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

ISSN 2349-8870
Volume: 5
Issue: 8
435-440
Year: 2018

SYNADENIUM GRANTII LEAF EXTRACT DECREASES THE AGGREGATION, NUCLEATION AND FORMATION OF URINARY CRYSTALS

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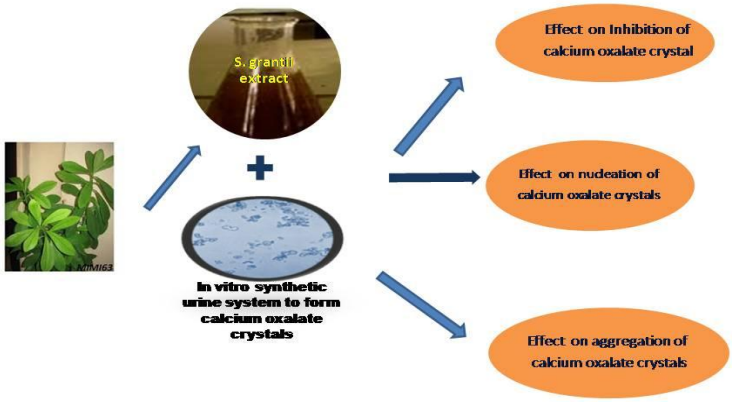
Article Received on 16/06/2018 **Article Revised on 06/072018** **Article Accepted on 26/07/2018**

ABSTRACT

Background: Urolithiasis and Nephrolithiasis is a worldwide disease characterized by formation of stones in urinary tract and kidney due to supersaturation of urine with minerals like calcium oxalate. The phyto-inhibitors are being extensively studied to decrease the risk of kidney stone formation and associated symptoms. **Objective:** In this paper we studied the anti-urolithiatic potential of *Synadenium grantii* aqueous extract on nucleation, aggregation and growth of calcium oxalate crystallization by in vitro assays. **Methods:** *In vitro* calcium oxalate crystals were synthesized using synthetic urine system and effect of plant extract on nucleation, aggregation and growth of crystal was studied under *in vitro* conditions. Results were compared with positive and negative controls. **Result:** The *in vitro* studies show that the aqueous leaf extract inhibits nucleation, aggregation and growth of calcium oxalate crystals. The percent inhibition (87.4%) and inhibition of aggregation (81%) was highest at 100 mg/ml concentration of extract. The crystal nucleation also got delayed with increase in concentration of the extract. **Conclusions:** The plant leaf extract has anti-urolithiatic potential when tested in *in vitro* assays. However, *in vivo* and *in silico* studies would be needed to confirm the activity.

KEYWORDS: Urolithiasis, Synadenium grantii, Nucleation, Aggregation, Simulation, Calcium oxalate monohydrate.

Graphical Abstract



INTRODUCTION

The occurrence of urolithiasis has steadily risen, affecting more than 12% of world's population with an increasingly high recurrence rate in males than in females.[1] Supersaturation of urine with mineral salts is byfar the major cause of urolithiasis, as it leads to

nucleation, crystal aggregation to form stones. Depending upon the urinary saturation levels, the stone types can vary, however the majority of the kidney stones are predominantly composed of calcium oxalate (CaOx).[2,3] Certain metabolic abnormalities such as hypercalciuria, hypocitraturia, hyperoxaluria, hyperuricosuria and hypomagnesuria can also profoundly affect stone formation.[4] Prolong retention of salts in kidney causes ulceration of the renal papillary surface forming a stone nidus[5,6] which eventually supports crystal nucleation even at low levels of supersaturation.

The medicinal plants have played a significant role in various ancient traditional systems like Ayurveda. Ayurvedic formulations are widely used for the management of kidney stones. Recent studies have reported more than 107 antiurolithiatic plants, majority of which are from Asteraceae, Rosaceae, Euphorbiaceae, Malvaceae and Cucurbitaceae families. The beneficiary effects of plants are attributed to presence of secondary metabolites.[7,8]

Synadenium grantii, a monoecious shrub from Euphorbiaceae family is a native plant to tropical eastern. Several species of this genus have been pharmacologically evaluated and are used in folk medicines to treat diseases like cancer[9,10], peptic ulcers and other health problems.[11,12] However the use of this plant for prevention of urolithiasis has not been reported. In this paper we report the anti-urolithiatic effects of this plant on formation of CaOx crystals in *in vitro* assays.

MATERIALS AND METHODS

Preparation of Plant Leaf Extract

The leaves of *S. grantii* were collected from Hislop College garden during the month of September, 2017. The plant was authenticated by taxonomist and a voucher specimen (PRS-2161) has been deposited at Department of Botany, RTM Nagpur University, Nagpur. The leaves were collected, dried in shade and extracts were prepared using decoction method.[13] 20 g of leaves were boiled in 100 ml of deionized water and concentrated to 5 ml and used as plant extract. The extract was concentrated under reduced pressure and a final concentration of 100 mg/ml was obtained.

Synthesis of calcium oxalate crystals

CaOx crystals were synthesized using synthetic urine system composed of 5 mM calcium chloride and 0.5 mM sodium oxalate in 90 mM Tris- HCl and 10 mM NaCl buffer.[14] The reaction mixture was incubated at 25°C overnight and CaOx crystals were resuspended in methanol. After centrifugation at 2000 x *g* for five minutes, the crystals were pelleted out and dried at room temperature.

Inhibition assay

Inhibition assay was carried out as per the method reported by P. Rajeshwari *et al.*[15] with slight modification. The plant leaf extract (50 ml) was placed in the beaker and the two salt forming solutions calcium chloride and sodium oxalate were allowed to run into it (drop wise) through burettes and boiled for 10 min. Mixture was cooled at room temperature and the precipitate was collected into a pre-weighed centrifuge tube by centrifuging small volumes at a time. The precipitate was dried in oven, cooled to room temperature and weighed till constant weight was obtained. Simultaneous experiments with water in place of extracts were used as blank. Inhibitory potential of the leaf extract was calculated using following formula:

$$\% \text{ Inhibition} = \frac{(\text{Wt of ppt in expt.set} - \text{Wt of ppt in Blank set})}{\text{Wt of ppt in Blank Set}} \times 100$$

Nucleation assay

Nucleation assay[16] was performed using buffered solutions of calcium chloride (5 mmol/L) and sodium oxalate (7.5 mmol/L) in Tris–HCl 0.05 mol/l and NaCl 0.15 mol/L (pH 6.5). Calcium chloride solution (9 ml) was mixed with 1 ml of herb extracts at different concentrations (10, 25, 50, 75 and 100 mg/ml). Crystallization was initiated using 1250 ml of sodium oxalate solution. The absorbance was read at 620 nm after every 30 min at 37°C. The rate of nucleation was estimated by comparing the induction time in the presence of extract with that of poly herbal drug Cystone as control.

The CaOx crystals were formed due to following reaction:

$$CaCl_2 + Na_2C_2O_4 \longrightarrow CaC_2O_4 + 2\ NaCl$$

Aggregation assay

Calcium chloride and sodium oxalate solutions (50 mmol/L) were equilibriated to 60°C in a water bath for 1 hour and then cooled to 37°C overnight. The crystals formed were then harvested by centrifugation, evaporated at 37^0C. Buffered crystal solution (0.8 mg/ml) was prepared using Tris–HCl 0.05 mol/L and NaCl 0.15 mol/L (pH 6.5). Experiments were conducted at 37°C in the absence and presence of the plant extract.[17] The percent inhibition of aggregation was calculated by comparing the turbidity in the presence of extract at different concentrations (10 –100 mg/ml) with that obtained in the positive control (Cystone) and the negative control (distilled water), using following formula:

$$\% \text{ Inhibition of Aggregation} = \frac{(1 - \text{Turbidity of Sample})}{\text{Turbidity of Negative Control}} \text{ x } 100$$

Simulation of the sedimentary crystal formation

A simulation study for sedimentary crystal formation was performed.[18] The crystal size development was monitored in sample drops every five minutes by polarizing microscope. A drop of sample was put on hemocytometer counting chamber and it was observed under the microscope after 15 minutes. The number of crystals were calculated and photographed. A series of experiments using plant extract of varied concentrations (10 – 100 mg/ml) were conducted. The number of

crystals and size was monitored after every 15 minutes. The percentage of Inhibition (I %) was calculated by the formula:

I% = [(TSI- TAI) / TSI] * 100

TSI- represents the number of calcium oxalate monohydrate (COM) crystals without inhibitor.

TAI- represents the number of COM crystals after addition of inhibitor.

RESULTS

The nucleation and aggregation of CaOx crystals are important steps in the growth and formation of kidney stones. Any drug or chemical intervention which delays or inhibits these processes will help in the management of kidney stones. The leaf extracts of *S. grantii* were tested for the anti-urolithiatic activities using *in vitro* assays and the results were compared with a marketed poly-herbal combination, Cystone, under identical conditions. Crystals generated in the urine were also analyzed by light microscopy. Statistical differences and percentage inhibitions were calculated and assessed.

Inhibition Assay

Leaf extract of *S. grantii* has significantly reduced the formation of CaOx crystals (P<0.05). The inhibitory effect increased from 26.70% (at 10 mg/ml of extract) to 87.47% (at 100 mg/ml of extract). The result is presented in the Table 1.

Table 1: Effect of *S. grantii* leaf on CaOx crystals.

	Concentration of Plant Extract				
	10 mg/ml	**25 mg/ml**	**50 mg/ml**	**75 mg/ml**	**100 mg/ml**
% Inhibition Cystone (Std)	29.05%	38.50%	59.60%	78.80%	98.50%
% Inhibition *S. grantii*	26.07%	39.14%	49.33%	64.92 %	87.47%

Values are expressed as mean ±sem, n=6, where, statistical significance P<0.05.

Nucleation Assay

The formation of crystal starts with nucleation process during which molecules rearrange into cluster and grows into macroscopically larger size. We studied the effect of *S. grantii* extract on the nucleation process of CaOx crystallization (Fig**. 1**).

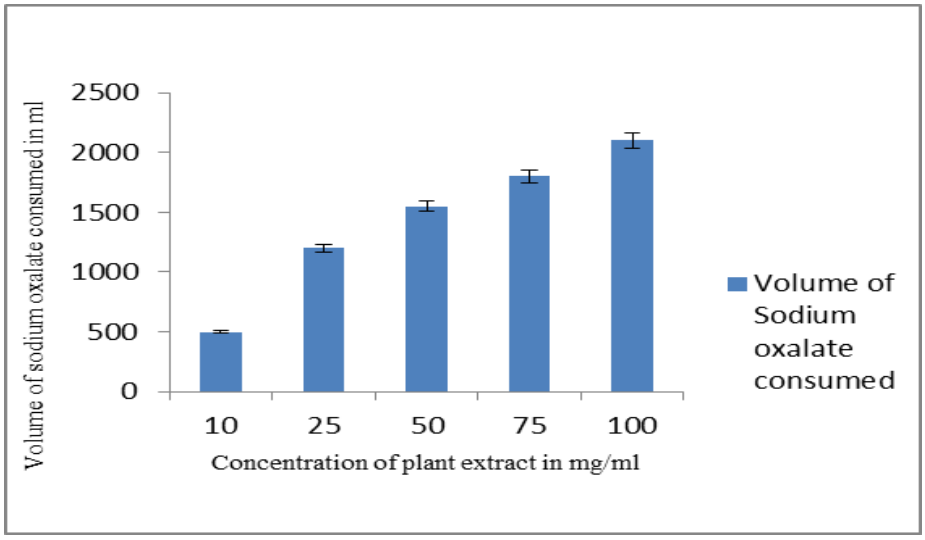


Fig. (1): Effect of *S. grantii* extract on Nucleation of CaOx crystals.

The amount of sodium oxalate needed for nucleation, increased with the increasing concentration of extract. This indicates that the presence of *S. grantii* extract causes a delay in the nucleation process of CaOx crystals.

Aggregation Assay

The percent inhibition of aggregation was calculated by comparing the turbidity obtained in the presence of extract at different concentrations (10 –100 mg/ml) with the control. The turbidity decreased with the increase in concentration of plant extract indicating inhibitory effect of extract on aggregation of CaOx crystals. The OD was highest in control and lowest at the highest concentration of leaf extract (100 mg/ml). The maximum percent inhibition of aggregation was found at 100 mg/ml concentration (Fig **2**).

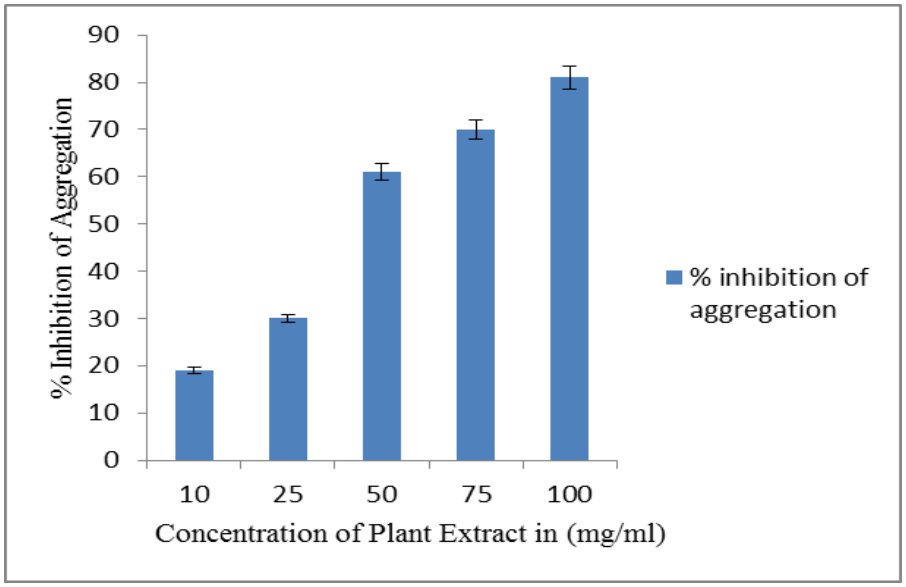


Fig. (2): Percent Inhibition of Aggregation in presence of *S. grantii.*

Simulation Study

The number of CaOx crystals were monitored under polarizing microscope at a time interval of 15 minutes upto 1 hour in presence of extract and compared to the control (Fig. **3**). Results indicated a reduction in crystal formation with the increasing concentration of *S. grantii* extract. Maximum reduction was found at 100 mg/ml concentration. The data is presented in Table **2.**

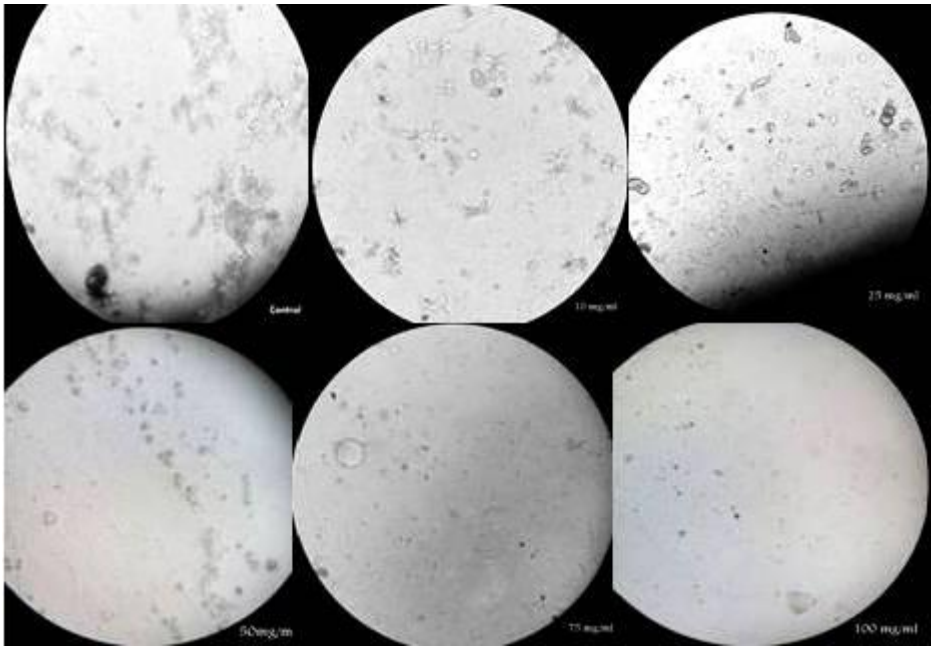


Fig. (3): Polarized microscopy image of sedimentary crystal formation.

Table 2: Effect of leaf extract on no. of CaOx crystals.

Time in minutes		Number of CaOx Crystals at Different Time Intervals				
		0	**15**	**30**	**45**	**60**
Control		668	780	788	930	1000
Concentration of Plant Extract in mg/ml	10	430	510	660	600	830
	25	380	390	4800	53	676
	50	285	280	363	388	539
	75	108	133	138	145	260
	100	59	82	125	138	110

DISCUSSION

Urolithiasis is a widespread disease which has afflicted mankind since centuries. Lithiasis or stone formation occurs mainly due to supersaturation of salts predominantly of CaOx. Urolithiasis is a multi step process starts with the supersaturation of salts in urine and followed by nucleation, aggregation and growth of crystals. Many of the plants like *Didymocarpus pedicellata*, *Saxifraga ligulata* and *Tribulus terrestris* have been extensively used as polyherbal medicines for treatment. Most of the plants which are reported to have anti lithiatic activity are found to be rich in saponins and divalent cations like magnesium. In 2012, Paras *et al.* have reported that the anti-urolithiatic activity of *Solanum xanthocarpum* fruit extract was due saponins through *in vitro* and *in vivo* studies. Saponin rich fractions of other plants like, *Herniaria hirsute*[19] and *T. arjuna* have also been reported to have antilithiatic activity.[20]

In the current study, *S. grantii* extract was used to evaluate the antilithiatic activity by *in vitro* crystallization assays. The nucleation of crystal formation got delayed in presence of *S. grantii* plant extract. Further evaluation confirmed that the aqueous extract of *S. grantii* has inhibitory effect on the aggregation and growth of CaOx crystals. Aqueous extracts of *S. grantii* were found to be rich in saponins and magnesium. Therefore the antilithiatic activity of *S. grantii* can be attributed to the presence of saponins and high magnesium levels.[21] However further *in vivo* and *in silico* studies of *S. grantii* would be needed for the confirmation of the activity.

CONCLUSION

S. grantii leaf extract shows antilithiatic activity in *in vitro* assays. The plant extract inhibits nucleation, aggregation and growth of CaOx crystals and therefore can be used for management of kidney stones. However such use of this plant can be confirmed only after *in vivo* and *in silico* assays. This is the first report about antilithiatic property of this plant. Future research is needed for the confirmation of the property.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Kundal, Head, Department of Geology for simulation studies and Dr. Iyer from VNIT and Principal, Dr. Dipti Christian for facilitating the research work.

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