	0.5
Aqueous leaf extract of *Tinospora cordifolia*	0.2
Methanolic leaf extract of *Psidium guajava*	1.2
Aqueous leaf extract of *Psidium guajava*	1
Methanolic Fruit extract of *Psidium guajava*	0.4
Aqueous fruit extract of *psidium guajava*	0.4
Blank solution	0.3

Table 3. The comparative study of various herbal plants with standard drug cystone

Sample	Concentration (ug)	Absorbance @620nm	Percentage Inhibition
Methanolic fruit extract of *Phyllanthus acidus*	100ug	1.13	14%
Methanolic leaf extract of *Tinospora cordifolia*	100ug	1.18	10%
Aqueous leaf extract of *Tinospora cordifolia*	100ug	1.11	15%
Methanolic leaf extract of *Psidium gujava*	100ug	1.12	14%
Aqueous leaf extract of *Psidium gujava*	100ug	1.09	17%
Methanolic fruit extract of *Psidium gujava*	100ug	1.17	10%
Aqueous fruit extract of *Psidium gujava*	100ug	1.10	16%
Cystone (Standard drug)	100ug	1.2	35%

Table 4. The spectroscopic value of herbal plants with extracts

Sample	Absorbance@620nm
Methanolic fruit extract of *Phyllanthus acidus*	0.323
Methanolic leaf extract of *Tinospora cordifolia*	0.176
Aqueous leaf extract of *Tinospora cordifolia*	0.607
Methanolic leaf extract of *Psidium gujava*	0.686
Aqueous leaf extract of *Psidium gujava*	0.657
Methanolic fruit extract of *Psidium gujava*	0.316
Aqueous fruit extract of *Psidium gujava*	0.365

6. CONCLUSION

Numerous plants are said to be helpful in treating urinary stones in the extensive Ayurvedic literature; nevertheless, many more plants still need to be used for their pharmacological effects. Despite extensive study into the mechanisms underlying stone formation, dietary management, the assessment of medicinal plants, and other agents, as well as the use of these agents in the treatment of urinary stones, no conventional medication is currently on the market. As this review shows, many therapeutic herbs are tested primarily using different experimental models of urolithiasis against kidney stone types caused by calcium oxalate and magnesium ammonium phosphate. The majority of these research were exploratory, conducted on animals, and insufficient to support the creation of a pharmaceutical medication. While they might not completely replace these methods, plant materials and their lead component derivatives might undoubtedly aid in lowering the rate at which renal calculi reoccur. Due to the negative effects of modern medicine combined with the superiority and effectiveness of activity offered by natural ingredients in herbs, there is growing interest in the use of herbal treatments for the prevention and cure of illnesses.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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