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Original Article

STUDY ON *IN VITRO* ANTI-LITHIATIC ACTIVITY OF *PHYLLANTHUS NIRURI* LINN. LEAVES BY HOMOGENOUS PRECIPITATION AND TURBIDITORY METHOD

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ABSTRACT

Objective: Kidney stone is one of the most prevalent diseases worldwide and calcium oxalate has been shown to be the main component of the majority of stones formed in the urinary system of the patients. Traditional knowledge on use of medicinal plants for curing chronic diseases is proving its worth to modern society too. In this regards, study was conducted with an objective to find out the role of *Phyllanthus niruri* Linn. leaves extracts to inhibit stone formation and dissolution of already exiting stone in renal system using *in vitro* methods in the laboratory

Methods: Leaves powder was serially extracted in petroleum ether, ethyl acetate, methanol and water. *In vitro* study was conducted to assess anti-urolithiatic effect of plant for all the extracts with two standard drugs namely, Neeri and Cystone as control. Two methods, turbidity method and calcium oxalate dissolution methods were practiced to access the inhibition of stone formation and dissolution of stone crystals respectively. Microscopic study was done for the comparative evaluation of crystal density and size in each treatment in turbidity method.

Results: Water extract of plant leaves proved its potential statistically equal to the standard drug, cystone in dissolving the exiting calcium oxalate crystals. Water extract could dissolve 56.8% crystals while cystone dissolved 58.4% crystals in *in vitro* study and found statistically at par. Water extract also could inhibit up to 53.09% aggregation of calcium oxalate crystals as compared to the cystone with 76.54%. Among other extracts, methanol extract got second position in anti-lithiatic activity.

Conclusion: Common medical practice recommends use of cystone as effective medicine for preventing as well as treating renal stone which found at par with the water extract of plant leaves to inhibit the stone formation even in its crude form. Results guide us for the further detailed investigation and development of new drugs from this medicinal plant.

Keyword: - Kidney stone, Kidney diseases, Renal stone

INTRODUCTION

All over the world especially in developing countries, approximately 80% of population continues to use traditional medicine in primary medical problems. In the past decade, therefore, research has been focused on scientific evaluation of traditional drugs of plant origin. There is an urgent need to systematically evaluate the plants used in traditional medicine. Such research could lead to new drug discovery or advance use of indigenous herbal medicines for treatment. This revival of interest in plant derived drugs is mainly due to the current widespread belief that green medicine is safe and more dependable than the costly synthetic drugs many of which have adverse side effects [1].

Urolithiasis is characterized by the formation of a stone in the kidneys or urinary tracts. A large number of people, nearly 4–15% of the human populations are suffering from urinary stone problem all over the globe [2].The crystals of calcium oxalate (CaOx) are the primary constituent of more than 60% of the majority of human kidney stones; they exist in the form of CaOx monohydrate (COM) and CaOx dihydrate (COD) [3]. The pathogenesis of calcium oxalate stone formation is a multi-step process and in essence includes-nucleation, crystal growth, crystal aggregation and crystal retention. The stone formation requires supersaturated urine. Super-saturation also depends on urinary pH, ionic strength, solute concentration and complexations [4]. In spite of substantial progress in the pathophysiology and treatment of urolithiasis, there is no satisfactory drug being used in clinical therapy. Endoscopic stone removal and extracorporeal shock wave lithotripsy are prohibitively costly and recurrence is quite common with these procedures [5]. Thus a drug for the prevention of this disease or its recurrence would be of great interest. *Phyllanthus niruri* Linn. (Bhui amla) has occupied an important place in Indian culture and folk medicines. It has been used in all most all the traditional systems of medicine viz., *Ayurveda, Unanai* and *Sidha*. From the ancient time the tribal and rural 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 to find out the stone formation inhibitor effect and stone dissolving effect of *P. niruri* extracts.

MATERIAL AND METHOD

Material collection and extract preparation

P. niruri leaves were collected in the month of October-November from the State Forest Research Institute, Jabalpur, Madhya Pradesh (M.P.), India and identification of the plant species was done in collaboration with Dept. of Botany, Govt. Model Science College, Jabalpur, M.P., India. Collected leaves were shade dried for 2 week and powdered using grinder. Weighed 500gm powdered material was loaded in Soxhlet extraction assembly. Extraction was done serially in petroleum ether, ethyl acetate, methanol up to 60 cycles and finally it was suspended in to the water for getting aqueous extract. All the extracts collected were first distilled and filtered with Whatman filter paper#1 in a Buchner funnel under vacuum pressure. The filtrate was evaporated using rotatory evaporator under reduced pressure to dry the material. In this way different crude extracts of *P. niruri* leaves were obtained.

For the control treatments, Poly herbal formulation, cystone and neeri were used and their stock solutions were prepared by suspending them in DMSO solution (50mg/ml) and filtered through a 0.22 mm pore size filter paper. ANOVA (Analysis of Variance) was

performed on mean data obtained on percentage inhibition in nucleation of CaOx and percentage dissolution of CaOx crystals.

***In vitro* anti-lithiatic activity test by turbidity method**

In vitro anti-lithiatic activity of the extract was tested in terms of inhibition of calcium oxalate formation by the extracts in the presence and absence of inhibitors (standard drugs and extract). The Precipitation of calcium oxalate at 37°C and pH 6.8 has been studied by the measurement of turbidity at 620nm. A spectrophotometer UV/Vis (Shimadzu 1800) was employed to measure the turbidity caused due to formation of calcium oxalate in treatments[3].The method used was similar to that described by Burns and Finlayson[9] with some minor modifications.

First of all, growth of stone nucleus *in vitro* in the absence of any inhibitor was done. For this, a volume of 1.0 ml of 0.025M $CaCl_2$ and 2ml of Tris-buffer (pH 7.4) were added in a test tube.

Then 1.0 ml of 0.025M sodium oxalate was added. Formation of the turbidity results immediately after mixing of above chemicals and then the measurement of turbidity formed (in terms of absorption at 620 nm in UV/Vis spectrophotometer) was started immediately up to period of 10 min (600 seconds) after the mixing of the chemicals. This control experiment was done in six replications.

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.

Table 1: Shows reduction of CaOx nucleus formation by plants extracts compared with control drugs.

Treatment Groups	Absorbance at 620nm (after 180 min)	Regression equation	Reduction in turbidity (In %)
Control	1.0	y = 0.005x + 0.041	0.0[a]
Cystone	0.26	y = 0.001x + 0.011	76.54[f]
Neeri	0.21	y = 0.001x + 0.020	81.23[f]
Petroleum ether extract	0.93	y = 0.005x + 0.014	7.12[b]
Ethyl Acetate extract	0.79	y = 0.004x + 0.018	24.95[c]
Methanol extract	0.63	y = 0.003x + 0.019	43.71[d]
Water extract	0.43	y = 0.002x + 0.013	53.09[e]
$F_{(P<0.01)}$	-		106.35
Df	-		30
$SE_{(d)}\pm$	-		0.391
$LSD_{(P<0.05)}$	-		6.28

[a,bc]..Values designated with different alphabets are significantly different from each other.

Table 2: Shows dissolution of calcium oxalate by all extracts of *Phyllanthus niruri* and Standard drugs, Cystone and Neeri.

Treatments	Absorbance At 570nm (Mean ± Sd)	Amount of Calcium in solution (mg/dl)	Reduction in Calcium (mg/dl)	CaOx Dissolution (In %)
Control	0.760±0.06[e]	1.562	-	0.0
Cystone	0.235±0.07[b]	0.650	0.9125	58.4
Neeri	0.167±0.06[a]	0.550	1.0125	64.8
Petroleum ether extract	0.620±0.05[d]	1.313	0.2495	15.9
Ethyl Acetate extract	0.638±0.09[d]	1.325	0.2375	15.2
Methanol extract	0.346±0.08[c]	0.8375	0.7250	46.4
Water extract	0.247±0.05[b]	0.6750	0.8875	56.8
$F_{(P<0.01)}$	126.47	-	-	-
df	30	-	-	-
$SE_{(d)\pm}$	0.002	-	-	-
$LSD_{(P<0.05)}$	0.061	-	-	-

Now the study was continued to know the effect of plants extracts against stone nucleus formation *in vitro.* In this experiment four sets of test tubes with 1ml of 0.025M Calcium Chloride, 2ml Tris-buffer and 1ml (50 mg/ml solution) of four plant extracts were taken. Two more sets of test tubes were prepared same as the above in which synthetic drug of the poly herbal formulation, Cystone and Neeri were administered. Then 1 ml volume of 0.025M sodium oxalate was added to each test tube. Each set was replicated six times. Immediately after the mixing of sodium oxalate, measurement of change in turbidity of the solution was done up to the period of 10 min post mixing. Inhibition in stone nucleus formation was calculated by the graphical method using the following mathematical formula:

$$\text{Inhibition \%} = \{1-[Si / Sc]\} \times 100$$

Where;

Si: slope of graph in the presence of inhibitor (drugs/extracts).

Sc: slope of without Inhibitor (Control).

Light microscopy of the crystals formed in the solution with and without the administration of the inhibitors was also done. Photographs of CaOx were taken using the objective of 40X (Plate 1).

***In vitro* anti-lithiatic activity test by calcium oxalate dissolution method**

Behind this second experiment the idea was to know the role of plant extract in dissolving the already formed stones nucleus in renal system. For this artificial calcium oxalate crystal were prepare in the laboratory by standard method [10,11,12]. Also semi permeable membrane was prepared from egg using standard methods [13,14]. 5 mg of artificially prepared calcium oxalate crystals were weighed and were mixed with 1 ml (50 mg/ml solution) of the petroleum ether extract, ethyl acetate extract, methanol extract, water extract, cystone and neeri and packed in separate egg based semi permeable membranes by suturing. Each treatment was repeated six times. Now the material packed in semi permeable membrane was allowed to suspend in separate conical flasks containing 100 ml 0.1 M Tris buffer.

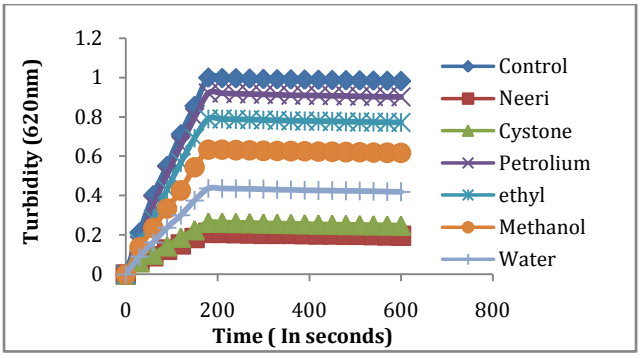

Fig. 1: Shows change in turbidity without and with plant extracts at 620nm.

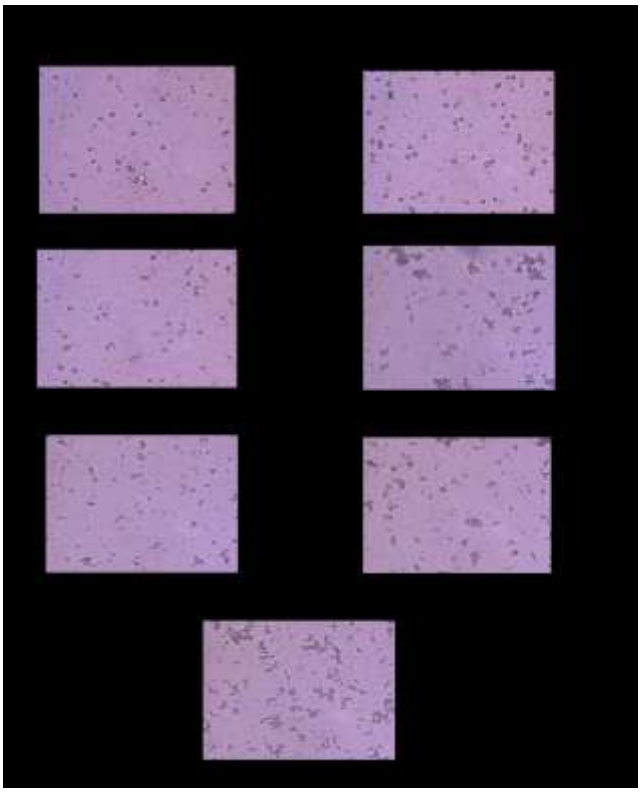

Plate 1: Shows formation of CaOx nucleus and inhibition by plant extracts and standard drugs in turbidity method.

One group served as negative control (contained only 5 mg of calcium oxalate in membrane). All the flasks were placed in incubator at 37±1^{0}C for 7 hrs. After 7 hrs, flasks with membrane were kept in magnetic stirrer for three days. After starring, membranes were taken out of the flask and content of each membrane was collected in different test tubes. Each tube then was added with 2 ml of 1 N sulfuric acid. From each test tube, 0.1ml of the suspension was taken into separate test tube and was added with 2 ml O-Cresolphthalein Complexone (O-CPC) indicator, which produces a purple color in solution[15,16]. Intensity of color produced was measured by spectroscopy method at 570 nm and using the corresponding absorption readings, concentration of calcium was determined from the standard calibration curve of calcium solution [10]. There is inverse relationship between the dissolution of calcium oxalate crystals and concentration of calcium ions in solutions.

RESULTS

In all the treatments concentration dependent initial steep rising in turbidity (nucleation) followed by decrease turbidity (aggregation) was seen. Maximum inhibition 53.09% in stone nucleus formation (turbidity) was seen by the water extract of the plant leaves after 180 seconds of chemical reaction started as compared to the control ($F_{(P<0.01)}$ = 106.3, df = 30, $SE_{(d)}$± = 0.39, $LSD_{(P<0.05)}$ = 6.28) which is significantly different from other extract. Methanol extract of the plant leaves got second position among plant leaves extracts in bio activity test which inhibited 43.71% of stone nucleus (turbidity) formation, followed by ethyl acetate extract which inhibited 24.95% in turbidity and are significantly different from each other. Least inhibition in nucleation was seen by petroleum ether extract which accounted only 7.12% inhibition. Two control drugs, Neeri and Cystone inhibited 81.23 and 76.54% in stone nucleation respectively and are significantly at par (P>0.05) to each other as shown in Table 1 and fig 1.

Photographs in Plate 1 show aggregation of calcium oxalate crystals in different treatments. Most of the nucleation and aggregation was visible in control and Petroleum ether extract treated tubes.

In Calcium oxalate dissolution study, effect of water extract was statistically equal to the effect of standard drug being used for dissolving the existing renal stone. Water extract of plant could dissolve 56.8% artificial calcium oxalate crystals packed in semi permeable and cystone could dissolve 58.4% artificial stone crystals and are statistically at par (P>0.05) ($F_{(P<0.01)}$ = 126.47, df = 30, $SE_{(d)}$± = 0.002, $LSD_{(P<0.05)}$ = 0.061). Methanol extract could dissolve 46.4% calcium oxalate crystals and got second rank among all plant extracts in dissolving effect. Petroleum ether and ethyl acetate extracts of plant leaves could dissolve 15.9 and 15.2% calcium oxalate crystals respectively and are significantly at par (P>0.05). Standard antilithiatic drug, Neeri could dissolve 64.8% of calcium oxalate crystals and was found superior over all the treatments in effect (Table 2).

DISCUSSION

Since the initial events of nucleation occurs in the first few minutes, the graphs were re-plotted within the first 3-min for each extract as well as for control[20]. In this study all the extracts of target plant leaves inhibited the stone crystal formation and dissolved the CaOx crystal also and thus proved the presence of some active antilithiatic compounds. Ability of extract to reduce the nucleation increases the metastable limit of oxalate in urine and prevents the precipitation of the CaOx crystal. Organic inhibitory compounds adsorb to the surface of the crystal, thereby inhibiting crystal nucleation, growth and aggregation [4]. Kidney stone formation is a complex process that results from a succession of several physico-chemical events including super saturation, nucleation, nucleus growth, nucleus aggregation, and retention within renal system and to prevent this physiological malfunction, variety of drugs are being used [17,18,19]. Target plants leaves have proved to have tremendous capacity to prevent the stone nucleus formation as well as extracts showed great potential to dissolve the existing stone crystal *in vitro*.

The exiting fact came out of the study is that the water extract of plant showed statistically equal potential as compared to standard drug cystone in dissolving the artificial stone crystals even in the crude form. Also the results came out of the turbidity inhibition experiment have great importance as water extract in crude form got second position in effect after the standard drugs, neeri and cystone. Up to 53.09% reduction in turbidity as compared to control and up to 56.8% crystal dissolution by the water extract is of great medical science interest.

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