Anti-urolithiasis activity of *Vaccinium macrocarpon* fruits: An in vitro study

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DOI: [https://doi.org/10.22271/plants.2020.v8.i5a.1191](https://doi.org/10.22271/plants.2020.v8.i5a.1191)

**Abstract**

**Objective:** To perform the phytochemical screening and *in vitro* Calcium Oxalate anti-urolithiatric activity of *Vaccinium macrocarpon* aqueous and ethanolic fruit extracts.

**Methods:** The aqueous and ethanolic extracts from fruit pulp was subjected to qualitative phytochemical screening and the anti-urolithiasis activity was evaluated on *in vitro* models like Calcium Oxalate dissolution assay, Calcium Oxalate nucleation and aggregation assay.

**Results:** Following the phytochemical screening it was concluded that the ethanolic and aqueous extracts were found to be rich in flavonoids, steroids, phenols, coumarins, terpenoids and cardiac glycosides. The anti urolithiatic activity was found to be dose dependent. A 35.6±0.06 and 33.43±0.02 percentage of Calcium Oxalate nucleation was exhibited by the 40mg/ml aqueous and methanolic extracts respectively, while percentage inhibition of Calcium Oxalate aggregation was found to be 32.2±0.06 and 34.6±0.02 by 40mg/ml dilution of aqueous and methanolic extracts respectively. However the aqueous and methanolic extracts exhibited a higher capacity to dissolve the Calcium Oxalate crystals prepared homogenously. Percentage inhibitions of 44.2±0.06 and 47.3±0.02 were shown by the aqueous and methanolic extracts respectively.

**Conclusions:** The study indicates that the aqueous and methanolic extracts of *Vaccinium macrocarpon* fruits showed inhibition against the important phases of Calcium Oxalate urolithiasis like nucleation and aggregation. It also aids the dissolution of the Calcium Oxalate crystals prepared. Owing to the rich presence of polyphenols and flavanoids, *Vaccinium macrocarpon* proves to be an easily available and a beneficial alternative or adjunctive treatment for Calcium Oxalate urolithiasis. Further *in vivo* and clinical explorations are required to confirm the efficacy of *Vaccinium macrocarpon* as an antiurolithiatic.

**Keywords:** Urolithiasis, calcium oxalate, polyphenols, *Vaccinium macrocarpon*, phytochemicals

1. **Introduction**

Kidneys being the major organs responsible for excretion of toxins, any abnormalities disrupting this normal physiology will cause major problems for human beings. Formation of stones in the kidneys is termed as nephrolithiasis, while calculi if formed in the urinary bladder, ureter or anywhere in the urinary tract is discerned as urolithiasis. It is the oldest and excruciatingly painful urologic disorder affecting 5-7 million individuals. 10-12% of the population in the industrialized counties is victimized to urinary stone formation, with 20–40 year aged individuals showing the highest incidence [1,2]. The word “Urolithiasis” stems from the Greek as “Urone” for urine and “Lithos” for stones. The pathogenesis of stone formation can be explained by numerous theories like super saturation and inhibitors theory. For instance, according to the super saturation theory, stone formation is promoted when there is an overabundance of solute in the solvent [3]. Urinary stones are classified on the basis of size, location, X-ray characteristics, and aetiology of formation, composition, and risk of recurrence [4-7]. Based on aetiology, stones are mainly of infective, non-infective, genetic and drug induced origins. Calcium-containing stones, especially Calcium Oxalate monohydrate, Calcium Oxalate dihydrate and Calcium Phosphate are the most commonly occurring ones to an extent of 75-90% followed by magnesium ammonium phosphate (Struvite) to an extent of 10-15%, uric acid 3-10% and cystine 0.5-1% [8]. The formation of the kidney stones involves multiple events beginning with the crystal nucleation, aggregation, and ends with retention of the formed stones within urinary tract [9]. Supersaturation preceds crystal nucleation. Urinary PH, Ionic strength, Solute concentration are factors affect the supersaturation process [10]. Urolithiasis treatment majorly focuses on the dissolution and prevention of stone relapse.

**Keywords:** Urolithiasis, calcium oxalate, polyphenols, *Vaccinium macrocarpon*, phytochemicals

1. **Introduction**

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Therapeutic intervention is essential of pain resolution and the stones pass out on their own. Standard pharmaceutical drugs such as allopurinol, citrate, cystine and thiazide diuretics are used to prevent and treat urolithiasis, but these are not effective in all patients, owing to common kidney stone recurrence and potential side effects \(^{[11]}\). Stones larger than 5 mm or stones that fail to pass through should be treated by some interventional procedures such as extracorporeal shock wave lithotripsy (ESWL), ureteroscopy (URS), or percutaneous nephrolithotomy (PNL) \(^{[12]}\). However, the propensity for stone recurrence is not altered by removal of stones with ESWL and stone recurrence is still about 50% \(^{[13]}\).

In Ayurveda, ‘Pashanabheda’ group plants, claimed to be useful in the treatment of urinary stones. ‘Pashanabheda’ is the Sanskrit term used for a group of plants with diuretic and antiurolithiatic activities \(^{[14]}\). *Vaccinium macrocarpon* commonly known as American cranberry belongs to the family Ericaceae. American cranberries are small pink blossoms that appear in the spring. *Vaccinium macrocarpon* (American cranberry) and *Vaccinium oxyccocus* (European cranberry) are the two major species \(^{[15]}\). Owing to the presence of rich bioactive polyphenols, cranberries find potential applications in therapeutic intervention diseases for improving cardiac, urinary and anticancer health \(^{[16-18]}\). Polyphenols that constitute cranberries encompass anthocyanins, flavonols, phenolic acids and isoflavones \(^{[19-23]}\). Cranberry juice has been long used for the prevention of UTI’s bolstered by anecdotal evidence. 1980’s saw the emergence of evidence demonstrating the ability of cranberry juice to prevent attachment of *E. coli* bacteria to uroepithelial cells. Studies from Cape Town evaluated the effect of cranberry juice in a randomized crossover trial on Calcium Oxalate calculi. This study concluded the anti-lithogenic properties of cranberry. According to a study published in 2003, consumption of cranberry juice had favorable effects on three potential urinary risk factors: decreased Oxalate and phosphate excretion with an increase in citrate excretion. Kebler et al. further evaluated the influence of plum, cranberry and blackcurrant juice on factors that influence urinary stone formation in 12 healthy males involved in a standardized diet formulation. Although polyphenols have prevented ethylene glycol assisted Calcium Oxalate crystallization in rats, the mechanistic processes underlying this pharmacological activity are yet to be explored. The reduced Oxalate excretion following cranberry juice consumption makes its *in vitro* anti-urolithiatic activity worth exploring. Based on the above information and considering the complete lack of any scientific basis in literature, a methodical approach using a simple *in vitro* models was undertaken to evaluate the efficacy of *Vaccinium macrocarpon* as an antiurolithiatic agent. The present study was designed to investigate: (i) Phytochemical screening of *Vaccinium macrocarpon*; (ii) anti urolithiatic activity on experimentally prepared Calcium Oxalate crystals by homogeneous precipitation method in the laboratory, Calcium Oxalate nucleation and aggregation assay. Although a difference between naturally occurring kidney stones and the experimentally prepared stones exists, this study was undertaken as the first step in experimental drug discovery.

2. Materials and methods

2.1 Chemicals

Calcium Chloride dehydrate, Tris buffer and Sodium Oxalate were procured from Sisco Research Laboratories Pvt. Ltd. All other chemicals employed were of analytical grade and purchased from usual sources.

2.2 Preparation of the fruit extract

Fruits of *Vaccinium macrocarpon* were purchased from a local authenticated store. Following which they were rinsed under running tap water and dried at 40°C in a hot air oven. The dried fruits were subjected to powdering. For extraction, 10 g of dried powder was placed in 100 ml each of triple distilled water and ethanol in respective beakers and heated for 15 mins on a magnetic stirrer. The extracts were further allowed to soak overnight at room temperature. Subsequently, the extracts were subjected to filtration through a what man filter paper (size 41) to eliminate smaller particles and obtain final extracts for the study. The filtered solutions were evaporated in a ventilated hot air oven at 40°C to constant weight and then dissolved in respective solvents to obtain 10, 20, 30 and 40 mg/ml extracts. The final extract was emptied into a dark screw capped sterile container (as the phenols are photosensitive) and stored at 4°C for 24 hours before further evaluation.

2.3 Phytochemical screening

Preliminary phytochemical screening was carried out for the presence of secondary metabolites; alkaloids, sterols, terpenoids, phenols, flavonoids, glycosides, tannins, saponins, and fixed oils by standard methods \(^{[24, 25]}\).

2.4 Evaluation for Anti-urolithiatic Activity

The purpose behind this activity was to know the role of aqueous and methanolic extracts of *Vaccinium macrocarpon* in dissolving the already formed stones in renal system. For this artificial Calcium Oxalate crystal were prepared in the laboratory according to standard methods \(^{[26]}\). Also semi permeable membrane was prepared from farm eggs using standard methods \(^{[27]}\).

2.4.1 Preparation of Calcium Oxalate crystals by homogeneous precipitation method

Calcium Chloride dehydrate (4.41g) dissolved in distilled water and Sodium Oxalate (4.02g) dissolved in 2N Sulphuric acid were taken into separate beakers and both solutions were mixed together to react with stirring until Calcium Oxalate precipitate formed. Excess Sulphuric acid was removed by washing with Ammonia solution and distilled water respectively. It was allowed to dry at 60°C for 4 h.

2.4.2 Preparation of semi-permeable membranes from farm eggs

The apex of eggs was punctured by a glass rod to remove the entire content. Empty egg shells were washed thoroughly with distilled water and placed in a beaker consisting 2M HCl for overnight which caused complete decalcification. Then membranes were washed with distilled water and they were placed in ammonia solution for neutralization of acid traces in the moistened condition for a while. Then they were rinsed with distilled water and stored in a refrigerator at a pH of 7-7.4.

2.4.3 Evaluation of anti-urolithiatic activity by the titrimetric method

Totally 9 semi-permeable membranes were prepared and exactly 5 mg of Calcium Oxalate crystals, four different concentrations (10 mg, 20 mg, 30 mg, 40 mg) of extracts and standard (Positive control) were placed in separate
membranes and they were sutured carefully. One sample which contained Calcium Oxalate crystals only was used as the negative control. These were allowed to suspend in the separate conical flasks which containing 100 ml of tris buffer solution (0.1M). All the conical flasks were incubated at 37°C for 7 h. Then the content in the semi-permeable membrane was transferred into a test tube and 2 ml of 1N Sulphuric acid was added. The resulting mixture was titrated against the 0.9494 N standard KMnO4 solution until the light pink colour was observed. This whole procedure was carried out in triplicate to obtain accurate results. The dissolution percentages of the Calcium Oxalate crystals were calculated for each sample to evaluate the activity.

2.5 Calcium Oxalate nucleation assay
Calcium Chloride (CaCl2) (5 mmol/l) and Sodium Oxalate (Na2C2O4) solution (7.5 mmol/l) were prepared in Tris-HCl (0.05 mol/l) and Sodium Chloride (0.15 mol/l) buffer (pH 6.5). Dilutions of aqueous and methanolic extracts Vaccinium macrocarpon extracts ranging from 10–40mg/ml were used for the study One milliliter of each extract concentration was mixed with 3 ml CaCl2 solution followed by the addition of 3 ml Na2 C2 O4 solution. Final mixtures were incubated for 30 min at 37°C. The optical density (OD) of the mixtures was then measured at 620 nm wavelength. Percent inhibition of nucleation by DCRE was calculated using the under mentioned formula and compared to that calculated for the standard drug, Cystone.

\[
\left(\frac{{\text{Turbidity}}_{\text{control}} - \text{Turbidity}_{\text{sample}}}{\text{Turbidity}}_{\text{control}}\right) \times 100.
\]

2.6 Calcium Oxalate Aggregation assay
Calcium Oxalate crystals were prepared by mixing Calcium Chloride and Sodium Oxalate at a concentration 50 mmol/L. The solutions were equilibrated to 60 °C in water bath, cooled to 37 °C and kept overnight. The solution was centrifuged to yield Calcium Oxalate crystals and evaporated at 37 °C. The reaction mixture consisted of Calcium Oxalate crystals at a concentration of 0.8 mg/mL, 0.05 mol/L Tris-HCl and 0.15 mol/L Sodium Chloride at pH 6.5. The experiment was conducted at 37 °C in the presence of plant extract/fractions at final concentrations in the range of 10-40mg/mL and incubated for 30 min. OD of the final mixtures was then read at 620 nm wavelength and percent inhibition of aggregation was then calculated as described for nucleation assay. All samples were assayed in triplicate. Cystone® was used as positive control [28]. The percentage inhibition of aggregation was calculated using the formula:

\[
\left(\frac{{\text{Turbidity}}_{\text{control}} - \text{Turbidity}_{\text{sample}}}{\text{Turbidity}}_{\text{control}}\right) \times 100.
\]

3. Results
3.1 Phytochemical screening
In the present study phytochemical screening was performed for ethanol, chloroform and aqueous fruit extracts of cranberry. The ethanolic and aqueous extracts were found to be rich in flavonoids, steroids, phenols, coumarins, terpenoids and cardiac glycosides.

Table 1: Phytochemical screening of cranberry extracts

<table>
<thead>
<tr>
<th>Phytochemicals tested</th>
<th>Cranberry (Vaccinium macrocarpon) extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>Coumarins</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: + indicates positive; - indicates negative and +/- indicates semi positive

3.2 Percentage of Calcium Oxalate dissolving capacity by titrimetry
On basis of the above phytochemical screening, in vitro Antiurolithiatic activity Experimental in vitro model as shown in figure 1 and 2 was performed by comparing different extracts of Vaccinium macrocarpon with standard Cystone. The percentage of Calcium Oxalate dissolution was found to be concentration dependent. A slight difference was found between aqueous and methanolic extracts in terms of Calcium Oxalate dissolution capacity. 40mg/ml of each of the extracts showed the highest inhibitory capacity of 47.3±0.01 and 44.2±0.06 respectively. Cystone showed the highest inhibitory potential of 85±0.05 at a concentration of 40mg/ml

Fig 1: Experimental in vitro anti urolithiatic model (a) Decalcified eggs b) Aqueous and methanolic extracts of Vaccinium macrocarpon fruits (c) Egg membrane for urolithiasis activity

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3.3 Inhibition of Calcium Oxalate nucleation

Here both the aqueous and methanolic extracts were subjected to a spectrophotometric assay to evaluate the ability to inhibit Calcium Oxalate nucleation. The extracts were compared with the standard drug cystone. The results revealed that 40mg/ml concentrations showed good activity with percent inhibitions of 32.2±0.06 and 34.63±0.02 for aqueous and methanolic extracts respectively, although less than the standard drug. The concentrations 10mg/ml and 20mg/ml were found to be least active.

Table 2: In-vitro percent inhibition (%) of Calcium Oxalate nucleation of cystone and Vaccinium macrocarpon extracts.

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Percentage inhibition of Calcium Oxalate nucleation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10mg/ml</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>19.5±0.04</td>
</tr>
<tr>
<td>Methanolic extract</td>
<td>19.7±0.02</td>
</tr>
<tr>
<td>Cystone</td>
<td>49.5±0.02</td>
</tr>
</tbody>
</table>
3.4 Inhibition of Calcium Oxalate aggregation

Here both the aqueous and methanolic extracts were subjected to a spectrophotometric assay to evaluate the ability to inhibit Calcium Oxalate aggregation. The extracts were compared with the standard drug Cystone. The results revealed that 40mg/ml concentrations showed good activity with percent inhibitions of 32.2±0.06 and 34.6±0.02 for aqueous and methanolic extracts respectively, although less than the standard drug. The concentrations 10mg/ml and 20mg/ml were found to be least active.

Table 3: *In-vitro* percent inhibition (%) of Calcium Oxalate aggregation of cystone and *Vaccinium macrocarpon* extracts.

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>10mg/ml</th>
<th>20mg/ml</th>
<th>30mg/ml</th>
<th>40mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td>18.2±0.04</td>
<td>21.3±0.05</td>
<td>29.4±0.01</td>
<td>32.2±0.06</td>
</tr>
<tr>
<td>Methanolic extract</td>
<td>19.5±0.02</td>
<td>20.2±0.05</td>
<td>31.6±0.01</td>
<td>34.6±0.02</td>
</tr>
<tr>
<td>Cystone</td>
<td>47.4±0.02</td>
<td>59.2±0.01</td>
<td>60.9±0.01</td>
<td>64.5±0.01</td>
</tr>
</tbody>
</table>

4. Conclusion

In the present investigation, we analyzed the anti-urolithiatic activity of aqueous and ethanolic extracts of *Vaccinium macrocarpon* fruits using Calcium Oxalate dissolution assay, Calcium Oxalate nucleation assay and Calcium Oxalate aggregation assay models. From testimony and knowledge of clinical trials, cranberries aid the dissolution and expulsion of stones from urinary tract following a few days of consumption [29]. Calcium Oxalate urolithiasis is the most prevalent type of urinary stone disease. Key events involved in its pathological biomineralization include crystal nucleation, growth and aggregation [30]. Present study was designed to address these key events involved in Calcium Oxalate stone formation as a means to investigate the efficacy of *Vaccinium macrocarpon* fruits as an antiurolithiatic. Initially, Calcium Oxalate dissolution property was evaluated on homogenously synthesized Calcium Oxalate crystals by employing a titrimetric method. The percentage of Calcium Oxalate dissolution was found to be concentration dependent. 40mg/ml concentrations of aqueous and methanolic fruit extracts showed the highest inhibitory potential. However it was still lower compared to the standard drug Cystone. Nucleation is a prerequisite in the pathogenesis of Calcium Oxalate urolithiasis. Nucleation basically marks an event thermodynamically driven for phase change wherein dissolved substances in a supersaturated solution spontaneously crystallize [31]. Similar phase change and formation of Calcium Oxalate crystals was witnessed while carrying out nucleation assay. Significant inhibition in the nucleation of Calcium Oxalate crystals was observed in the...
presence of 40mg/ml extracts which was even better than in the presence of Cystone. This suggests the anticyrstallization activity of Vaccinium macrocarpon against Calcium Oxalate crystallization. One possible mechanism of anti crystallization activity of Vaccinium macrocarpon could be its ability to complex with free Calcium and Oxalate ions, thus preventing the formation of Calcium Oxalate complexes. Aggregation of crystals marks the process wherein numerous crystals in the solution come together and adhere forming large crystal agglomerates. Aggregation is a key determinant of crystal retention as large crystal agglomerates are the ones that produce renal tubular obstruction thereby promoting stone formation. Vaccinium macrocarpon extracts showed a moderate inhibitory effect on Calcium Oxalate crystal aggregation. Findings of the present study clearly demonstrate antiurolithic potential of Vaccinium macrocarpon against Calcium Oxalate urolithiasis in vitro. It showed significant inhibition of the pivotal phases of Calcium Oxalate stone formation viz. nucleation, aggregation and aided the dissolution of the formed Calcium Oxalate crystals. Although further in vivo and clinical explorations are required to confirm the efficacy of Vaccinium macrocarpon as an antiurolithic, still, considering its vast consumption and availability all around the globe together with its antiurolithic potential, Vaccinium macrocarpon could serve as an easily accessible and beneficial alternative or adjunctive treatment for Calcium Oxalate urolithiasis.

5. Acknowledgments
The authors wish to express their gratitude to the Principal and Management of Sarojini Naidu Vanita Pharmacy Maha Vidyalaya for undertaking the research work.

Conflicts of Interest: The authors declare no conflict of interest

6. References

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