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IN VITRO ANTIUROLITHIATIC ACTIVITY OF VARIOUS EXTRACTS USING *PONGAMIA PINNATA*LINN. LEAVES

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ABSTRACT

Urinary kidney stone formation is a multifaceted complex process involving accumulation and aggregation of oxalates and phosphates of calcium, magnesium, ammonium and uric acid. Traditional systems of healing like Ayurveda and Unani have played a praiseworthy role from time immemorial in the treatment of kidney stones. One such plant is *Pongamia pinnata* Linn. Belonging to the Leguminosae family. In Ayurveda, it is described as 'Paashanabhedha' meaning 'stone breaking'; It is highly endorsed with diuretic, antioxidant properties and used in the treatment of kidney stones. The present study was taken up toevaluate the antiurolithiatic potential of the leaves by a methodical approach, using a simple *In-vitro*method. Calcium oxalates mainly compose kidney stones. Hence, the study involved the preparation of Calciumoxalate and its dissolution in the presence of *Pongamia pinnataLinn.* The successive solvent extractswerescreened for *In-vitro* antiurolithiatic activity using the turbidity method. The ethanolic extract had a highdissolving potential of 86.2% for Ca-Ox. A trend of dose-dependent dissolution was identified with increasing the concentration of ethanolic extract. The ethanolic extract shows the presence of phenolic compounds, flavonoids, tannins, amino acids, terpenoids, and saponins. The LC-MS studies were performed to identify the presence of chemical constituents and mainly identification of flavonoid group compounds such as "Karanjin" responsible for the activity. The present study reveals the promising evidence of *Pongamia pinnata* Linn. belonging to the family *Leguminosae* used as 'Paashanabhedha' in the traditional system of medicine.

INTRODUCTION

In the Ayurvedic system of medicine, the term Urolithiasis is explained as "Mutrashmari" [1].Urinary stone formation is one of the serious and painful urological diseases that is seen in 12% of the worldwide population and its reoccurrence rate in males is high (70-80%) when compared to females (47-60%)[2]. The cause of urolithiasis is still unknown but probably may be due to positive family history, overweight obesity or increased body mass index (BMI)[3].

"Pashanabheda" is the Sanskrit term used for a group of plants with diuretic and antiurolithiatic activities. A total of 503 species, 365 genera, and 119 families were cited for treating kidney stones[4,5]. The most cited families are Asteraceae (41), Leguminosae (34), Lamiaceae (26), Apiaceae (21), Rosaceae (19) and Poaceae (16)[6]. There are different types of kidney stones which are composed of calcium oxalate, uric acid, calcium phosphate, cystine and struvite [7]. The evaluation of the stone formation in

the kidney can be studied by the urinalysis, which includes measurement of pH, albumin, glucose, 24-hour urine calcium, phosphate, magnesium, creatinine, oxalate, uric acid, citrate, and cystine [8]. Some of the theories involved in the stone formation are free particle theory, vascular theory and blocked lymphatic theory [9]. The events that trigger the stone formation are nucleation, growth, aggregation, and retention of crystals within the kidneys [10]. Plant sources contain several phytoconstituents and exhibit their effects by several mechanisms by diuretic activity, crystallization inhibitory activity, regulation of oxalate mechanisms, improvement of renal functions, ACE and Phospholipase A2 inhibition and antimicrobial activity[11].It is reported that flavonoids, triterpenoids, and saponins such as α-amyrin, β-amyrin, lupeol from different plants showed antiurolithiatic and diuretic activity. Many plant extracts and different fractions possessing these active constituents have been screened for antiurolithiatic activity[12]. The *Pongamia pinnata* is commonly known as 'Karanj', has been recognized in a different system of traditional medicines for the treatment of different diseases and ailments of human beings [13]. Pongamia is a genus having one species only *Pongamia pinnata (L.)* [Syn. *Pongamia glabra* (Vent); *Derris indica* (Lamk.)] which belongs to family Leguminosae and subfamily Papilionaceae. It is a medium-sized glabrous, the perennial tree grows in the littoral regions of South-Eastern Asia and Australia. Traditionally, its bark is used in piles treatment. Leaves are effective as medicated baths and rheumatic pains, seeds are used in the treatment of hypertension, bronchitis, whooping cough, skin diseases, and rheumatic arthritis. In primitive areas of Malaysia and India, root extracts are applied to abscesses; other plant parts such as crushed seeds and leaves are regarded as having antiseptic properties. In India, seeds were used for skin ailments. Today the oil is used as a liniment for rheumatism; their juice is used for colds, coughs, diarrhea, dyspepsia, flatulence, gonorrhea and leprosy. Roots are used for cleaning gums, teeth, and ulcers also effective in fistulous sores. Ayurvedic medicine described the root and bark as alexipharmic, anthelmintic and useful in abdominal enlargement, diseases of the eye, skin, vagina, itch, piles, splenomegaly, tumors, ulcers and wounds.The leaves are used as anthelmintic, digestive, laxative, inflammations, piles and wounds.The fruit and seed for keratitis, urinary discharges, diseases of the brain, eye, head, and skin [14]. Unani uses the ash of leaves to strengthen the teeth, the seed, carminative, depurative, for chest complaints, chronic fevers, earache, hydrocele, and lumbago [15]. Scientific studies of the plant include for its antibacterial [16], antifungal [17], anti-inflammatory [18], antioxidant [19], anticancer [20], antipyretic, antinociceptive [18], antidiarrheal [21]and anti-lice activity. Hence the present study was carried out to find the efficacy of *Pongamia pinnata* leaves claiming the traditional use and its bioactivity guided fractionation for antiurolithiatic activity.

MATERIALS AND METHODS:

The leaves of the *Pongamia pinnata* were collected in May 2019 at T. John college of pharmacy campus, Bangalore south district, Karnataka. The plant was authenticated and confirmed leaves of *Pongamia pinnata* belonging to the Leguminosae family by Dr. V Rama Rao, Research officer (S-2) Department of Botany, RARIMD, Uttarahalli, Bangalore, Karnataka. The leaves were washed with tap water and dried under shade.Air-dried leaves were coarsely ground using a Remi Laboratory Mixer and sieved to #24 and stored in airtight containers. 10gm of the powdered plant material was extracted using 250ml of the solvents. The extracts were prepared by successive solvent extraction in a soxhlet assembly with solvents in the increasing order of polarity. Petroleum ether 60-80º C, chloroform 55-70º C, ethanol 60-80º C and water 100º C. The process of extraction was carried out up to 6-7 cycles, till the solvent in siphon tube of an extractor becamecolorless. The extracts were filtered separately, concentrated in a Rotavapor (Buchi) at 30̊ C. The color, consistency, and %yield of the extracts was noted and stored at 4± 1.0 ºC for further analysis.

Preliminary phytochemical screening: Qualitative analysis of ethanolic extractsof *Pongamia pinnata* leaves was performed for the identification of various classes of active chemical constituents like alkaloids, carbohydrates, glycosides, proteins, amino acids, steroids using different methods of Harborne [24]. Petroleum ether extract shows the presence of alkaloids and glycosides. Chloroform extract does not show the presence of any phytochemicals. Ethanol extract shows the presence of saponins, phenolic compounds, tannins, flavonoids, terpenoids, and amino acids. The aqueous extract shows the presence of saponins and terpenoids.

***In-vitro* Anti-urolithiatic Activity by turbidity method:** *In-vitro* anti-urolithiatic activity of *Pongamia pinnata* leavesof various leaves extract were tested in terms of inhibition of calcium oxalate formation by the extracts and absence of inhibitors. The method was taken as a reference from the research articles [25]. The precipitation of calcium oxalate at37º Cand pH 6.8 has been studied by the measurement of turbidity by UV/Vis spectrophotometer at 620nm. It was employed to measure the turbidity caused due to formation of calcium oxalate. To evaluate calcium oxalate inhibition of plant extracts by absorptions was noted and the uncontrolled growth of the stone nucleus for the comparison of growth in the presence of plant extracts was also observed.

Study without inhibition: Take 1ml of 0.025M calcium chloride dihydrate solution, 2ml of tris-buffer (pH7.4) was added in a test tube. Following, 1.0ml of 0.025M sodium oxalate was added.The white turbid solution is formed in the test tube. Mix gently, then place the test tube without disturbing for 10 minutes to measure the turbidity of the solution by UV/Vis spectrophotometer at 620nm. This control experiment was done in three trials.

Study with an inhibitor: In this experiment, 1ml of 0.025M calcium chloride dihydrate, 2ml Tris-buffer (pH7.4), 1ml of 0.025M sodium oxalate and 1ml (0.1,0.2,0.3,0.4,0.5,0.6,0.7,0.8,0.9,1.0mg/ml concentrations) of petroleum ether, chloroform, ethanolic and aqueous plant extracts were added in a four sets of test tubes. Two more test tubes were added with 1ml of 0.025M calcium chloride dihydrate, 2ml Tris-buffer(pH7.4). 1ml of 0.025M sodium oxalate was added to each test tube and then place the test tube without disturbing for 10 minutes to measure the change in turbidity of the solution by UV/Vis spectrophotometer at 620nm. Each procedure was performed three trials. The % Dissolution of each extract was calculated using the formula.

Yield Calculation: % Inhibition= x/y x 100

X= Sample + Calcium chloride dihydrate +Tris buffer + Sodium oxalate, Y= Calcium chloride dihydrate +Tris buffer + Sodium oxalate.

TLC PROFILING OF ETHANOLIC EXTRACT: The TLC profile of aqueous extract was developed by the method of optimization using different solvent systems to show maximum separation. 2mg of the ethanolic fraction was dissolved in Toluene: ethyl acetate: methanol. The sample was applied as thin bands on silica gel 60 F254 (0.25µ) pre-coated plates (Merck). The plates were developed in different solvent systems and air-dried.Visualization was done in daylight, 254 and 366nm. Later the plates sprayed with vanillin-sulphuric acid and heated in hot air oven (maintained at 105^0 C) for 15minutes. The Rf values were calculated and recorded.

Characterization analysis using liquid chromatography-mass spectroscopy:

The LC-MS studies were performed to separate the chemical constituents simultaneously to understand the molecular weight of the compound. These help in the prediction of the chemical structure for further understanding of compounds.

Instrumentation and analytical conditions: The LC-MS studies were performed using Agilent 1200 series HPLC and Agilent 6120 single quadrupole mass spectrometric detector with atmospheric pressure chemical ionization (APCI) technique (Agilent technology, CA, USA).

Table 1: Nature and % yield of extracts

Extract	% yield	Colour	Consistency
Petroleum Ether	1.03+0.05	Yellowish green	Sticky
Chloroform	1.86+0.04	Dark green	Sticky
Ethanol	2.14+0.05	Brownish green	Sticky
Aqueous	1.43+0.07	Dark brown	Sticky

Table 2: Phytochemical tests for successive extracts of *Pongamia pinnata* Leaves:

Tests	Petroleum ether extract	Chloroform extract	Ethanolic extract	Aqueous extract
1)Alkaloids				
a) Mayer's test	+ve	-ve	-ve	-ve
b) Hagner's test	+ve	-ve	-ve	-ve
2)Glycosides				
a) Keller killani test	+ve	-ve	-ve	-ve
b) Legal test	+ve	-ve	-ve	-ve
3) Flavonoids				
a) Shinoda test	-ve	-ve	+ve	-ve
b) Lead acetate test	-ve	-ve	+ve	-ve
4) Tannins/ Phenolic compounds				
a) Iodine test	-ve	-ve	+ve	-ve
b) 5% $FeCl_3$ test	-ve	-ve	+ve	-ve
5) Terpenoids				
a) Salkowski's test	-ve	-ve	+ve	+ve
b) Liebermann Burchard's Reaction	-ve	-ve	+ve	+ve
6)Amino acids				
a) Ninhydrin test	-ve	-ve	+ve	-ve
b) Tyrosine test	-ve	-ve	+ve	-ve
7) Saponins				
a) Froth test	-ve	-ve	+ve	+ve

Phytochemical Tests Of Ethanolic Extract Of Pongamia Pinnata Linn.

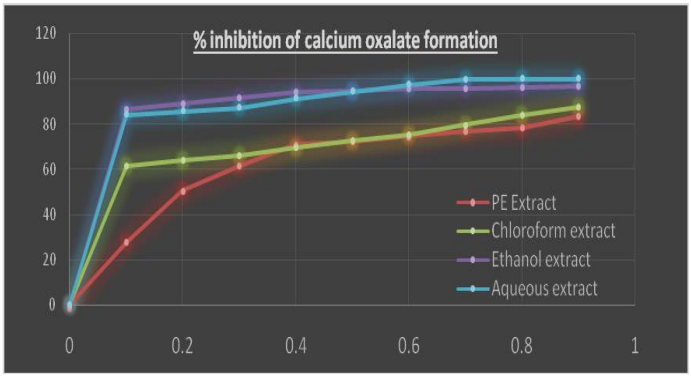
"+" ve represents Present and "-"ve represents Absent

Table.3: The percentage inhibition of various extracts of *Pongamia pinnata* at different concentrations.

SL No	Concentration mg/ml)	Petroleum Ether extract	Chloroform Extract	Ethanolic extract	Aqueous extract
1.	0.1mg/ml	27.7	61.2	86.2	84.3
2.	0.2mg/ml	50.6	63.7	89.0	85.7
3.	0.3mg/ml	61.4	65.7	91.7	87.3
4.	0.4mg/ml	71.0	69.3	94.1	91.4
5.	0.5mg/ml	72.2	72.4	94.8	94.3
6.	0.6mg/ml	74.6	75.2	95.5	97.3
7.	0.7mg/ml	76.8	79.4	95.8	99.8
8.	0.8mg/ml	78.3	83.9	96.2	100
9.	0.9mg/ml	83.5	87.3	96.6	100
10.	1.0mg/ml	86.0	91.2	97.0	100

Table 3: Optimization of solvent system ethanolic extract

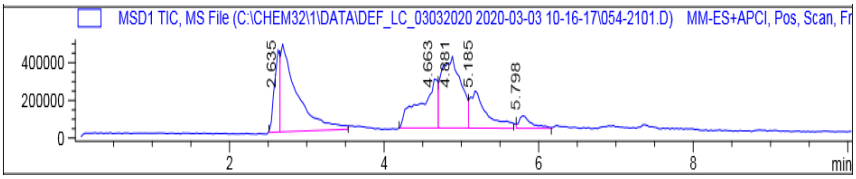
Solvent system	Detection by UV@254nm		Detection by Vanillin sulphuric acid	
	No. of bands	Rf value	No. of bands	Rf value
Toluene: EA: Methanol (45:3.5:1.5)	2	0.39, 0.43	7	0.04,0.11,0.16,0.72, 0.76,0.81,0.83
Toluene: EA (8:2)	1	0.39	5	0.05, 0.15, 0.21, 0.61, 0.79.
Petroleum ether: Chloroform (7:3)	-	-	3	0.04, 0.10, 0.22



% Inhibition of crystals vs Concentration of extract

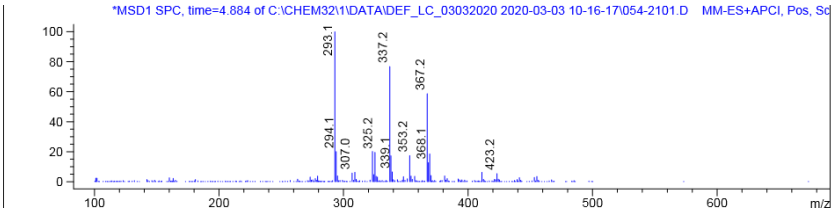
Figure 1: Inhibition of calcium oxalate formation by various extracts:

Figure 2: TLC profiles of *Pongamia pinnata* ethanolic extract.



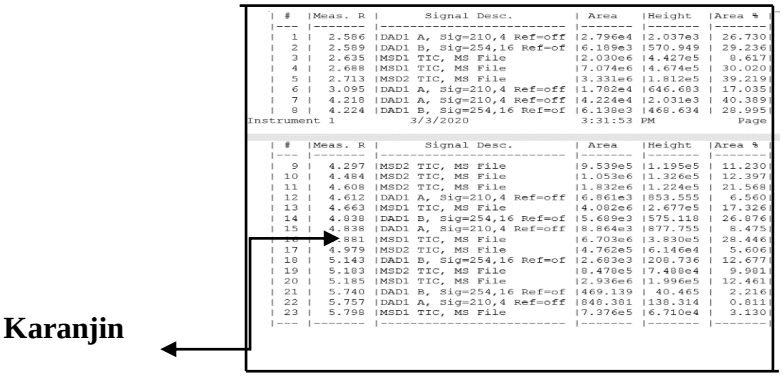
Relative intensity (AU) vs time (min)

Figure 3: Chromatogram acquired from the ethanolic extract of *Pongamia pinnata* leaves



Relative abundance vs mass/charge ratio (m/z)

Figure 4: Mass spectrum acquired from ethanolic extract of *Pongamia pinnata* leaves.



#	Meas. R	Signal Desc.	Area	Height	Area %
1	2.586	DAD1 A, Sig=210,4 Ref=off	2.796e4	2.037e3	26.730
2	2.589	DAD1 B, Sig=254,16 Ref=of	6.189e3	570.949	29.236
3	2.635	MSD1 TIC, MS File	2.030e6	4.427e5	8.617
4	2.688	MSD1 TIC, MS File	7.074e6	4.674e5	30.020
5	2.713	MSD2 TIC, MS File	3.331e6	1.812e5	39.219
6	3.095	DAD1 A, Sig=210,4 Ref=off	1.782e4	646.683	17.035
7	4.218	DAD1 A, Sig=210,4 Ref=off	4.224e4	2.031e3	40.389
8	4.224	DAD1 B, Sig=254,16 Ref=of	6.139e3	468.634	28.995

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#	Meas. R	Signal Desc.	Area	Height	Area %
9	4.297	MSD2 TIC, MS File	9.539e5	1.195e5	11.230
10	4.484	MSD2 TIC, MS File	1.053e6	1.326e5	12.397
11	4.608	MSD2 TIC, MS File	1.832e6	1.224e5	21.568
12	4.612	DAD1 A, Sig=210,4 Ref=off	6.861e3	853.555	6.560
13	4.663	MSD1 TIC, MS File	4.082e6	2.677e5	17.326
14	4.838	DAD1 B, Sig=254,16 Ref=of	5.609e3	575.118	26.876
15	4.838	DAD1 A, Sig=210,4 Ref=off	8.864e3	877.755	8.475
16	4.881	MSD1 TIC, MS File	6.703e6	3.830e5	28.446
17	4.979	MSD2 TIC, MS File	4.762e5	6.146e4	5.606
18	5.143	DAD1 B, Sig=254,16 Ref=of	2.683e3	208.736	12.677
19	5.183	MSD2 TIC, MS File	8.470e5	7.488e4	9.981
20	5.185	MSD1 TIC, MS File	2.936e6	1.996e5	12.461
21	5.740	DAD1 B, Sig=254,16 Ref=of	469.139	40.465	2.216
22	5.757	DAD1 A, Sig=210,4 Ref=off	848.381	138.314	0.811
23	5.798	MSD1 TIC, MS File	7.376e5	6.710e4	3.130

Figure 5: LC-MS profiles of ethanolic extractof *Pongamia pinnata* leaves.

STRUCTURE OF KARANJIN:

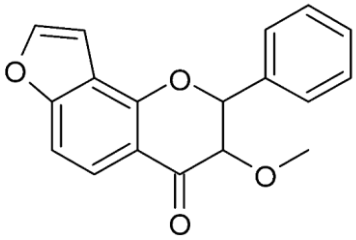

Figure 6: Chemical structure of 'Karanjin"

Data were acquired employing Agilent 6120 Quantitative Analysis using Mass hunter workstation software. The operation conditions were followed such as Capillary voltage for positive 4000V and negative 2500V. The source temperature was maintained 200^{0}C, drying gas flow 12.01/min, the current is maintained at 10.0µAand nebulization pressure was maintained at 35 psi. Chromatographic separation was performedon AgilentInertsil ODS-3V C18 150 mm × 4.6 mm i.d., 3.5µm) column with a Security Guard (4.0x3.0 mm i.d., 5 mm) The mobile phase "A" was acetonitrile, and the mobile phase "B" was 0.1% aqueous formic acid. The linear gradient elution program was performed as follows: 0–1 min, 20% A; 1–1.2 min, 20–90% A; 1.2–3.5 min, 90% A and 3.5–6.6 min, 90–20% A. The mobile phase was delivered at a flow rate of 1.2 mL/min by a post-column split ratio of 1: 1 with a three-way joint. To assure the reproducibility of the retention time the column temperature was maintained at 25^{0}C,injection volume of 20µl and Diode array detector was used in the instrument at wavelength 254nm for maximum absorbance.

LC-MS ANALYSIS: LC–APCI–MS parameters were tuned in both positive and negative ionization modes for karanjin. A good response was achieved in positive ionization mode. Figure 4 shows the protonated molecular ions of karanjin (m/z 293.1). Owing to the high responses of the protonated molecular ions of analytes,

quantitation of karanjin was carried out through MSD mode at m/z 293.1. The chromatographic retention times were 2.635 min, 4.663 min, 4.881, 5.185 and 5.798 minutes seen in figure 3. The maximum retention time of the Karanjin was found at 4.881 min shown the maximum mass/charge ratio as 293.1, which proved the presence of Karanjin in ethanolic leaves extract of *Pongamia pinnata*. The internal standard reference was taken from the article which was proven, showed maximum retention time at 4.8 minutes and molecular weight at positive mode was shown as 293.2 for Karanjin[23].

RESULTS: In the present study, the leaves of *Pongamia pinnata Linn*. were selected to study the antiurolithiatic potential by a methodical approach of using simple in-vitro studies based on its traditional use. The nature and yield of successive solvent extracts were recorded which gave a maximum yield of ethanolic extract of 2.14gm followed by chloroform, Aqueous and petroleum ether extract.The preliminary screening for*in-vitro* antiurolithiatic activity by the turbidity method was carried out on all the extracts accordingly.The percentage dissolution of Calcium oxalate by the successive solvent extracts was found to be 27.7%, 61.2%, 86.2%, 84.3% for petroleum ether, chloroform, ethanol, and aqueous extracts respectively in Table 2.Further, the preliminary phytochemical screening of Ethanolic extracts shown the presence ofsaponins, phenolic compounds, tannins, flavonoids, terpenoids, and amino acids.As a step ahead towards identifying the principal constituents the TLC profiles in table 3 is established in the present investigation.The TLC studies inmobile phase toluene: ethyl acetate: methanol(45:3.5:1.5) showed the presence of maximum bands in UV 254 nm with Rf value 0.39, 0.43 two bands with Rf value 0.53 and 0.66 in UV 366nm. After spraying with vanillin-sulphuric acid, seven bands with Rf values 0.04,0.11,0.16,0.72, 0.76,0.81,0.83 were obtained. The LC-MS analysis was performed by Agilent instrument which shown the retention time at 4.881 minutes. The positive mode of ionization shown the molecular weight 493.1 in figure 4, which confirmed the presence of karanjin in the ethanolic extract of *Pongamia pinnata*Leaves.

DISCUSSION: Urolithiasis is the third most common disorder of the urinary tract the others being frequently occurring urinary tract infections and benign prostatic hyperplasia/ prostate diseases.Since the main intention of treatment is enhancing the excretion of stone-forming constituent elements and altering the physicochemical environment which influences formation many drugs are lined up in the market. However, with current therapy, the risks of reoccurrence are too high. Thus, there is retrieval of interest in safer plant drugs. The presented study on the in-vitro antiurolithiatic activity on*Pongamia pinnata Linn*. leavesshows that there is a greater dissolution of Ca-Ox by the ethanolic extract than the other extracts.The diuretic property attributed to the plant can be inferred from the presented in-vitro antiurolithiatic turbidity study.From the TLC studies, it is evident that ethanolic extract contains many constituents that are also likely to contribute to its property of "Paashanabheda- the stone breaker". An attempt was made to develop the LC-MS parameters to separate the chemical constituents and find the molecular weight of the components. The method that could effectively separate and accurately quantify karanjin in the ethanolic extract, which also shown the other chemical constituents but the maximum ionization of the components was failed in Mass spectroscopy. In the future bymethod validation and development by APCI ionization or other ionization techniques which can be tried to attain proper separation and ionization of the other components. Other constituents which may be also responsible for the antiurolithiatic potential are under investigation.

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