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CHEMICAL COMPOSITION AND *IN VITRO* ANTILITHIATIC POTENTIAL OF BIOFLAVONOIDS FRACTIONATED FROM *PHYLLANTHUS NIRURI* SEED

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ABSTRACT

Kidney stone (Lithiasis) is the formation of calculi or urinary stone which is a concretion of material mainly mineral salts in any part of the body. One of the major causes of acute and chronic renal failure is lithiasis (Stone formation) which includes both nephrolithiasis (stone formation in the kidney) and urolithiasis (stone formation in the ureter or bladder or both). Of many types of stones that are formed, the most common are calcium oxalate. The formation of such concretion encompasses several physicochemical events beginning with crystal nucleation, growth, aggregation, and ending by retention within urinary tract. The present study is to reveal the in vitro antilithiatic activity of Phyllanthus niruri (Fam: Phyllanthaceae) seeds and its chemical characterization. Phyllanthus niruri (Fam: Phyllanthaceae) is a widespread tropical plant commonly known as stone breaker. It is a plant used for the treatment of kidney stone by tribal people. The seeds of this plant are suggested for the patients of the kidney stone. From this project it can be concluded that the phenolic compounds isolated from seed extract is able to inhibit CaC_2O_4 crystallization in vitro and thus it can be confirmed to have antilithiatic property.

KEYWORDS: ***Lithiasis, calculi, urinary stone, nephrolithiasis, urolithiasis, nucleation, aggregation.***

INTRODUCTION

In developing country, about 80% of population use traditional medicine against primary medical problem. In past decade, research in plant sciences is of interest. Due to the development in technology new tools are innovated; which results in novel drugs, isolated and synthesized from plant origin. There is necessary to complete evaluation of medicinal plants used in folk medicine, it could be lead to advanced drug discovery. Now a days the trend of characterization of such green medicine is underutilized, which is safe and more dependable. [1]. Urolithiasis is characterized by the formation of a stone in thekidneys or urinary tracts. A large number of people, nearly 4–15% ofthe human populations are suffering from urinary stone problem allover the globe [2].The crystals of calcium oxalate (CaOx) are theprimary constituent of more than 60% of the majority of humankidney stones; they exist in the form of CaOx monohydrate (COM)and CaOxdihydrate (COD) [3].

Nucleation, crystal growth, crystal aggregation and crystal retention are the major steps for pathogenesis of calcium oxalatestone formation.The stone formation requires supersaturated urine. Supersaturation also depends on urinary pH, ionic strength, solute concentration and complexations [4]. There is nosatisfactory drug being used in clinical therapy for removal of stone, inspite it is done by shock wave lithotripsy which is prohibitivelycostly and recurrence [5].Thus a drug for the prevention of this disease or its recurrence would be of great interest. *Phyllanthus niruri* Linn. (Bhuiamla) hasoccupied an important place in Indian culture and folk medicines. Ithas been used in all most all the traditional systems of medicine viz.,*Ayurveda*, *Unanai* and *Sidha*. From the ancient time the tribal andrural people of our country commonly used this herb in treating various disorders. *P. niruri* has also been used traditionally for treating liver problems like hepatitis, elimination of mucous, kidney stones and diuretic problems [6,7,8].Keeping above knowledge in the mind, current study was done tofind out the stone formation inhibitor effect and stone dissolving effect of bioflavonoids and identification of bioactive molecule fractionated from*P. niruri*seeds.

MATERIALS AND METHODS

Collection and identification of the plant species: Collection of the *Phyllanthus niruri* seeds was done from forest area of Sangamner tehsil. Identification of plant was done in Department of Botany, Sangamner College, Sangamner by using Flora of Maharashtra.

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Extraction and fractionation: Collected seeds of the plant will be shade dried for 15 days and grind to fine powder. Hydromethanolic extract will be prepared by maceration technique. The Phenols will be isolated using standard fractionation method. Each of the fraction will be filtered through four layers of gauze, and then filtrates will be passed through a Whatman No. 1 filter paper. The resulting double filtrates were then concentrated in a rotary evaporator.

Phytochemical Screening:The seed powder was used for the preliminary phytochemical screening for the identification of the various classes of active chemical constituents, using standard prescribed methods [9.10]. The positive tests were noted as weak (+), moderate (++), strong (+++) and absent (-).

Alkaloids: 1ml of the seed powder filtrate was mixed with 2 ml of Dragendoff s reagent; a turbid orange colourindicated the presence of alkaloids. The confirmation test was done using Mayer's reagent; a yellow precipitate indicated the presence of the alkaloids.

Tannins: 1 ml of the filtrate was mixed with 2 ml of $FeCl_3$; a dark green colour indicated a positive test for the tannins.

Saponins: 1 ml of the plant filtrate was diluted with 2 ml of distilled water, the mixture was vigorously shaken and left to stand for 10 min during which time, the development of foam on the surface of the mixture lasting for more than 10 min, indicates the presence of saponins.

Anthraqmnones: 1 ml of the plant filtrate was shaken with 10 ml of benzene; the mixture was filtered and 5 ml of 10% (v/v) ammonia was added, then shaken and observed. A pinkish solution indicates a positive test.

Flavonoids: 1 ml of leaves filtrate was mixed with 2 ml of 10% lead acetate; a brownish precipitate indicated a positive test for the phenolic flavonoids. While for flavonoids, 1 ml of the plant filtrate was mixed with 2 ml of dilute NaOH; a golden yellow colour indicated the presence of flavonoids.

Steroid: 1ml of extract was dissolve in 10 ml of chloroform and equal volume of conc.H_2SO_4 was added from the side of the test tube. Upper layer turns red and H_2SO_4 layer turns yellow with green fluroscence shows the presence of steroid.

Anthocyanin: 2ml of methanolic extract was mixed with 2N HCl and equal volume of NH_3. Pink red colour turns to blue voiolet indicates the presence of anthocyanin.

Caumarin: 3ml of 10% NaOH was mixed with 2ml of methanolic extract the formation of yellow colour indicates the presence of coumarin.

Emoidin: 2ml of NH_4OH and 3ml of benzene was mixed with extract confirmation test indicates the appearance of red colour.

Protein: Few drop of conc.HNO_3 was mixed with methanolic extract formation of yellow colour indicates the presence of protein.

Reducing sugar: 10 drop of the copper acetate solution was mixed in the filtrate. Formation of emerald green colour indicates the presence of reducing sugar.

Phytosterol: The mixture of extract and chloroform was filtrate using whatman no.1 filter paper shaken well after adding few drop of conc. H_2SO_4 and allow standing. The appearance of golden yellow colou indicates the presence of Phytosterol.

Phenol: Test extract was treated with 4 drop of alcoholic $FeCl_3$ solution formation of bluish black colour indicates the presence of phenol.

Phlobatannin: Aqueous extract of plant powder was boiled with 1% aqu. HCl. Deposition of red precipitate indicates the presence of Plobatatnnin.

Leucoanthocyanin: Equal volume of isoamyl alcohol and aqu. Extract was mixed. The red upper layer indicates the presence of Leucoanthocyanin.

Cardinal glycoside: 2ml of glacial acetic acid containing a drop of $FeCl_3$ was mixed with a filtate appearance of brown colour ring indicates the presence of Cardinal glycoside.

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SCREENING FOR ANTILITHIATIC ACTIVITY

Preparation of synthetic urine: The artificial urine was prepared according to the method of Burns and Finlayson (1980) and had the following com-position: 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 oxalate0.32 mmol/L, ammonium hydroxide 17.9 mmol/L, and ammonium chloride 0.0028 mmol/L. The synthetic urine was freshly prepared each day and pH adjusted to 6.0.

Nucleation Assay: The inhibitory activity of the fractions on nucleation of CaC_2O_4crystals were determined based on the spectrophotometric assay [11]. The calcium chloride solution (50 mmol/L) and sodium oxalate solution (50 mmol/L) were prepared in a buffer containing Tris 0.05 mol/L and NaCl 0.15 mol/L at pH 6.5 at a temperature of 37◦C. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that have reached a critical size and thus become optically detectable) in the presence of the extract and that of the control without extract. The extract was prepared in distilled water, filtered and concentrations of 1, 2, 5, 7.5 and 10 mg/ml were obtained. The absorbance was recorded at 620 nm using UV-Visible spectrophotometer. The percent inhibitions of plant extracts and cystone will be calculated.
Percent Inhibition = 1 – Si/Sc × 100
Where; *S*i: Slope of graph in the presence of inhibitor (extract),
*S*c: Slope of control (without inhibitor).

Gas chromatography and mass spectrophotometric analysis: The extract, was dissolved in analytical grade methanol and analyzed by gas chromatography (GC) coupled with a mass spectrometer (MS) using a THERMO GC (TRACE 1300) with a fused silica capillary column, PE-5 (50m× 0.32mm, film thickness 0.25µm) and a triple quadrapole Thermo MS (TSQ 8000) mass spectrometer. A sample of 5.0µl was injected in the split mode with split ratio 10:1. An electron ionization (EI) system, with electron energy of 70 eV and emission current 200 µA was used for GC-MS detection. Helium was used as a carrier gas at a flow rate of 1 ml/min and ionization temperature was kept at 200^{o}C. The GC-MS was equipped with Dyna Max XR detection system having discrete dynode electron multiplier and electrometer. The mass scanning range was varied over 40-550 Da and for run time of 40 min. The components of the extract was identified by their retention time and compared with mass spectrum data from the National Institute Standard and Technology (NIST) library available with the GC-MS system.

Statistical analysis: The results are expressed as mean ± SEM. Statistical analysis and linear regression analysis was performed using Graph-Pad Instat, software, and version 3.0. The values were analyzed by one-way Analysis of Variance (ANOVA) at a significance level of p <0.05. The IC_{50}values were calculated by analysis using Chi-square test.

RESULT AND DISCUSSION

Phytochemical tests: Phytochemical Analysis (Table 01) shows the presence of Sapponin, tannin, Protein, Flavanoid, Phenol, Cardiac glycosidase. While the absence of Alkaloid, Phobatannin, Leucoanthocyanin, and Steroid.

Table 01: Phytochemical Tests.

Test	*Phylanthusniruri*
Steroid	-
Tannin	-
Sapponin	+++
Anthocynanin	-
Caumarin	+++
Emoidin	-
Alkaloid	+++
Proteins	+++
Flavonoid	+++
Phytosterol	+++
Phenol	+++
Phobatatnnin	-
Leucoanthocyanin	-
Cardiac glycoside	+++
Reducing Sugar	+++

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Table 02:Nucleation Assay.

	Phyllanthus niruri		
	PNDEE	PNEA	Cystol
Conc. (mg)	% inhibition	% inhibition	% inhibition
0	0	0	0
1	15.03±0.64	17.67±0.34	16.54±0.15
2	32.64±0.15	36.48±0.96	33.01±0.94
5	44.03±0.15	51.97±0.01	49.87±0.19
7.5	59.15±0.63	67.84±0.44	61.04±0.67
10	74.03±0.51	96.45±0.06	82.94±0.64

Nucleation assay: In the nucleation assay, the number of crystals formed was estimated in terms of the turbidity of the solution. The absorbance of control recorded was subtracted from that obtained with the seed extract. There was a steep decrease in the absorbance with the increase in the concentration of the extract and cystone when incubated along with sodium oxalate (50 mM). The percent inhibition of the extract was in the range of 15-74% (Table: 02). However, cystone showed the percent inhibition in the range of 16-82%. There was a dose dependent increase in percent inhibition of nucleation by the extract (r^2= 0.899) and cystone (r^2= 0.917).

GCMS Analysis: The PNDEE fraction of bioflavonoid shows the presence of 3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene1,-1',4,4'-tetrone,9-2',2'Dimethylpropanoilhydrazono)-3,6-dichloro-2,7bis-[2-(diethylamino)-ethoxy]Fluorine,2-Myristynoyl Pantetheine, ButylatedHydroxytoluene, Fumaric acid, 2-chlorophenyl ethyl ester, Benzoic acid, 2-(1-phenylethyl)-Naphthalene, 1,6-dimethyl-4-(1-methylethyl)- Azulene, 1,4-dimethyl-7-(1-methylethyl).As well as PNEA fraction shows the presence of Benzene, Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-Phenol,2-(1-phenylethyl)- 6-Isopropyl-1,4-dimethylnaphthalene,Dibutyl phthalate, Phthalicacid,butyl hept-4-yl ester, Phthalic acid, butyl hex-3-yl ester, Octadecenoic acid, Acetic acid n-octadecyl ester, Eicosyl acetate, Heneicosyl acetate, N,N-Dimethyldodecanamide, 9-Octadecenamide, 13-Docosenamide, 2,4-bis(1-phenylethyl)-Methanone, [1,4-dimethyl-7-(1-methylethyl)- 2-azulenyl] Phenyl-Methanone, 4-(1,3-Diphenylbutyl) phenol, 1,3-Diphenyl-1-(2-hydroxyphenyl) Butane, Diphenyl-cyclohexyl-methanol, Non-7-enoic acid, Phenol, 2,4-bis(1-phenylethyl)- Methanone, [1,4-dimethyl-7-(1methylethyl) -2-azulenyl] Phenyl-Methanone, Phthalic acid, di(2-propylpentyl) Ester, Diisooctyl phthalate, Bis(2-ethylhexyl) Phthalate, 4-(1,3-Diphenylbutyl) Phenol, 1,3Diphenyl-1-(2-hydroxyphenyl) Butane, Cyclopropanecarboxylic acid, Cannabinol, 2,4-Bis[2-(4-methoxyphenyl-2-propyl)]methoxybenzene.

CONCLUSION

Plants and other natural substances have been used as the rich source of medicine. All ancient civilizations have documented medicinal uses of plant in their own ethnobotanical texts. The list of drugs obtained from plant source is fairly extensive. Ayurveda, an indigenous system of Indian medicine, offers vast scope for the successful treatment of urolithiasis. Many remedies have been employed during ages to treat urolithiasis. Most of the remedies were taken from plants and proved to be useful, though the rationale behind their use is not scientifically established except for a few plants and some proprietary composite herbal drugs. Considering the present view, many plants are employed for the treatment of kidney stone by the tribes. This can be a good source for alternative therapeutic agents for future lead drugs. It can be concluded that the phenolic compounds isolated from seed extract is able to inhibit CaC_2O_4 crystallization *in vitro* and thus it can be confirmed to have antilithiatic property. However, further *in vivo* studies in animal models and clinical trials are needed for evaluating its potential therapeutic values.From this workit is concluded that the chemical compounds which are responsible to dissolve the crystals of calcium oxalate with effective dose.

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