Assessment of Antiurolithic Potential of Crude Extracts of Berberis lycium by In-vitro Inhibition of Calcium Oxalate Crystallization

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Abstract

Developing non surgical methods for removal and prevention of reoccurrence of urolithiasis is challenge for researchers of the present time. Traditional systems of medicine utilize teas, decoction or powdered plant parts for removal of kidney stones. *Berberis lycium* is among the plants which find its traditional use in urolithiais along with other therapeutic effects. Crude aqueous and crude methanol extracts of dried root bark of the plant were studied at 10µg/ml, 50µg/ml, 100µg/ml and 1000µg/ml concentrations for inhibition assay, nucleation assay and aggregation assay. Assays were carried out at 37°C in the presence of buffer containing Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l, pH 6.5. Both extracts had significant *in vitro* antiurolithic activity but methanol extract of the plant was found more effective in preventing crystallization in all the three assays. Thus, *Berberis lycium* can serve as Potential source of active ingredient for antiurolithiais treatment agents.

Keywords

Urolithiasis, Calcium oxalate (CaOx), *Berberis lycium*, inhibition assay, nucleation assay, aggregation assay, Tris buffer, crystallization.

1. Introduction

Urolithiasis is the process of formation of calculus (stone) in the urinary tract which is a painful disorder. Urolithiasis affects approximately 12% of the world's population and has the tendency of frequent recurrence. Its recurrence rate is 70-80 % in males and 47-60 % in females (Soundararajan et al., 2006). Primary urolithiasis and its recurrence is a major challenge for urologists today as urolithiasis is one of the major cause of morbidity in later age (OW, 2006). Four types of calculi exist in kidneys; calcium oxalate, uric acid, cystine stones and struvite. Among these, calcium oxalate

monohydrate (COM) and calcium oxalate dehydrate (COD) has the highest incidence i.e. between 75-90% followed by struvite (10-15%), uric acid (3-10%), and cystine (0.5-1%) (Harsoliya MS, 2011). Management and treatment of urolithiasis includes high intake of water i.e 2.5 to 3 L/day as first step, followed by use of analgesics and monitoring of salts that are responsible for increase or decrease in calculus formation (Sean and Darren, 2005). Kidney stone exceeding the size of 8mm must undergo intervention like extracorporeal shockwave lithotripsy or nephrolithotomy. Use of these techniques successfully removes the stone but do not eliminate the chances of recurrence (Matlaga and Assimos, 2002).

Traditional systems of medicine have served humanity for centuries. There is revival of interest among researchers to investigate the new bioactive components of medicinal plants because of the affordability, less or no side effects and easy availability of herbal medicines (Jain, 2003). Use of plant based medicines has been proven effective in removal of urolithiasis and number of plants have been studied scientifically and proven effective for treatment of various diseases. Species of genus Berberis finds its use in most of the herbal and homeopathic formulations used for treating urolithiasis (Arayne et al., 2007). *Berberis lycium* is a plant of genus Berberis known commonly as Sumbloo or Sumbal and Kashmal in Pakistan. It is found in semi temperate and temperate regions of Pakistan, India, Bangladesh and Afghanistan. The plant is used as an anti infectious crude drug in Unani system of medicine and is also used as an anti urolithic agent in Homeopathic system of medicine. Studies on the plant have proven its antidiabetic, anti hyperlipidemic, hepatoprotective, antimicrobial and antimutagenic potential. Potent active constituents of the plant are berbrine (Fig.1.), berbamine (Fig. 2), and oxyacanthine along with less potent constituents punjabine, baluchistanamine, palmitine, karakoramine, chinabine, gigitine, and jhelumine (Zahid Munawar, 2017, Arayne et al., 2007, Gulfraz, 2004, Shabbir et al., 2012). Therefore, the present study was designed to investigate *in vitro* anti urolithic activity of crude extracts of root bark of *Berberis lycium*.

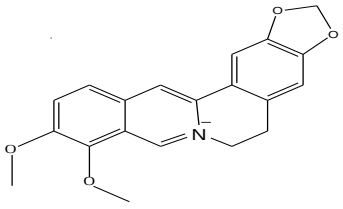


Fig. 1. Berberine

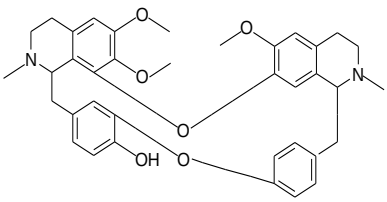


Fig. 2. Berbamine

2. Materials and Methods

Whole plant of *Berberis lycium* was collected from Gilgit-Baltistan region of Pakistan in the month of July, 2017 and identified by Department of Pharmaceutical Chemistry Hamdard Institute of Pharmaceutical Sciences, Islamabad. Plant was dried under shade until there was no difference in the weights obtained between two different time intervals. Root bark of the plant was separated and crushed to powder form for extraction using mechanical grinder.

2.1 Extraction of Crude Extracts

Crude aqueous and methanol extracts of root bark of *Berberis lycium* were obtained using Soxhlet extractor at 90°C. 5 g of powder was used for extraction in 200ml of each solvent and extraction was carried out for 48 hours. Obtained extract was dried using Rotary evaporator at 50°C and weighed. Activity was determined using various concentrations of crude extracts.

2.2 Antiurolithic Activity

In vitro antiurolithic activity models have been developed simulating naturally occurring processes of nucleation, aggregation and growth of calcium oxalate in kidney (Kalpana et al., 2013, Srinivasa et al., 2013). Inhibition of stone formation is estimated using following models:

2.2.1 Inhibition Assay

Investigation of antiurolithic activity of crude aqueous, and methanol extracts of root bark of *Berberis lycium* was carried out according to the method developed by N. A. M. Farrook et.al. with some minor modifications (Farook et al., 2004). 10µg/ml, 50µg/ml, 100µg/ml, and 1000µg/ml concentrations of aqueous and methanol extract were prepared in distilled water and named A1, A2, A3, and A4, respectively for aqueous extracts and M1, M2, M3, and M4, respectively for methanol extracts. 5 ml of calcium acetate (20mmol/l) in buffer containing Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l, pH 6.5 and 5ml of sodium oxalate (50mmol/l) in buffer containing Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l, pH 6.5 were added drop wise to 50 ml of extract solutions at 37°C. Calcium oxalate precipitates were allowed to settle down. Crystals were collected by

centrifugation and dried to measure weight. Same procedure was repeated in the absence of plant extracts and calcium oxalate precipitates were prepared. Percentage inhibition in formation of calcium oxalate crystals is measured according to following formula:

Percentage inhibition = $W_{control}$- W_{sample}/ $W_{control}$×100

Where,

$W_{control}$ = Weight of control

W_{sample} = Weight of sample (test)

2.2.2 Nucleation Assay

Nucleation assay was performed according to the method proposed by Hennequin et. al with some minor modifications (Hennequin et al., 1993). Different solutions were prepared (Table 1).

Table1. Solutions for Nucleation Assay

Solutions	Concentration	Solvent
Calcium chloride	3mmol/l	Buffer containing Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l, pH 6.5
Sodium oxalate	0.5mmol/l	Buffer containing Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l, pH 6.5
Plant extracts Aqueous Methanol	10, 50, 100 mg/ml	Distilled water

Calcium chloride solution 3mmol/l and extract solution were mixed in 9:1 for all concentration of extract solutions. Solution of sodium oxalate is added drop wise in the above mixture. Reaction occurs between calcium chloride and sodium oxalate according to the following equation:

$$CaCl_2+Na_2C_2O_4 \rightarrow CaC_2O_4+2NaCl$$

Absorbance of the mixture was measured on 620nm wavelength. Experiment was repeated with blank and absorbance was measured. Percentage inhibition was measured using following formula:

Percentage Inhibition = $A_{control}$- A_{sample}/ $A_{control}$×100

Where,

$A_{control}$ = Absorbance of control

A_{sample} = Absorbance of sample

2.2.3 Aggregation Assay

Assay simulating aggregation step of calcium oxalate stone formation as proposed by Hess et al. was used with minor modifications (Phatak and Hendre, 2015, Hess et al., 1989). Seed crystals of calcium oxalate were prepared by mixing 50mmol concentration of calcium chloride and sodium oxalate prepared in Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l, pH 6.5. Mixture was kept at 60°C in water bath for 1 hour and then allowed to stand at 37°C overnight. CaOx crystals were separated by centrifugation and dried by evaporation of water at 37°C. Separated seed crystals were used for aggregation assay at final solution concentration of 0.8 mg/ml, in buffer containing Tris–HCl 0.05mol/l and NaCl 0.15 mol/l at pH 6.5. Procedure was carried out at

37°C both in the absence and presence of the plant extracts. Percentage inhibition in aggregation was estimated by comparing the turbidity of mixture in the presence and absence of extract solutions using following formula:

$$I.A = (1\text{-}turbidity_{sample}/turbidity_{control}) \times 100$$

Where,

I.A = percentage inhibition in aggregation

2.4 Statistical Analysis

Each sample was replicated thrice for each assay. The mean of percentage inhibitions and standard deviation were calculated using Microsoft Excel.

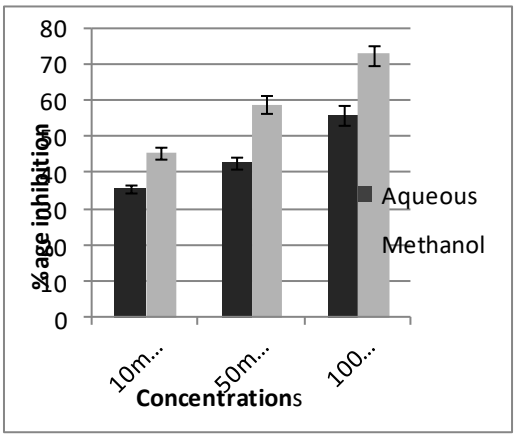
3. Results

Soxhlet extraction of 5 g of powdered plant material carried out for extraction of crude aqueous, and methanol extracts, yielded dry mass of 1.2g and 1.01g, respectively. The inhibition assay for Crude aqueous extract showed significant inhibitory effect on calcium oxalate formation. Inhibitory activity increased with increase in concentration of extract and the maximum percentage inhibition shown was 61.22±1.87 at 1000µg/ml concentration. Crude methanol extract had better inhibitory effect than aqueous extract. Percentage inhibition value shown by Cr. methanol extract was 75.59±2.45 at 1000µg/ml. Percentage inhibition showed increase with increase in extract concentration (Table 2).

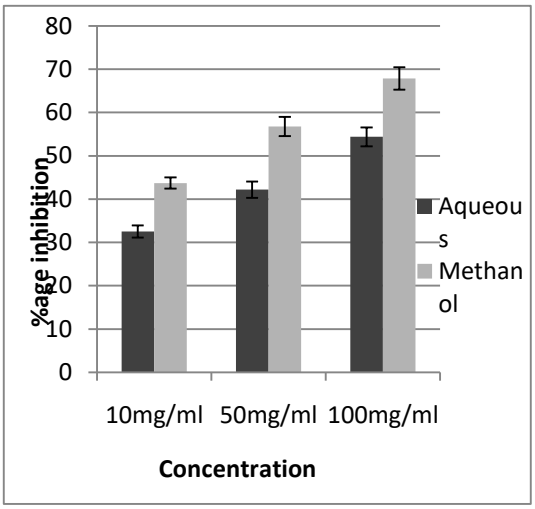
Table 2: Results for Inhibition assay of cr. aqueous and cr. methanol extracts of *Berberis lycium*

Sample	10µg/ml (%)	50µg/ml (%)	100µg/ml (%)	1000µg/ml (%)
Aqueous	35.43 ± 1.13	41.56 ± 0.45	55.75 ± 1.65	61.22 ± 1.87
Methanol	39.78 ± 0.64	51.24 ± 0.86	60.65 ± 1.29	75.59 ± 2.45

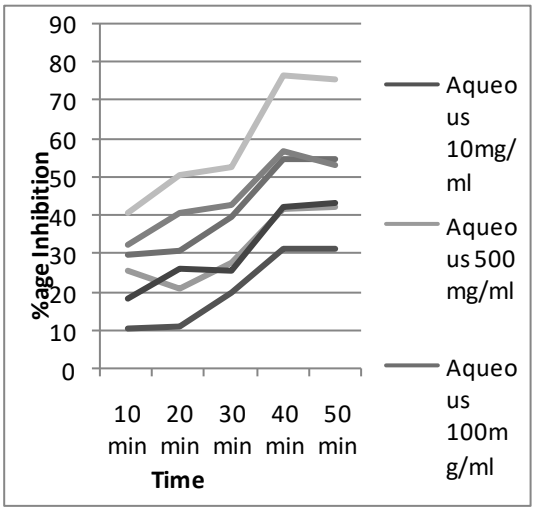
Nucleation assay showed up to 59% inhibition in nucleation for cr. aqueous extract and 72% inhibition for cr. methanol extract at 100mg/ml concentration as demonstrated in (Graph 1). Both extracts were found effective in preventing nucleation process of CaOx crystallization. Similar result was observed for aggregation assay where inhibition in aggregation demonstrated by cr. methanol extracts was slightly greater than inhibition shown by cr. aqueous extract, although both extracts had noticeable activity (Graph 2). Activity increased with the passage of time provided for aggregation (Graph 3).



Graph 1: Result for nucleation assay of cr. aqueous and methanol extracts of *Berberis lycium*



Graph 2: Result for aggregation assay of cr. aqueous and methanol extracts of *Berberis lycium*



Graph 3: Percentage inhibition in aggregation with time

4. Discussion

The results of the present investigation showed that crude aqueous and crude methanol extracts of *Berberis lycium* significantly inhibited nucleation and aggregation processes of CaOx crystallization *in vitro*. Cr. methanol extract had slightly better activity than aqueous extract. A similar *in vitro* antiurolithic study was performed on root bark extract of *Berberis lycium* examining the inhibition in CaOx crystallization in artificial urine. Decoction of the *Berberis lycium* root bark was found highly effective in inhibition of CaOx crystallization in artificial urine (Shah et al., 2014). The results of this study were consistent with our findings because both the studies showed inhibition in CaOx crystallization although the *in vitro* examination models used were different. Drawback in this study was that nucleation and aggregation processes were not separately studied for inhibitory action. Many plants of genus Berberis have been studied for their antiurolithic potential. In another study performed on aqueous-methanol root bark extract of *Berberis vulgaris*, an *in vivo* Wistar albino rat's model was used to investigate antiurolithic effect. Results showed decrease in CaOx crystal formation in kidney cross sections of rat (Bashir et al., 2010). This result is also similar to our findings which prove the effectiveness of genus Berberis against urolithiasis. Berberine is the most useful active constituent of genus Berberis having antibacterial, antidiabetic and antihyperlipidemic properties. A study evaluated the effectiveness of berberine against urolithiasis was performed on Wistar albino rat's model. The compound was found highly effective in

minimizing crystallization of CaOx (Bashir and Gilani, 2011).

Many traditionally used plants for antiurolithic effect are scientifically studied for confirmation of their activity and isolation of active constituents. *Kalanchoe pinnata* is a traditionally used plant in India for dissolution of kidney stone. This plant was evaluated *in vitro* using nucleation and aggregation assays on various concentrations of crude extracts. Results of the experiment confirmed the traditional use of the plant for antiurolithiasis (Phatak and Hendre, 2015). Investigation on aqueous extract of *Costus arabicus* was carried out to study inhibition in CaOx crystallization and cytotoxicity of the plant. Plant was found effective in minimizing crystal growth and found safe at minimum effective concentration (de Cógáin et al., 2015). *Hernia hirsuta, Aerva lanata, Dolichos biflorus, Bergenia ligulata,* and *Hedychium coronarium* are among other plants which are traditionally used for dissolving kidney stones in traditional systems of medicine. Studies on these plants confirmed their effectiveness in inhibiting calcium oxalate crystallization (Atodariya et al., 2013, de Cógáin et al., 2015, Soundararajan et al., 2006, Harsoliya MS, 2011, Atmani and Khan, 2000, Bahuguna and Kumar, 2014).

5. Conclusion

The study concluded that *Berberis lycium* is found effective in inhibiting CaOx crystal formation in *in vitro* models. Findings of the study support the use of *Berberis lycium* in homeopathic, unani and ayervedic medicine systems for antiurolithiasis. *In vivo* studies are recommended to confirm its role as a future potential drug for non surgical removal of urolith. Separation and identification of active constituent should be carried out for future drug development.

Competing Interest

Authors declare no conflict of interest.

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