INVITRO ANALYSIS OF PHYTOCHEMICAL AND ANTIUROLITHIATIC ACTIVITY OF VARIOUS EXTRACTS OF MELIA DUBIA LEAVES.

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ABSTRACT

Objective: To study the antiurolithiatic activity of various solvent extracts of Melia dubia leaves. Methods: The inhibition of in-vitro calcium oxalate crystal formation by various solvent extracts of M. dubia leaves were investigated by synthetic urine assay. In synthetic urine method, the calcium oxalate formation is induced by the addition of 0.01 M sodium oxalate solution and the inhibition percentage of the calcium oxalate crystals from synthetic urine at different concentrations of various solvent extracts of M. dubia Leaves (10, 50 and 100mg) was investigated by the time course measurement of turbidity changes due to the crystal nucleation and aggregation in the synthetic urine at 620nm by means of a spectrophotometer. Results: The aqueous, acetone and ethanol extracts of M. dubia leaves remarkably inhibits the crystal formation in dose dependent manner in the calcium oxalate urinary lithiasis. Conclusion: Acetone extract of M. dubia leaves showed maximum inhibitory effect on calcium oxalate crystallisation in synthetic urine than ethanol and water extracts. Thus acetone extract could be further analyzed in vivo and characterization of its active compound could lead to the discovery of a new drug for urolithiasis.

KEY WORDS: Antiurolithiatic activity, Acetone extract, Melia dubia, Synthetic urine assay, Calcium oxalate crystallisation.

INTRODUCTION

Stone formation in the kidney is one of the oldest and most wide spread diseases known to man. In India people living in different states utilize different plants for curing urolithiasis.\[1]
Urolithiasis is derived from the Greek words “ouron” (urine) and “lithos” (stone). It is considered as the third most common affliction of the urinary tract. Urolithiasis is deposition or formation of stones in any part of the kidney like ureters or the urinary bladder.

A stone is an aggregation of solute materials from urine such as calcium, oxalate, phosphate and uric acid which forms stone. In India, calcium oxalate is found to be the most predominant constituent of urolithiasis. Stone formation is the culmination of a series of physiochemical events i.e supersaturation and nucleation, growth of the crystal and aggregation that occurs as the glomerular filtrate traverses through the tubules of nephron.

Urine is normally supersaturated with most stone forming salt components, as well as contains chemicals that prevent or inhibit crystal development in urinary tract. However, the presence of certain molecules raise the level of supersaturation of salts needed to initiate crystal nucleation or reduce the rate of crystal growth or aggregation and prevents stone formation. Calcium oxalate stones represent up to 80% of analyzed stones.

Calcium phosphate account for 15-25%, while 10-15% is mixed stones. The others are struvite 15-30%, cystine 6-10%, and uric acid stones 2-10%. Calcium oxalate stones are of primary two types, calcium oxalate monohydrate (whewellite) and calcium oxalate dihydrate (weddellite). The occurrence frequency of whewellite is 78% while that of weddellite is 43%. Though technological advancements have made dramatic improvement in the removal of urinary stones still some of the drawbacks of these methods exists which includes their being too costly for a common man and recurrence of stone formation along with a number of other side effects.

Many medications and remedies have been used during the past many years to treat urinary stones. Endoscopic stone removal and extracorporeal shock wave lithotripsy have revolutionized the treatment of nephrolithiasis, but do not avoid the possibility of new stone formation. Various therapies including thiazide diuretics and alkali-citrate are being used in an attempt to prevent the recurrence of hypercalciuria and hyperoxaluria induced calculi, but scientific evidence for their efficacy is less convincing. Medicinal plants have played as significant role in various ancient traditional system of medication. Even today, plants provide a cheap source of drugs for majority of world’s population. Several pharmacological investigations on the medicinal plants used in traditional antiurolithiatic therapy have revealed their therapeutic potential in the in-vitro or in-vivo models.
Hence search for new antilithiatic drugs from natural sources has assumed greater importance as herbal drugs are cost effective and cause least side effects. In ayurveda many plants having the property of disintegrating and dissolving the stone are referred to as “pashanabheda”. ‘Pashanabheda’ is the Sanskrit term used for a group of plants with diuretic and antiurolithiatic activities.\textsuperscript{[12,13]} Drugs with multiple mechanisms of protective action may be one way forward in minimizing tissue injury in human disease.\textsuperscript{[14]} Herbal medicines contain several phytoconstituents and exert their beneficial effects by multiple mechanisms like diuretic activity, crystallization inhibition activity, lithotriptic activity, antioxidant activity, antimicrobial activity, analgesic and anti-inflammatory activity.

\textit{Melia dubia} belonging to the family Meliaceae has shown great potential for the best management in terms of secondary plant chemistry. It is a large deciduous and native tree species to India. Its timber is mainly used for furniture and agricultural implements.\textsuperscript{[15]} Every part of the plant is being used as traditional herbal medicines, such as anthelmintics, treatment of leprosy, eczema, asthma, malaria, fevers and veneral diseases,\textsuperscript{[16]} as well as cholelithiasis, acarasis and pain.\textsuperscript{[17]} Fruits of \textit{M. dubia} are considered to be important in colic and skin diseases and also as anthelmintic.\textsuperscript{[18]} It is well known as rich and valuable source of bioactive limonoids.\textsuperscript{[19]} Although hundreds of limonoids have been isolated from various plants but, their occurrence in the plant kingdom is more abundantly in Meliaceae and Rutaceae. Ongoing studies show that limonoids are highly oxygenated, modified terpenoids and have recently attracted attention because compounds belonging to this group have exhibited a range of biological activities like insecticidal, insect antifeedant especially on some of the forest insect pests and growth regulating activity on insects as well as antibacterial, antifungal, antimalarial, anticancer, antiviral and a number of other pharmacological activities on humans.\textsuperscript{[20,21,22]}

Literature on traditional medicines show the use of fresh decoction of leaves of \textit{M. dubia} in treating urinary stones but no such \textit{in-vitro} study has been undertaken. Thus the aim of the present study is to evaluate the effectiveness of aqueous, acetone and ethanolic extract of leaves of \textit{M. dubia} for its antiurolithiatic activity using \textit{in-vitro} methods: synthetic urine assay.
MATERIALS AND METHODS

Collection and preparation of plant extract

*M. dubia* leaves were collected from this tree in and around Mayiladuthurai, Tamilnadu, India, where it was found naturally. The leaves were washed thoroughly in running tap water to remove soil particles and other adhered debris then shade dried for 14 days and ground well into fine powder. The powdered materials were stored in air tight container until the time of use. 50 grams of this powdered material were soaked in 250 ml of various solvents (aqueous, acetone and ethanol) separately and kept at room temperature for 12 hours and kept at shaker for 3 hours. The samples were filtered and used for phytochemical screening and excess filtrate was filtered through a single layer of muslin cloth, and then final filtrate was collected by passing it through a Whatman grade 1 filter paper in a Buchner funnel under vacuum. The filtrate was evaporated to dryness. The crude extract of *M. dubia* was obtained.\[^{23,24}\]

Phytochemical screening

The aqueous, acetone and ethanolic extracts of this plant leaves was screened qualitatively for the presence of various phytochemical constituents such as alkaloids, flavonoids, phlobatannins, anthroquinones, steroids, tannins, phenols, terpenoids, saponins, resins, carbohydrate, protein and amino acids by standard procedure.\[^{25}\]

Test for alkaloids

0.5 to 0.6 ml of aqueous extract was mixed with 8 ml of 1% HCl, warmed and filtered. 2 ml of the filtrate was treated separately with both reagents (Maeyer’s and Dragendorff’s), after which it was observed whether the alkaloids were present or absent in the turbidity or precipitate formation.

Test for flavonoids

0.5ml of aqueous extract was shaken with petroleum ether to remove the fatty materials (lipid layer). The defatted residue was dissolved in 20 ml of 80% ethanol and filtered. The filtrate was used for the following tests: 3 ml of the filtrate was mixed with 4 ml of 1% potassium hydroxide in a test tube and the colour was observed. A dark yellow colour indicated the presence of flavonoids.
Test for glycosides
5 ml aqueous extract was hydrolysed with 5 ml of Conc.HCl and boiled for few hours on a water bath and hydrolysates were subjected to the following test: A small amount of alcoholic extract of sample was dissolved in 1 ml water and then aqueous 10% sodium hydroxide was added. Formation of a yellow colour indicated the presence of glycosides.

Test for steroids
0.5 ml of the aqueous extract mixed with 2 ml of acetic anhydride followed by 2 ml of sulphuric acid. The colour changed from violet to blue or green in sample indicated the presence of steroids.

Test for tannins
2.5 ml of aqueous extract was dissolved in 10 ml distilled water and filtered. 1% aqueous ferric chloride (FeCl3) solution was added to the filtrate. The appearance of intense green, purple, blue or black colour indicated the presence of tannin.

Test for phenols
To 1 ml of aqueous extract of sample, 2 ml of distilled water followed by a few drops of 10% aqueous ferric chloride solution was added. Formation of blue or green colour indicated the presence of phenols.

Test for terpenoids
5 ml of aqueous extract was mixed with 2 ml of chloroform followed by the careful addition of 3 ml Conc.H2SO4. A layer of the reddish brown colouration was formed at the interface thus indicating a positive result for the presence of terpenoids.

Test for saponins
To 1 ml of aqueous extract was diluted to 5 ml of water and the tubes were shaken vigorously, formation of 1 cm layer of foam indicate the presence of saponins.

Test for resins
To 1 ml of aqueous extract was treated with few drops of acetic anhydride solution followed by 1 ml of concentrated sulphuric acid. Resins give colouration ranging from orange to yellow.
Test for carbohydrate
Aqueous extract (1 ml) was added to 1 ml of water and 20 drops of boiling Fehling’s solution (A and B) in a test tube was added. The formation of a precipitate red-brick in the bottom of the tube indicates the presence of carbohydrate.

Test for protein and amino acid
To 1 ml of the aqueous extract was treated with few drops of ninhydrin reagent. Appearance of purple colour shows the presence of amino acids.

Test for phlobatannins
When aqueous extract was boiled with 2% aqueous HCl. The deposition of a red precipitate was taken as evidence for the presence of phlobatannins.

Test for anthraquinones
5 ml of the aqueous extract solution was hydrolysed with diluted Conc. H$_2$SO$_4$ extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones. Acetone and ethanolic extracts also screened in the same procedure.

invitro anti-urolithiatic activity
Experimental protocol
The effect of *M. dubia* extracts on calcium oxalate crystallisation was determined by the time course measurement of turbidity changes due to the crystal nucleation and aggregation in the synthetic urine on addition of 0.01M sodium oxalate. The Precipitation of calcium oxalate at 37°C and pH 6.8 has been studied by the measurement of turbidity at 620 nm. A spectrophotometer UV/Vis was employed to measure the turbidity of the formation of calcium oxalate.\(^{26}\)

Preparation of artificial urine
The artificial urine (AU) was prepared according to the method of Burns and Finlayson\(^{27}\) and had the following composition: sodium chloride 105.5 mmol/l, sodium phosphate 32.3 mmol/l, sodium citrate 3.21 mmol/l, magnesium sulfate 3.85 mmol/l, sodium sulfate 16.95 mmol/l, potassium chloride 63.7 mmol/l, calcium chloride 4.5 mmol/l, sodium oxalate 0.32 mmol/l, ammonium hydroxide 17.9 mmol/l, and ammonium chloride 0.0028 mmol/l. The AU was prepared fresh each day and pH adjusted to 6.0.
Study without inhibitor
A volume of 1.0 ml of AU was transferred into the cell and 0.5 ml of distilled water added to it and blank reading was taken. 0.5 ml of 0.01 M sodium oxalate was added, to the previous volume, and the measurement is immediately started for a period of 400 sec.[26]

Study with inhibitor
Extract was resuspended in distilled water (200 mg/ml), filtered and used at a final concentration of 10, 50 and 100 mg/0.5 ml were prepared. A mixture of 1 ml of AU and 0.5 ml of plant extract solution is versed in the cell. A blank reading was taken and then volume of 0.5 ml of 0.01M sodium oxalate was added and the measurement is immediately started for a period of 400 sec.[26]

The percentage of inhibition was calculated using the following formula.

\[ \text{% inhibition} = \left\{ 1 - \frac{\text{Si}}{\text{Sc}} \right\} \times 100 \]

Where; Si: slope of graph in the presence of inhibitor (Plant extract),
Sc: slope of graph without inhibitor (Control).

RESULTS
Table - 1 : Phytochemical constituents in various extracts of M. dubia Leaves

<table>
<thead>
<tr>
<th>S.No</th>
<th>Phytoconstituents</th>
<th>Aqueous extract</th>
<th>Acetone extract</th>
<th>Ethanolic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Glycosides</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>Steroid</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Tannins</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7.</td>
<td>Terpenoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8.</td>
<td>Saponin</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td>Resins</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10.</td>
<td>Carbohydrate</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11.</td>
<td>Protein and aminoacids</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12.</td>
<td>Phlobatannins</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13.</td>
<td>Anthroquinones</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = presence, - = absence
Figure 1. Change in turbidity without and with aqueous extract of *M. dubia* Leaves (Inhibitor)

Figure 2. Change in turbidity without and with acetone extract of *M. dubia* Leaves (Inhibitor)

Figure 3. Change in turbidity without and with ethanolic extract of *M. dubia* Leaves (Inhibitor)
The effect of various solvent extracts like aqueous, acetone and ethanolic of *M. dubia* on various phases of calcium oxalate crystallisation was determined by time course measurement of turbidity in the synthetic urine. Figure 1 shows an initial detectable increase in the turbidity after induction of the crystallisation with sodium oxalate, was observed. In the control experiment, the initial step rise in turbidity; the nucleation phase, on attaining its maximum, it was followed by a decrease; the aggregation phase. Aqueous extract of *M. dubia* Leaves, inhibited the slope of turbidity in concentration dependent manner and followed by very slow decrease as shown in Figure 1. Acetone and ethanolic extracts of *M. dubia* Leaves were also inhibited the slope of turbidity in concentration dependent manner and followed by very slow decrease as shown in Figure 2 and 3 respectively.

Figure 4 shows the percentage of inhibition of calcium oxalate crystallisation by *in vitro* anti-urolithiatic activity of various solvent extracts of *M. dubia* leaves. An acetone extract at 100mg concentration produced higher inhibition of calcium oxalate crystallization as compare to other extracts. Results showed that the decrease in number of crystal as well as % inhibition of the formation of calcium oxalate crystals was directly proportional to the increase in concentration of plant extract, with minimum inhibition 25.31% in 10 mg of ethanolic extract while maximum inhibition of 98 % at 100 mg of acetone extract concentration.
DISCUSSION
Kidney stones are hard, solid particles that form in the urinary tract. In many cases, the stones are very small and can pass out of the body without any problems. However, if a stone (even a small one) blocks the flow of urine, excruciating pain may result, and prompt medical treatment may be needed. Many remedies have been used to removed and dissolved the urinary stones but overuse of synthetic drugs, which results in higher incidence of adverse drug reactions, has motivated humans to return to nature for safe remedies. Antilithiatic drugs from natural sources has assumed greater importance as herbal drugs are cost effective and less or no side effects.

*In vitro* crystallization systems are widely used for different purposes in urolithiasis research. Since kidney stone formation is a complex process that results from a succession of several physico-chemical events including supersaturation, nucleation, growth, aggregation and retention within renal tubules. The supersaturation of urine with calcium oxalate is an important factor in crystallisation, with later factors being nucleation, growth and aggregation. Thus if supersaturation or initial stages in crystallisation can be prevented, then lithiasis could be avoided. The present *in-vitro* study revealed that the plant extract has potent antiurolithiatic ability in synthetic urine assay (with maximum inhibition of 98 % at 100 mg of acetone extract concentration). Along with this in synthetic urine assay maximum crystals formation was observed in control while minimum was formed at 100 mg concentration of acetone extract. However these *in-vitro* results should be confirmed *in-vivo* in order to develop a potent antilithic agent from this plant.

Phytochemical screening revealed the presence of alkaloids, flavonoids, amino acids, phlobatannins, anthroquinones, steroids, tannins, phenols, terpenoids, saponins, resins, carbohydrate, protein and amino acids compounds (Table 1). Different activities observed in the plant crude extract might be due to the presence of these phytochemicals. For example, flavonoids are known to possess antispasmodic and Ca$^{2+}$ channel blocking, antioxidant and antidiuretic activities. Saponins are known to possess anti-crystallization property by disaggregating the suspension of mucoproteins, promoters of crystallization. Antiurothiatic activity of this plant may be due to the presence of these phytocompounds.

CONCLUSION
The results exhibited by the aqueous, acetone and ehanolic extracts of *M. dubia* leaves showed significant antiurolithiatic activity. Acetone extract showed maximum inhibition of
calcium oxalate crystallization than other extracts. Further studies need to isolation and purification of active phytoconstituents with potent antiurolithiatic activity.

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