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Investigating the Effect of Hydroalcoholic Extract of *Ocimum sanctum* on *In-vitro* Calcium Oxalate Crystallization

Chetna Faujdar[1], Priyadarshini[1*]

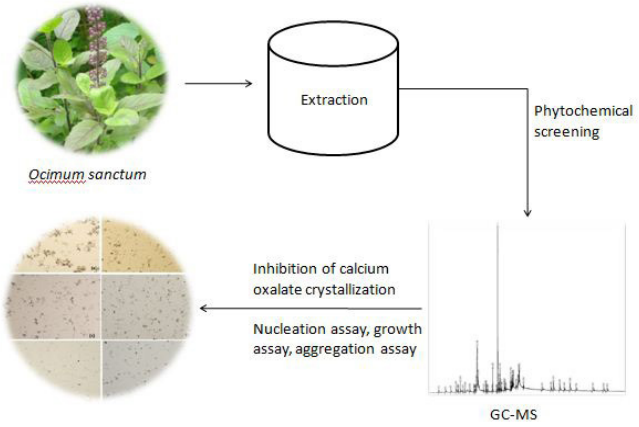
[1]Jaypee Institute of Information Technology, Noida, India
* Corresponding author: priya.juit@gmail.com

Abstract

Leaves of *Ocimum sanctum* have been used in traditional Indian medicinal system since ancient times for its preventive and curative properties for various diseases. The aim of our study was to investigate the effect of Hydroalcoholic extract of *Ocimum sanctum* (OLE) on Calcium oxalate crystallization process. Phytochemical profiling of extract was done using GC-MS to confirm the presence of different therapeutically important phytocompounds. In-vitro effect of different concentrations (1-8 mg/ml) of extract was evaluated at different stages of crystallization i.e. nucleation, growth of crystals and aggregation. The data indicates that upto 40.32% inhibition of calcium oxalate nucleation was observed with maximum concentration (8 mg/ml) of extract used in the study. Microscopic observation revealed that the extract decreased the number and size of crystal nuclei with increase in concentration. Maximum inhibition of aggregation (33.9%) was observed at 1 mg/ml concentration. Significant inhibition of crystal growth was observed at 1 mg/ml and 2 mg/ml of the extract to 94.59% and 97.21% respectively. On the basis of available data, it can be concluded that *Ocimum sanctum* leaf extract possess the potential to inhibit all the three phases of calcium oxalate crystallization and can be investigated further for the treatment and prevention of urolithiasis.

Keywords *Ocimum sanctum*; Phytocompounds; Calcium oxalate; Crystallization

Graphical Abstract



Introduction

Urolithiasis is a condition caused by crystal concretion within the urinary system affecting more than 12% of the world's population (1,2). Recent reports indicated that in past few decades prevalence of urolithiasis has been increased in both developed and developing countries. The increased incidences are believed to be associated with a number of factors including lack of physical activities, unhealthy dietary habits (3-5) and global warming (6). About 12% of Indian population is estimated to suffer from kidney stones, 50% of which may even experience improper kidney functions (7). It is a well known fact that more than 70% of kidney stones are composed of calcium oxalate (8). The major challenge in the treatment of kidney stones is high recurrence rate of more than 30% within 10 years (9-11). Prevention and treatment of the disease require better understanding of molecular mechanism of stone formation and invention of safer medication which can be used for longer duration to avoid recurrence (12,13). The etiology of kidney stone is a multifactorial process resulting from several physicochemical events. Urinary supersaturation provides the microenvironment for different phases of crystal formation such as nucleation, growth and aggregation and eventually crystal retention within tubular cells. Thus preventing crystal formation at different phases could be an effective approach to reduce the chances of crystal retention and further formation of kidney stone.

Unfortunately, despite the advancements in the field of medical science, no satisfactory treatment is available to reduce the recurrence or cure urolithiasis completely. Plants possess a strong historical background of providing a basis for several therapeutic agents. Reportedly 75 % of synthetic drugs are derived from plants even after great development of synthetic drugs industry (14). Each part of the plants including roots, stem, leaves, fruits and seeds are known to possess bioactive components and have been used as a primary source of therapeutics. Among various medicinal plants *Ocimum sanctum* also known as *Ocimum tenuiflorum* or holy basil has been traditionally used as a medicine provided by nature due to its therapeutic potential in almost every part of the plant (15). Although *Ocimum sanctum* has been traditionally used in Ayurveda to treat various ailments but the plant is not very well utilized and explored for its potential in preventing or treating urolithiasis. Several scientific reports reveal the use of *Ocimum sanctum* in treating various ailments but no reports on the effect of the plant on nucleation, aggregation and growth stages of calcium oxalate crystallization is reported as per our knowledge.

Therefore the aim of present study was to evaluate the affect of hydroalcoholic extract of *Ocimum sanctum* leaves (OLE) on calcium oxalate (CaOx) crystallization which can be correlated with the antiurolithiatic properties of the plant. Extract was investigated for its impact on different phases of crystallization i.e. nucleation, growth

and crystal aggregation.

Materials and Methods

Materials

Plant material

Ocimum sanctum plants were collected from a nursery. Identification and authentication of plant was done by Department of Botany, University of Delhi. A voucher specimen was deposited (DUH14478) in the herbarium for future reference.

Chemicals and reagents

Sodium oxalate was purchased from Merck, Germany. Gallic acid was purchased from Sigma-Aldrich USA. Calcium chloride dihydrate and Folin ciocalteu reagent were purchased from CDH fine chemicals, India.

Methods

Preparation of plant extract

Fresh Leaves (10 gm) of *Ocimum sanctum* were ground to paste and then mixed with 100 ml of water and ethanol (30:70) for 3 days with frequent agitation to prepare hydroalcoholic extract. The extract was then filtered through Whatman filter paper and concentrated using rotary evaporator. *Ocimum sanctum* leaves extract (OLE) was then lyophilized to obtain powdered extract and stored at 4^0C for further use. Distilled water was used to dissolve powdered OLE before each experiment (16). The percentage yield (%, w/w) was calculated using the given formula.

$$\% \text{ Yield} = [(W\_1\ )/W\_2\ ]X\ 100$$

Where, W_1 is weight of the extract obtained after solvent evaporation, W_2 is weight of the leaves taken.

Phytochemical screening of the plant extract

OLE was subjected to preliminary phytochemical screening to test the presence or absence of different classes of phytoconstituents such as tannins, flavonoids, saponins, alkaloids, glycosides, terpenes and steroids using standard procedures (17).

Total phenolic content (TPC) was also estimated spectrophotometrically using a method explained by Koncic et al (18). Gallic acid was used as a reference standard and TPC was quantified and expressed as gallic acid equivalent (GAE) mg/g of the dry extract. Following linear equation, derived from gallic acid standard curve was used to calculate total phenolic content: $Y = 0.007x + 0.048$, $R^2 = 0.990$
Where, Y is absorbance and X is concentration of gallic acid in µg.

Afterwards, GC-MS analysis was done using Gas Chromatograph mass Spectrometer (Model QP-2010 Plus; Shimadzu, Japan).

Briefly, one µl of sample dissolved in ethanol (1 mg/ml) was injected into an Rtx-5 MS capillary column (30m length X 0.25mm i.d X 0.25 µm film thickness) for analysis.• The injector and detector temperatures for the gas chromatograph were 250 and 280 , respectively. The stepped temperature program was held at 80 for 3 min, and then from 80 to 280 with a ramp rate of 10 /min. Components were identified by their retention time and compared with standard components provided by different databases such as WILEY8LIB and NIST11LIB.

Evaluation of anti-crystallization activity of the extract

Anti-crystallization activity of the extract was done on different stages of crystallization i.e. nucleation, growth and aggregation of the crystals.

Nucleation assay

This assay serves as a model to study calcium oxalate crystallization in the presence or absence of crystallization inhibitor. Calcium chloride (5 mM) and sodium oxalate (7.5 mM) solutions were prepared in a buffer containing Tris (0.05 mol) and NaCl (0.15 mol) at pH 6.5. Reaction mixture was prepared by mixing 950 µl of calcium chloride solution with 100 µl of different concentrations of OLE (1-8 mg/ml). To initiate crystallization, 950 µl of sodium oxalate solution was added. Solutions were then incubated for 30 minutes at 37^0C and absorbance was recorded at 620 nm (19). Following formula was used to calculate the percentage inhibition.

Microscopic study of crystals formed in nucleation assay was done by taking 10 µl of solution on microscopic slide and observed under the microscope at 10x magnification

$$\% \text{ Inhibition} = [(OD\ (Control)-OD(Experimental))/(OD\ (Control)\ )]X\ 100$$

Growth assay

A method described by Hess et al was used to study the effect of OLE on CaOx crystal growth (20). COM crystals (1.5 mg/ml) were dissolved in Tris buffer (10 mM) containing 90 mM NaCl (pH 7.2) to prepare stone slurry. Stone slurry was mixed with 4 mM $CaCl_2$ and 4 mM sodium oxalate to initiate the reaction. Deposition of calcium oxalate on the surface of crystal will result in the growth of the crystal thereby depleting the free oxalate from the solution. This depletion of free oxalate can be detected spectrophotometrically at 214 nm. There will be decrease in the depletion of free oxalate if there is an inhibition of crystal growth. Experiment was performed in the presence or absence of extract and following formula was used to calculate the relative inhibitory activity:

Where, C is reduction rate of free oxalate in the absence of OLE and S is the reduction rate of oxalate in the presence of OLE.

$$\% \text{ Relative inhibitory activity} = [(C(Control)-S(Experimental))/(C\ (Control)\ )]X\ 100$$

Aggregation assay

A method described by Atmani and Khan with slight modifications was used to perform aggregation assay (21). Calcium oxalate monohydrate (COM) crystals were used as seed to study aggregation. Solutions of calcium chloride (50 mM) and sodium oxalate (50 mM) were mixed and then equilibrated at 60°C in a water bath for 1 hour followed by overnight incubation at 37°C to prepare COM crystals. Crystals were harvested by centrifugation followed by solvent evaporation. Harvested COM crystals (0.8 mg/ml) were dissolved in a buffer containing Tris (0.05 mol) and NaCl (0.15 mol) at pH 6.5 before conducting the experiment. Experiment was performed in presence and absence of OLE at 37^0C. Following formula was

used to calculate the percentage inhibition (22).

% Inhibition = [1-(Turbidity (Sample))/(Turbidity (Control))]X 100

STATISTICAL ANALYSIS

Each experiment was performed in triplicates (n=3) and results were presented as mean ± SD. Graphpad prism 6.0 software (GraphPad Software, San Diego, CA, USA) was used for data presentation. Associated probability (P) value of < 0.05 was considered as statistically significant.This formula should come at the end of the Aggregation assay. It is appearing in the statistical analysis section.

% Inhibition = [1-(Turbidity (Sample))/(Turbidity (Control))]X 100

RESULTS AND DISCUSSION

Results

Preparation of plant extract

The hydroalcohalic extract was prepared and percentage yield was calculated. The percentage yield of the lyophilized OLE was found to be 6.86% (w/w).

Phytochemical screening of the plant extract

Phytoconstituents are responsible for the bioactivity of any plant material. • Preliminary screening of OLE was done and the results indicated the presence many of important biologically active classes of phytoconstituents viz. tannins, alkaloids, flavonoids, glycosides and saponins etc (Table 1).

Table 1: Preliminary phytochemical screening of Hydroalcoholic extract of *Ocimum sanctum* leaves

Phytochemical test	Name of the test	OLE
Tannins	FeCl3 test	+
Flavonoids	Shinoda test	+
Cardiac glycosides	Keller Killiani test	-
Saponins	frothing test	+
Alkaloids	Mayer's test	+
Steroids	Salkowski test	-

+ : Presence of phytochemical; -- : Absence of phytochemical

Quantitative estimation of total phenolic content was done using the Folin–Ciocalteu reagent. Total phenolic content in OLE was found to be 41.14±0.004 mg/g GAE. Phenolic compounds are believed to be responsible for antioxidant activity of any herbal product (23). Antioxidants are also found to be responsible for protecting the renal epithelial cell damage and thus possess antiurolithiatic property. Khan et al reported that crude extract of Origanum vulgare holds antiurolithiatic properties due to presence of strong antioxidants and its ability to inhibit CaOx crystallization (24).

GC-MS phytochemical screening reveals the presence of several bioactive phytoconstituents in OLE. These compounds can be responsible for its ability to inhibit crystallization (Fig. 1). Table 2 provides the details, relative retention time and percentage of compounds present in OLE (Table 2).

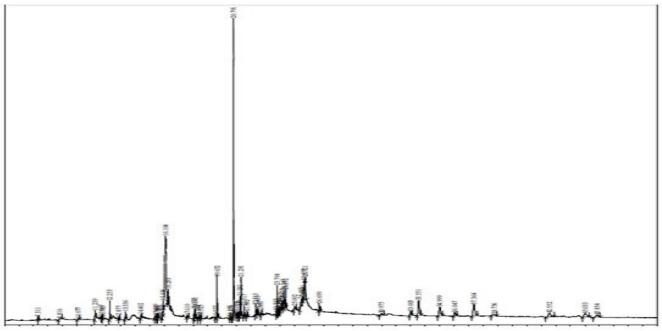


Fig. 1: GC-MS chromatogram of the constituents of hydroalcoholic leaf extract of *Ocimum sanctum*

Evaluation of anti-crystallization activity of the extract

The preventive effect of OLE against the calcium oxalate crystallization has been studied in vitro by the turbidometric methods including nucleation assay, growth assay and aggregation assay.

Nucleation assay

The number of crystal nuclei formed was estimated by nucleation assay in terms of the turbidity of the solution. The affect of extract on nucleation was evaluated at different concentrations of the extract ranging from 1mg/ml to 8 mg/ml. The results of nucleation assay revealed concentration dependant inhibition of calcium oxalate

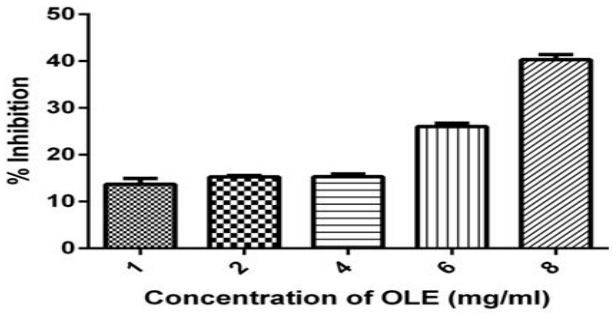


Figure 2: Effect of different concentration of *Ocimum sanctum* extract on CaOx crystal Nucleation

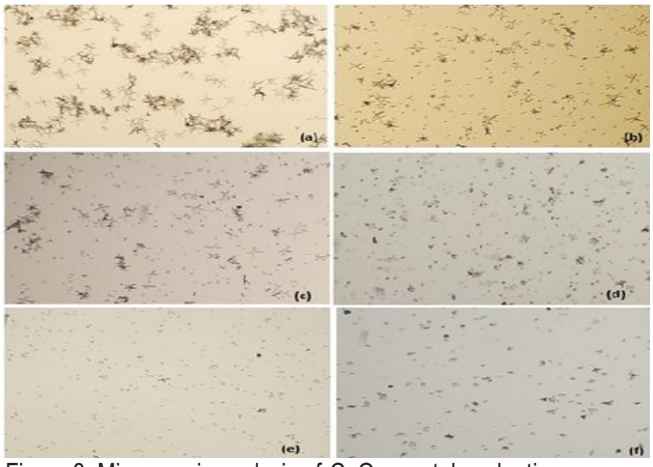


Figure 3: Microscopic analysis of CaOx crystal nucleation
a—Control, b-1 mg/ml, c-2 mg/ml, d-4 mg/ml, e-6 mg/ml and f-8 mg/ml) of OLE

Table 2: Chemical compositions of OLE by GC-MS analysis

S.No.	Name of Compound	Retention time	Area%
1.	Eucalyptol	7.311	0.19
2.	3,4 Dehydro-dl-proline	8.816	0.45
3.	Ketone, 2,2-dimethylcyclohexyl methyl	10.077	0.15
4.	1-Acetylproline	11.239	1.37
5.	2-Isopropyl-5-methyl-1-heptanol	11.667	0.25
6.	Terpinyl acetate	12.253	1.41
7.	Beta elemen	12.877	0.13
8.	β Caryophyllene	13.336	0.60
9.	Phenol, 3,5-bis(1,1-dimethylethyl)	14.402	0.35
10.	6-[1-(hydroxymethyl)vinyl]-4,8a-dimethyl-1,2,4a,5,6,7,8,8a-octahydro-2-naphthalenol	15.419	0.64
11.	Duvatriendiol	15.507	0.08
12.	2-Octene, 2-methoxy	15.589	0.07
13.	Megastigmatrienone	15.863	0.09
14.	5-Ethyl-1,3-dioxane-5-methanol, tert-butyldimethylsilyl ether	15.936	0.88
15.	Ethyl .alpha.-d-glucopyranoside	16.104	21.39
16.	Beta.-D-Lactose	16.293	2.42
17.	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	17.610	0.37
18.	Neophytadiene	18.077	0.98
19.	2-Pentadecanone, 6,10,14-trimethyl	18.141	0.55
20.	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	18.364	0.37
21.	2-Hydroxycyclopentadecanone	19.577	0.11
22.	Hexadecanoic acid, ethyl ester	19.652	3.24
23.	n-Nonadecanol-1	20.557	0.09
24.	7-Hexadecenal	20.679	0.13
25.	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl	20.791	26.03
26.	Methyl (Z)-5,11,14,17-eicosatetraenoate	21.023	0.31
27.	Trans,trans-9,12-Octadecadienoic acid, propyl ester	21.232	1.18
28.	Ethyl (9z,12z)-9,12-octadecadienoate	21.291	2.48
29.	Octadecanoic acid, ethyl ester	21.517	0.87
30.	Phytol, acetate	21.692	0.40
31.	3-Cyclopentylpropionic acid, 2-dimethylaminoethyl ester	22.313	3.10
32.	Glycidyl palmitate	22.433	0.57
33.	2,2-Dimethyl-5-(3-methyl-2-oxiranyl)cyclohexanone	22.660	0.17
34.	1,8,11-Heptadecatriene	23.883	0.19
35.	9,12,15-Octadecatrienoic acid	23.941	0.38
36.	2-Ethylbutyric acid, eicosyl ester	24.062	0.90
37.	N-[1-(4,6-dimethyl-benzothiazol-2-ylamino)-2,2,2-trifluoro-1-trifluoromethyl-ethyl]-3-phenyl-propionamIde	24.157	0.46
38.	2-Palmitoylglycerol	24.247	2.06
39.	1,2-Benzenedicarboxylic acid	24.372	1.17
40.	Acetylisoeugenol	25.002	1.00
41.	2,5-Octadecadiynoic acid, methyl ester	25.463	1.95
42.	Dehydrodieugenol	25.590	0.23
43.	E,Z-1,3,12-Nonadecatriene	25.657	1.25
44.	2-Monolinolenin	25.721	0.03
45.	Squalene	26.699	0.74
46.	Vitamin E	30.975	1.3
47.	Ergost-5-en-3-ol, (3.beta.,24r)	33.003	1.06
48.	Stigmasterol	33.551	3.90
49.	Gamma.-Sitosterol	34.999	2.48
50.	Olean-12-en-3-one	36.047	0.93
51.	Alpha.-Amyrin	37.364	4.00
52.	Methyl bayogenalate	42.552	3.78
53.	Ursolic aldehyde	45.854	0.98

crystallization measured in terms of percent inhibition of nucleation (figure 2). Inhibition of nucleation increased from 13.68% to 40.32% with the increase in concentration of OLE from 1mg/ml to 8 mg/ml. Interestingly, microscopic analysis of the crystals showed that the morphology of the crystals was completely changed after exposure to the extract. There was significant change in both size and shape of the crystals (Figure 3) against control (without extract).

Growth assay

Crystal growth is another very important phase of calcium oxalate

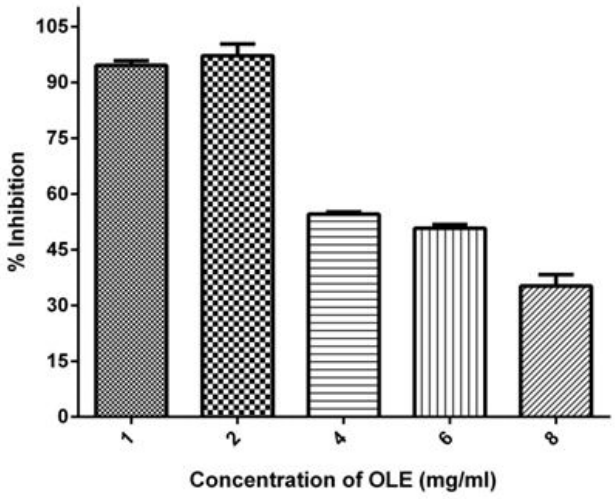


Figure 4: Effect of different concentration of OLE on CaOx crystal growth

(CaOx) kidney stone formation. Growth assay was performed to evaluate the affect of OLE on growth of crystals. The results indicated that different concentrations of OLE inhibited the *in-vitro* calcium oxalate growth. There was significant increase in inhibition of crystal growth at lower concentrations up to 2 mg/ml followed by concentration dependant fall in inhibition. OLE resulted in 94.59% and 97.21% inhibition in the crystal growth with OLE concentration of 1 mg/ml and 2 mg/ml respectively. Percentage inhibition declined with increase in concentration till 8 mg/ml to 35.24% (Figure 4).

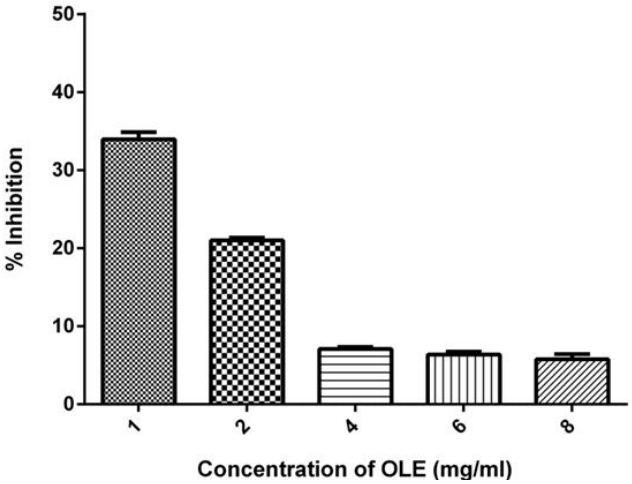


Figure 5 should be placed after "Aggregation assay" section (column 1, page 5)

Aggregation assay

Aggregation assay was used to estimate the degree of COM crystal aggregation. Surprisingly, increase in the concentration of OLE resulted in decrease in percent inhibition of aggregation (Figure 5). OLE exhibited maximum inhibition at lower concentration (1 mg/ml) followed by concentration dependant decrease in inhibition with increased concentration of OLE. Minimum inhibition was observed at 8mg/ml concentration of OLE.

Discussion

Anti-crystallization activity of OLE was studied by using in-vitro models for nucleation, aggregation and growth assay of calcium oxalate (CaOx) crystals to evaluate the antiurolithiatic property of the extract. Since nucleation is a critical step to initiate crystal formation, it was observed that OLE potentially inhibited the process at nucleation stage resulting in less turbidity as compared to control. Inhibition of nucleation was increased from 13.68% to 40.32% with increase in the concentration of OLE from 1mg/ml to 8 mg/ml. the results were further supported by microscopic analysis. Microscopic data showed a significant change in size, shape and number of crystals against control (without extract).

Similarly, OLE also showed inhibitory activity at aggregation and crystal growth stage also but at lower extract concentration. The results can be correlated with the presence of pharmacologically active phytoconstituents from the class of tannins, alkaloids, flavonoids, glycosides and saponins. Although, exact mechanism of the activity is still not very clear but previous reports suggest that different phytoconstituents may be responsible for the activity. Zhong et al found that flavonoids inhibit calcium oxalate crystallization both in human urine and in the animal models (25). Similarly, Gurocak reported that saponins also posses anti-crystallization action. Saponins can disaggregate the suspensions of mucoproteins which act as promoters of crystallization (26). In our study OLE was also found to possess high total phenolic content (>40 mg/g GAE). Poly phenols including tannins and flavonoids are important source of natural antioxidants (27). Among all the phytochemicals found to be present in OLE through GC MS analysis, most of the constituents are either biologically active or act as key components for the production of bioactive compounds via different biological pathways. This CaOx crystallization inhibitory property of OLE can be beneficial in preventing lithiasis at its earlier stage. Above observations support the applicability of extract of *Ocimum sanctum* as an inhibitor of calcium oxalate crystallization.

Conclusion

Nature has gifted us with a lot of medicinal plants possessing excellent pharmacological activities. Here, we evaluated the affect of hydroalcoholic extract of *Ocimum sanctum* on in-vitro crystallization of calcium oxalate. Based on the data it can be concluded that *Ocimum sanctum* hydroalcohalic extract can effectively inhibit the nucleation, growth as well as aggregation of CaOx crystallization process. Hence, it can be used as a phytotherapeutic agent to manage urolithiasis at its early stage and can be effective to reduce the chances of its reoccurrence. Molecular events taking place during the inhibition of different phases of crystallization needs to be investigated further.

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