JOURNAL OF BASIC PHARMACOLOGY AND TOXICOLOGY

Original Research Article

Antiurolithiatic activity of *Bacopa monnieri* by *in vitro* calcium oxalate crystallization methods

Kishore Bandarapalle[1*], Rajasekhar KK[2], Bhavitha J[2]

[1]*Department of Pharmaceutics, Sri Padmavathi School of Pharmacy, Tiruchanoor, Tirupati, 517503, India*

[2]*Department of Pharmaceutical Chemistry and Analysis, Sri Padmavathi School of Pharmacy, Tiruchanoor, Tirupati, 517503, India*

Abstract

The primary objective of this research was to investigate the antiurolithiatic effect of the aqueous extract of *Bacopa monnieri* (AEBM) on *in vitro* crystallization methods. The antiurolithiatic behaviour was observed in the presence and absence of AEBM at the concentration range of 100-1000 µg/mL by employing crystal nucleation, crystal aggregation, and crystal growth assay methods. Cystone (Himalaya Drug Company) was used as positive control in the concentration range of 100-1000 µg/mL. Inhibition efficiencies of AEBM on crystal nucleation, crystal aggregation and crystal growth were estimated spectrophotometrically. The percentage inhibition rates of crystal nucleation, crystal aggregation and crystal growth by AEBM and cystone were found to be dose-dependent. The half maximal inhibitory concentration (IC_{50}) values of cystone on crystal nucleation, crystal aggregation and crystal growth were estimated to be 355 ± 45.4, 598 ± 20.1 and 590 ± 63.3 µg/mL respectively, while the IC_{50} values of AEBM were found to be 854 ± 24.5, 1008 ± 155 and 926 ± 25.1 µg/mL, respectively. The findings of this study disclosed that an aqueous extract of *Bacopa monnieri* showed a promising calcium oxalate crystal inhibition activity on crystal nucleation, crystal aggregation, and crystal growth stages of urine stone formation.

Keywords: *Bacopa monnieri,* Antiurolithiatic activity, Crystal nucleation, Crystal aggregation, Crystal growth

1 Introduction

Urolithiasis is the third pervasive renal disorder. Calcium oxalate calculi is the predominant and frequently noticed calculi among different types of calculi [1-2]. Pathogenesis of renal calculi emergence involve sequential steps include supersaturation, crystal nucleation, crystal aggregation, crystal retention and crystal growth [3]. Urine supersaturation is a prerequisite step that contributes to the eventual formation of strong crystal particles and facilitates crystallization in the urine. These further assist in crystal nucleation, and these crystals consolidate into crystal aggregates. The resultant aggregates impair the renal tissue. These aggregates are preserved and deposited for the progress of calculi establishment [4-5]. Further, studies demonstrate that oxalate-induced renal damage originates by the collaboration of reactive oxygen species (ROS) in urolithiasis [6-8]. Therefore, urolithiasis can be prevented by inhibition of key steps in the crystallization process followed by inhibition of ROS-influenced renal damage.

Numerous medicinal plants have been documented for the management of renal calculi since pre historic times. In the current context, the world population sheds light on medicinal plants for their multiple pharmacological actions, mitigating complications, side effects, cost effectiveness and easily accessible. *Bacopa monnieri* is generally referred to as "jalabrahmi" and belongs to Scrophulariacae family. The *B. monnieri* draws interest owing to its variety of phytochemical constituents such as brahmine, herpestine, nicotine, D-mannitol, hersaponin and monnierin. The leaves contain saponin glycosides known as bacoside-A and bacoside-B. The leaves also contain saponins A, B and C, triterpenoid saponins, stignastanol, beta-sitosterol, D-mannitol, aspartic acid, glutamic acid and serine [9-10]. It is broadly used for depression, memory enhancement and convulsions. Further, it is used as diuretic, antioxidant, antimicrobial, antiinflammatory, and analgesic. Literature survey reveals that no research work has been reported on the antiurolithiatic behavior of aqueous extract of *B. monnieri.* In continuation of our previous works, the antiurolithiatic intervention of an aqueous extract of *B. monnieri* (AEBM) was investigated using of an *in vitro* crystallization model [11-12].

2 Materials and methods

2.1 Chemicals

Analytical grade chemicals (Merck India Ltd., Hi-media, and Sigma Aldrich) were procured from Bros Scientifics,

*Corresponding author; E-mail: kishore.brr89@gmail.com; Tel: +91 7995536331

Tirupati, India. Cystone (Himalaya Drug Company, Bangalore, India) was procured from the Apollo pharmacy, Tirupati.

2.2 Plant material and preparation of aqueous extract of B. monnieri

B. monnieri was procured from the Sri Srinivasa Ayurvedic Pharmacy, Tirupati, India. It was coarsely powdered and used for extraction. The 100 g of coarse powder was macerated with 1 L of distilled water for 24 hr at room temperature. The extract was filtered, concentrated, and dried. The dried semisolid mass of 20 g was preserved in an airtight container free of excessive heat, moisture, and air.

2.3 In vitro calcium oxalate (CaOx) crystallization model

2.3.1 Crystal nucleation assay

The solutions of 7.5 mM of sodium oxalate and 5 mM of calcium chloride were prepared using buffer consisting of 0.05 M/L of trisaminomethane hydrochloride (Tris-HCl) and 0.15 M of sodium chloride at pH 6.5. Calcium chloride solution of 8 mL was blended separately with 1 mL AEBM at concentrations of 100, 200, 400, 600, 800 and 1000 µg/mL. Crystallization was triggered by the introduction of 1 mL of sodium oxalate solution and the absorbance shift was recorded at 620 nm in a UV spectrophotometer (UV-1800, Shimadzu Pvt. Ltd.) for 30 min at 37 °C. Same procedure was followed for the control, substituting distilled water instead of the extract. All samples were inspected in triplicate. Standard drug cystone was used as a positive control for comparison at concentrations of 100, 200, 400, 600, 800 and 1000 µg/mL. Percentage inhibition of nucleation rate was computed by equating the turbidity slope of different concentrations of cystone or AEBM with the control by the following formula [13].

[1-(Tsi /Tsc)] x100

Tsi was the nucleation turbidity slope in the existence of inhibitor sample, i.e, cystone/(AEBM) and Tsc was the nucleation turbidity slope in the absence of the inhibitor (control).

2.3.2 Crystal aggregation assay

The extent of crystal aggregation of CaOx was investigated by the procedure of Atmani and Khan with modest adjustments [14-15]. The calcium oxalate monohydrate crystals were developed by combining 50 mM solutions of sodium oxalate and calcium chloride. The solutions were adjusted to 60 °C in a water bath, cooled to 37 °C and held overnight. The solution was then centrifuged and evaporated at 37 °C. CaOx crystals were used at a final concentration of 0.8 mg/mL, formulated with buffer containing 0.05 M of Tris-HCl and 0.15 M of sodium chloride at pH 6.5. The test was tracked at 37 °C in the existence and absence of AEBM at concentrations of 100, 200, 400, 600, 800 and 1000 µg/mL. The absorbance was recorded for one hour for every 10 min time duration at 620 nm. All samples were inspected in triplicate. Cystone was used as a positive control. Percentage inhibition of aggregation rate was determined by comparing the turbidity slope of different concentrations of cystone/AEBM with the turbidity slope of the control by the following formula.
[1-(Tsi/ Tsc)] × 100

Where, Tsi was the turbidity slope of aggregation in the presence of inhibitor sample, i.e., cystone/ AEBM and Tsc was the turbidity slope of aggregation in the absence of inhibitor.

2.3.3 Crystal growth assay

The crystal growth assay was performed based on the frame work stated by Nakagawa et al. with few necessary modifications [16-17]. COM stone slurry 0.2 mg/mL was processed with 50 mM sodium acetate buffer of pH 5.7. Calcium chloride 1 mM and sodium oxalate 1 mM were prepared with buffer containing 10 mM of Tris-HCl and 90 mM of NaCl and regulated to pH 7.2. COM crystal seed (0.2 µL) was applied to the solution comprising 1 mM of calcium chloride and 1 mM of sodium oxalate. The concentration of free oxalate declines with the introduction of COM slurry owing to the initiation of the consumption of oxalate. The drop in free oxalate concentration was measured by spectrophotometry at wavelength 214 nm. In order to assess the inhibitory potential of AEBM on CaOx crystal growth, one ml of AEBM at different concentrations of 100, 200, 400, 600, 800 and 1000 µg/mL was applied to the above described COM slurry containing calcium chloride and sodium oxalate and cystone was used as a positive control. The similar procedure was repeated for the control by substituting distilled water in place of the AEBM/cystone. All experiments were inspected in triplicate. The relative reduction rate of free oxalate was determined using the baseline value and the value after 30 seconds in gestation with or without cystone or AEBM. The relative percentage inhibition of crystal growth was computed as follows.

$[(C - I)/C] \times 100$

Where I is the relative rate of depletion of free oxalate in the presence of the inhibitor sample, i.e, cystone/(AEBM), C is the relative rate of depletion of free oxalate without any inhibitor sample.

2.4 Statistical analysis

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

3 Results and discussion

Phytochemical studies disclosed the presence of alkaloids, tannins, flavonoids, steroids, phenols, proteins, amino acids, saponins, and carbohydrates in the aqueous extract of *Bacopa monnieri* heart wood.

3.1 Effect of AEBM on crystal nucleation

Percentage inhibition of crystal nucleation of standard drug cystone and AEBM at different concentrations of 100, 200, 400, 600, 800 and 1000 µg/mL improved from 25.35 ± 1.72 % to 79.28 ± 4.22% and 13.72 ± 2.80% to 56.31 ± 4.04%, respectively (Table 1). The results indicate that cystone and AEBM exhibited dose-dependent crystal nucleation inhibition. The IC_{50} values of cystone and AEBM on crystal nucleation were calculated to be 355 ± 45.4 and 854 ± 24.5 µg/mL, respectively (Fig. 1).

3.2 Effect of AEBM on crystal aggregation

Similar dose-dependent consequences were observed in the crystal aggregation assay. Percentage inhibitions of crystal aggregation of cystone and AEBM were calculated as 25.63 ± 2.28% to 65.24 ± 3.20% and 9.54 ± 3.22% to 51.57 ± 2.36%, respectively (Table 1) and the IC_{50} values of cystone and AEBM were found to be 598 ± 20.1 and 1008 ± 155 µg/mL, respectively (Fig. 2).

3.3 Effect of AEBM on crystal growth

A substantial rise in the percentage inhibition of crystal growth was found in the presence of cystone and AEBM at different concentrations in ascending sequence, intensified from 27.49 ± 2.81% to 67.35 ± 2.95% and 11.58 ± 4.26% to 52.56 ± 3.74%, respectively, which was symbolized by a reduction in the free oxalate levels in the presence of cystone and AEBM (Table 1). The IC_{50} values of cystone and AEBM on crystal growth were found to be 590 ± 63.3 and 926 ± 25.1 µg/mL, respectively (Fig. 3).

Results suggest that percentage inhibition of crystal nucleation, crystal aggregation, and crystal growth in presence of AEBM and cystone are dose-dependent. Though inhibitory activity of AEBM was lower relatively to the reference drug cystone, but it was found to be effective in inhibiting crystallization. Several investigations demonstrated that distinct mechanistic pathways were involved in the crystal inhibition in distinct aspects of crystallization. In general, this interruption of crystallization could be the modifications that occur at COM surface, to form defective or unhealthy crystals during the crystallization cycle or through the development of soluble metal complexes to insoluble calcium salts by distinct phytoconstituents in the extract [18].

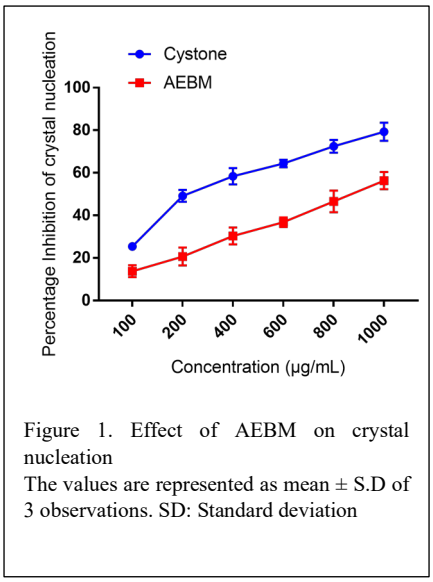

Figure 1. Effect of AEBM on crystal nucleation
The values are represented as mean ± S.D of 3 observations. SD: Standard deviation

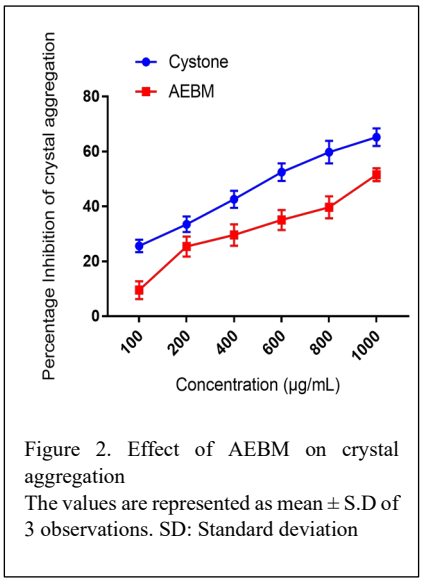

Figure 2. Effect of AEBM on crystal aggregation
The values are represented as mean ± S.D of 3 observations. SD: Standard deviation

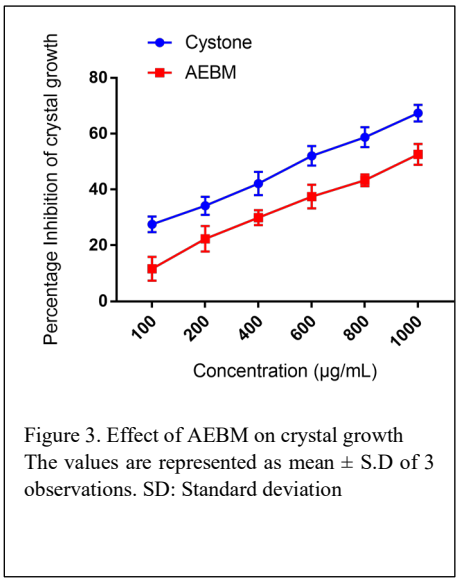

Figure 3. Effect of AEBM on crystal growth
The values are represented as mean ± S.D of 3 observations. SD: Standard deviation

Table 1. Effect of AEBM on *In vitro* calcium oxalate crystallization

Concentration (µg/mL)	% Inhibition of crystal nucleation		% Inhibition of crystal aggregation		% Inhibition of crystal growth	
	Cystone	AEBM	Cystone	AEBM	Cystone	AEBM
100	25.35 ± 1.72	13.72 ± 2.80	25.63 ± 2.28	9.54 ± 3.22	27.49 ± 2.81	11.58 ± 4.26
200	49.14 ± 2.79	20.65 ± 4.19	33.53 ± 2.81	25.42 ± 3.64	34.16 ± 3.23	22.31 ± 4.56
400	58.40 ± 3.83	30.33 ± 3.95	42.60 ± 3.10	29.61 ± 3.88	42.12 ± 4.16	29.90 ± 2.69
600	64.41 ± 1.75	36.76 ± 2.24	52.52 ± 3.20	35.07 ± 3.65	52.05 ± 3.51	37.44 ± 4.23
800	72.48 ± 3.00	46.51 ± 5.09	59.79 ± 4.10	39.70 ± 3.97	58.72 ± 3.58	43.29 ± 2.10
1000	79.28 ± 4.22	56.31 ± 4.04	65.24 ± 3.20	51.57 ± 2.36	67.35 ± 2.95	52.56 ± 3.74
IC_{50}	355 ± 45.4	854 ± 24.5	598 ± 20.1	1008 ± 155	590 ± 63.3	926 ± 25.1

All the values are represented as mean ± SD of 3 observations. IC_{50} was calculated from the logistic regression analysis, AEBM: Aqueous extract of *B. monnieri*, and SD: Standard deviation

4 Conclusion

The current investigation showed evidence of inhibition of calcium oxalate crystallization *in vitro* by AEBM by authorized methods; including crystal nucleation, crystal aggregation, and crystal growth. So further *In vivo* studies need to be carried out to explore and manifest antiurolithiatic activity of AEBM.

Acknowledgements

The authors express deepest gratitude to the management and Dr. D. Ranganayakulu, M. Pharm., Ph.D., Principal, Sri Padmavathi School of Pharmacy, Tiruchanoor, Andhra Pradesh, India, for presenting all the necessary laboratory demands of the research and constant support. The first author is thankful to Dr. N. Sree Lakshmi, P. Aparna, S. Vijay, S. S. Deepa, T. Shobha and K. Subhiksha for their timely help and support.

References

1. Strope SA, Wolf JS, Hollenbeck BK. Changes in gender distribution of urinary stone disease. Urol. 2010;75(3):543-6.
2. Bensatal A, Ouahrani MR. Inhibition of crystallization of calcium oxalate by the extraction of *Tamarix gallica* L. Urol Res. 2008;36(6):283-7.
3. Kavanagh JP. *In vitro* calcium oxalate crystallization methods. Urol Res. 2006;34(2):139-45.
4. Prasad KVSRG, Sujatha D and Bharathi K. Herbal drugs in urolithiasis: a review. Pharmacog Rev. 2007;1(1):175-8.
5. Spivacow FR, Negri AL, Polonsky A and Del Valle EE. Long-term treatment of renal lithiasis with potassium citrate. Urol. 2010;76(6):1346-9.
6. Davalos M, Konno S, Eshghi M, Choudhury M. Oxidative renal cell injury induced by calcium oxalate crystal and renoprotection with antioxidants: a possible role of oxidative stress in nephrolithiasis. J Endourol. 2010;24(3):339-45.
7. Basavaraj DR, Biyani CS, Browning AJ, Cartledge JJ. The Role of urinary kidney stone inhibitors and promoters in the pathogenesis of calcium containing renal stones. EAU-EBU update series. 2007;5:126-36.
8. Thamilselvan S, Khan SR, Menon M. Oxalate and calcium oxalate mediated free radical toxicity in renal epithelial cells: effect of antioxidants. Urol Res. 2003;31(1):3–9.
9. Apel K., Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Ann Rev Plant Biol. 2004;55:373-399.
10. Kawagishi H, Finkel T. Unraveling the truth about antioxidants: ROS and disease: finding the right balance. Nat Med. 2014;20(7):711.
11. Kishore Bandarapalle, Prasanna Raju Yalavarthi, Chandra Sekhar Kothapalli Bannoth. Antiurolithiatic activity of Berberis asiatica by In vitro calcium oxalate crystallization methods. Int J Res Pharm Sci. 2020;11(4):6233-6237.
12. Kishore Bandarapalle, Prasanna Raju Yalavarthi and Chandra Sekhar Kothapalli Bannoth, Evaluation of Antiurolithiatic Activity of Asparagus Racemosus on In Vitro Calcium Oxalate Crystallization Methods. Int J Life Sci Pharma Res. 2020;10(5):56-62.
13. Aggarwal S, Tandon CD, Forouzandeh M, Singla SK, Kiran R, Jethi RK. Role of biomolecules from human renal stone matrix on COM crystal growth. Mol Cell Biochem. 2000;210(1-2):109–119.
14. Hess B, Nakagawa Y, Coe FL. Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am J Physiol. 1989;257(1-2):99-106.
15. Aggarwal KP, Narula S, Kakkar M, Tandon C. Nephrolithiasis: molecular mechanism of renal stone formation and the critical role played by modulators. Bio Med Res Int. 2013;1–21.
16. Hennequin C, Lalanne V, Daudon M, Lacour B, Drueke T. A new approach to studying inhibitors of calcium oxalate crystal growth. Urol Res 1993;21:101-8.
17. Mohamed N, Farook P, Mozhiyarasi, Nalini R. Inhibition of Mineralization of Urinary Stone Forming Minerals by Medicinal Plants. E-Journal of Chemistry. 2006;3(3):182-5.
18. Sree Lakshmi N, Sujatha D, Bharathi K, Prasad KVSRG. Effect of *Phaseolus vulgaris* on *in vitro* calcium oxalate crystallization. J Global Trends Pharm Sci. 2015;6(3):2817-2824.