ANNEXURES
Prof. S.K Singla
Deptt. of Biochemistry,
Panjab University,
Chandigarh.

Subject: Animal ethical clearance certificate for research project.

Dear Prof. Singla,

This has reference to your research project entitled Purification, characterization of most potent antilithiatic metabolite of *Bergenia ligulata* and its mechanism of action to be sent to DST for possible funding.

The project has been approved by the Institutional Animal Ethics Committee in its meeting held on 14.3.2011.

With best regards,

(A.R. Rao)
Prof. Rajat Sandhir
Convener

Ms. Deepika
Department of Biochemistry,
Panjab University
Chandigarh

Subject: Animal Ethics clearance Certificate

This has reference to your project titled “Characterization & validation of most potent antioxidant and anticalcifying compound(s) from Bergenia Ligulata rhizome extract” under the supervision of Prof. S.K. Singla and funding from ICMR, New Delhi.

The project has been approved in anticipation to the Institutional Animal Ethics Committee of Panjab University, Chandigarh.

With Best Regards

(Prof. Rajat Sandhir)
Convener, IAEC
Convener,
Institutional Animals Ethics Committee
Panjab University
Chandigarh
March 11, 2011

AUTHENTICATION CERTIFICATE

Name of the party: - Prof. S.K. Singla
Address: - Department of Biochemistry (DST-FIST Sponsored Department),
Panjab University, Chandigarh 160014
Reference: - No.255/ Bch; dated 01.02, 2011
Name of the sample: - Bergenia ligulata
Sample size: - Dried sample, about 350 g
Date of receipt: - 21.02.2011

Report:-

The sample has been critically studied with macroscopic, microscopic, organoleptic characters and TLC profile. We hereby authenticate that the sample belongs to rhizomes of Bergenia ciliata (Haw.) Sternb. forma ligulata Yeo (Family-Saxifragaceae)
As per request, the sample is deposited and voucher number allotted is as below:

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Family</th>
<th>Deposited on</th>
<th>Voucher number allotted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bergenia ciliata (Haw.)</td>
<td>Saxifragaceae</td>
<td>08/03/2011</td>
<td>R-126</td>
</tr>
<tr>
<td>Sternb. forma ligulata Yeo</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This certificate is issued at his request and is given only for the academic use.

(A. S. Upadhye)
PUBLICATIONS AND CONFERENCES

Published article


Conference Papers

- “A comprehensive study of bergenia ligulata rhizome confirmed bergenin as its most potent anticalcifying agent” presented at ISNCON organized by Indian Society of Nephrology 18-21st December 2014
- “Evaluation of Antiurolithiatic Efficacy of Bergenia ligulata Rhizome” Presented at 100th meeting of Indian science congress organized by Indian science congress association 3-7th, January 2013.
- “Antiurolithiatic activity of Bergenia ligulata rhizome: comparison of different solvent extracts” Presented at Prospects and perspectives in biochemistry (Department of Biochemistry, PU Chandigarh) 10-11th February 2012.

Workshops Attended

- **Workshop on HPTLC** organized by the STEP, Thapar University, Patiala 21-22nd November, 2011.
Research Paper

The most potent antilithiatic agent ameliorating renal dysfunction and oxidative stress from Bergenia ligulata rhizome

Deepika Aggarwal a, Rajeev Kaushal a, Tanzeer Kaur b, Rakesh Kumar Bijarnia c, Sanjeev Puri a,d, Surinder Kumar Singla a,*

a Department of Biochemistry, Panjab University, Chandigarh 160014, India
b Department of Biophysics, Panjab University, Chandigarh 160014, India
c Department of Nephrology, Hypertension and Clinical Pharmacology, University Hospital Bern, Inselspital, Freiburgstrasse 15, Bern 3010, Switzerland
d Biotechnology Branch, University Institute of Engineering and Technology, Panjab University, Chandigarh 160014, India

ABSTRACT

Ethnopharmacological relevance: The rhizome of Bergenia ligulata is referred by the Ayurvedic system for the treatment of kidney stone since decades and a few, in vitro and in vivo studies also support it. To identify the main phytochemical constituent(s) responsible for antilithiatic activity of its rhizome.

Materials and methods: In order to identify the most potent antilithiatic metabolite, the crude extract of rhizome was fractionated using in vitro Calcium oxalate (CaOx) crystal growth inhibitory activity guided fractionation followed by its characterization via LC-MS, FTIR and NMR. Further, the antioxidant potential of purified molecule was assessed using in vitro assays (FRAP and H2O2 scavenging). In vivo activity of the metabolite was evaluated in hyperoxaluric rats given 0.4% ethylene glycol (EG) and 1.0% ammonium chloride (NH4Cl) for 9 days.

Results: Activity guided fractionation led to the isolation of most potent antilithiatic metabolite from the rhizome of Bergenia ligulata and spectroscopic analysis revealed it as bergenin. Bergenin showed reducing ability and H2O2 scavenging activity comparable with commercially available antioxidant, α-tocopherol. At a dose of 10 mg/kg body weight of the treated rat, it protected against deleterious effects of lithogenic treatment including weight loss, impaired renal function and oxidative stress, manifested as increased malondialdehyde, reduced redox ratio and decreased antioxidant enzyme activities in the kidneys of hyperoxaluric rats. The creatinine clearance and kidney damage were more improved by bergenin as compared to crude extract of rhizome.

Conclusions: Since, bergenin maintained oxidant/antioxidant balance in hyperoxaluric rats, thus mechanistic insight of its antilithiatic activity was attributed to the antioxidant capability of bergenin. The results of the present study provide significant evidence that bergenin is an active component present in the rhizome of Bergenia ligulata for managing CaOx calculi.

© 2014 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

The current lack in regimen of management and therapy of renal tissue calcification, the side-effects of synthesized allopathic medicine and importance of natural products derived molecules in therapeutics, shifted the focus of scientists towards the traditional system of herbal therapy. Bergenia ligulata (family, Saxifragaceae) is one such perennial herb commonly used in South Asia and has been shown to possess antidiabetic, diuretic, astringent, cardiotoxic, wound healing, antipyretic and anti-hemorrhoidal activities (Kirtikar and Basu, 1933; Zhang et al., 2011). It grows against rocks and popularly known as Pushambhedu (dissolve the stone), signifying its use in herbal formulations for urolithiasis (Garinella et al., 2001). The rhizome of Bergenia ligulata is one of the major ingredient of Cystone® (Himalaya) and Calcury (Charak pharma), the two herbal formulations commonly used for treating kidney stones (Singh et al., 2007). In an in vitro study, the rhizome of Bergenia ligulata is reported to inhibit homogeneous precipitation and growth of calcium phosphate (CaP) and calcium oxalate (CaOx) crystals (Joshi et al., 2005a, 2005b) and in vivo (hyperoxaluric rat model) antilithiatic efficacy of its rhizome had been attributed to the combination of antioxidant, diuretic and hypermagnesuric features (Bashir and Gilani, 2009). Albeit, the mechanistic base of its activity remained unknown.
Bashir and Gilani (2009) proposed that crude extract of Bergenia ligulata has the ability to reduce hyperoxaluria induced oxidative damage and maintain antioxidant enzymes in rat kidneys. Many studies revealed that urine from CaOx stone patients had significantly increased markers of oxidative damage, tubular injury and inflammation, indicating that renal impairment in these patients is most probably caused by reactive oxygen species (Aggarwal et al., 2013; Boonla et al., 2007; Mustaq et al., 2007).

Therefore it appears that antioxidants and anti-inflammatory therapy may impede CaOx growth and inhibit stone recurrence. Eminent antioxidant vitamin E (α-tocopherol) supplementation to stone forming patients, significantly normalized the kinetic properties of Tamm–Horsfall protein (Sumitra et al., 2005), and in normal patients, increased urinary citrate level (Theka et al., 2012).

So far, various metabolites have been identified from Bergenia ligulata like bergenin, afzelechin, leucocyanidine, gallic acid, methyl gallate, catechin, and paashaanolactone etc. showing diverse biological activities (Dharmender et al., 2010; Sajad et al., 2010). But the antilithic activity of any of its metabolites has not been reported yet. To move one step further, it becomes important to identify the main phytochemical constituent(s) responsible for anti-calciifying activity of its rhizome. So, at present, a blind study was conducted to identify the most potent antilithic metabolite (on the basis of in vitro CaOx inhibitory potency of extract at each step of purification) from the rhizome of this plant. As a result of purification and characterization, of many purified metabolites, bergenin showed maximum inhibitory activity. To strengthen the data further, the in vivo efficacy of bergenin, for renal functioning and injury in a hyperoxaluric rat model (induced by 0.4% EG and 15%NH₄Cl) was evaluated.

2. Material and methods

2.1. Collection and identification of plant materials

The rhizomes of Bergenia ligulata were procured from an accredited herbal shop in Chandigarh (India) and certified from the plant drug authentication centre of the botany division at Agharkar Research Institute, Pune (India). A voucher specimen (R-126) was submitted to the herbarium of the institute. Plant material was thoroughly washed, dried in shade and ground into fine powder by using an electric grinder. Powdered material was stored in air sealed container at an ambient temperature.

2.2. Preparation of plant extracts

Powdered material was successively extracted with different organic solvents in the increasing order of polarity (Jeyaseelan et al., 2012). The powder (100 g) was sequentially extracted with hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol and water using Soxhlet apparatus (Singh et al., 2009). Each extract was dried under reduced pressure, yield documented and finally stored at 4 °C until used.

2.3. Chromatographic separation

Ethanolic extract was further subjected to column chromatography. A glass column (size, 150 cm × 4 cm) was packed with 500 g silica (mesh size, 230–400) and equilibrated with toluene (three times of bed volume) before loading sample. Ethanolic extract (10 g) was loaded onto the column and eluted with absolute toluene followed by gradient of toluene and ethyl acetate in the ratio of 4:1, 3:2, 2:3, 1:4 and absolute ethyl acetate, respectively. Later, a gradient of ethyl acetate and methanol in ratio of 4:1, 3:2, 2:3, 1:4 and absolute methanol was used for elution. A total of 168 fractions, each of 50 ml was collected. Thin layer chromatography (TLC) was performed on pre coated silica plates (silica gel 60F₂₅₄, 0.25 mm, Merck) to check the homogeneity. Based on the TLC profile, similar fractions were pooled, dried, weighed and their 0.5% (w/v) solution were prepared in 10% dimethyl sulfoxide (DMSO) to check in vitro CaOx crystal growth inhibitory activity. The most active fraction was further subjected to another silica gel (mesh size 230–400) column (60 cm × 3 cm) and eluted with chloroform–methanol in the ratio of 6:4 followed by absolute methanol. The eluted fractions were further subjected to TLC and similar fractions were pooled, dried and weighted. Finally, three major sub fractions were obtained and used to check in vitro CaOx crystal growth inhibitory activity. All samples were stored at –20 °C until used.

2.4. Spectroscopic techniques

Most active sub-fraction was characterized by NMR, FTIR and LC-MS. The isolated sub-fraction was characterized by nuclear magnetic resonance (NMR) spectroscopy, ¹H and ¹³C (400 MHz) NMR spectra were recorded on a Bruker DRX-400 with trimethylsilane (TMS) as the internal standard. IR spectra were recorded on FT-IR Spectrophotometer Model RZX (Perkin-Elmer). High performance liquid chromatography was performed using WaterTM 2795 mode with column C₁₈ Extrelut™ and the eluant was subsequently subjected to integrated Q_TOF micromass spectroscopic analysis. The isolated sub-fraction was scanned with UV spectrophotometer (UV-1800 Shimadzu Corporation).

2.5. Assay to measure inhibitory activity against CaOx crystal growth

Inhibitory activity against CaOx crystal growth was measured using the seeded solution-depletion assay (Chutipongtane et al., 2005). Briefly, CaOx monohydrate clinical kidney stone was procured from Department of Urology, PGIMER, Chandigarh and stone particles were added to the solution containing 1 mM CaCl₂ and 1 mM sodium oxalate (Na₂C₂O₄). A decrease in depletion of free oxalate ions (detected at λ₂₃₄ nm) in the presence and absence of test samples indicated the inhibition of CaOx crystal growth by the test sample. The relative inhibitory activity was calculated as follows: % relative inhibitory activity=[(C–S)/C] × 100, where C is the rate of reduction of free oxalate without any test compound and S is the rate of reduction of free oxalate with a test compound.

2.6. Total reduction capability by Fe³⁺–Fe²⁺ transformation

Total reducing powers of separated fraction and crude extract were determined with different concentrations of all samples (10, 20, 30, and 40 μg/ml) in 0.75 ml of distilled water mixed with phosphate buffer (1.25 ml, 0.2 M, pH 6.6) and potassium ferrocyanide [K₃Fe(CN)₆] (1.25 ml, 1%) (Huang et al., 2003). α-tocopherol was used as a positive control in this assay. Higher absorbance at 700 nm of the reaction mixture indicated greater reducing power.

2.7. Hydrogen peroxide scavenging

The hydrogen peroxide scavenging capacity was measured according to the method of Ruch et al. (1989). The test samples were prepared at concentration of 30 μg/ml in 50 mM phosphate buffer (pH 7.4). After 10 min of the addition of 0.6 ml hydrogen peroxide solution, tubes were vortexed and absorbance recorded at 230 nm, against a blank. Hydrogen peroxide scavenging ability was calculated by the formula: % scavenging=[1–A₀/Aₐ] × 100, where A₀ is the absorbance without sample, and Aₐ is absorbance with sample. Hydrogen peroxide concentration was determined spectrophotometrically at 230 nm using the molar extinction
2.8. Ethylene glycol-induced damage in rats

The animal experimental protocols were approved by Institutional Animal Ethics Committee (reference no. IAEC/130 Dated 31/03/2011) and conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals. Twenty-four male Wistar rats (200 ± 20 g) of equivalent age were procured from central animal house facility at Panjab University, Chandigarh (India). All animals were acclimatised for 15 days in cages before starting the experiment and randomly divided into four groups consisting of six rats in each group. Normal (NRM) rats served as vehicle-treated control. In order to induce hyperoxaluria, the second, third and fourth group rats received ethylene glycol (0.4%, v/v) and ammonium chloride (NH₄Cl, 1%, w/v) in drinking water for 9 days (Lee et al., 2011). Additionally, third and fourth group rats were treated with pure bergenin (BRG) and Bergenia ligulata ethanolic extract (BLE) respectively at a dosage of 10 mg/kg body weight, in saline for 9 days. The standardization of the hyperoxaluric rat model was already done in the lab from previous studies (Kaur et al., 2009).

2.9. Sample collection

At the end of 8th day, rats were placed in metabolic cages for 24 h and urine samples were collected using 20 μl of 20% sodium azide as a preservative. After measuring urine volume, an aliquot of it was acidified with 3 N HCl for the determination of urinary creatinine. Blood was collected into clean centrifuge tubes by puncturing inferior vena cava under anesthesia and allowed to clot for 1 h. Serum was separated as a supernatant after centrifugation at 3,000 × g for 15 min.

2.10. Measurement of serum urea level

Serum urea level was estimated by using commercially available kits (Erba diagnostic, Manheim, Germany) as per manufacturer’s instructions.

2.11. Measurement of creatinine in serum and urine

The creatinine in both serum and urine was estimated by the commercially available kit (Erba, Mannheim, Germany). Creatinine clearance was calculated according to standard clearance formula, C = U/V × S, where U is the urinary concentration of creatinine, S is the concentration of creatinine in the serum and V is the urinary flow in ml/min.

2.12. Measurement of urinary enzyme

Urinary LDH was measured using commercially available kits (Erba Mannheim, Germany) as per the manufacturer’s instructions. LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. The rate of NADH formation is directly proportional to the LDH catalytic activity and is determined by measuring the increased absorbance at 340 nm. The activity was expressed in units of enzyme/mg protein. The protein was measured by the method of Lowry et al. (1951). Urinary Alkaline phosphatase (ALP) was measured using commercially available kits (Reckon diagnostics Pvt. Ltd) as per the manufacturer’s instructions. ALP hydrolyzes para-nitrophenyl phosphate into para-nitrophenol and phosphate, in the presence of magnesium ions. The rate of increase in absorbance of the reaction mixture at 405 nm due to liberation of para-nitrophenol is proportional to the alkaline phosphatase activity. The activity was expressed in units of enzyme/mg protein.

2.13. Measurement of oxidant/antioxidant status in renal tissue

Lipid peroxidation was assessed by quantifying malondialdehyde (MDA) level in kidney tissue homogenate using the method of Buege and Aust (1978). Reduced glutathione (GSH) was estimated as total non-protein thiol (SH) group by the method described by Moron et al. (1979). For the purpose of quantization, a calibration curve was prepared using GSH as a standard. Catalase (CAT) activity was determined by the UV spectrophotometer method described by Luck (1963) and expressed as molar extinction coefficient of NADPH oxidized per minute per milligram protein. The assay for superoxide dismutase (SOD) was performed according to the method of Kono (1978). The activity of enzyme was expressed as units per milligram of protein. Glutathione peroxidase (GPx) activity was quantified as the rate of oxidation of GSH by H₂O₂ catalyzed by the GPx. The rate of formation of GSSG was monitored at 340 nm for 3 min. GPx activity was calculated using molar extinction coefficient of NADPH (6.22 × 10⁶ M⁻¹ cm⁻¹) and the results were expressed as nanomoles of NADPH oxidized per minute per milligram protein (Rotruck et al., 1973). The activity of the kidney tissue enzymes was expressed as units of enzyme/mg protein which was determined by the method of Lowry et al. (1951).

2.14. Urinary crystal study

A drop of urine obtained from bladder is spread on a glass slide and visualized under polarized light using Leica DM3000 light microscope. The crystals were counted in five fields and the results represent their mean.

2.15. Statistical analysis

All quantitative measurements were expressed as mean ± SD. Analyses were performed using one-way analysis of variance (ANOVA) and the group means were compared by Tukey’s multiple comparison post hoc tests. P < 0.05 was considered as statistically significant.

3. Results and discussion

The rhizome of Bergenia ligulata is one of the important constituents of commercially available formulation, Cystone® used for the treatment of kidney stone. An in vitro study reported antilithic activity of the rhizome of Bergenia ligulata (Bashir and Gilani, 2009), yet there is no study about the active metabolites responsible for this activity. To explore the scientific basis further, a blind study was designed to find potent active metabolites responsible for its calcium oxalate crystal growth inhibitory activity.

The complete sequential strategy of extraction and yield at each respective step from rhizome of Bergenia ligulata has been shown in Fig. 1a. Out of all extracts of the rhizome of Bergenia ligulata, its ethanolic extract (BLE) exhibited maximum inhibitory activity towards in vitro CaOx crystal growth assay, so it was further profiled for its constituent metabolites. After first chromatography, the elutes were pooled on the basis of their TLC profile into 28 fractions and fraction 13–15 presented highest activity towards in vitro CaOx inhibitory assay. This fraction was further subjected to another silica gel column resulting in three sub-fractions (SFR) having inhibitory activities, 63.34% (SFR1), 58.44% (SFR2) and 49.07% (SFR3). The SFR1 was a white crystalline material and it showed maximum inhibitory potential. Characterization of SFR1...
using NMR, FTIR and LC-MS (Fig. 1b–d) revealed it as purified bergenin (BRG) molecule (Fig. 1e) (De Abreu et al., 2008). The complete spectra are shown in Supplementary information (Figs. 1–3). The UV spectrum of SFR1 illustrated two characteristic absorption bands at 220 (1) and 275 (2) nm (Supplementary Fig. 3b). Qin et al. (2010) has reported similar absorption peaks for BRG in a previous study.

The antilithiatic potential of BRG and crude ethanolic extract (BLE) were compared with Cystone®, where BRG showed the highest activity i.e. 63% in comparison with BLE (41%) and Cystone® (52%) towards in vitro CaOx crystal growth inhibitory assay (Fig. 2). This activity of BRG could be attributed to its structural property, being a gallic acid derivative. Lee et al. (2011) had shown that 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG), a water soluble Gallotannin can alter the CaOx crystal adhesion to cells and oxalate induced renal cell injury. Role of Gallotannin from green tea has also been assessed as a protectant of renal cells against CaOx induced injury (Lee et al., 2012).

Bergenin is a C-glycoside of 4-O-methyl gallic acid and its hydrolytic product contains 4-O-methylglycoside (4-OMG) as the main species (Fig. 3) having acidic properties and hence may undergo ionization in neutral and alkaline physiological environments (Kobayashi and de Mejia, 2005). The ionizing ability of BRG in buffer under neutral pH could explain its mechanism of interference with the precipitation and growth of CaOx crystals. Taller et al. (2007) had reported that linear aspartic acid rich peptide preferentially binds to {121} faces of CaOx crystals. The acidic amino acids having negatively charged side chains are attracted to positively charged calcium ions of CaOx crystals (Bijarnia et al., 2009). It is augmented that proteins rich in γ-carboxy glutamic acid possessing two negative carboxylate groups have better binding ability with calcium sites of CaOx crystal (Sheng et al., 2005). These reports supported that the hydrolysis of BRG to 4-OMG having free carboxylate group makes it capable of actively regulating and inhibiting CaOx crystal growth and also explains its mechanism of interference with the growth of CaOx in the in vitro assay system. Crystal growth inhibitors are believed to act by adsorbing onto sites at which lattice-ion addition is energetically favored (Bijarnia et al., 2009). Hydrogen bonding...
with freely available growing sites of CaOx crystals ie oxalate ion (carbon and oxygen) as shown in Fig. 3, implicates the modulation of calcium oxalate crystal growth. Chen et al. (2010) suggested that phenolic group rich tea extract can form hydrogen bonds with electronegative oxalate in CaOx crystal. The phenolic groups of BRG might also be able to effectively bind via hydrogen bonding electronegative oxalate in CaOx crystal. The phenolic groups of BRG might also be able to effectively bind via hydrogen bonding electronegative oxalate in CaOx crystal.

The in vivo efficacy of BRG was further examined on rat hyperoxaluric (HYO) model and compared with BLE. It has been observed that ethylene glycol administration to rats caused significant reduction (p < 0.05) in rat body weight (Table 1) whereas the rats treated with BRG and BLE showed no significant difference between initial and final body weights.

Among various risk index of kidney stone disease, crystalluria (presence of crystals in urine) is suggested to be an important factor to determine severity and recurrence of stones disease. Crystalluria, can predict the risk of stone recurrence in calcium stone formers, if it is repeatedly found in early morning urine samples (Daudon et al., 2005).

Urine of HYO rats showed significant presence of CaOx monohydrate crystals (Fig. 4a), whereas urine from both BRG and BLE treated rats was either devoid of crystals or showed debris of crystals in some instances (Fig. 4b, c). The decrease in crystalluria of BRG treated rats (Fig. 4d), reflected its in vitro antilithogenic potential.

EG administration to rats caused marked elevation in urinary risk factors associated with stone. As shown in Table 1, HYO rats showed significant (p < 0.001) increase in serum urea and serum creatinine as compared to NRM rats and the treatment with BRG normalized serum urea levels more significantly as compared to BLE treated rats. Similarly, the contents of urea and creatinine in urine showed enhanced ability of BRG in maintaining their levels as compared to BLE.

LDH, a cytosolic enzyme, is partly involved in the synthesis of oxalate. It catalyzes the coupling of oxidation and reduction of glyoxylate, resulting in the formation of glycolic acid and oxalate. Increased activity of LDH activity has already been reported in hyperoxaluria (Pragasam et al., 2005). In addition, to ascertain the extent of injury in renal tissue, LDH release in urine was observed (Fig. 5a).

Intergroup analysis confirmed that LDH activity was 19% (p < 0.001) higher in HYO rats as compared to NRM rats. BRG and BLE had 67% and 41% reduction in LDH activity respectively as compared to HYO group. Moreover, their LDH activity was comparable to that of NRM rats, which suggested the protective action of BLE against hyperoxaluria.

Table 1. Body weight, serum urea and serum creatinine after 9 days of treatment.

<table>
<thead>
<tr>
<th></th>
<th>NRM</th>
<th>HYO</th>
<th>BRG</th>
<th>BLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial weight (g)</td>
<td>155.8 ± 35.6</td>
<td>150.0 ± 32.9</td>
<td>143.3 ± 38.3</td>
<td>145.0 ± 32.7</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>35.6 ± 150.0</td>
<td>31.6 ± 131.7</td>
<td>26.0 ± 114.1</td>
<td>35.0 ± 140.0</td>
</tr>
<tr>
<td>Urea (mg/dl)</td>
<td>38.3 ± 57.7</td>
<td>32.9 ± 143.3</td>
<td>35.8 ± 148.3</td>
<td>35.0 ± 148.3</td>
</tr>
</tbody>
</table>

* n = 6 [p < 0.05] indicates significant difference between initial and final weight in each group.

** p < 0.05 indicates significant change in comparison with NRM group.

*** p < 0.001 indicates significant change in comparison with HYO group.

### p < 0.001 indicates significant difference between initial and final body weights.

![Fig. 3. Possible way of hydrolysis of bergenin into C-glycoside of 4-O-methyl gallic acid and interaction bergenin with calcium oxalate crystal.](image-url)
restored by BRG and BLE treatment though the BRG was more effective in restoring the values to normal levels (Fig. 5c). There are reports pertaining to normalization of CrCl by plant metabolites, like a plant alkaloid-berberine treatment to hyperoxaluric rats, improved kidney functions reflected by the levels of CrCl (Bashir and Gilani, 2011).

Fig. 4. Crystalluria depicted by polarization micrographs of experimental rat’s urine. (a) Urine of HYO showing number of dumbbell shaped COM crystals. (b) Urine of BRG treated rats showing crystal debris. (c) Urine of BLE treated rats showing crystal debris. Original magnifications of 100×. (d) Average number of crystals observed at five different fields in the urine of treated rats. HYO: Ethylene glycol and ammonium chloride treated; COM: Calcium monohydrate; BRG: Bergenin treated urolithiatic rats; BLE: crude extract treated urolithiatic rats.

Fig. 5. Renal function tests in hyperoxaluric rats treated with BRG and BLE. (a) Lactate dehydrogenase (LDH) activity (U/min/mg protein). (b) Alkaline phosphatase (ALP) activity (U/min/mg protein). (c) Creatinine clearance (ml/min). NRM: control rats; HYO: Ethylene glycol and ammonium chloride treated; BRG: Bergenin treated urolithiatic rats; BLE: crude extract treated urolithiatic rats. The results are compared by one way ANOVA with Tukey’s multiple comparison post hoc test. n=6, a: p < 0.01 significantly different from the NRM group, b: p < 0.05 significantly different from HYO group.
Instigation of oxidative stress following oxalate load in renal tissue is a pivotal step in pathophysiology of kidney stone and various antioxidants have been employed to reduce its consequences. Bashir and Gilani (2009) suggested ethanolic extract of *Bergenia ligulata* showed potential to ameliorate renal dysfunction in hyperoxaluric rats due to its antioxidant capability.

Fig. 6. In vitro antioxidant activities. (a) Comparison of antioxidant activity of BRG with BLE and α-tocopherol using FRAP assay. (b) Comparison of percentage scavenging activity between 30 μg/ml solution of BRG, BLE and α-tocopherol. BRG: Bergenin treated urolithiatic rats; BLE: crude extract treated urolithiatic rats. The results are compared by one way ANOVA with Tukey’s multiple comparison post hoc test. n = 6, a: p < 0.01 significantly different from the BRG group, b: p < 0.05 significantly different from BLE group.

Fig. 7. Oxidative stress markers in renal tissue of hyperoxaluric rats treated with BRG and BLE. (a) Lipid peroxidation (LPO). (b) Redox ratio (GSH/GSSG). (c) Superoxide dismutase (SOD) activity (U/min/mg protein). (d) Catalase activity (U/min/mg protein). (e) Glutathione peroxidase (GPx) activity (U/min/mg protein). NRM: control rats; HYO: Ethylene glycol and ammonium chloride treated; BRG treated urolithiatic rats; BLE: crude extract treated urolithiatic rats. The results are compared by the one way ANOVA with Tukey’s multiple comparison post hoc test. n = 6, a: p < 0.01 significantly different from the NRM group, b: p < 0.05 significantly different from HYO group.
The previous reports suggesting the antioxidant potential of bergenin accentuated to explore its ability to reduce ROS (Nazar et al., 2011; Roselli et al., 2012). The total reducing potential of BRG was found to be comparable with α-tocopherol (Fig. 6a). Also, H₂O₂ scavenging capacity of BRG was similar to α-tocopherol (Fig. 6b). The free radical scavenging capacity of BRG could be primarily attributed to the high reactivity of hydroxyl substituents on benzene ring as they form aromatic conjugated dienes which can stabilize the free radical by resonance. Bergenin can form complexes with Fe (II) and hence prevent the production of hydroxyl radicals in Fenton reaction (De Abreu et al., 2008). The galloyl moieties of the hydrolyzed BRG could be responsible for the chelating and radical scavenging properties of this compound. This implies that the antioxidant and antiarthritic capability of Bergenia ligulata suggested by Bashir and Gilani (2009) might be prevailing in bergenin.

Further, in vivo antioxidant potential of BRG was also assessed in hyperoxaluric rats. The malondialdehyde (MDA) levels were found to be elevated significantly in HYO rats indicating oxalate induced oxidative stress. The levels of MDA were found to be reduced by 58% and 51% in BRG and BLE treated rats respectively (Fig. 7a). Redox ratio (GSH/GSSG) is another indicator of reducing intracellular environment and is significantly maintained by BRG while BLE treatment could not significantly restore the reducing environment (Fig. 7b).

Reduced levels of enzymatic antioxidants in HYO rats as compared to NRM rats suggested that oxalate induces oxidative stress in the renal tissue. Treatment with BRG and BLE improves the activities of SOD, CAT and GPX significantly as compared to the HYO rats (Fig. 7c–e). These results unveiled the better potential of BRG as compared to BLE treatment as a free radical quencher. The antioxidant potential of BRG along with its direct binding to CaOx crystals might be the mechanism of its antiarthritic activity. Present study firmly supported the role of bergenin for combating calcium oxalate crystallization and reducing free radical damage both in vitro and in vivo. The animal study put forth the idea that bergenin is the active constituent of Bergenia ligulata possessing activity to maintain renal functionality following hyperoxaluric exposure.

4. Conclusion

The existence of bergenin in Bergenia ligulata is reported in various publications but a scientific study indicating its in vitro CaOx inhibitory activity and in vivo efficacy in the management of hyperoxaluria induced alterations in rat kidneys is first of its kind and novel. The present study suggests bergenin as an effective therapeutic candidate possessing both antiarthritic and antioxidant capabilities required to manage hyperoxaluria induced kidney damage.

Financial Interest

The authors declare no competing financial interest.

Acknowledgment

The present study has been supported by the financial assistance provided by the Department of Science and Technology, Government of India (SR/SO/BB-10/2010) in the form of a major research project. We thank central instrument facility, Panjab University Chandigarh for assistance with mass spectrometry, NMR and IR spectra.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.10.013.

References


