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Berberis vulgaris root bark extract prevents hyperoxaluria induced urolithiasis in rats.

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**INTRODUCTION**

Urinary stone disease occurs worldwide with some geographical and racial variation and is constantly rising in parallel with socio-economic development. Calcium oxalate (CaOx) has been shown as the most common constituent of renal calculi and hyperoxaluria is one of the major risk factors for CaOx kidney stone formation (Tiselius, 2003). The mechanisms involved in the formation of calcific stones are not fully understood but it is generally agreed that urinary lithiasis is a multifaceted process involving events leading to crystal nucleation, aggregation and the growth of insoluble particles (Hess and Kok, 1996). Urine is always supersaturated with common stone forming minerals, however, the crystallization inhibiting capacity of urine does not allow urolithiasis to happen in most individuals, whereas this natural inhibition is in deficit in stone formers (Tiselius et al., 2001). Studies have also shown that tubular cell injury facilitates CaOx crystal formation and deposition in the renal tubules (Khan and Hackett, 1991; Selvam, 2002). Animal and tissue culture studies have demonstrated that both oxalate and CaOx crystals directly induce renal epithelial cell injury mediated through lipid peroxidation and involve oxygen free radical generation (Scheid et al., 1996; Thamilselvan et al., 1997, 2000).

Urolithiasis is largely a recurrent disease with an approximate relapse rate of 50% in 5–10 years and 75% in 20 years (Moro et al., 2005). Endoscopic stone removal and extracorporeal shock wave lithotripsy have revolutionized the treatment of nephrolithiasis but do not affect the likelihood of new stone formation (Pak, 1989). Various therapies including thiazide diuretics and alkali-citrate are being used in an attempt to prevent the recurrence of hypercalciuria- and hyperoxaluria-induced calculi but scientific evidence for their efficacy is less convincing (Hess, 2003).

Medicinal plants have played a significant role in various ancient traditional systems of medication. Even today, plants provide a cheap source of drugs for the majority of the world’s population (Gilani and Atta-ur-Rahman, 2005). Several in vitro and in vivo studies on medicinal plants used in traditional antiurolithic therapy have proven that these remedies are effective (Atmani et al., 2003; Barros et al., 2006). *Berberis vulgaris* Linn. (family, Berberidaceae) commonly known as ‘berberry’ (Usmanghani et al., 1997) occurs in most areas of Central and Southern Europe, the Northeastern regions of the United States (Dorfler and Roselt, 1989) and in South Asia including Northern areas of Pakistan (Shinwari et al., 2003). In folk medicine, *Berberis vulgaris* has been used for various conditions including kidney stones and other urinary tract diseases (Usmanghani et al., 1997; Duke et al., 2002). In the homeopathic system of medicine, it is the most widely used drug for kidney pain and for removal of kidney stones (Arayne et al., 2007).

The current work was aimed to investigate the in vivo antiurolithic effect of the crude extract prepared from *Berberis vulgaris* root bark and its fractions to rationalize its medicinal use in urolithiasis.

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MATERIALS AND METHODS

Drugs and standards. Ethylene glycol, thymol, reduced glutathione, 5'-dithiobis, 2-nitrobenzoic acid (DTNB), thiobarbituric acid, H₂O₂, trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), 1,1,3,3-tetraethoxy propane, nuclear fast red and silver nitrate (AgNO₃) were obtained from Sigma Chemical Co., St Louis, MO, USA. Kits used in this study for the determination of calcium, magnesium, blood urea nitrogen (BUN), creatinine, superoxide dismutase (SOD) and glutathione peroxidase(GPx) were purchased from Randox Laboratories Ltd, Ardmore, Diamond Road, Crumlin, Co., Antrim, UK. Oxalate estimation was done by the kit from Trinity Biotech Plc, IDA Business Park, Bray, Co., Wicklow, Ireland and for citrate estimation, the kit was purchased from R-Biopharm AG, D-64293 Darmstadt, Germany.

Plant material, extraction and fraction. Roots of Berberis vulgaris were collected from District Swat, N.W.F.P., Pakistan. A sample of the plant material was submitted to the herbarium of Natural Products Research Unit at the Department of Biological and Biomedical Sciences of the Aga Khan University, with a voucher number BV-RB-08-03-66. The root bark was removed and cut into pieces. Approximately 850 g of the root bark was soaked in aqueous-methanol at room temperature for 3 days with occasional shaking. After filtering through a single layer of muslin cloth, the final filtrate was collected by passing it through a Whatman 1 filter paper. This procedure of soaking the root bark residue and filtration was repeated twice. All the filtrates were combined and evaporated to dryness on a rotary evaporator under reduced pressure to a thick and dark brown material (110 g), the crude extract of Berberis vulgaris (Bv.Cr), the approximate yield was 12.9%. Bv.Cr was completely soluble in distilled water and normal saline for in vivo and in vitro experiments, respectively.

For activity-guided fractionation, approximately 70 g of Bv.Cr was dissolved in 100 mL of distilled water (Williamson et al., 1998). n-Butanol (100 mL) was then added and it was shaken well in a separating funnel. The mixture was allowed to separate into layers. The upper layer, the n-butanol fraction, was separated and the lower aqueous phase was remixed with a fresh volume of n-butanol. The process was repeated twice more and all the n-butanol fractions were combined and then evaporated on a rotary evaporator to a thick yellow mass, the n-butanol fraction (Bv.Butn). The aqueous layer left after extraction with organic solvent was evaporated on a rotary evaporator to a thick yellow mass, the aqueous fraction (Bv.Aq). The approximate yield of Bv.Butn and Bv.Aq fractions was 15.8% and 71.5%, respectively.

Animals. Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996) and were approved by the Ethical Committee for Research on Animals (ECRA) of Aga Khan University, Karachi, Pakistan. Wistar albino rats (180–220 g) were sourced from the Ethical Committee for Research on Animals (ECRA) of Aga Khan University, Karachi, Pakistan. Wistar albino rats weighing 180–220 g were divided with matched body weights into six groups of eight animals each, which were then randomly selected to receive various treatments. Rats in group I served as a vehicle treated control. Group II, serving as the untreated group, received stone-inducing treatment, for up to 21 days, comprising 0.75% (w/v) ethylene glycol (EG) with 1% (w/v) ammonium chloride (AC) for 5 days, following this, the water supply was switched to 0.75% EG alone in water (Atmani et al., 2003). Treated groups III, IV and V received intraperitoneal injection of Bv.Cr (50 mg/kg), Bv.Aq (40 mg/kg) and Bv.Butn (10 mg/kg), respectively, once in 24 h and simultaneously received stone-inducing treatment similar to group II, while group VI received Bv.Cr (50 mg/kg) alone thus serving as the Bv.Cr control. Doses of the fractions were selected based on their respective percent yields. The animal weights and water intake were monitored weekly during the study. At the end of 21 days of treatment, the animals were housed individually in metabolic cages. After collecting 3 h morning urine for the crystalluria study, 24 h urine was collected. Following volume and pH determination, part of each urine sample was acidified to pH 2 with 5 μL HCl. Both acidified and non-acidified urine samples were then centrifuged at 1500 × g for 10 min to remove debris and supernatants were stored at −20°C until analysed. Acidified urine samples were tested for oxalate (Ox), calcium (Ca²⁺) and magnesium (Mg²⁺), while non-acidified urine samples were used to determine the contents of citrate, uric acid (UA) and creatinine using commercially available kits.

Animals were anesthetized with diethyl ether and blood was collected through cardiac puncture. It was allowed to clot at room temperature and serum was collected in order to assess serum creatinine and BUN.

The creatinine clearance (CC) was calculated using the formula

\[
CC(\text{mL/min}) = \frac{(\text{mg creatinine/dL urine})(\text{mL urine/24 h})}{(\text{mg creatinine/dL serum}) \times 1440}
\]

The animals were killed and both the kidneys were excised, rinsed in ice cold physiological saline and weighed. The right kidney was fixed in 10% neutral buffered formalin, processed through various grades of alcohol and xylene, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin (H & E) and by the method of Pizzolato (1971) for light microscopic examination. To count the number of crystalline deposits, a sagittal section of each renal specimen was divided into eight readings. The left kidney was condensed to eight readings. The left kidney was

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worked into a 10% homogenate in ice cold PBS (50 mm, pH 7.4), centrifuged at 1500 × g, and the supernatant was used to assess malondialdehyde (MDA) and protein carbonyl contents, reduced glutathione and activities of various antioxidant enzymes.

The MDA content was estimated by the thiobarbituric acid reactive method (Wong et al., 1987). Briefly, 0.5 mL of the supernatant was mixed in succession with an equal volume of trichloroacetic acid (28% w/v) and 0.75 mL of 1% thiobarbituric acid (TBA) containing 0.01% BHT, and the mixture was heated at 90°C for 20 min. The pink coloured MDA–TBA complex formed was extracted with n-butanol (3 mL). After centrifugation to remove precipitated protein, the colour intensity was measured at 532 nm using a spectrophotometer and the amount of MDA was determined from the standard curve of 1,1,3,3-tetraethoxy propane.

Reduced glutathione was estimated as the total non-protein thiol (SH) group by the method described by Moron et al. (1979). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined by using commercially available methods. Catalase activity was determined by monitoring the decomposition of H_{2}O_{2} at 240 nm with a spectrometer (Aebi, 1987). The protein contents of kidney homogenates were determined by the method of Lowry et al. (1951).

Data analysis. The values are presented as mean ± SEM and expressed as mg/24 h for urinary electrolytes and respective units/mg protein for markers of oxidative stress. All statistical comparisons between the groups were made by means of one-way analysis of variance (ANOVA) with post hoc Dunnett’s test. A value of *p* < 0.05 was regarded as significant.

RESULTS AND DISCUSSION

In view of its medicinal use as an antiurolithic, *Berberis vulgaris* root bark extract was studied to evaluate its potential to prevent calcium oxalate urolithiasis. Renal calcium oxalate deposition induced by ethylene glycol and ammonium chloride in rats is frequently used to mimic the urinary stone formation in humans (Khan, 1997; Atmani et al., 2003). Therefore, the therapeutic effect of *Berberis vulgaris* on urolithiasis was evaluated using this model. In the normal course, liver metabolizes ethylene glycol into oxalic acid thus leading to hyperoxaluria, while ammonium chloride accelerates the process through urinary acidification (Khan, 1997; Atmani et al., 2003).

Body weights, water intake and urine volume, pH and composition recorded before the start of treatment were not significantly different among the groups (data not shown). Parameters recorded from groups of animals at the end of 21 days of treatment period are given in Table 1. In the 3 h morning urine, lithogenic treatment induced significantly abundant and visibly bigger CaOx crystals predominately of COD compared with the control group, while treatment of animals with Bv.Cr significantly decreased the urinary crystal count as well as visibly reduced the crystal size (Fig. 1). Crystalluria can occur similarly in both healthy and stone-forming individuals where the latter tend to excrete larger and aggregated particles than the former (Robertson et al., 1969). Calcium oxalate crystal agglomerates tend to be retained in kidney by being trapped in the renal tubules and developing into renal stones (Atmani et al., 2003).

After 21 days on a stone-inducing regimen, volumes of 24 h urine and water intake were higher in the untreated group compared with those of the control animals and the urine pH was slightly reduced, which is consistent with previous studies (Fan et al., 1997). A co-treatment with Bv.Cr prevented these urinary changes. In parallel with crystalluria, oxalate excretion was also significantly enhanced in untreated animals with stone-inducing treatment while calcium excretion was decreased. Bv.Cr also prevented hyperoxaluria and reduction in urine Ca^{2+} content known to be induced by hyperoxaluric agents (Fan et al., 1997; Park et al., 2008). The presence of significantly less oxalate content in the urine of Bv.Cr treated rats is the obvious reason for smaller and less abundant crystals but the presence of crystallization inhibitory constituents in Bv.Cr cannot be ignored. The urinary contents of citrate, UA and magnesium excretion remained unchanged. In the group receiving Bv.Cr, ethylene glycol treatment increased oxalate excretion but not significantly higher than those of the control animals and similarly calcium excretion was not altered.

Lithogenic treatment impaired renal function, as evident from increased BUN and serum creatinine and decreased creatinine clearance, which was prevented in animals receiving a simultaneous treatment with Bv.Cr. A significant loss in body weight by the stone-inducing treatment was observed with the untreated group during the study period, while in the Bv.Cr treated group there was a net gain in body weight over 21 days of treatment similar to that of the control animals.

**Figure 1.** CaOx crystals, viewed under microscope (400×), in 3 h morning urine from (A) control animals, (B) stone-forming group (C) group receiving Bv.Cr (50 mg/kg) and the stone-inducing treatment simultaneously.
Kidneys excised from the untreated group were found enlarged while they were not significantly different in the Bv.Cr treated group from those of the control animals. The surface of the kidneys of untreated rats had a speckled appearance. In histological preparations of kidneys removed from untreated animals, many intratubular birefringent crystalline deposits were seen under polarized light in all regions of the kidneys (cortex, medulla and papilla) of all animals, but were found in only two of eight rats treated with Bv.Cr. The crystals were also visibly small and less abundant compared with those in the untreated kidneys (Fig. 2, Table 1). The crystals were shown to be CaOx when stained black with Pizzolato’s stain. The renal tubules were markedly dilated in the entire kidneys of all rats in the untreated group, and this might have been caused by distal obstruction of renal tubular flow by large crystals. Hyperoxaluria is a major risk factor for calcium oxalate nephrolithiasis. Several in vivo and in vitro studies have demonstrated that high levels of oxalate may have a toxic effect on renal epithelium mediated through intracellular oxidative stress (Thamilselvan et al., 1997; Kwak et al., 2002; Selvam, 2002; Jeong et al., 2006), followed by changes in membrane integrity, membrane lipid peroxidation and cell death (Thamilselvan and Menon, 2005; Jeong et al., 2006). These changes facilitate CaOx crystal adherence and retention in renal tubules (Khan and Hackett, 1991; Khan, 1995; Thamilselvan et al., 2000).

The stone-inducing treatment enhanced MDA content of kidneys (11.4 ± 3.24 vs 0.71 ± 0.21 nmol/mg protein), and decreased reduced glutathione levels and activity of the antioxidant enzymes in the kidneys of group II (Table 2). The Bv.Cr treatment protected against the changes associated with oxidative stress. Bv.Cr did not cause any change in the urine composition of the Bv.Cr control group compared with that of the vehicle treated control, which suggests that antioxidant and crystallization inhibitory properties of Berberis vulgaris may have accounted for its prophylactic effect on CaOx urolithiasis.

Among the fractions tested, the frequency and extent of CaOx crystal deposition in rat kidneys in the Bv.Aq treated group was similar to the parent crude extract (Table 1). The Bv.Aq also exhibited a protective effect against impairment of renal function, oxidative damage to kidney and biochemical changes in urine composition, comparable to Bv.Cr. In the Bv.Butn treated group, crystalline deposits were found in the kidneys of all animals though less extensively (p < 0.05) than in the untreated group (Table 1). There was also an impairment of renal function in the Bv.Butn group as indicated by BUN and creatinine clearance values but significantly less than in the untreated rats. Kidney weights were similar but there was a net gain in body weights though it was not significant compared with the untreated group, while oxidative damage associated with lithogenesis was also relatively reduced by the Bv.Butn treatment. Activity-directed fractionation thus reveals that the antiurolithic activity of Bv.Cr is concentrated in the aqueous fraction.

CONCLUSION

Berberis vulgaris root bark contains constituents that can prevent the formation of renal stones mediating their effect possibly through inhibition of calcium oxalate crystallization and antioxidant activity. The data provide a rationale for the medicinal use of Berberis vulgaris in nephrolithiasis and identify this plant as a potential source of new antiurolithic drugs.

Acknowledgements

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Conflict of Interest

The authors have declared that there is no conflict of interest.
Table 1. Various parameters in animal model study recorded from rats after 21 days exposure to different treatments

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gain in body weight (%)</td>
<td>14.53 ± 1.34</td>
<td>-5.89 ± 1.15</td>
<td>9.3 ± 2.18</td>
<td>8.59 ± 2.27</td>
<td>2.0 ± 3.52</td>
<td>10.02 ± 1.63</td>
</tr>
<tr>
<td>CU (count/mm³)</td>
<td>45 ± 9</td>
<td>150 ± 24a</td>
<td>79 ± 13a</td>
<td>83 ± 15a</td>
<td>135 ± 18</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.71 ± 0.01</td>
<td>1.49 ± 0.03b</td>
<td>0.83 ± 0.24a</td>
<td>0.85 ± 0.26a</td>
<td>1.34 ± 0.17</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>CD/field (10⁻⁶)</td>
<td>0</td>
<td>50.1 ± 4.8b</td>
<td>6 ± 4.85</td>
<td>6.4 ± 6.68d</td>
<td>33.2 ± 4.3a</td>
<td>0</td>
</tr>
<tr>
<td>Water intake (mL)</td>
<td>6.4 ± 0.86</td>
<td>16.5 ± 1.1b</td>
<td>6.75 ± 0.81a</td>
<td>6.3 ± 0.46a</td>
<td>11.8 ± 0.5b</td>
<td>6.5 ± 0.78</td>
</tr>
<tr>
<td>Urine/24 h Volume (mL)</td>
<td>8.49 ± 1.7</td>
<td>15.63 ± 1.3a</td>
<td>7.18 ± 1.24a</td>
<td>6.93 ± 0.9a</td>
<td>12.05 ± 0.85</td>
<td>7.63 ± 0.86</td>
</tr>
<tr>
<td>pH</td>
<td>6.48 ± 0.13</td>
<td>5.97 ± 0.07</td>
<td>6.49 ± 0.26</td>
<td>6.67 ± 0.26</td>
<td>5.9 ± 0.09</td>
<td>6.56 ± 1.9</td>
</tr>
<tr>
<td>Ca²⁺ (mg)</td>
<td>3.28 ± 0.45</td>
<td>1.28 ± 0.06b</td>
<td>2.76 ± 0.41</td>
<td>2.58 ± 0.45</td>
<td>1.76 ± 0.40</td>
<td>3.02 ± 0.83</td>
</tr>
<tr>
<td>Mg²⁺ (mg)</td>
<td>3.9 ± 0.58</td>
<td>3.15 ± 0.35</td>
<td>4.1 ± 0.23</td>
<td>4.23 ± 0.42</td>
<td>3.77 ± 0.53</td>
<td>3.19 ± 0.35</td>
</tr>
<tr>
<td>Ox (mg)</td>
<td>0.34 ± 0.09</td>
<td>2.12 ± 0.41a</td>
<td>0.56 ± 0.17a</td>
<td>0.60 ± 0.19a</td>
<td>1.4 ± 0.27a</td>
<td>0.35 ± 0.06a</td>
</tr>
<tr>
<td>Citrate (mg)</td>
<td>31 ± 3.7</td>
<td>29 ± 3.5</td>
<td>30 ± 2.9</td>
<td>31 ± 4.2</td>
<td>28 ± 4.4</td>
<td>33 ± 6.8</td>
</tr>
<tr>
<td>UA (mg)</td>
<td>0.85 ± 0.21</td>
<td>0.67 ± 0.19</td>
<td>0.58 ± 0.25</td>
<td>0.67 ± 0.18</td>
<td>0.61 ± 0.26</td>
<td>0.63 ± 0.41</td>
</tr>
<tr>
<td>Kidney function tests</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN</td>
<td>26.8 ± 1.19</td>
<td>70 ± 6.1b</td>
<td>23 ± 1.6d</td>
<td>22.3 ± 2.4d</td>
<td>50.7 ± 1.8d</td>
<td>28 ± 0.96</td>
</tr>
<tr>
<td>SC mg/dL</td>
<td>0.92 ± 0.03</td>
<td>1.95 ± 0.15b</td>
<td>0.96 ± 0.03d</td>
<td>0.90 ± 0.06d</td>
<td>1.45 ± 0.04d</td>
<td>1.1 ± 0.07</td>
</tr>
<tr>
<td>CC mL/min</td>
<td>0.85 ± 0.04</td>
<td>0.5 ± 0.03b</td>
<td>0.8 ± 0.06b</td>
<td>0.82 ± 0.05d</td>
<td>0.64 ± 0.04</td>
<td>0.87 ± 0.04</td>
</tr>
</tbody>
</table>

Values are compared with group I, a p < 0.01.
Values are compared with group II, b p < 0.01.
Values are compared with group III, c p < 0.05, d p < 0.01.

Table 2. Effects of various treatments on markers of oxidative stress in kidneys of rats

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>0.71 ± 0.21</td>
<td>11.4 ± 3.2a</td>
<td>2.49 ± 1.2a</td>
<td>2.22 ± 1.02a</td>
<td>9.51 ± 1.96a</td>
<td>0.89 ± 0.17a</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>6.27 ± 0.58</td>
<td>4.31 ± 1.75a</td>
<td>5.91 ± 0.62</td>
<td>5.84 ± 0.53</td>
<td>4.50 ± 0.68a</td>
<td>6.33 ± 0.61a</td>
</tr>
<tr>
<td>GPX (U/mg protein)</td>
<td>0.52 ± 0.12</td>
<td>0.34 ± 0.06b</td>
<td>0.48 ± 0.04</td>
<td>0.45 ± 0.08a</td>
<td>0.35 ± 0.02a</td>
<td>0.49 ± 0.03a</td>
</tr>
<tr>
<td>Catalase (H2O2/min/mg protein)</td>
<td>40.3 ± 4.9</td>
<td>29.8 ± 5.7a</td>
<td>38.4 ± 10.3a</td>
<td>40.2 ± 5.7a</td>
<td>34.0 ± 3.4a</td>
<td>41.0 ± 5.31a</td>
</tr>
<tr>
<td>GHS (nmol/mg protein)</td>
<td>19 ± 2.5</td>
<td>14.9 ± 2.5a</td>
<td>18.2 ± 2.7</td>
<td>17.3 ± 0.82</td>
<td>16.1 ± 2.91</td>
<td>18.5 ± 2.21</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for eight animals in each group.
MDA, malondialdehyde; SOD, superoxide dismutase; GPX, glutathione peroxidase.
Values are compared with group I, *p < 0.05, †p < 0.01.
Values are compared with group II, ‡p < 0.05, §§p < 0.01 vs group II.

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