Antiurolithiatic activity of *Berberis asiatica* by *In vitro* calcium oxalate crystallization methods

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**ABSTRACT**
The primary objective of this research was to investigate the antiurolithiatic effect of the aqueous Heartwood extract of *Berberis asiatica* (AEBA) on *in vitro* crystallization methods. The antiurolithiatic behaviour was carried out in the presence and absence of AEBA at the concentration range of 100-1000 μg/ml by employing crystal nucleation, crystal aggregation, and crystal growth assay methods. Standard drug Cystone was made use of positive control in the concentration range of 100-1000 μg/ml. Inhibition efficiency of AEBA on crystal nucleation, crystal aggregation and crystal growth was spectrophotometrically validated. The percentage inhibition rate of crystal nucleation, crystal aggregation and crystal growth by AEBA and standard drug cystone was endorsed to be dose-dependent in nature. The half maximal inhibitory concentration (IC₅₀) values of standard drug cystone on crystal nucleation, crystal aggregation and crystal growth were estimated to be 415.30±21.35, 573.7±65.53 and 566.20±62.06 μg/ml, respectively, while the AEBA, IC₅₀ values were reckoned to be 839±69.13, 927.10±69.98 and 851±68.60 μg/ml, respectively. The findings of *in vitro* crystallization study disclosed that an aqueous Heartwood extract of *Berberis asiatica* possesses calcium oxalate crystal inhibition activity on crystal nucleation, crystal aggregation, and crystal growth recommended it as a potent and promising antiurolithiatic activity.

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establishment (Lakshmi et al., 2015; Spivacow et al., 2010). Further, studies demonstrate that oxalate obliged renal damage originates by the collaboration of reactive oxygen species (ROS) in urolithiasis (Davalos et al., 2010; Basavaraj et al., 2007; Thamilselvan et al., 2003). Therefore, urolithiasis can be prevented by inhibition of vital aspects in the crystallization process followed by ROS influenced renal damage.

Numerous medicinal plants have been documented for the management of renal calculi since before 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 effective and easily accessible. Berberis asiatica is generally referred to as Daruhaldi / Kilmora in berberidaceae family. The Heartwood of B. asiatica draws interest owing to a variety of phytochemical constituents such as alkaloids, carbohydrates, proteins, steroids, phenols, flavonoids, amino acids, saponins and tannins (Swati et al., 2012; Srivastava et al., 2004). It is broadly used for antimicrobial, analgesic, anti-infective properties, diuretic, anticancer, anti-inflammatory, antioxidant, antidiabetic, anti-rheumatic, hepatoprotective, strong wound healer and antipyretic (Amritpal et al., 2010; Saeidnia et al., 2014). Until now no research work has been performed on the antiurolithiatic behavior of aqueous Heartwood extract of B. asiatica. Thus, in the current research, the antiurolithiatic intervention of an aqueous Heartwood extract of B. asiatica (AEBA) was investigated by implementation of an in vitro crystallization model.

Graph 1: Consequence of AEBA on crystal nucleation

Graph 2: Consequence of AEBA on crystal aggregation

Graph 3: Consequence of AEBA on crystal growth

MATERIALS AND METHODS

Chemicals
Analytical grade chemicals (Merck India Ltd., Himedia, and Sigma Aldrich) procured from Bros Scientifics, Tirupati, India, were utilized in the study. Cystone, (Himalaya Drug Company, Bangalore, India) was procured from the Apollo pharmacy, Tirupati.

Plant Material
Heart wood of B. asiatica has been procured from the Sri Srinivasa Ayurvedic Pharmacy, Tirupati. It was recognized and authenticated by Dr. K. Madhava chetty, Assistant professor, Department of Botany, Sri Venkateswara University, Tirupati. Voucher specimen (voucher No: 0663) were submitted to the research centre. The heart wood was coarsely grated and used for extraction.

Preparation of aqueous Extract of B. asiatica
The 200 g Heartwood powder was macerated with 1 L of distilled water for 24 h at room temperature. The extract was concentrated, and the concomitant semisolid mass of 20 g was preserved in an airtight container free of excessive heat, moisture, and air.

Preliminary Phytochemical Screening
AEBA was Pre-screened for the existence of alkaloids, tannins, sterols, phenolic compounds, carbohydrates, flavonoids, and saponins using standard procedures.

In vitro CaOx crystallization model

Crystal Nucleation assay
The solutions of 7.5 mM of Sodium oxalate and 5 mM of Calcium chloride solutions 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 AEA at distinct 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 minutes at 37 °C. The 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 distinct concentrations include 100, 200, 400, 600, 800 and 1000 µg/ml. Percentage inhibition of nucleation rate was then accessed by comparing the turbidity slope of different concentrations of Cystone/AEA with the control by the succeeding formula (Aggarwal et al., 2000).

\[1 - \frac{(Tsi)}{(Tsc)}\] × 100

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

**Crystal Growth assay**

The crystal growth assay is exhibited based on the frame work stated by Nakagawa et al. with few necessary modifications (Farook et al., 2006; Henequin et al., 1993). 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 was 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 was measured by spectrophotometry at wavelength 214 nm. In order to assess the inhibitory potential of AEA on CaOx crystal growth One ml at different concentrations of 100, 200, 400, 600, 800 and 1000 µg/ml was applied.

### Table 1: Consequence of AEA on In vitro calcium oxalate crystallization

| Concentration (µg/ml) | % Inhibition of crystal nucleation Cystone | % Inhibition of crystal nucleation AEA | % Inhibition of crystal aggregation Cystone | % Inhibition of crystal aggregation AEA | % Inhibition of crystal growth Cystone | % Inhibition of crystal growth AEA |
|----------------------|------------------------------------------|----------------------------------------|------------------------------------------|----------------------------------------|----------------------------------------|--------------------------------|
| 100                  | 25.28±3.16                               | 12.32±5.44                             | 28.77±3.18                               | 8.68±1.89                              | 28.08±2.13                             | 12.48±2.45                          |
| 200                  | 52.30±6.23                               | 19.67±2.13                             | 35.18±6.12                               | 17.23±2.42                             | 37.31±3.37                             | 24.42±3.13                          |
| 400                  | 60.52±4.68                               | 30.38±3.83                             | 47.29±3.44                               | 27.63±1.72                             | 45.45±6.72                             | 30.39±1.87                          |
| 600                  | 67.49±6.84                               | 37.11±6.02                             | 56.26±2.76                               | 34.90±3.03                             | 54.54±2.93                             | 38.26±5.75                          |
| 800                  | 76.96±5.17                               | 46.82±3.66                             | 63.10±4.86                               | 42.59±2.86                             | 63.22±4.18                             | 48.03±2.78                          |
| 1000                 | 82.31±7.26                               | 58.40±2.26                             | 67.90±3.47                               | 53.41±3.21                             | 72.04±4.46                             | 55.49±5.01                          |
| IC<sub>50</sub>      | 415.30±21.35                             | 69.13±56.20                           | 573.70±65.53                             | 92.60±62.00                            | 66.20±62.08                             | 86.80±51                             |

All the values are represented as mean±SD of 3 observations. IC<sub>50</sub> was calculated from the logistic regression analysis.

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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 AEBA/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 AEBA. The relative percentage inhibition of crystal growth was computed as follows,

\[\left(\frac{(C - I)}{C}\right) \times 100\]

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

**Statistical analysis**

All values were exhibited 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.

**RESULTS AND DISCUSSION**

Phytochemical studies disclosed the existence of alkaloids, tannins, flavanoids, steroids, phenols, proteins, amino acids, saponins, and carbohydrates in the aqueous extract of *Berberis asiatica* heart wood.

**Effect of AEBA on crystal nucleation**

Percentage inhibition of crystal nucleation of standard drug cystone and AEBA at different concentrations 100, 200, 400, 600, 800 and 1000 μg/ml improved from 25.28±3.16 % to 82.31±7.26 % and 12.32±5.44 % to 58.40±2.26 %, respectively (Table 1). It was established that cystone and AEBA exhibited dose dependent crystal nucleation inhibition. The IC\(_{50}\) values of cystone and AEBA on crystal nucleation were reckoned to be 415.30±21.35 and 839±69.13 μg/ml, respectively (Graph 1).

**Effect of AEBA on crystal aggregation**

Similar dose dependent consequences were ascertained in the crystal aggregation assay. Percentage inhibition of crystal aggregation of cystone and AEBA was calculated as 28.77±3.18 % to 67.90±3.47 % and 8.68±1.89 % to 53.41±3.21 %, respectively (Table 1) and the IC\(_{50}\) values of cystone and AEBA were accounted to be 573.70±65.53 and 927.10±69.98 μg/ml, respectively (Graph 2).

**Effect of AEBA on crystal growth**

A substantial rise in the percentage inhibition of crystal growth was found in the presence of cystone and AEBA at diverge concentrations in ascending sequence, intensified from 28.08±2.13 % to 72.04±4.46 % and 12.48±2.45 % to 55.49±5.01 %, respectively, which was symbolized by a reduction in the free oxalate levels in the presence of cystone and AEBA (Table 1). The IC\(_{50}\) amounts of cystone and AEBA on crystal growth were found to be 566.20±62.06 and 851±86.80 μg/ml, respectively (Graph 3).

Results suggest that percentage inhibition of crystal nucleation, crystal aggregation, and crystal growth in presence of AEBA and cystone are dose-dependent. Though inhibitory activity of AEAR was lower relatively to the reference drug cystone, but it was found to be effective in inhibiting crystallization. Several investigations demonstrated that distinct mechanistic view points were involved in the crystal inhibition in distinct aspects of crystallization. In general, this interruption of crystallization can 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 (Lakshmi et al., 2015).

**CONCLUSIONS**

The current investigation portrayed a statistical evidence of inhibition of calcium oxalate crystallization of AEBA 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 AEBA.

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Conflicts of interest

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