

ANTI-UROLITHIASIS ACTIVITY OF SELECTED PLANT EXTRACTS BY TITRIMETRY METHOD AND AGGREGATION ASSAY

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 antiurolithiatic 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 *Phyllanthusacidus*(fruits) *Tinosporacordifolia*(leaves)and*Psidiumgujava*(fruits and leaves) and also Preliminary Phytochemical Investigation of *Phyllanthusacidus*(fruits), *Tinosporacordifolia*(leaves) and *Psidiumgujava*(fruits and leaves).

Key words : Kidney stones, Aggregation, Cystone, spectroscopy, Calcium oxalate crystals.

INTRODUCTION

One of the major kidney illnesses that necessitates a well-targeted therapeutic approach is urolithiasis. For the treatment of lithiasis, 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 antilithiatic medications, and the hunt for novel molecules has expanded to include herbal medications that provide improved protection and a lower risk of relapse. 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 antilithiatic medications, and the hunt for novel molecules has expanded to include herbal medications that provide improved protection and a lower risk of relapse. Plant-based medications are becoming more and more well-liked and are being researched for a variety of illnesses, including lithiasis.[1][2] “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”.[4][6]

“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”.[3][7]

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. CYSTINE

- “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".[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.

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)".[3]

➤ 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".[3]

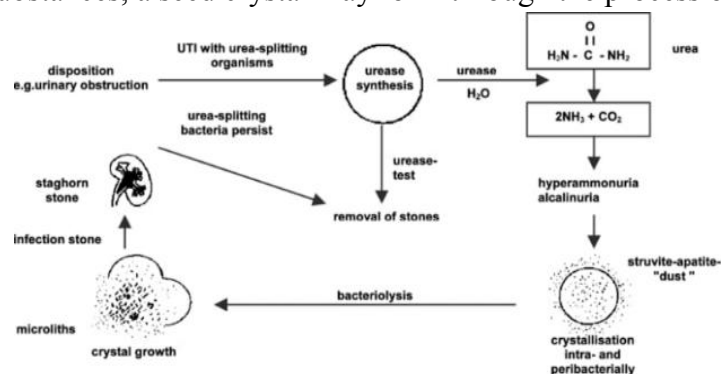


Fig 1 :Supersaturation of urine

PLANT PROFILE

PHYLLANTHUS ACIDUS

PLANT INTRODUCTION:

Botanical name : *Phyllanthusacidus*

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 constituents : Alkaloids, flavonoids, tannins, glycosides, lignin, terpenes, sterols.

BOTANICAL CLASSIFICATION:

Phyllanthusacidus 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 : *Tinosporacordifolia* Family : Menispermaceae.
Indian Name : Guduchi, Giloy.
Habitat : Distributed throughout tropical regions of India that are located 1200 meters above from sea level of Kumaon to Assam. Native to India, Myanmar and Sri Lanka.
Parts Used : Leaves.
Phytochemical constituents : Alkaloids, steroids, glycosides, tannins, flavonoids

BOTANICAL CLASSIFICATION:

Tinosporacordifolia 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 : *Psidiumguajava* Family : Myrtaceae.
Indian Name : Amrudh, Amarood, Peyara.
Habitat : Guava is successfully grown under tropical and subtropical climate.
The quality of fruit is better in areas having distinct winters. Guava tolerates drought, protective irrigation facilities are required.
Parts Used : Fruits and Leaves.
Phytochemical constituents : The fruit contains Saponins, Oleanolic acid, Lycopyranside, Araboyranside, and Flavonoids.

BOTANICAL CLASSIFICATION:

Psidiumguajava belongs to the family of "Myrtaceae."

CLASSIFICATION:

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

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 Chandraprabhabati (Baidyanath, India) have been used extensively in clinical settings. Some of the herbal plants with Urolithiatic activity are *Phyllanthusacidus*, *Tinosporacordifolia*, *Psidiumguajava*, *Cyperusrotundus*.

PHYTOCHEMICAL TESTS

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 occurrence 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.

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) occurrence of foam results test /The formation of foam indicates saponins.

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.

METHODS AND METHODOLOGY

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 *Phyllanthusacidus*, *Tinosporacordifolia*, *Psidiumgujava* 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 (%) = $\text{Weight of dried extract} / \text{Weight of plant material} \times 100$

METHOD

TITRIMETRY METHOD

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 $2N H_2SO_4$. 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 ($2N H_2SO_4$), was allowed to react in sufficient quantity of distilled water in a beaker. The resulting precipitate was calciumphosphate. Both precipitates freed from traces of H_2SO_4 by ammonia solution. Washed the precipitates with distilled water and dried at $60^{\circ}C$ for 4 hours”. [3]

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 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”. [3]

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 only 1 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 $37^{\circ}C$ 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 $0.9494N KMnO_4$ till a light pink colour end point obtained. The amount of remaining undissolved calcium oxalate is subtracted 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 $0.9494N KMnO_4$ equivalent to 0.1898mg of Calcium oxalate”. [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 dissolved 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 $(1-S_1/Sc)/100$, where S_1 is the slope of the plot in the presence of inhibitor (extract) and Sc the slope of the control plot (with no inhibitor)”. [3]

RESULT 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”. [3] 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. [5][15] These results could be considered positives because the herb extracts inhibit crystallization and prevent stone formation. [3][8]

Results of preliminary Phytochemical Investigation of *Phyllanthusacidus*, *Tinosporacordifolia* and *Psidiumgujava*

On preliminary phytochemical analysis of *Phyllanthusacidus* (Usiri) fruits, *Tinosporacordifolia* (Giloy) leaves and *Psidiumgujava* (Guava) fruits, leaves have shown the presence of flavonoids, saponins and tannins. All the results are shown in the table.

Table: 1 PHYTOCHEMICAL STUDIES OF PHYLLANTHUS ACIDUS, TINOSPORA CORDIFOLIA AND PSIDIUM GUJAVA

| Test | Flavonoids | Alkaloids | Glycosides | Tannins | Saponins | Phenolics |
|-------------------------------------|------------|-----------|------------|---------|----------|-----------|
| <i>Phyllanthusacidus</i> (Fruit) | + | - | - | + | + | - |
| <i>Tinosporacordifolia</i> (Leaves) | + | - | - | + | + | - |

| | | | | | | |
|-------------------------------|---|---|---|---|---|---|
| af) | | | | | | |
| <i>Psidiumguajava</i> (Fruit) | + | - | - | + | + | - |
| <i>Psidiumguajava</i> (Leaf) | + | - | - | + | + | - |

IN-VITRO ANTI-UROLITHIASIS ACTIVITY BY TITRIMERTY METHOD AND AGGREGATION ASSAY

In-vitro anti-urolithiasis activity of *Phyllanthusacidus*, *Tinosporacordifolia*and *Psidiumguajava*Methanolic and Aqueous extract by Titrimerty method

Table: 2 The titrimetric value of the sample extract

| SAMPLE | TITRIMETRY VALUE |
|---|------------------|
| Methanolic fruit extract of <i>Phyllanthusacidus</i> | 0.2 |
| Methanolic leaf extract of <i>Tinosporacordifolia</i> | 0.5 |
| Aqueous leaf extract of <i>Tinosporacordifolia</i> | 0.2 |
| Methanolic leaf extract of <i>Psidiumguajava</i> | 1.2 |
| Aqueous leaf extract of <i>Psidiumguajava</i> | 1 |
| Methanolic Fruit extract of <i>Psidiumguajava</i> | 0.4 |
| Aqueous fruit extract of <i>psidiumguajava</i> | 0.4 |
| Blank solution | 0.3 |

In-vitro anti-urolithiasis activity of *Phyllanthusacidus*, *Tinosporacordifolia*and *Psidiumguajava*Methanolic and Aqueous extract by Aggregation assay

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

| Sample | Concentration(ug) | Absorbance@620nm | Percentage Inhibition |
|---|-------------------|------------------|-----------------------|
| Methanolic fruit extract of <i>Phyllanthusacidus</i> | 100ug | 1.13 | 14% |
| Methanolic leaf extract of <i>Tinosporacordifolia</i> | 100ug | 1.18 | 10% |
| Aqueous leaf extract of | 100ug | 1.11 | 15% |

| | | | |
|--|-------|------|-----|
| <i>Tinosporacordifolia</i> | | | |
| Methanolic leaf extract of <i>Psidiumgujava</i> | 100ug | 1.12 | 14% |
| Aqueous leaf extract of <i>Psidiumgujava</i> | 100ug | 1.09 | 17% |
| Methanolic fruit extract of <i>Psidiumgujava</i> | 100ug | 1.17 | 10% |
| Aqueous fruit extract of <i>Psidiumgujava</i> | 100ug | 1.10 | 16% |
| Cystone (Standard drug) | 100ug | 1.2 | 35% |

In-vitro anti-urolithiasis activity of *Phyllanthusacidus*, *Tinosporacordifolia* and *Psidiumgujava* Methanolic and Aqueous extract by Spectroscopy

Table: 4 The spectroscopic value of herbal plants with extracts

| Sample | Absorbance@620nm |
|---|------------------|
| Methanolic fruit extract of <i>Phyllanthusacidus</i> | 0.323 |
| Methanolic leaf extract of <i>Tinosporacordifolia</i> | 0.176 |
| Aqueous leaf extract of <i>Tinosporacordifolia</i> | 0.607 |
| Methanolic leaf extract of <i>Psidiumgujava</i> | 0.686 |
| Aqueous leaf extract of <i>Psidiumgujava</i> | 0.657 |
| Methanolic fruit extract of <i>Psidiumgujava</i> | 0.316 |
| Aqueous fruit extract of <i>Psidiumgujava</i> | 0.365 |

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.

REFERENCES

1. Kyada A, Mansuri N, Patel P. In vitro investigation of some alternative therapeutic agents for antiurolithiatic activity. *J Pharm Res.* 2017;11:955-61.
2. Mosquera DM, Ortega YH, Quero PC, Martínez RS, Pieters L. Antiurolithiatic activity of *Boldoapurpurascens* aqueous extract: An in vitro and in vivo study. *Journal of ethnopharmacology.* 2020 May 10;253:112691.
3. Lolla S, Peddinti H, Gojuvaka S, Saba S, Dasari UD, Pillalamarri M. Evaluation of Anti-urolithiasis Activity by Nucleation Assay. *International Journal of TROPICAL DISEASE & Health.* 2024 Apr 8;45(6):13-20.
4. Yadav RD, Jain SK, Alok S, Mahor A, Bharti JP, Jaiswal M. Herbal plants used in the treatment of urolithiasis: a review. *IJPSR.* 2011 Jun 1;2(6):1412-20.
5. Pillalamarri M, Harika P, Gojuvaka S, Saba S, Dasari UD, Lolla S. Evaluation of Periodontitis Prevention by Using Traditional Medicinal Plants. *International Journal of TROPICAL DISEASE & Health.* 2024 Apr 3;45(5):45-51.
6. KVSRG P, Sujatha D, Bharathi K. Herbal drugs in urolithiasis-a review. *Pharmacog Rev.* 2007 Jan;1(1):175-8.
7. Shukla AK, Shukla S, Garg A, Garg S. A review on anti-urolithiatic activity of herbal folk plants. *Asian Journal of Biomaterial Research.* 2017;3(2):1-1.
8. Ram J, Moteriya P, Chanda S. An overview of some promising medicinal plants with in vitro anti-urolithiatic activity. *IOSR J. Pharm.* 2015;5:23-8.
9. Vargas S R, Perez G RM, Perez G S, Zavala S MA, Perez G C. Antiurolithiatic activity of *Raphanussativus* aqueous extract on rats. *Journal of ethnopharmacology.* 1999 Dec 15;68(1-3):335-8.
10. Baheti DG, Kadam SS. ANTIUROLITHIATIC ACTIVITY OF SOME TRADITIONAL MEDICINAL PLANTS AGAINST CALCIUM OXALATE INDUCED UROLITHIASIS IN RATS. *International Journal of Pharmaceutical, Chemical & Biological Sciences.* 2013 Oct 1;3(4).
11. Arya P, Pandey S, Verma V. Kidney stone formation and use of medicinal plants as antiurolithiatic agents. *Universal Journal of Pharmaceutical Research.* 2017;2(4):42-8.
12. Shelke T, Wayal S, Gunjegaokar S, Gaikwad S, Shirsath A, Hadke S. An overview on Indian medicinal plants with antiurolithiatic activity. *J. Pharm. Res. Clin. Pract.* 2014 Jul;4:33-40.
13. Raj S, Rajan MS, Ramasamy S, Goldy RI, Ariyamuthu R, Sudhagar M, Gandhi S, Shoba P, Gurusamy M. An in vitro Anti-urolithiasis Activity of a Herbal Formulation: *Spinaciaoleracea* L. and *Coriandrumsativum* L. *Clinical Complementary Medicine and Pharmacology.* 2024 Mar 1;4(1):100124.

14. Tiwari A, Soni V, Londhe V, Bhandarkar A, Bandawane D, Nipate SO. An overview on potent indigenous herbs for urinary tract infirmity: urolithiasis. Asian J Pharm Clin Res. 2012;5(1):7-12.
15. Gaybullaev A, Kariev S. Phytotherapy of calcium urolithiasis with extracts of medicinal plants: Changes of diuresis, urine pH and crystalluria. Applied Technologies & Innovations. 2012 Jun 1;7(2).

UNDER PEER REVIEW