EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 4
Issue: 7
340-344
Year: 2017

CALCIUM OXALATE CRYSTAL INHIBITION POTENTIAL OF *PHYLLANTHUS NIRURI, SYNADENIUM GRANTII AND CORIANDRUM SATIVUM* AS AN INDICATOR OF ANTI-UROLITHIATIC ACTIVITY

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Article Received on 08/05/2017 **Article Revised on 28/05/2017** **Article Accepted on 18/06/2017**

ABSTRACT

The aim of study was to assess inhibitory potential in the aqueous leaf extracts of *Synadenium grantii*, *Phyllanthus niruri*, and *Coriandrum sativum* against calcium oxalate crystal formation by *in vitro* agar gel assay method. This study gave useful insight about the anti-urolithiatic potential of these plants. Urolithiasis or renal calculi is also known as kidney stone. The calculi formation is a multistep process which involves supersaturation of calcium oxalate followed with nucleation, aggregation and retention of calcium oxalate crystals. The process is favored by imbalance between stone promoters and stone inhibitors. The predominant stone promoter is calcium oxalate and effective inhibitors are magnesium and citrate. Plants rich in stone inhibitors can act as phytoremedies for this condition. In the current study, formation of calcium oxalate crystals under *in vitro* conditions was performed using calcium chloride and ammonium oxalate in presence and absence of plant extracts. The extracts were prepared by decoction method of concentrations 10, 25, 50, 75 and 100 mg/ml. The results were compared against Magnesium chloride and Cystone as positive controls. The result showed that the different leaf extracts had significantly decreased the precipitation reaction in the gel assay. It also confirmed that the percentage inhibition is highest in *Coriandrum sativum* dried leaf decoction at concentration of 100 mg/ml among three selected medicinal plants as compared to Cystone. The mineral analysis has confirmed the presence of high levels of magnesium, phosphorus and iron in the leaf extracts by Atomic absorption spectrophotometry and inductively coupled plasma mass spectrometry.

KEYWORDS: Anti-urolithiasis, Synadenium grantii, Phyllanthus niruri, Calcium oxalate crystals.

INTRODUCTION

Urolithiasis is commonly known as kidney stone is known to human since ages. Sedentary life style is one of the major causes for a large number of human populations to suffer from urological disorders like this. Stones are formed due to imbalance between stone promoters and inhibitors available in body. The stone promoters are calcium oxalate, calcium phosphate, struvite, cystineuria etc. whereas magnesium and citrate are stone inhibitors. Majority of urinary stone exists in the form of calcium oxalate monohydrate (COM). Along with these factors one of the additional causes is that we are nowadays consuming more green leafy vegetables and citrus fruits which are very rich in oxalates. On a regular basis very small amount of dietary oxalates are absorbed. The ingested oxalates bind to calcium in the intestinal track or gets degraded by oxalate degrading bacteria present in the gut.[1,2,3,4,5]

The pathogenesis of calcium oxalate (CaOx) stone is a multistep process. It includes super saturation of urine with calcium oxalate, nucleation, aggregation and retention of oxalate crystal.[6] Treatment approaches for kidney stones includes both synthetic drugs and herbal remedies. Synthetic drugs are associated with several known side effects and therefore herbal treatments are preferred over synthetic drugs due to their lesser adverse effects.[7] Although several plants are known to have anti-urolithiatic activity but they all are used in combination with several other plants as none of them has been 100% effective. Most widely used herbal formulation for kidney stone is cystone which has both antilithiatic and lithotriptic potential.[8]

Synadenium granti (family; Euphorbiaceae) is a native plant of Africa and is commonly known as “Janauba”. It is a monoecious shrub which has been pharmacologically evaluated to have potent anti-cancer and anti-inflammatory activity in latex. It is widely used in folk

medicine to treat diseases like cancer, peptic ulcers and other health problems.[9.10]

The herb *Phyllanthus niruri* also belongs to family Euphorbiaceae and is distributed throughout the tropical and subtropical regions. It is commonly known as "Bhuiamla". It is a renowned medicinal plant used in folk medicines to treat liver diseases and different viral infections. This plant is reported to have inhibitory effect on calcium oxalate crystal formation.

Coriandrum sativum commonly known as Dhania in India, Cilantro or Chinese parsley and belongs to family Apiaceae. It is considered as an important medicinal plant with anti-hyperglycemic, anti-inflammatory, antioxidant, hypolipidemic, anti-mutagenic, anti-cancer, diuretic and anti-microbial potential.[11,12,13]

In this article we present a comparative study of inhibitory effects of three plant extracts: *Phyllanthus niruri, Synadenium grantii and Coriandrum sativum* on the formation of CaOx crystals by a simple Agar Gel Assay (AGA). This method is an *in vitro* qualitative and quantitative method to assess the anti-lithiatic potential in the plant extracts and it can be used for large scale screening and selection of anti-urolithiatic plants.

MATERIAL AND METHODS

Extract preparation

Plant leaves of *S. grantii*, *P. niruri* and *C. sativum* were collected in the month of December 2016 to February 2017 from Hislop College, Nagpur. The leaves of *S. grantii*, *P. niruri* and *C. sativum* were collected and washed thoroughly under tap water and shade dried. 20g of fresh and dried leaves of selected plants were separately boiled in 100ml deionized distilled water and concentrated. The decoction of fresh and dried leaves were collected separately by squeezing through muslin cloth, evaporated and dried completely at room temperature and then stored in deep freezer till further use.[14] The extracts were diluted with deionized distilled water to obtain a concentrations range of 10, 25, 50, 75 and 100 mg/ml.

Mineral Analysis

Acid digestion of sample

The method opted for the sample digestion was nitric acid and hydrochloric acid in the ratio of 1:3. The plant leaf extracts were prepared in deionized distilled water. To the sample 9 ml of freshly prepared acid mixture of 65% nitric acid and 35% hydrochloric acid was added and allowed the mixture to boil at 95^{o}C in water bath for 4 to 5 hours and make up the volume to 25 ml.

Metal analysis by atomic absorption spectrophotometer (AAS)

Magnesium (Mg), Calcium (Ca), Phosphorus (P) and Iron (Fe) were analyzed in the acid digested plant leaf extracts by AAS. The instrument is equipped with flame and graphite furnace. Air acetylene flame was used for determination of metal content. The instrument was operated in flame mode of acetylene 1.8 L/min, air 15 L/min, the inert argon gas flow and temperature parameters were followed as recommended by manufacturer. The absorption wavelength for determination of each metal together with its linear working range and correlation coefficient of calibration curve were obtained. The determination was repeated in triplicate.[15,16]

The presence of the minerals is also confirmed by Inductive coupled plasma mass spectrometry (ICP- MS method). ICP- MS method is a modern and widely used technique for multi-elemental analysis with quadruple mass analyzer and multichannel detector. This reconfirmed the presence of Mg in the acid digested leaf sample.

Screening the anti-lithiatic activity in fresh and dried leaves of *S. grantii, P. niruri and C. sativum*

Gel Assay Method

A gel assay method for screening anti-lithiatic metabolites was performed as described. Bactoagar solution (MERCK, 25 ml, 1%) was prepared in double distilled water, heated to liquefy and then poured into petri plates. The agar was allowed to solidify. In each plate, two wells opposite to each other and two troughs perpendicular to the longitudinal distance between the wells, were made (spacing between wells was 0.5 cm distance longitudinally × 1.25 cm distance perpendicularly).[17] The petri plates were divided into negative control (D/W only), positive controls (Magnesium chloride and Cystone) and test (aqueous leaf extracts of *S. grantii, P. niruri, C. sativum*).

The opposite wells were filled with 40 µl of 0.2 M calcium chloride dihydrate (MERCK) and 40 µl of 0.2M ammonium oxalate (HIMEDIA) respectively. The calcium and oxalate ions diffuse towards each other to form visible CaOx precipitin line. The presence of thick precipitin line in the negative control showed the CaOx crystal formation. In tests, the longitudinal wells contain 70 µl of varied concentrations of leaf extracts of *S. grantii, P. niruri and C. sativum*. In positive controls, the longitudinal wells contain 70 µl of $MgCl_2.6H_2O$ (MERCK) and Cystone (Himalaya) separately. All the petri plates were kept in the moist chamber for two hours. Statistically significant differences within the area of the crystals formation between negative control, positive control and test were studied.

The results of the aqueous extracts and positive control on the *in vitro* growth of CaOx crystals compared with the negative control by using the inhibition index (I) and percentage inhibition (PI). The inhibition index and percentage inhibition is calculated by:

Inhibition index (I) = 1- (As/Ac)

Percentage inhibition = [1- (As/Ac)] x 100

Where,

As = area of calcium oxalate crystals in presence of sample tested

Ac = area of calcium oxalate crystals formed for the corresponding blank

I = 0; shows absence of inhibition

I = 1; shows complete inhibition

RESULT AND DISCUSSION

Medicinal plants are used as folk medicines in the treatment of kidney stones. In kidney stones the prevalent form is the formation of CaOx crystals. In this article comparative study of *S. grantii*, *P. niruri* and *C. sativum* plants with respect to anti-urolithiatic potential is reported. Leaf extracts of the selected plants were prepared by four different methods: fresh leaf extract, fresh leaf decoction, dry leaf extract and dry leaf decoction. All the extracts were then tested for anti-urolithiatic property.

Initially all the extracts were tested for mineral composition. It is reported in the previous studies that plants rich in Mg, Fe, P and Ca are natural phytoinhibitors of stone formation.[12] Mineral analysis of *S. grantii*, *P. niruri* and *C. sativum* were performed and results is given in table no. 1.

Table No. 1: Metal analysis in leaf extract of *S. grantii, P. niruri, C. sativum*.

Metal Conc. in ppm	*S. Grantii*	*P. Niruri*	*C. Sativum*
Calcium (Ca)	423.10	3467.00	124.6
Magnesium (Mg)	80.00	327.00	69.4
Phosphorus (P)	0.019	96.25	48.1
Iron (Fe)	9.87	172.73	5.67

On the basis of the above analysis it was confirmed that all three plants are rich in Ca, Mg, P and Fe. Therefore can be a potent inhibitor of CaOx crystal formation as supported by previous reports.[13]

The plant extracts were prepared in four different ways hence to ascertain whether method of extract preparation has any effect on anti-urolithiatic activity. A comparative study was done to screen the inhibitory potential of CaOx crystal formation. The gel assay method provides an opportunity to study anti-lithogenesis potential in *S. grantii*, *P. niruri* and *C. sativum* in a suitable manner. In the present study the extracts were prepared in water and thus is safe for human consumption (Figure No. 1, 2, 3).

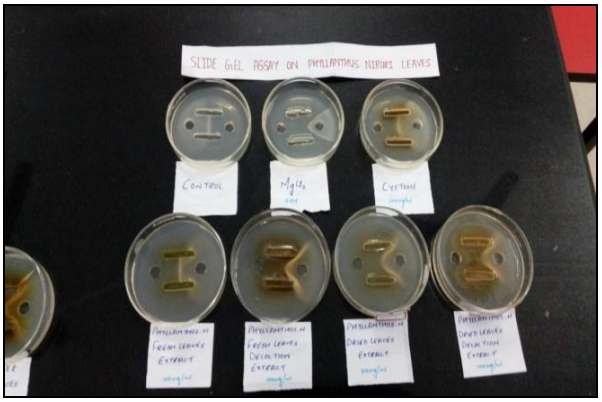

Fig.1: Inhibitory effect of *P. niruri* extracts on CaOx crystallization by agar gel assay.

Fig. 2: Inhibitory effect of *S. grantii* extracts on CaOx crystallization by agar gel assay.

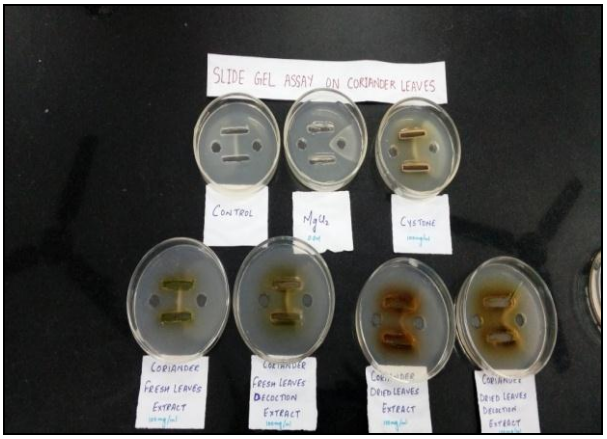

Fig. 3: Inhibitory effect of *C. sativum* extracts on CaOx crystallization by agar gel assay.

Gel assay method is a significant qualitative and quantitative technique in which the data is obtained by measuring the visible disc area which denotes the area of zone of inhibition. The percent inhibition (PI) for all three therapeutic plants were calculated using PI formulae. Cystone and $MgCl_2$ were taken as positive control. The result confirmed that out of all the experimental extracts, dried leaf decoction is best for all three selected plants given in table no. 2.

Table No. 2: Effect of mode of extract preparation on calcium oxalate crystal formation by leaf extracts.

S. No.	Name of Sample	Type of Extract (100mg/ml)	Percent inhibition (mean± S.D.)
1.	Cystone	Aqueous extract	52.18 ±0.23
2.	$MgCl_2$	0.2 M	68.82 ±0.25
3.	*S. grantii*	Fresh leaf extract	10.25 ± 0.23
		Fresh leaf decoction	37.68 ± 0.42
		Dried leaf extract	37.68 ± 0.32
		Dried leaf decoction	60.15 ±0.057
4.	*P. niruri*	Fresh leaf extract	19.95 ± 0.23
		Fresh leaf decoction	37.68 ± 0.26
		Dried leaf extract	50.00 ± 0.23
		Dried leaf decoction	58.98 ± 0.096
5.	*C. sativum*	Fresh leaf extract	24.30 ± 0.063
		Fresh leaf decoction	24.30 ± 0.05
		Dried leaf extract	54.20 ± 0.23
		Dried leaf decoction	66.04 ±0.25

The effect of varied concentrations of plant extracts on calcium oxalate crystal formation was tested. Maximum inhibition of CaOx crystal formation was found at a concentration of 100mg/ml and the inhibition was dose dependent for all the three plants. The dried leaf decoction of all the three plants were more potent inhibitor than other extracts (Figure no. 4). However *C. sativum* extract showed highest percent inhibition potential than *P. niruri* and *S. grantii*.

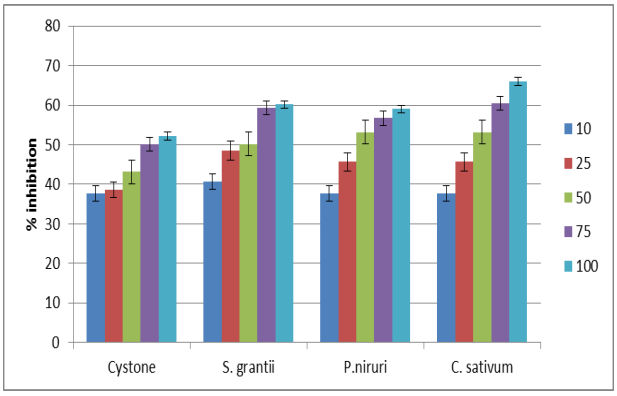

Fig. 4: Percent inhibition of crystals by different concentrations of extracts (Coloured bar represents concentration in mg/ml).

CONCLUSION

In the present work, the inhibition of calcium oxalate crystal formation by aqueous extracts of *S. grantii*, *P. niruri* and *C. sativum* leaves were studied. The dried leaf extract decoction showed highest percentage inhibition of CaOx crystal formation. It was also observed that the anti-lithiatic potential is directly proportional to the increasing extract concentration. Toxicity assessments are needed to ascertain the exact dose. Extracts prepared by decoction is most suitable and it is suggested that isolation of anti-lithiatic compounds can be done from the decoction of dried leaf.

ACKNOLEDGEMENT

The authors would like to acknowledge Dr. Dipti Christian, Principal, Hislop College, Nagpur for providing the necessary research facilities. We are also grateful to Qualichem Laboratory and NEERI, Nagpur for facilitating mineral analysis.

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