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

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ISSN: 0975-7538
DOI: [https://doi.org/10.26452/ijrps.v11i4.3303](https://doi.org/10.26452/ijrps.v11i4.3303)

**INTRODUCTION**

Urolithiasis is the third pervasive renal disorder. Calcium oxalate calculi is predominant and frequently noticed calculi among different types of calculi (*Strope et al.*, 2010). Calcium oxalate calculi are managed to accessible in two forms stated calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) (*Bensatal and Ouahrani*, 2008).

Pathogenesis of renal calculi emergence involve sequential steps include supersaturation, crystal nucleation, crystal aggregation, crystal retention and crystal growth (*Kavanagh*, 2006). 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 and are preserved and deposited for the progress of calculi.
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 / Kilmorai berberidaceae family. The Heartwood of B. asiatica is 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, antirheumatic, hepatoprotective, strong wound healer and antipyretic (Amritpal et al., 2010; Saeidnia et al., 2014). Until now no research work has been performed on the antiurolithic behavior of aqueous Heartwood extract of B. asiatica. Thus, in the current research, the antiurolithic intervention of an aqueous Heartwood extract of B. asiatica (AEBA) was investigated by implementation of an in vitro crystallization model.

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, steroids, phenolic compounds, carbohydrates, flavonoids, and saponins using standard procedures.

In vitro CaOx crystallization model
Crystal Nucleation assay
Table 1: Consequence of Aeba on In vitro calcium oxalate crystallization

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% Inhibition of crystal nucleation</th>
<th>% Inhibition of crystal aggregation</th>
<th>% Inhibition of crystal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cystone</td>
<td>Aeba</td>
<td>Cystone</td>
</tr>
<tr>
<td>100</td>
<td>25.28±3.16</td>
<td>12.32±5.44</td>
<td>28.77±3.18</td>
</tr>
<tr>
<td>200</td>
<td>52.30±6.23</td>
<td>19.67±2.13</td>
<td>35.18±6.12</td>
</tr>
<tr>
<td>400</td>
<td>60.52±4.68</td>
<td>30.38±3.83</td>
<td>47.29±3.44</td>
</tr>
<tr>
<td>600</td>
<td>67.49±6.84</td>
<td>37.11±6.02</td>
<td>56.26±2.76</td>
</tr>
<tr>
<td>800</td>
<td>76.96±5.17</td>
<td>46.82±3.66</td>
<td>63.10±4.86</td>
</tr>
<tr>
<td>1000</td>
<td>82.31±7.26</td>
<td>58.40±2.26</td>
<td>67.90±3.47</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>415.30±21.35839±69.13</td>
<td>573.70±65.53927.10±69.98 566.20±62.08851±86.80</td>
<td></td>
</tr>
</tbody>
</table>

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 Aeba 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 triplicates. 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/Aeba with the control by the succeeding formula (Aggarwal et al., 2000).

\[ \frac{1 - (Tsi / Tsc)}{100} \]

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

Crystal Aggregation assay

The extent of crystal aggregation of CaOx was investigated by the procedure of Atmani and Khan with modest adjustments (Hess et al., 1989; Aggarwal et al., 2013). The COM 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 solutions were 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 Aeba at distinct concentrations of 100, 200, 400, 600, 800 and 1000 µg/ml. The absorbance was recorded for one hour for every 10 minutes time duration at 620 nm. All samples were inspected in triplicate. Cystone was used as a positive control. Percentage inhibition of aggregation rate was then determined by comparing the turbidity slope of different concentrations of cystone/Aeba with the turbidity slope of the control by the ensuing formula,

\[ \frac{1 - (Tsi / Tsc)}{100} \]

Where Tsi was the turbidity slope of aggregation in the presence of inhibitor sample, i.e, cystone/ plant extract (Aeba) 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; Hennequin 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 Aeba on CaOx crystal growth One ml 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 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,

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

Where \(I\) is the relative rate of depletion of free oxalate in the presence of the inhibitor sample, i.e., cystone/ (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 \(\mu\)g/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 \(\mu\)g/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 \(\mu\)g/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 \(\mu\)g/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.

ACKNOWLEDGEMENTS
The first author wishes to 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. C. Sridhar, M. Pharm., Ph.D, Professor, Dept. of Pharmaceutical analysis, Sri padmavathi school of pharmacy, Tiruchanoor, Andhra Pradesh, India, Dr. D. Sujatha, M. Pharm., Ph.D., Assistant Professor, Institute of pharmaceutical technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati, Andhra Pradesh, India, and Dr. N. Sree Lakshmi, Dept. of Pharmacology, Gokaraju Rangaraju college of pharmacy, Hyderabad, Telangana, India, for their valuable inputs and support.

Funding support
The authors declare that they have no funding support for this study.

Conflicts of interest
The authors declare that they have no conflict of interest for this study.

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