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EFFECT OF *PHASEOLUS VULGARIS* ON *IN VITRO* CALCIUM OXALATE CRYSTALLIZATION

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ABSTRACT

The objective of the present study was to evaluate antiurolithiatic activity of ethanolic seed extract of *Phaseolus vulgaris* (EPV) on *in vitro* calcium oxalate (CaOx) crystallization. *In vitro* CaOx crystallization was assessed by employing crystal nucleation, aggregation and growth assay methods in the presence and absence of EPV at the concentration range of 100-1000 µg/ml. Cystone (100-1000 µg/ml) was used as positive control. Effect of EPV on the rate of crystal nucleation, aggregation and growth were measured spectrophotometrically and their percentage inhibition was calculated. Morphological characteristics of the crystals in control, cystone and EPV were observed under phase contrast microscope. EPV and cystone produced dose-dependent inhibition of crystal nucleation, aggregation and growth. IC_{50} values of cystone on nucleation, aggregation and growth were found to be 166.40±22.31, 343.69±27.51 and 360.10±17.52 µg/ml respectively, whereas with EPV, IC_{50} values were 489.10±49.27, 580.11±53.16 and 725.70±29.33 µg/ml respectively. Results of the present *in vitro* study suggest that ethanolic seed extract of *P. vulgaris* possess calcium oxalate crystal inhibition activity on crystal nucleation, aggregation and growth suggesting its antiurolithiatic activity.

INTRODUCTION

Urolithiasis is characterized by the formation of stones in the urinary tract. Approximately 4-20% of humans are affected by urinary stone disease. Among various stones formed, calcium stones are more predominant (up to 60%) of the human kidney stones [1]. The common form of calcium stones are calcium oxalate, which exist in two forms, calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) [2, 3].

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Pathogenesis of stone formation in the kidney involves a series of steps such as supersaturation, crystal nucleation, crystal aggregation, crystal retention and crystal growth [4]. Supersaturation of urine promotes crystallization in urine with subsequent formation of solid crystal particles within the urinary tract. This further leads to crystal nucleation, then these crystals combine with each other to form the crystal aggregates. Later these aggregates damage the renal tissue, where they get retained and accumulated to develop in to stones [5, 6]. Further, studies suggest that oxalate assisted renal damage occurs through involvement of reactive oxygen species (ROS) in the urolithiasis [7-9].Therefore, if the progression of crystallization steps and ROS induced renal damage can be prevented, urolithiasis can also be prevented.

Many medicinal plants have been used to treat urinary stones since ancient, though the rationale behind their use is not well established. Plant medicines are in great demand all over the world for the primary health care due to their wide biological and medicinal activities, higher safety margin and lesser cost. *Phaseolus vulgaris*, also known as kidney beans, called Raajmah in India, is a common Indian dish. The seeds of *P. vulgaris* are gaining increasing attention as a functional or nutraceutical food, due to its rich variety of phytochemicals such as proteins,amino acids, complex carbohydrates, dietary fibers, oligosaccharides, phenols, saponins, flavonoids, alkaloids and tannins with potential health benefits [10, 11]. The seeds of *P. vulgaris* possess biological activities like enhancement of the bifidogenic effect [12], antioxidant [13] and anticarcinogenic effects [14]. The seeds were claimed to possess diuretic activity and are commonly used to treat water retention in pregnant women [15]. Till now no studies were carried out on the antiurolithiatic activity of *P. vulgaris.* Hence, in the present study, antiurolithiatic activity of the ethanolic seed extract of *P. vulgaris* (EPV) was investigated employing an *in vitro* model.

MATERIALS AND METHODS

Plant Material

Seeds of *P. vulgaris* were purchased from the local market, authenticated by the Dr. B. Sitaram, Professor, Department of Dravyaguna, S.V. Ayurvedic Medical College, Tirupat. Voucher specimens were kept in Sri Padmavati Mahila Visvavidyalayam, Tirupati. The seeds were coarsely powdered by mechanical blender and used for extraction.

Preparation of ethanolic seed extract of *P. vulgaris* (EPV)

The seed powder (200 g) was macerated with 95% ethanol (1 L) for 24 hours, followed by Soxhlet extraction for 6 hours. The extract was concentrated under reduced pressure and the obtained semisolid mass (11 g) was stored in an air tight container.

Preliminary phytochemical screening

Preliminary phytochemical screening of EPV was carried out for the presence of alkaloids, carbohydrates, flavonoids, phenolic compounds, saponins, sterols and tannins using standard procedures [16].

Quantification of Phytoconstituents

Total phenolic content

Quantification of total phenols in EPV was done according to modified Folin-Ciocalteu method with some minor modifications [17]. A calibration curve was prepared using gallic acid as standard (50-1000 µg/mL). The standard/extract solution (100 µL) was mixed with 200 µL Folin-Ciocalteu's reagent (diluted ten-fold with water) and 2 mL of 0.7 M sodium carbonate. The absorbance was measured after incubation for 2 hours at room temperature at 765 nm with a UV-visible spectrophotometer. All determinations were carried out in triplicate. The concentration of phenolic compounds in EPV was determined from the standard gallic acid calibration curve. The total content of phenolic compounds in EPV was expressed as µg gallic acid equivalents (GAE)/g of dry extract.

Total flavonoid Content

Total flavonoid content in the EPV was measured by aluminum chloride colorimetric assay [18]. A calibration curve was constructed using quercetin. At the concentration range of 50-1000 µg/mL of quercetin (0.5 mL) were mixed separately with 1.5 mL methanol in 10 mL volumetric flask containing 4ml of distilled water and 0.3 mL of 5% of sodium nitrate. After five minutes, 0.3 mL of 10% aluminum chloride was added. At sixth minute, 2 mL of 1M sodium hydroxide was added and then the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. All the determinants were in triplicate. Total flavonoid content of EPV was calculated from calibration curve of quercetin was expressed as µg of quercetin equivalents/ g of dry extract.

***In vitro* CaOx crystallization model**

Crystal Nucleation assay

The method used was similar to that described by Hennequin et al 1993 with some minor modifications [19]. Solutions of calcium chloride (5 mmol/L) and sodium oxalate (7.5 mmol/L) were prepared in buffer containing Tris-HCl (0.05 mol/L) and sodium chloride 0.15 mol/L at pH 6.5. Calcium chloride solution (8 mL) was mixed separately with 1 mL of EPV at different concentrations (100, 200, 400, 600, 800 and 1000 µg /mL). Crystallization was started by adding sodium oxalate solution (1 mL) and the change in absorbance was measured at 620 nm in a UV spectrophotometer (UV-1800, Shimadzu Pvt. Ltd) for 30 mints. The temperature was maintained at 37 ºC. Similar procedure was repeated for the control with distilled water in place of the extract. All samples were assayed in triplicate. Cystone was used as a positive control. Percentage inhibition of Nucleation rate was then calculated by comparing the turbidity slope of different concentrations of cystone or EPV with the control by the following formula.

$$[1-(Tsi\ /Tsc)]\ x100$$

Where Tsi was the turbidity slope of nucleation in the presence of inhibitor sample i.e, cystone/plant extract (EPV) and Tsc was the turbidity slope of nucleation in the absence of inhibitor (control).

Crystal Aggregation assay

The rate of aggregation of the CaOx crystals was determined by the method of Atmani and Khan [20] with slight modifications. The COM crystals were prepared by mixing both the solutions of calcium chloride and sodium oxalate at 50 mmol/L. The solutions were equilibrated to 60 ºC in water bath, cooled to 37 ºC and kept overnight. Then the solution was centrifuged and evaporated at 37 ºC. CaOx crystals were used at a final concentration of 0.8 mg/mL, buffered with 0.05 mol/L Tris-HCl and 0.15 mol/L sodium chloride at pH 6.5. The experiment was conducted at 37 ºC in the presence and absence of EPV at concentration range of 100, 200, 400, 600, 800 and 1000 µg /mL. The absorbance was recorded at 620 nm for a period of one hour for every 10 minutes time interval. All samples were assayed in triplicate. Cystone was used as positive control. Percentage inhibition of aggregation rate was then calculated by comparing the turbidity slope of different concentrations of cystone/EPV with the turbidity slope of the control by the following formula.

$$[1-(Tsi/\ Tsc)] \times 100$$

Where Tsi was the turbidity slope of aggregation in the presence of inhibitor sample i.e, cystone/ plant extract (EPV) and Tsc was the turbidity slope of aggregation in the absence of inhibitor.

Crystal Growth assay

The assay followed was the method described by Nakagawa et al. with some essential modifications [21]. COM stone slurry (0.2 mg/mL) was prepared in 50 mM sodium acetate buffer (pH 5.7). Solution of 1 mM calcium chloride and 1 mM sodium oxalate was prepared with 10 mM Tris-HCl containing 90 mM NaCl was adjusted to pH 7.2. COM crystal seed (0.2 µL) was added to a solution containing of 1 mM of calcium chloride and 1 mM of sodium oxalate. Consumption of oxalate starts immediately after COM slurry addition and decreasing free oxalate was detected by spectrophotometry at wavelength 214 nm. One ml of EPV (100, 200, 400, 600, 800 and 1000 µg /mL) was added to the above COM slurry containing calcium chloride and sodium oxalate, to evaluate the inhibitory capacity of EPV on CaOx crystal growth. Similar procedure was repeated for the control with distilled water in place of the extract. All experiments were performed in triplicate. Cystone was used as positive control. The relative rate of reduction of free oxalate was calculated using the baseline value and the value after 30 seconds incubation with or without cystone or EPV. The relative percentage inhibition of crystal growth was calculated as follows. Where *S* is the relative rate of reduction of free oxalate with in the presence of inhibitor sample i.e, cystone/ plant (EPV), *C* is the relative rate of reduction of free oxalate without any inhibitor sample.

$$[(C - S)/C]\ x\ 100$$

Statistical analysis

All values were expressed as Mean ± SD of (n = 3) observations. The 50% inhibitory concentration (IC_{50}) value was evaluated by logistic regression analysis by using Graph pad prism software version 5.0.

RESULTS

Preliminary phytochemical analysis revealed the presence of flavonoids, polyphenols, saponins and tannins in the EPV. Total phenolic content of EPV, determined from the calibration curve of gallic acid ($y = 0.030x+0.055$, $R^2 = 0.990$) by Folin-Ciocalteu method was found to be 418.78 µg/g gallic acid equivalents. The standard curve of gallic acid was shown in Figure 1. Total flavonoid content of EPV was determined from the calibration curve of quercetin ($y = 0.052x+0.032$, $R^2 = 0.996$) by spectrophotometric method and was 181.47 µg/g quercetin equivalents. The standard curve of quercetin was shown in Figure 2. Different concentrations (100, 200, 400, 600, 800 and 1000 µg/ml) of cystone and EPV showed dose dependent inhibition of crystal nucleation (Table 1). The IC_{50} values of cystone and EPV on crystal nucleation were found to be 166.40±22.31 and 489.10±49.27 µg/ml respectively (Figure 3). Similar dose dependent effects were observed in crystal aggregation assay (Table 1) and the IC_{50} values of cystone and EPV were found to be 343.69±27.51 and 580.11±53.16 µg/ml respectively (Figure 4).

A significant increase in the percentage inhibition of crystal growth was observed in presence of cystone and EPV and it was denoted by a decrease of free oxalate levels in the presence of cystone and EPV (Table 1). The IC_{50} values of cystone and EPV on crystal growth were found to be 360.10±17.52 and 725.70±29.33 µg/ml respectively (Figure 5). The Phase contrast microscopical images of crystals in the control and in presence of cystone (1000 µg/ml) and EPV (100, 400, 800 and 1000 µg/ml) were shown in Figure 6 a-f respectively. Figure 6 a showed sharp, large size and small crystal aggregates, denotes crystallization was more in the control. Figure 6 b showed decrease in the crystal mass and crystal aggregations by cystone (1000 µg/ml). Figure 6 c-f showed dose dependent inhibitions of crystal growth were observed in the presence of EPV (100, 400, 800 and 1000 µg/ml).

DISCUSSION

Urolithiasis with multifactorial etiology and high rate of recurrence is a medical challenge for its management [22]. To reduce the incidence of urolithiasis, elucidation of lithogenesis mechanism/s, through fundamental research is essential. Basic steps involved in crystallization process are nucleation, aggregation and growth. Crystallization process is initiated in solutions or in urine by supersaturation of solutes or salts on reaching their metastable levels. Crystal nucleation is the initial step in a supersaturated solution. It occurs due to transformation of liquid to solid phase. The first formed crystal nuclei have anchored surfaces to form crystal lattice; later crystal size is increased by addition of new components or clusters to the nucleus. These crystals stick to each other to form large particles, called crystal aggregates and is the critical step in stone formation [23]. The whole process potentiates crystal stone. In the present *in vitro* study, percentage inhibition of CaOx crystallization on nucleation, aggregation and growth were assessed in presence of EPV and standard cystone.

Results of the present study suggest that percentage inhibition of crystal nucleation, aggregation and growth in presence of EPV was less when compared to the standard, but the inhibitory effects are dose-dependent on crystallization. Phase contrast microscope images denote dense solid crystals in the control group. Treatment with cystone and EPV reduced the crystal mass. Earlier studies reveal that different mechanistic approaches were involved in the crystal inhibition at different phases of crystallization. In general, this inhibition of crystallization is due to changes that occur in COM surface, to form defected or unhealthy crystals in the process of crystallization. Other mechanisms suggested were the sequestering of insoluble calcium salts to form soluble metal complexes by various phytoconstituents [24].

Table 1: Effect of EPV on *In vitro* calcium oxalate crystallization

Concentrations (µg/ml)	Nucleation Percentage inhibition of crystal nucleation (Mean± SD)		Aggregation Percentage inhibition of crystal aggregation (Mean± SD)		Growth Percentage relative inhibition of growth (Mean± SD)	
	Cystone	EPV	Cystone	EPV	Cystone	EPV
100	23.01±5.29	09.59±3.22	30.45±3.43	10.24±5.19	25.01±2.41	10.23±6.30
200	49.18±4.33	20.12±6.30	37.18±4.51	18.39±4.11	36.13±1.54	18.63±5.41
400	59.12±7.30	29.17±8.31	49.12±5.88	26.45±2.28	49.23±4.63	29.57±3.22
600	64.20±6.25	37.81±6.21	56.20±3.47	30.56±4.21	53.34±3.61	33.89±4.29
800	75.14±8.41	42.70±8.34	61.14±2.44	37.78±3.17	60.22±2.54	41.11±3.32
1000	81.03±2.27	50.23±7.29	66.03±3.49	41.33±6.10	68.20±2.44	49.80±2.45
IC_{50}	166.40±22.31	489.10±49.27	343.69±27.51	580.11±53.16	360.10±17.52	725.70±29.33

All the values are expressed as mean ± SD of 3 observations.
IC_{50} were calculated from the logistic regression analysis

Figure 1: Calibration curve of gallic acid

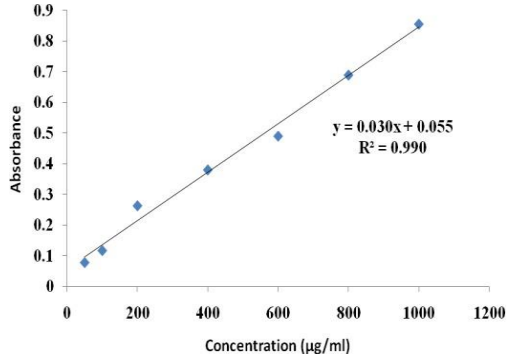


Figure 2: Calibration curve of quercetin

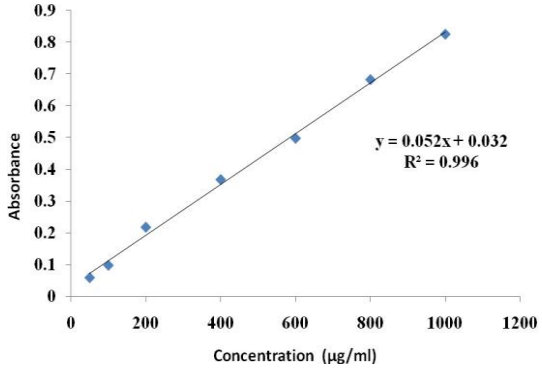


Figure 3: Effect of EPV on crystal Nucleation

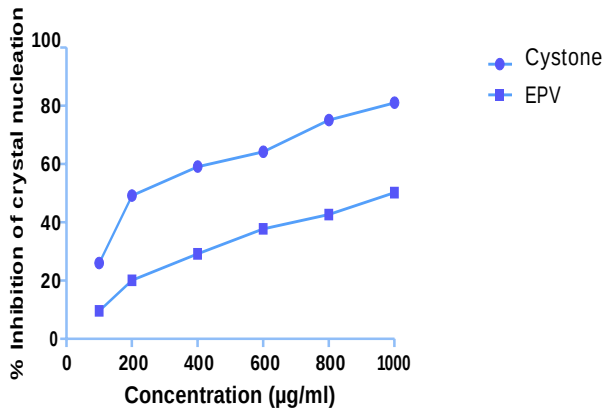
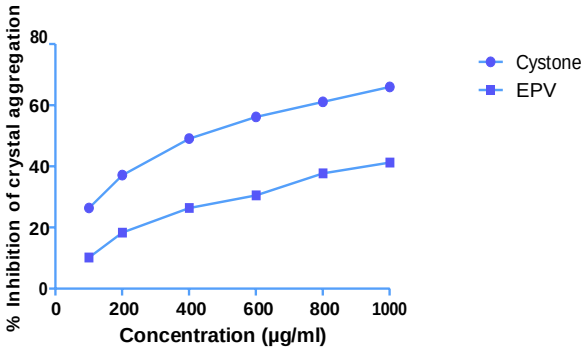


Figure 4: Effect of EPV on crystal Aggregation



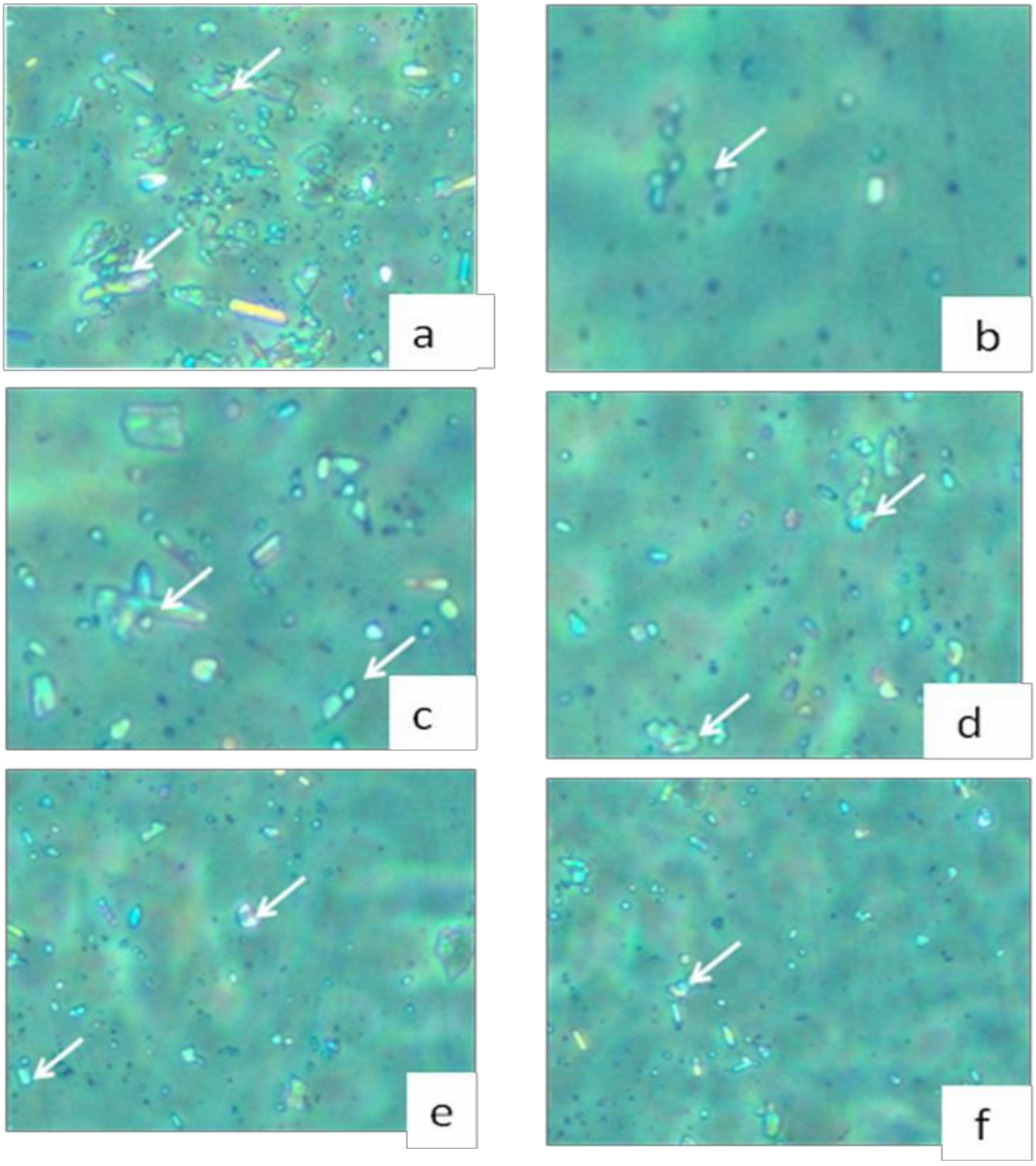
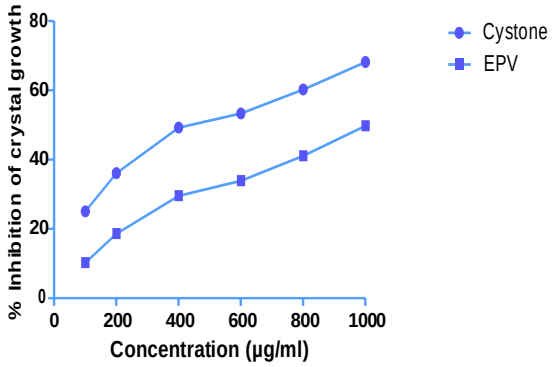


Figure: 1 (a-f): Phase contrast images of CaOx crystallization: a) Control (without inhibitor): showed large sized and dense mass of CaOx crystals, b) Cystone (1000 µg/ml): showed inhibition of CaOx crystallization by cystone, c-f) showed dose dependent inhibition of CaOx crystallization in the presence of EPV at doses of 100 µg/ml, 400 µg/ml and 1000 µg/ml

Figure 3: Effect of EPV on crystal Growth



Studies revealed that plant phytoconstituents such as saponins, flavonoids, polyphenols, poly carbolic acids and terpenoids inhibit the crystallization of stone forming constituents in human urine as well as in animal models. Saponins show anticrystallization properties by disaggregating the suspension of mucoproteins [25]. In the present study, preliminary phytochemical screening of EPV revealed the presence of flavonoids, polyphenols, tannins and saponins. Hence, CaOx crystallization inhibitory effect produced by EPV may be due to the presence of phytoconstituents like flavonoids, polyphenols, tannins and saponins that possess the crystal inhibitory capacity.

CONCLUSION

In the present study the seeds of *Phaseolus vulgaris* showed *in vitro* calcium oxalate crystal inhibitory effect at the level of crystal nucleation, aggregation and growth. Further *in vivo* studies are needed to explore the mechanism of action and phytoconstituents responsible for the antiurolithiatic activity.

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