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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(4): 900-904

www.thepharmajournal.com
Received: 17-02-2019
Accepted: 20-03-2019

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In-vitro anti urolithiatic assessment of *Syzygium cumini* seed extract

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Abstract
The aim of the study was to evaluate *in vitro* anti urolithiatic activity *of Syzygium cumini* seed extract. Urolithiasis is the formation of urinary stones, which are calculi formed or located anywhere in the urinary system. Introduction of herbal medicines extracted from various medicinal plants improved health and quality of people's life. The *in vitro* anti urolithiatic activity was carried out by turbidity method (nucleation assay) and calcium oxalate dissolution method (aggregation assay). The findings of the study revealed that the hydroethanolic extracts of *Syzygium cumini* seeds has shown significant anti-urolithiatic activity. The observed anti-urolithiatic activity of the hydroethanolic extract of the seed of *Syzygium cumini* might be due to the various phytoconstituents present in it such as alkaloids, triterpenoids, flavonoids, Tannins and polyphenols etc.

Keywords: Anti urolithiatic activity, *Syzygium cumini*, extract, calcium oxalate, turbidity method, aggregation

Introduction
Herbal medicine may also refer to phytomedicine, phytotherapy, or paraherbalism, which are alternative and pseudoscientific practices of using unrefined plant or animal extracts as supposed medicines or health-promoting agents. In general, Herbal medicines are used for cure, mitigation, treatment and prevention of diseases especially those endemic to the local environment of the herbs [1]. Plants have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today [2]. Modern medicine makes use of many plant-derived compounds as the basis for evidence-based pharmaceutical drugs. Introduction of herbal medicines extracted from various medicinal plants improved health and quality of people's life.
Urolithiasis is the formation of urinary calculi (urinary stones), which are calculi formed or located anywhere in the urinary system. Urinary stones are typically classified by their location or by their chemical composition (calcium-containing, struvite, uric acid, or other compounds). In humans, calcium oxalate is a major constituent of most urinary stones. Dehydration from low fluid intake is a major factor in stone formation. Kidney stones can result from an underlying metabolic condition, such as distal renal tubular acidosis, Dent's disease, hyperparathyroidism, primary hyperoxaluria, or medullary sponge kidney.
The aim of the present study was to assess the *in vitro* anti-urolithiatic potential of the hydroethanolic extract of the seed of *Syzygium cumini.*

Materials and Methods
Collection of plant part
The seeds of *Syzygium cumini* were collected from the local market of Kodad, Telangana.

Drying, extraction and fractionation
The seeds of *Syzygium cumini* were cleaned, dried and then it was grinded to obtain coarse powder of standard size suitable for extraction. The powdered seed was subjected to hydroethanolic (Water: Ethanol= 60: 40) extraction by Soxhlet apparatus. After that the hydroethanolic extract was defatted with petroleum ether to remove the chlorophylls. Finally the extract was air dried. The practical percentage yield will be calculated.

Methods for evaluation of *in-vitro* anti-urolithiatic activity
The *in-vitro* anti-urolithiatic activity of the extract was evaluated by using two models namely turbidity method (inhibition of calcium oxalate formation) and Calcium oxalate dissolution method (Aggregation assay).

Method A: Turbidity method (Nucleation assay)
In this method the *in vitro* anti-urolithiatic activity of the extract was tested in terms of inhibition of calcium oxalate formation by the method of Prachi Khare *et al*. with modification [3]. The inhibition of calcium oxalate formation in the presence of the extract was compared with the inhibition of calcium oxalate formation in the presence of the standard (Cystone). The precipitation of calcium oxalate at 37°C and pH 6.5 was studied by the measurement of turbidity at 620nm using UV/Vis spectrophotometer. The turbidity caused due to formation of calcium oxalate by the reaction of calcium chloride ($CaCl_2$) with sodium oxalate.
In the control, turbidity due to the formation of calcium oxalate was determined in the absence of any inhibitor. For this, a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of water were added in a test tube. Then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. Formation of the turbidity results immediately after mixing of above chemicals. The measurement of turbidity was done by measuring the absorption by UV/Vis spectrophotometer at 620 nm after shaking the mixture for 1 min. Then the measurement of the absorbance was carried out after 1 min interval up to a period of 5 min. Absorptions were noted down.
The study was continued to know the effect of plants extracts against stone nucleus formation (formation of calcium oxalate) *in vitro*. In this experiment the effect of the extract on inhibition was carried out in three concentrations of the extract. For this, in one test tube a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 100µg/ml extract in water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. In another test tube a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 250µg/ml extract water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. In another test tube a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 500µg/ml extract water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. The measurement of turbidity was done by measuring the absorption by UV/Vis spectrophotometer at 620 nm after shaking the mixture for 1 min. Then the measurement of the absorbance was carried out after 1 min interval up to a period of 5 min. Absorptions were noted down.
After that the effect of the standard (Cystone) on the inhibition of the formation of calcium oxalate was studied. The standard drug is a poly herbal formulation. For this in a test tube a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 100µg/ml of standard in water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. In another test tube 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 250µg/ml of standard in water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. In a third test tube a volume of 0.95 ml of 50mM $CaCl_2$ (in Tris buffer pH 6.5) and 1ml of 500µg/ml of standard in water were added, and then 0.95 ml of 75mM sodium oxalate (in Tris buffer pH 6.5) was added. The measurement of turbidity was done by measuring the absorption by UV/Vis spectrophotometer at 620 nm after shaking the mixture for 1 min. Then the measurement of the absorbance was carried out after 1 min intervals up to a period of 5 min. Absorptions were noted down.
Inhibition in stone nucleus formation was calculated by the graphical method using the following mathematical formula:

Inhibition % = {1-[Si / Sc]} x 100

Where;
Si: slope of graph in the presence of inhibitor (drugs/extracts).
Sc: slope of without Inhibitor (Control).

Method B: Calcium oxalate dissolution method (Aggregation assay)
In this second method the role of seed extract in dissolving the already formed calcium oxalate stones nucleus in artificial system. For this artificial calcium oxalate crystal were prepare in the laboratory by standard method.

Synthesis of calcium oxalate by the method of homogenous precipitation [3]
1.47gm of calcium chloride dihydrate was dissolved in 100ml distilled water. 1.34gm of sodium oxalate was dissolved in 100 ml of 2N H_2SO_4. Both the solution were mixed in a beaker and stirred continuously to precipitate out calcium oxalate. The calcium oxalate obtained was freed from traces of sulphuric acid by ammonia solution. After that it was washed with distilled water and was dried at a temperature 60 °C for a period of 2hours.

Preparation of the semi-permeable membrane from eggs [3]
The apex of eggs was punctured by a glass rod and entire content was squeezed out. Empty eggs were washed thoroughly with distilled water and then placed in a beaker consisting 4ml concentrated HCl in 200ml distilled water. It was kept for overnight that resulted in complete decalcification of semi permeable membrane. Next day the semi permeable membranes were removed carefully and washed thoroughly with distilled water. After that the membranes were placed in ammonia solution for neutralization of acid traces, and then rinsed it with distilled water. It was stored in refrigerator at a pH of 7-7.4 in the moistened condition.

Procedure
The experiment was carried out in four arrangements as given below according to the method of Prachi Khare *et al.* [3] with modification.

Control: 10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Test 100mg/ml: 10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 100mg/ml of the hydroethanolic extract in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2

times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Test 250mg/ml: 10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 250mg/ml of the hydroethanolic extract in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Test 500mg/ml: 10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 500mg/ml of the hydroethanolic extract in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Standard 100mg/ml: 10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 100 mg/ml of the cystone in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Standard 250mg/ml: 10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 250 mg/ml of the cystone in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

Standard 500mg/ml: 10 mg of prepared calcium oxalate crystals were weighed and were mixed with 2 ml of 500 mg/ml of the cystone in water and packed in semi permeable egg membranes by suturing. Each treatment was repeated 2 times and average was taken. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks that contained 150 ml 0.1 M Tris buffer.

All the flasks were placed for 24 hours. After that membranes were taken out of the flask and content of each membrane was collected in different test tubes. To each test tube 2 ml of 1 N sulphuric acid was added and titrated with 0.9494 N $KMnO_4$ till a light pink colour end point obtained. 1ml of 0.9494 N $KMnO_4$ equivalent to 0.1898mg of calcium.

Results and Discussion

Results

Method A: Turbidity method (Nucleation assay)

Addition of $Na_2C_2O_4$ solution to the reaction mixture consisting of $CaCl_2$ resulted in the formation of numerous calcium oxalate crystals. Calcium oxalate crystallizations without (control) and with extract in different concentrations were recorded (Figure 1, 2, 3). The percentage inhibition of turbidity (aggregation) in the presence of seed extract and standard was tabulated (table 1).

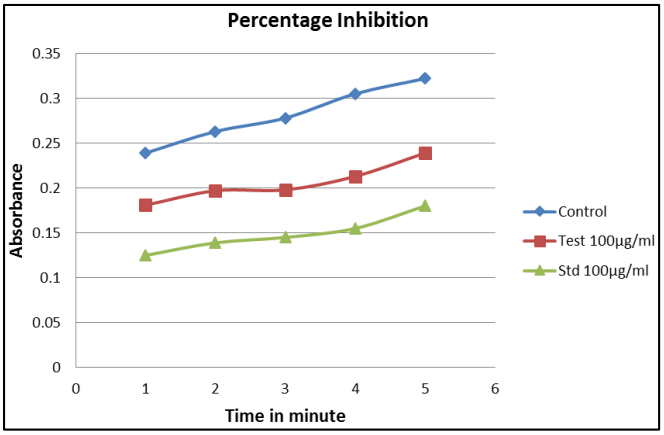

Fig 1: Graph showing change in turbidity in presence of Test 100µg/ml Standard 100µg/ml at 620nm

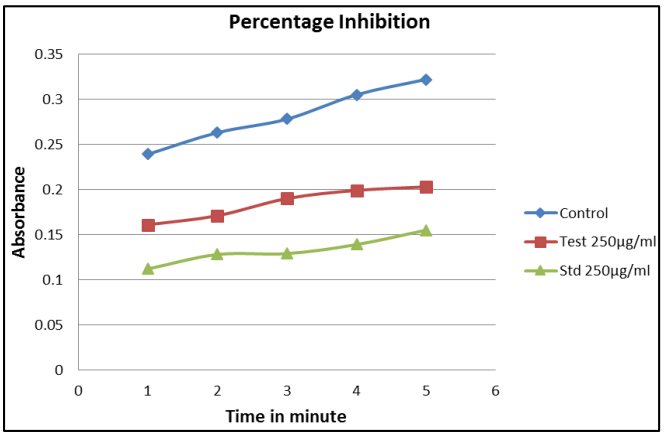

Fig 2: Graph showing change in turbidity in presence of Test 250µg/ml Standard 250µg/ml at 620nm

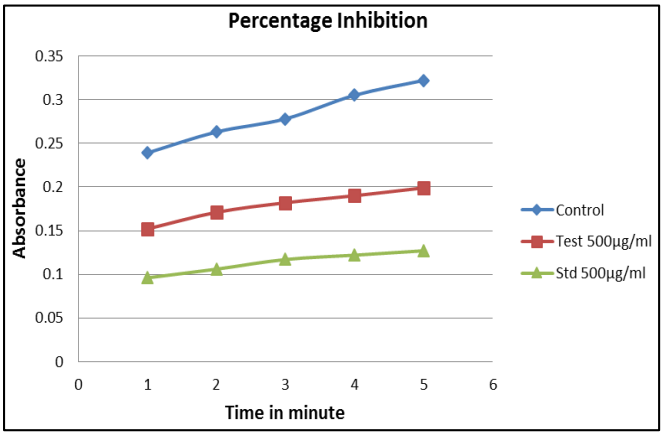

Fig 3: Graph showing change in turbidity in presence of Test 500µg/ml Standard 500µg/ml at 620nm

Table 1: Calculation of the percentage inhibition from the regression equation

Sample	Regression equation	Slope	% Inhibition
Control	y = 0.0208x + 0.219	0.0208	
Test 100µg/ml	y = 0.0132x + 0.166	0.0132	36.53
Test 250µg/ml	y = 0.0112x + 0.151	0.0112	46.15
Test 500µg/ml	y = 0.0113x + 0.144	0.0135	45.67
Standard 100µg/ml	y = 0.0126x + 0.111	0.0126	39.42
Standard 250µg/ml	y = 0.0097x + 0.1035	0.0097	53.36
Standard 500µg/ml	y = 0.0078x + 0.0902	0.0078	62.50

Method B: Calcium oxalate dissolution method (Aggregation assay)

In this method the role of seed extract in dissolving the already formed calcium oxalate stones nucleus in artificial system. For this artificial calcium oxalate crystal were prepare in the laboratory by standard method. The findings of the calcium oxalate dissolution method (Aggregation assay) is mentioned in the following table no 2.

Table 2: Comparison of percentage dissolution of the calcium oxalate by the extract and the standard

Concentration	% Inhibition	
	Test (Extract)	Standard (Cystone)
100µg/ml	23.61%	45.58%
250µg/ml	39.60%	66.58%
500µg/ml	65.58%	73.58%

Control represents zero inhibition

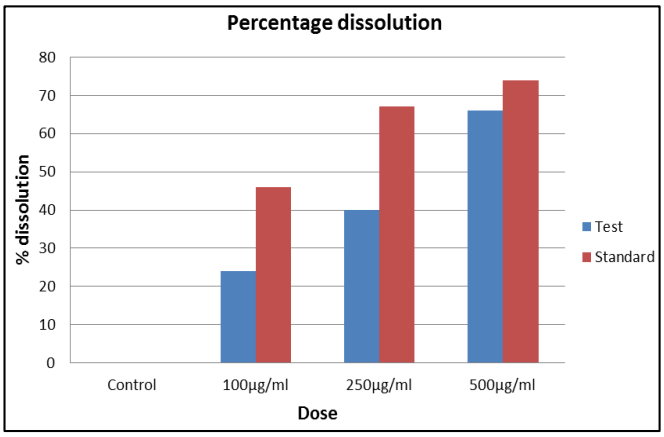

Fig 4: Graphical representation of dissolution of Calcium oxalate

Discussion

Turbidity method (Nucleation assay)

In the turbidity method i. e; the inhibition of calcium oxalate formation was measured in terms of turbidity by using UV/Visible spectrophotometer. The formation of calcium oxalate crystals were indicated by the formation of turbidity in the solution. More the inhibition less is the turbidity and less absorbance will be observed in UV/Visible spectrophotometer. The inhibition of calcium oxalate formation in the presence of the extract was compared with the inhibition of calcium oxalate formation in the presence of the standard (Cystone). The study carried out at 37 °C and pH 6.5. The measurement of turbidity was done at 620nm using UV/Vis spectrophotometer.

First of all, growth of stone nucleus *in vitro* in the absence of any inhibitor was done (control). The turbidity was formed immediately after mixing of chemicals according to the procedure of the method and then the turbidity formed was measured in terms of absorption at 620 nm in UV/Vis spectrophotometer for a period of 5 min at an interval of 1 min. Absorptions were noted down and data obtained was used as the un-controlled growth of the stone nucleus for the comparison of growth in the presence of the standard drugs and plant extracts. And then the study was continued to know the effect of plants extracts against stone nucleus formation *in vitro* according to the procedure mentioned in the materials and methods chapter. Inhibition in stone nucleus formation was calculated by the graphical method using the mathematical formula mentioned in materials and methods section.

As *in vitro* crystallization study was performed, since nucleation is an important first step for the initiation of crystals, which then grow and form aggregates. The seed extract of *Syzygium cumini* inhibited the crystallization by inhibiting nucleation of calcium oxalate through disintegrating into smaller particles with increasing concentrations of the fraction. From the results of the nucleation assay, it is confirmed that the extract contained nucleation preventing agents.

One possible mechanism of anticrystallization of the extract could be its ability to form complex with free calcium and oxalate ions, thus preventing the formation of calcium oxalate complexes, as has also been suggested for *Sarghassum wightti* [4].

In this study it is observed that the test 100µg/ml has shown 36.53% percentage of inhibition, test 250µg/ml has shown 46.15% percentage of inhibition, test 500µg/ml has shown 45.67% percentage of inhibition whereas the percentage of inhibition for standard 100µg/ml is 39.42%, percentage of inhibition for standard 250µg/ml is 53.36%, percentage of inhibition for standard 500µg/ml is 62.50% (table 1 and figure 1, 2, 3).

Calcium oxalate dissolution method (Aggregation assay)

In the second method i. e. dissolution of calcium oxalate crystals, the role of plant extract in dissolving calcium oxalate crystals was evaluated in artificial system in which calcium oxalate crystals are already formed.

It has been observed that the percentage dissolution for test doses 100µg/ml, 250µg/ml & 500µg/ml are 23.61%, 39.60%, 65.58% respectively whereas percentage dissolution for standard doses 100µg/ml, 250µg/ml & 500µg/ml are 45.58%, 66.58%, 73.58% respectively (Table 2, figure 4).

The observed anti-urolithiatic activity of the hydroethanolic extract of the seed of *Syzygium cumini* might be due to the various phytoconstituents present in it. The seeds of this plant *Syzygium cumini* contain several phytochemical constituents belonging to categories such as alkaloids, carbohydrates, glycosides, tannins, triterpenes and flavonoids etc. [5]. Previous studies on triterpenoids have been shown to possess anti-urolithiatic effect the lowering of oxalate excretion on treatment with the triterpene indicates the synthesis of oxalic acid from the glycollic acid is somehow inhibited [6]. Flavonoids containing plants have been reported to be associated with anti-urolithiatic activity [7].

Aggregation of crystals marks the process wherein numerous crystals in the solution come together and adhere forming large crystal agglomerates. Aggregation is a key determinant of crystal retention as large crystal agglomerates are the ones that produce renal tubular obstruction thereby promoting stone formation [8]. Saponins possess anti-urolithiatic properties [9] and are known to disintegrate mucoproteins that are crucial components of stone matrix [10].

Tannins and polyphenols inhibit calcium oxalate crystal formation as well as dissolve the preformed calcium oxalate crystals by aiding calcium complexation [11].

Conclusion

From the present study it was found that the hydroethanolic extracts of *Syzygium cumini* seeds has shown significant anti-urolithiatic activity. This study is a preliminary study; the results should be confirmed *in-vivo* in order to discover an effective anti-urolithiatic drug from the seeds of *Syzygium cumini*, as this property of the extract is advantageous in

preventing urinary stone formation by inducing the excretion of small particles from the kidney and reducing the chance of their retention in the urinary tract.

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