

## Exploring *in vitro* efficacy of roots of *Bergenia ligulata* for urolithiasis management

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### ABSTRACT

Urolithiasis is a metabolic disorder associated with formation of stones within the urinary tract. The physiochemical processes underlying stone formation involve precipitation, growth, aggregation and concretion of lithogenic salts in urine. An approach for the management of urinary stones, in lieu of surgical procedures, includes usage of herbal plants. *Bergenia ligulata* also known as "Paashanbheda" has been used for dissolving kidney stones traditionally. The objective of the present study is to investigate the calcium oxalate (CaOx) crystallization inhibition efficacy of aqueous root extract of *Bergenia ligulata* (BLAE) through *in vitro* experimental procedure- nucleation and aggregation assays. The inhibitory activity of the BLAE was determined by spectrophotometric assay for which percentage inhibition of calcium oxalate crystallization was calculated. BLAE showed dose dependent inhibition, i.e. more percentage inhibition of calcium oxalate crystallization was observed with the increase in the concentration of BLAE. Present study indicated significant efficacy of BLAE in inhibiting the formation of urinary stones displaying its inhibitory action in calcium oxalate formation in both nucleation and aggregation assays.

**Keywords:** Aggregation assay, *Bergenia ligulata*, calcium oxalate, nucleation assay, urolithiasis

### INTRODUCTION

Urolithiasis is a metabolic disease that is associated with multiple etiologies which occur as a result of complex interaction between genetic and environmental factors which leads to stone/urolith formation in the urinary tract (Yasui *et al.*, 2008). It is a wide spread condition that can affect individuals across all geographical regions and demographics (Bawari *et al.*, 2017; Thongprayoon *et al.*, 2020; Li *et al.*, 2023). About 12% of world population is affected by kidney stone disease and it is more common in males as compared to females (Sofia *et al.*, 2016). Several risk factors are associated with occurrence of urolithiasis which included consumption of ground water, obesity, lack of physical activity, high intake of salts of calcium, phosphorus, magnesium *etc.*, high intake of sugar, coffee and tea, increased consumption of red meat, less frequency of urination/ day and working in higher environmental temperature

(Sequeira *et al.*, 2023). Changes in urinary salt excretion are associated with calcium oxalate (CaOx) stone formation. Two processes of stone formation are described in *Sushruta Samhita*, one of which include stagnation and supersaturation of urine and the other is by crystallization of crystalloids in the urine (Das *et al.*, 2022). The conventional management of urolithiasis is based on surgical procedures that include external shockwave lithotripsy, ureteroscopy, percutaneous extraction but they are associated with certain drawbacks such as they are invasive procedures, there is higher rate of recurrence of the disease, associated with lifetime medical complications such as chronic kidney disease, hypertension *etc.* and involvement of high cost of treatment (Khan *et al.*, 2021). Along with surgical management, medical management of urolithiasis include usage of certain medicine such as diuretics, urinary alkalizer *etc.* but no such satisfactory treatment is available

(Jamshed *et al.*, 2022). One of the cheaper and safer alternatives for management of uroliths is the use of natural anti-urolithiatic agents derived from herbal plants (Khan *et al.*, 2021). Traditional herbal plants are known to have several beneficial effects in the urinary system such as aqueous extract of *Urticadioica* was helpful in mass reduction of calcium oxalate stones in *in vitro* studies (Belmamoun *et al.*, 2022). Another such important Himalayan plant is *Bergenia ligulata* also known as “*Paashanbheda*” in the Indian traditional system of medicine which is a perennial Himalayan herb belonging to the family Saxifragaceae and in the Indian subcontinent it is distributed along the high altitude of Himalayan regions (Goswami *et al.*, 2013). The roots and rhizomes of the plant are commonly consumed for management of wounds, sepsis, asthma, cough and cold, inflammation, stomach disorders and urinary related issues (Ruby *et al.*, 2012; Gurav *et al.*, 2014). *Ayurvedic* formulations are known to use *Bergenia* species since centuries to deliquesce kidney and bladder stones (Ahmad *et al.*, 2018). Some of the most important active phytoconstituents of *Bergenia ligulata* include bergenin, leucocyanidin, catechin, gallic acid, tannic acid, afzelechin,  $\beta$ -sitosterol (Roychoudhury *et al.*, 2022) which are responsible for the medicinal properties of the plant (Sadat *et al.*, 2015). Aqueous extract of *Bergenia ligulata* showed curative effects against ethylene glycol induced urolithiasis in rats as there was significant decrease in serum and urine markers, decreased CaOx deposits during histological examination and minimum damage in the kidney cells (Sharma *et al.*, 2017). The present study was conducted to evaluate the antilithiatic potential of aqueous roots extract of *Bergenia ligulata* (BLAE) by using different *in vitro* assays *i.e.* nucleation assay, aggregation assay, simultaneous flow static and dynamic model and reservoir static and dynamic model.

## **MATERIALS AND METHODS**

### **Collection of plant material**

The plant material (roots) utilized in this study was collected from Berinag, District Pithoragarh situated in the Kumaon region of Uttarakhand, India, located within 29.80° of North latitude and 80.07° of East longitude and authenticated by Dr.

D.S. Rawat, Assistant Professor, Department of Biological Sciences, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand, India (Voucher specimen GBPUH-1437).

### **Chemicals used in the study**

The study employed usage of analytical research grade chemicals that include calcium chloride, calcium acetate, sodium carbonate, sodium oxalate, sodium chloride, trisodium phosphate and Tris. These chemicals were purchased from Himedia (India) and SRL (India). Autoclaved distilled water was used to prepare different buffers and solutions.

### **Preparation of the extract**

The collected plant material was thoroughly rinsed with running tap water and subsequently by distilled water. The cleansed plant material was then shade dried and after complete drying, it was ground into fine powder. 50 grams quantity of the dried powder was soaked in 250 mL of double-distilled water which was then homogenised at a temperature of 37°C for duration of 72 hours in an incubator-shaker unit. Subsequently, the mixture was first strained through the muslin cloth and then passed through Whatmann filter paper No. 1. The resulting aqueous extract of *Bergenia ligulata* (BLAE) was then kept on water bath at 37°C-40°C to evaporate the water followed by lyophilisation and then it was stored at -20°C till further use.

### **Evaluation of anti-urolithiatic activity of the plant extract by using *in vitro* assays**

Different *in vitro* assays were conducted to study the anti-urolithiatic activity of BLAE *viz.* nucleation, aggregation and CaOx mineralization inhibition assays. Crystallization or inhibition assays were conducted with or without BLAE to evaluate the inhibitory potential of BLAE.

### **Nucleation assays**

The nucleation assay was conducted by the method as described by Patel *et al.* (2012) with slight modifications. A solution consisting of 5 mmol/L calcium chloride (CaCl<sub>2</sub>) and 7.5 mmol/L sodium oxalate (Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub>) was prepared in a buffer containing 0.05 mol/L Tris-HCl and 0.15 mol/L sodium chloride (NaCl) buffer adjusted at a pH of 6.5. Different dilutions of BLAE ranging from 25-

1000 µg/ml were prepared in distilled water. 100 µl of different dilution of BLAE was then mixed with 950 µl of CaCl<sub>2</sub> solution, which was followed by addition of 950 µl of Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> solution which initiated the crystallisation. The final mixture was incubated at a temperature of 37°C for a period of 1 hour. 100 µL of buffer was added to CaCl<sub>2</sub> solution which was used as a control in the experiment, and was similarly incubated at 37°C for a period of 1 hour. Subsequently, the nucleation of the crystals was observed under microscope at 40X magnification.

### Aggregation assay

The method used to perform aggregation assay was as described by Hess *et al.* (1989) with some modifications. To create 'seed' CaOx crystals, a solution was prepared by mixing calcium chloride, CaCl<sub>2</sub> (6.0 mmol/L) and sodium oxalate, Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> (6.5 mmol/L) in a buffer containing Tris-HCl (0.05 mol/L) and NaCl (0.15 mol/L) at a pH of 6.5. Then, 950 µL of CaCl<sub>2</sub> solution was mixed with 100 µL of different dilution of BLAE ranging from 100-1500 µg/mL to examine the degree of inhibition of aggregation by comparing the turbidity of samples in the presence of the plant extract at different concentrations. After this step, 950 µL of Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub> was added to it which initiated crystallization. 100 µL of buffer was added to CaCl<sub>2</sub> which was used as control in the experiment. The resulting mixture was then incubated for a period of 1 hour at 37°C.

The optical density (OD) of the crystallized suspension was measured at a wavelength of 620 nm. The inhibition of percentage aggregation was then calculated by comparing the turbidity observed in presence of the extract with that in the control.

The following formula was used to calculate percent aggregation inhibition:

$$\% \text{ Inhibition} = [1 - (\text{Turbidity of the sample} / \text{Turbidity of the control})] \times 100$$

### Inhibition of calcium oxalate mineralization

To evaluate the *in vitro* inhibition of CaOx mineralization by BLAE was examined using four different experimental models *viz.*, 'simultaneous flow static model' (S.S.M.), 'simultaneous flow dynamic model' (S.D.M.), 'reservoir static model' (R.S.M.) and 'reservoir dynamic model' (R.D.M.).

### Simultaneous flow static model (S.S.M)

The method used for S.S.M. was as described by Farook *et al.* (2004) with slight modifications. Three different burettes were filled with 50 mL of 0.01M of Na<sub>2</sub>C<sub>2</sub>O<sub>4</sub>, 50 mL of 0.01M of calcium acetate, Ca(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>, and 50 mL of the BLAE (1500 µg/ml). Subsequently, all the solutions were then allowed to fall simultaneously into a 250 mL beaker in a controlled drop wise manner and with equal speed. After the process was completed, the mixture was digested in a hot water bath for a period of 10 minutes and then cooled down to room temperature. The precipitate was then collected into a pre-weighed centrifuge tube by centrifuging small volumes at a time and discarding the supernatant liquid. The tube with the precipitate was subjected to drying in a hot air oven at 120°C, cooled to room temperature and weighed.

### Simultaneous flow dynamic model (S.D.M.)

The procedure was similar as S.S.M. except that the reaction mixture in the beaker was continuously stirred on a magnetic stirrer during the flow of salt forming solutions as well as the inhibitor (Farook *et al.*, 2004).

### Reservoir static model (R.S.M)

In this particular model, the entire volume of BLAE (50 mL) was placed in the beaker. Subsequently, the two salt forming solutions were allowed to run into it gradually through burettes and thus forming a reservoir of inhibitor into which the salt forming solutions ran down and the rest procedure was similar to that of simultaneous flow static model (Farook *et al.*, 2004).

### Reservoir dynamic model (R.D.M)

The procedure in the R.D.M. was similar to that of reservoir static model except that the reaction mixture was continuously stirred on a magnetic stirrer during the experiment (Farook *et al.*, 2004).

For the analysis of the data the observations were recorded in triplicates, and their mean ± standard deviation (SD) values were calculated for statistical analysis. Then the results were analysed using a one factorial Completely Randomized Design and to assess the significance of difference between different treatment means, a critical

difference was determined at a 5% level of significance.

## **RESULTS AND DISCUSSION**

### **Effect of BLAE on nucleation assay**

In the present study it was observed that as the concentration of BLAE was increased, there was a reduction in the aggregation of CaOx crystals and the percentage of inhibition of CaOx crystallization was also increased as presented in Figure 1.

### **Effect of BLAE on calcium oxalate crystallization through aggregation assay**

A dose dependent inhibition of CaOx crystallization was shown by the plant extract in the *in vitro* aggregation assay. As the concentration of BLAE was increased, there was an increase in the percent inhibition of aggregation. The highest percentage of inhibition of aggregation was obtained at a concentration of 1500 µg/ml which was 85.13%. The results are shown in Table 1 and Figure 2.

### **Effect of BLAE on Inhibition of calcium oxalate mineralization**

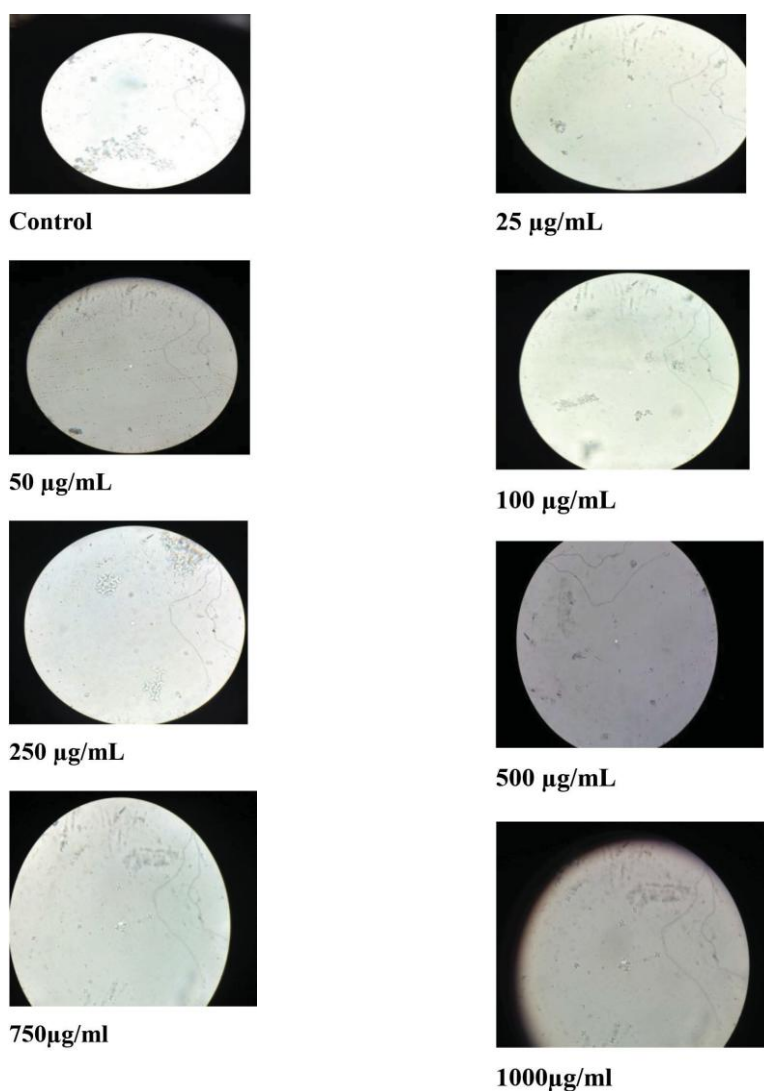
The inhibitory effect of BLAE on the formation of CaOx crystal has been studied in various dynamic models such as simultaneous flow static model, simultaneous flow dynamic model, reservoir static model and reservoir dynamic model. For this, BLAE at a concentration of 1500 µg/ml was taken and the above-mentioned procedure was followed. The formed precipitate was quantified at last and then percentage of inhibition of crystallization was calculated in various models. The percentage of inhibition of crystallization was found to be highest in reservoir static model (53.88%), followed by simultaneous flow dynamic model (32.27%), reservoir dynamic model (26.10%) and least in simultaneous flow static model (14.78%). The result showing the percent of inhibition by BLAE in various dynamic models is presented in Figure 3.

The oxalates can initiate a vicious cycle of oxidative stress in the renal epithelial cells which can lead to cellular injury and deposition of the crystal of CaOx on the injured cells which further cause oxidative damage and inflammation which can further lead to irrevocable damage of kidney

cells (Singh *et al.*, 2022). To prevent this kind of damage *Bergenia ligulata* is a traditional medicinal plant rich in antioxidants which is used for the management of kidney stones. Several reports suggest that the mechanism involved behind the antilithiatic effect of *Bergenia* sp. is mainly by diuresis, inhibiting formation and aggregation of CaOx crystals, antioxidant activity and hypermagnesemic effects (Nagal *et al.*, 2013). Aqueous extract of *Bergenia ligulata* showed inhibition of calcium oxalate monohydrate (COM) crystals (Joshi *et al.*, 2005). Methanolic extract of *Bergenia ligulata* as well as bergenin is known to inhibit, show significant anti-oxidant effect against 1, 1-diphenyl 2-picrylhydrazyl free radical as well as prevented lipid peroxidation in *in vitro* condition and prevented deposition of CaOx crystals in the renal tubules of rats (Bashir and Gilani, 2009). Ethanolic extract of *Bergenia ligulata* showed a dose dependent inhibition of nucleation and aggregation process of CaOx crystal formation and also showed a cytoprotective effect on renal epithelial NRK-52E cells against oxalate injury. It was also found that when the renal cells were exposed to the extract the COM crystals were converted to CaOx dihydrate crystals that are known to be less injurious (Singh *et al.*, 2021). Bergenin isolated from rhizome of *Bergenia ligulata* when given at a dose of 10 mg/kg body weight to hyperoxaluric rats-maintained oxidant/antioxidant balance, improved creatinine clearance and decreased kidney damage and thus helpful in managing the CaOx calculi (Aggarwal *et al.*, 2014). *Bergenia ligulata* shows its antiurolithiatic effect by decreasing oxidative stress, modulating structure of crystals and preventing adhesion of crystal thus, exhibiting cytoprotective effect (Singh *et al.*, 2022). *Bergenia ligulata* is one of the major ingredients of a polyherbal formulation named cystone which is commonly used in the management of uroliths. *Bergenia ciliata* is another species of the genus *Bergenia* which was found to inhibit the nucleation as well as aggregation of COM crystals in a dose dependent manner. 70% methanolic extract of rhizomes of *Bergenia ciliata* also prevented the histopathological changes in animal model of ethylene glycol induced hyperoxaluria (Saha and Verma, 2011). A crude phenolic compound when

**Table 1: Percentage inhibition of calcium oxalate crystallization by BLAE in aggregation assay**

S. No.	Different concentration of BLAE	Percent inhibition of Aggregation (Mean±S.D.)
1.	25 µg/ml	18.85±1.57
2.	50 µg/ml	31.91±2.10
3.	100 µg/ml	37.09±1.87
4.	250 µg/ml	43.38±1.77
5.	500 µg/ml	52.35±1.30
6.	750 µg/ml	58.77±1.95
7.	1000 µg/ml	72.93±2.06
8.	1250 µg/ml	75.74±1.72
9.	1500 µg/ml	85.13±1.74
	<b>Critical Difference</b>	3.112
	<b>Coefficient of Variation</b>	3.402



**Fig. 1: Nucleation assay at different concentration of BLAE observed under Microscope (40X)**

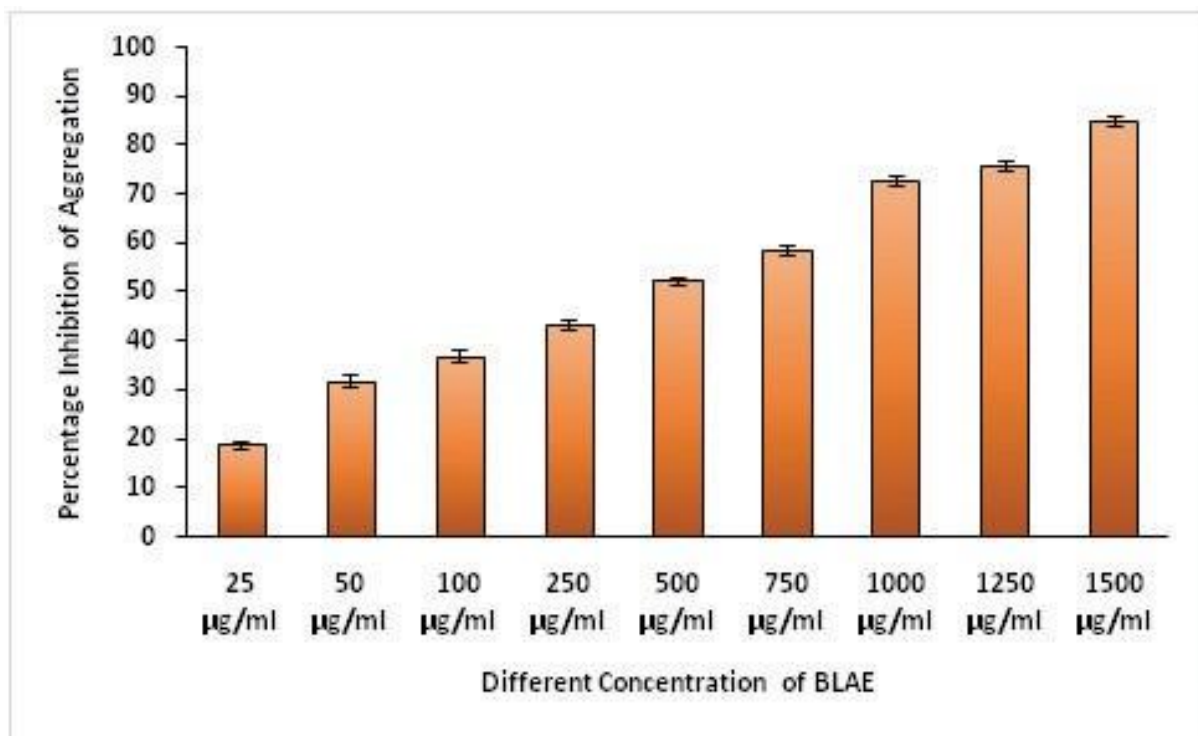


Fig. 2: Percentage inhibition of calcium oxalate crystallization by BLAE in aggregation assay

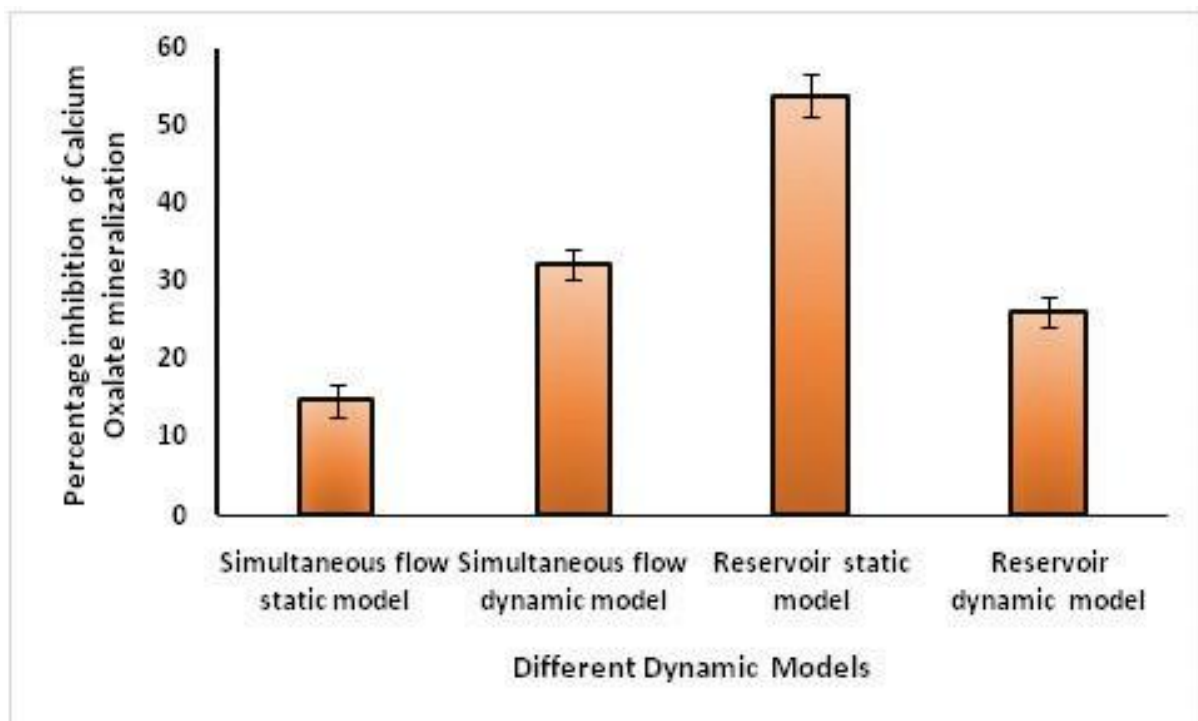


Fig. 3: Percentage inhibition of Calcium Oxalate mineralization by BLAE in different models

isolated from *Bergenia ciliata* showed highest dissolution of calcium phosphate and CaOx stones (Byahatti et al., 2010).

## CONCLUSION

Thus, the *in vitro* assays in the present study suggest that aqueous extract of roots of *Bergenia ligulata* has a significant anti-urolithiatic activity on the basis of results of nucleation assay, aggregation assays and inhibition of CaOx mineralization as seen in different dynamic models. There was inhibition of nucleation as well as aggregation of CaOx crystals which is a common component of kidney stones/uroliths. Significant efficacy was found in the highest concentration of the plant extract *i.e.*, 1500 µg/ml. Further *in vivo* studies are required to explore the mechanism of action of aqueous extract of *Bergenia ligulata*. The active phytoconstituents present in the plant extract are responsible for the anti-urolithiatic activity which will require further characterization and isolation of the active compounds.

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