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Antiurolithic effect of Bergenia ligulata rhizome: An explanation of the underlying mechanisms

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Antiurolithic effect of *Bergenia ligulata* rhizome: An explanation of the underlying mechanisms

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ABSTRACT

Ethnopharmacological relevance: Bergenia ligulata is widely used plant in South Asia, mainly India and Pakistan, as a traditional medicine for treatment of urolithiasis.

Aim of the study: To rationalize the *Bergenia ligulata* use in kidney stones and to explain the underlying mechanisms.

Materials and methods: The crude aqueous-methanolic extract of *Bergenia ligulata* rhizome (BLR) was studied using *in vitro* and *in vivo* methods.

Results: BLR inhibited calcium oxalate (CaC_2O_4) crystal aggregation as well as crystal formation in the metastable solutions and exhibited antioxidant effect against 1,1-diphenyl-2-picrylhydrazyl free radical and lipid peroxidation in the *in vitro*. BLR caused diuresis in rats accompanied by a saluretic effect. In an animal model of urolithiasis, developed in male Wistar rats by adding 0.75% ethylene glycol (EG) in drinking water, BLR (5–10 mg/kg) prevented CaC_2O_4 crystal deposition in the renal tubules. The lithogenic treatment caused polyuria, weight loss, impairment of renal function and oxidative stress, manifested as increased malondialdehyde and protein carbonyl contents, depleted reduced glutathione and decreased antioxidant enzyme activities of the kidneys, which were prevented by BLR. Unlike the untreated animals, EG intake did not cause excessive hyperoxaluria and hypocalciuria in BLR treated groups and there was a significant increase in the urinary Mg^{2+}, instead of a slight decrease.

Conclusions: These data indicate the antiurolithic activity in *Bergenia ligulata* mediated possibly through CaC_2O_4 crystal inhibition, diuretic, hypermagneseuric and antioxidant effects and this study rationalizes its medicinal use in urolithiasis.

1. Introduction

The formation of urinary tract stones is world wide, sparing no geographical, cultural or racial groups (Moe, 2006). Those composed of CaC_2O_4, either alone or mixed with calcium phosphate, are hitherto the most common uroliths accounting for more than 80% of the stones (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 growth of insoluble particles (Baumann, 1998). Urine is always supersaturated with common stone forming minerals, however, the crystallization inhibiting capacity of urine does not allow urolithi-asis to happen in most of the individuals, whereas, this natural inhibition is in deficit in stone formers (Tiselius et al., 2001). Studies have also shown that tubular cell injury facilitates CaC_2O_4 crystal formation and deposition in the renal tubules (Khan and Hackett, 1991). Animal and tissue culture studies have demonstrated that both oxalate and CaC_2O_4 crystals directly induce renal epithelial cell injury mediated through lipid peroxidation and involve oxygen free radical generation (Thamilselvan et al., 1997, 2003).

Urolithiasis is largely a recurrent disease with a relapse rate of 50% in 5–10 years, thereby exists with substantial economic consequences and a great public health importance (Moro et al., 2005; Moe, 2006). Endoscopic stone removal and extracorporeal shock wave lithotripsy have revolutionized the treatment of urolithiasis but do not prevent the likelihood of new stone formation (Pak, 1989). Various therapies including thiazide diuretics and alkali-citrate are being used in attempt to prevent 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 majority of world's population (Kraisintu,

2003). Several pharmacological investigations on the medicinal plants used in traditional antiurolithic therapy have revealed their therapeutic potential in the *in vitro* or *in vivo* models (Atmani et al., 2003; Barros et al., 2006). *Bergenia ligulata* (family, Saxifragaceae) has been used for centuries in South Asia, mainly India and Pakistan, for a wide range of ailments. However, the most important activities are its diuretic and lithotriptic effects. *Bergenia ligulata* is used as a cure for dysuria and strangury and for stones in the kidney and ureter (Kapoor, 1990; Usmanghani et al., 1997). Some preliminary studies have evaluated the antiurolithic potential of *Bergenia ligulata* rhizome. The aqueous extract of *Bergenia ligulata* rhizome inhibited homogenous precipitation of CaC_2O_4 crystals (Garimella et al., 2001) and *in vitro* growth of CaC_2O_4 and calcium hydrogen phosphate dihydrate crystals (Joshi et al., 2005a,b). In the *in vivo* studies, the alcoholic extract of *Bergenia ligulata* has been found effective in dissolving the calculi developed in the bladder of rats by foreign body insertion (Seth et al., 1974) and reduced idiopathic hyperoxaluria in stone formers (Pendse et al., 1984).

In this study we investigated the antiurolithic effect of *Bergenia ligulata* rhizome extract on CaC_2O_4 urolithiasis and the possible underlying mechanisms using both *in vitro* and *in vivo* models.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used were of analytical grade available. Ethylene glycol, thymol, reduced glutathione, 5-5′-dithiobis, 2-nitrobenzoic acid, thiobarbituric acid, Folin-Ciocalteu's phenol reagent, hydrochlorothiazide (HCT), potassium citrate tribasic hydrate, H_2O_2, trichloroacetic acid, 1,1,3,3,-tetraethoxy propane, sodium oxalate ($Na_2C_2O_4$), calcium chloride ($CaCl_2$) and guanidine hydrochloride were obtained from Sigma Chemical Company, St. Louis, MO, USA. Reagents used for histological preparations were eosin spirit soluble, hematoxylin and xylene from BDH Chemical Limited, Poole, England, paraffin wax from Merck, Darmstadt, Germany, silver nitrate ($AgNO_3$), nuclear fast red and poly-L-lysine from Sigma Chemical Company. Kits used in this study for the determination of calcium, magnesium, blood urea nitrogen (BUN), creatinine, superoxide dismutase and glutathione peroxidase 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.

2.2. Plant material, extraction and fraction

Bergenia ligulata rhizomes were purchased from an authentic herb supplier in the local market of Karachi, 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 BL-RT-09-06-67. The dried rhizome was made free of dirt and ground to powder, using commercial mill. Approximately 500 g of the ground rhizome was soaked in 70% methanol in water (v/v) 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 grade 1 filter paper. This procedure of soaking the rhizome 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 (145 g), the crude extract of *Bergenia ligulata* rhizome (BLR), the approximate yield was 29%.

2.3. Animals

Experiments were performed in compliance with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Research Council, 1996) and approved by the Ethical Committee for Research on Animals (ECRA) of the Aga Khan University, Karachi, Pakistan.

Wistar rats (180–220 g) of either sex used for this study were sourced locally and housed at the animal house of the Aga Khan University, kept in plastic cages (47 cm × 34 cm × 18 cm) with saw dust (renewed after every 48 h), under a controlled temperature of 23–25 °C and 12 h light–dark cycle. Animals were given standard diet consisting of flour (5 kg), bran (5 kg) molasses (150 g), salt (75 g), nutrivet L (33 g), potassium meta bisulphate (15 g), oil (500 g), fish meal (2.25 kg), and powdered milk (2 kg) for a total of 13 kg of the food material. Animals had access to food and water *ad-libitum* through out the study except 24 h before and during 6 h of diuretic study and while collecting 24 h urine samples, food was withdrawn.

2.4. In vitro studies

2.4.1. Determination of effect on CaC_2O_4 crystallization
The effect BLR on CaC_2O_4 crystallization was determined by the time course measurement of turbidity changes due to the crystal formation and aggregation in the metastable solutions of Ca^{2+} and oxalate. Stock solutions of $CaCl_2$ (8.5 mM) and $Na_2C_2O_4$ (1.5 mM), containing 200 mM NaCl and 10 mM sodium acetate were adjusted to pH 5.7, as described previously (Hess et al., 1995). An aggregometer (Chrono-Log Corporation, USA) devised for platelet aggregation studies based on the measurement of optical density at 620 nm was used to investigate the event of CaC_2O_4 crystallization (Ebisuno et al., 1997). The $CaCl_2$ solution (0.5 ml) was stirred constantly both in the absence and presence of different concentrations of the test material or reference drug: potassium citrate at 37 °C. After obtaining a stable base line, crystallization was induced by the addition of $Na_2C_2O_4$ solution (0.5 ml) to obtain the final concentration of Ca^{2+} as 4.25 and oxalate as 0.75 mM. The time course measurement of turbidity was simultaneously started on a chart, moving at the speed of 30 mm/h, and continued for 15 min with constant stirring of the solutions. All experiments were run in triplicate. Slopes of nucleation (S_N) and aggregation (S_A) phases were calculated using linear regression analysis. Using the slopes, the percentage inhibition was calculated as $[(1 - S_m/S_c) \times 100]$, where S_m is slope in the presence of modifier and S_c is slope of the control experiment.

To determine the effect of incubation with the test material on CaC_2O_4 crystal formation, stock solutions of $CaCl_2$ and $Na_2C_2O_4$ having composition similar to those in the kinetic study were used. $CaCl_2$ solutions, containing different concentrations of the test material or potassium citrate, were aliquoted (0.5 ml) to the flat bottomed tubes in a 24 well plate (Iwaki Microplate with lid, Scitech Div., Asahi Techno Glass, Japan). To each of these tubes $Na_2C_2O_4$ solution (0.5 ml) was added to obtain the final concentration of Ca^{2+} as 4.25 and oxalate as 0.75 mM (Guerra et al., 2004). Each concentration of the test material was prepared in triplicate. The plates were then incubated in a shaking water bath at 90 oscillations/min at a temperature of 37 °C for 45 min. Each tube was then observed under an inverted microscope (Nikon Corporation, Tokyo, Japan) for crystal morphology and count in five randomly selected fields (200×).

2.4.2. Determination of antioxidant effect
Antioxidant effect of BLR was estimated by free radical scavenging and lipid peroxidation inhibitory effects.

To determine the free radical scavenging activity, a 0.1 mM solution of DPPH radical in methanol was prepared and 1 ml of this

solution was added to 3 ml of the test material at different concentrations prepared in methanol (Huang et al., 2004). Solutions were incubated for 30 min at room temperature and then absorbance was measured at 517 nm. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated in the equation using DPPH solution as control:

$$\% \text{ DPPH radical} - \text{scavenging}$$
$$= 1 - \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

To assess lipid peroxidation inhibitory activity, the kidneys isolated from Wistar rat were homogenized with electric homgenzier (Zero-Max®), in ice cold 50 mM phosphate buffer saline (PBS) adjusted to pH 7.4. The homogenate was centrifuged at $10,000 \times g$ for 10 min and the supernatant was incubated without (control) and with different concentrations of (50 and 150 μg/ml) the test material in the presence of 10 μM $FeSO_4$ and 0.1 mM ascorbic acid at 37 °C for 1 h (Kang et al., 2003). The reaction was stopped by the addition of 0.5 ml TCA (28%) and 0.75 ml TBA (1%) in succession. The solution was then heated at 90 °C for 20 min. Pink coloured malondialdehyde–TBA complex was extracted with *n*-butanol (3 ml) and the colour intensity was measured at 532 nm using spectrophotometer (DU 730, Beckman Coulter). The inhibition ratio was calculated using the formula given for free radical scavenging activity.

2.5. In vivo studies

2.5.1. Determination of diuretic activity

The diuretic activity of the test material was studied on Wistar rats of either sex (180–220 g) as described previously (Consolini et al., 1999). Animals were divided with matched body weight and sex into groups of 6 animals each. Negative and positive control groups were given by gavage saline (20 ml/kg) and standard diuretic drug: hydrochlorothiazide (HCT), 10 mg/kg of body weight, respectively. The rest of the groups were given different doses of the test material dissolved in saline. Subsequently, the animals were placed individually in metabolic and diuretic cages (Techniplast, 21020 Buguggiate-Va-Italy). The urine was collected in graduated cylinders for 6 h at 1 h intervals. Total urine excreted out was collected and the volume was determined. The pH of the pooled urine from each animal was determined by using pH meter, Na^+ and K^+ concentrations on flame photometer (Flame Photometer 410, Corning, UK) and Ca^{2+} concentration by using commercially available kit.

2.5.2. Study on animal model of urolithiasis

Antiurolithic activity of BLR was determined using animal model of CaC_2O_4 urolithiasis as described by Atmani et al. (2003) with some modifications. Twenty-four male Wistar rats (weighing 180–220 g) were divided with matched body weights into 4 groups of 6 animals each, which were then randomly selected to receive various treatments.

To study the effect of test material on CaC_2O_4 urolithiasis, group I rats, serving as vehicle treated control, received intraperitoneal (i.p.) injections of normal saline (2.5 ml) once in 24 h, whereas, the untreated rats (group II) received stone inducing treatment for 21 days, comprised of 0.75% (w/v) EG with 1% (w/v) ammonium chloride for 5 days, following this the water supply was switched to 0.75% EG alone in water, along with saline treatment. The treated groups III and IV received i.p. injections of BLR, 5 and 10 mg/kg, respectively, dissolved in saline once in 24 h and simultaneously received stone inducing treatment similar to the untreated groups. The doses selected for the *in vivo* antiurolithic effect of BLR were those which had caused the significant increase in urine output. The weight and activity were regularly monitored to assess their overall health so that any animal looking lethargic or excessively losing weight could be excluded from the study. After collecting 3 h morning urine for the crystalluria study, 24 h urine samples were collected, in the presence of a few thymol crystals to prevent microbial growth in urine, immediately before and at the end of a total 21 days of treatment, by housing animals individually in the metabolic and diuretic cages. Water intake was also determined simultaneously.

Following volume and pH determination, part of each 24 h urine sample was acidified to pH 2 with 5 M HCl. Both acidified and non-acidified urine samples were then centrifuged at $1500 \times g$ for 10 min to remove debris and supernatants were stored at -20 °C until analysed. Blood was collected through cardiac puncture from animals under ether anaesthesia for serum separation in order to assess serum creatinine and BUN. Animals were sacrificed and both the kidneys were excised, rinsed in ice cold physiological saline and weighed. The right kidney was fixed in 10% neutral buffered formaline, processed in series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 μm and stained with Haematoxylin & Eosin for examination under polarized light or by Pizzolato's method; which selectively stains CaC_2O_4 (Pizzolato, 1971), for examination under light microscope. To count the number of crystalline deposits, a sagittal section of each renal specimen was divided into 8 equal sized regions by four virtual lines (Fig. 5A) according to the method of Tsai et al. (2008). A field of $100\times$ was then randomly selected from each region and CaC_2O_4 deposits were counted. The total number of CaC_2O_4 deposits in each specimen was reported as average of the eight readings. The left kidney was worked into 10% homogenate in PBS (50 mM, pH 7.4), centrifuged at $1500 \times g$ and the supernatants were used to assess in the kidneys various antioxidant enzymes activities, reduced glutathione (GSH) levels and markers of peroxidative injury to lipid and protein, malondialdehyde (MDA) and protein carbonyl content, respectively.

In acidified urine samples, oxalate, calcium (Ca^{2+}) and magnesium (Mg^{2+}) contents were determined by using commercially available kits, while inorganic phosphate excretion was determined by the method of Daly and Ertingshausen (1972). In non-acidified urine samples, citrate, creatinine and uric acid, while in serum, creatinine and BUN were estimated with the help of kit-based methods. Total protein in non-acidified urine was estimated by Lowry's method (1951). Creatinine clearance (CC) was calculated using the formula:

$$CC\,(\text{ml}/\min) = \frac{(\text{mg creatinine/dl urine}) \times (\text{ml urine/24 h})}{(\text{mg creatinine/dl serum}) \times 1440}$$

In the kidney homogenates, MDA content was estimated by thiobarbituric acid reactive method (Wong et al., 1987) and the amount of MDA was determined from the standard curve of 1,1,3,3,-tetraethoxy propane. The protein carbonyl content was estimated by the protein derivatization with dinitrophenyl hydrazine (DNPH) into chromophoric dinitrophenyl hydrazones by the method of Levine et al. (1990) and the carbonyl content was calculated using the DNPH molar extinction coefficient of 22,000 M^{-1} cm^{-1}. GSH was estimated as total non-protein thiol (SH) group following the method described by Moron et al. (1979). For the purpose of quantitation, a calibration curve was prepared using GSH as a standard. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined by using commercially available kits. Catalase activity was determined by monitoring the decomposition of H_2O_2 at 240 nm with a spectrometer (Aebi, 1987). Catalase activity was calculated by using ε_{240} (molar extinction coefficient) = 0.0394 $mmol^{-1}$ min^{-1} for H_2O_2 and expressed as μmoles of H_2O_2 decomposed per min under standard conditions at 25 °C. Protein contents of kidney homogenates were determined by Lowry's method (1951).

2.6. Statistical analysis

The data expressed are mean ± standard error of mean (S.E.M.) and the median inhibitory concentration (IC_{50} value) with 95% confidence intervals. All statistical comparisons between the groups are made by means of One Way Analysis of Variance with post hoc Dunnett's test or by Student's t-test. The p-value less than 0.05 is regarded as significant. The concentration–response curves were analysed by non-linear regression using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. In vitro studies

3.1.1. Effect on the CaC_2O_4 crystallization

The effect of BLR on various phases of CaC_2O_4 crystallization was determined by time course measurement of turbidity in the metastable solutions containing Ca^{2+} and oxalate at the final concentrations of 4.25 and 0.75 mM, respectively. Fig. 1A shows typical tracing of the experiment. After induction of the crystallization with $Na_2C_2O_4$, an initial detectable increase in the turbidity was observed subsequent to a delay of 17 ± 2.3 s; the induction time (T_i). In the control experiment, the initial rise in turbidity (8.3 ± 0.74 cm/min); the nucleation phase, on attaining its maximum after about 197 ± 18 s, was followed by a slow

decrease (0.22 ± 0.013 cm/min); the aggregation phase. BLR, inhibited the S_A with a median inhibitory concentration of 14.72 μg/ml (13.67–15.86; 95% confidence interval), similar to the reference drug, potassium citrate, which caused inhibition with an IC_{50} value of 0.4 mM (0.25–0.63) as shown in Fig. 1B. BLR did not affect the S_N and T_i, whereas potassium citrate, at 0.5, 1, and 2 mM, respectively, caused 26 ± 4.3, 43.5 ± 3.9, and $80.5 \pm 3.7\%$ inhibition of S_N and increased T_i to 21 ± 1.12 s, 23.8 ± 1.3 s ($p < 0.05$), and 32.5 ± 2.9 s ($p < 0.01$ vs. control).

Incubating the metastable solutions of Ca^{2+} and oxalate resulted in the formation of CaC_2O_4 crystals (Fig. 2A) composed predominately of hexagonal calcium oxalate monohydrate. The respective crystal count, observed under the light microscope (200×), in solutions incubated with BLR at 0.125, 0.25 and 0.5 mg/ml was 122 ± 10 ($p < 0.05$), 61 ± 10.0 and 45 ± 3 ($p < 0.01$) as shown in Fig. 2C–E. Similarly, potassium citrate, at 1 mM, reduced crystal count to 125 ± 7.6 ($p < 0.05$) as shown in Fig. 2B. BLR also caused a morphological change in calcium oxalate monohydrate crystals, which acquired more rounded polygonal shape, similar to potassium citrate.

3.1.2. Antioxidant effect

BLR caused scavenging of DPPH free radical with IC_{50} value of 2.0 (1.25–2.94) μg/ml (Fig. 3A) and inhibited in vitro lipid peroxidation induced in rat kidney homogenate by 41.33 ± 1.86 and $94.75 \pm 0.30\%$ at 50 and 150 μg/ml, respectively (Fig. 3B). The control chemical, BHT, similarly inhibited DPPH with IC_{50} value of 1.81 μg/ml
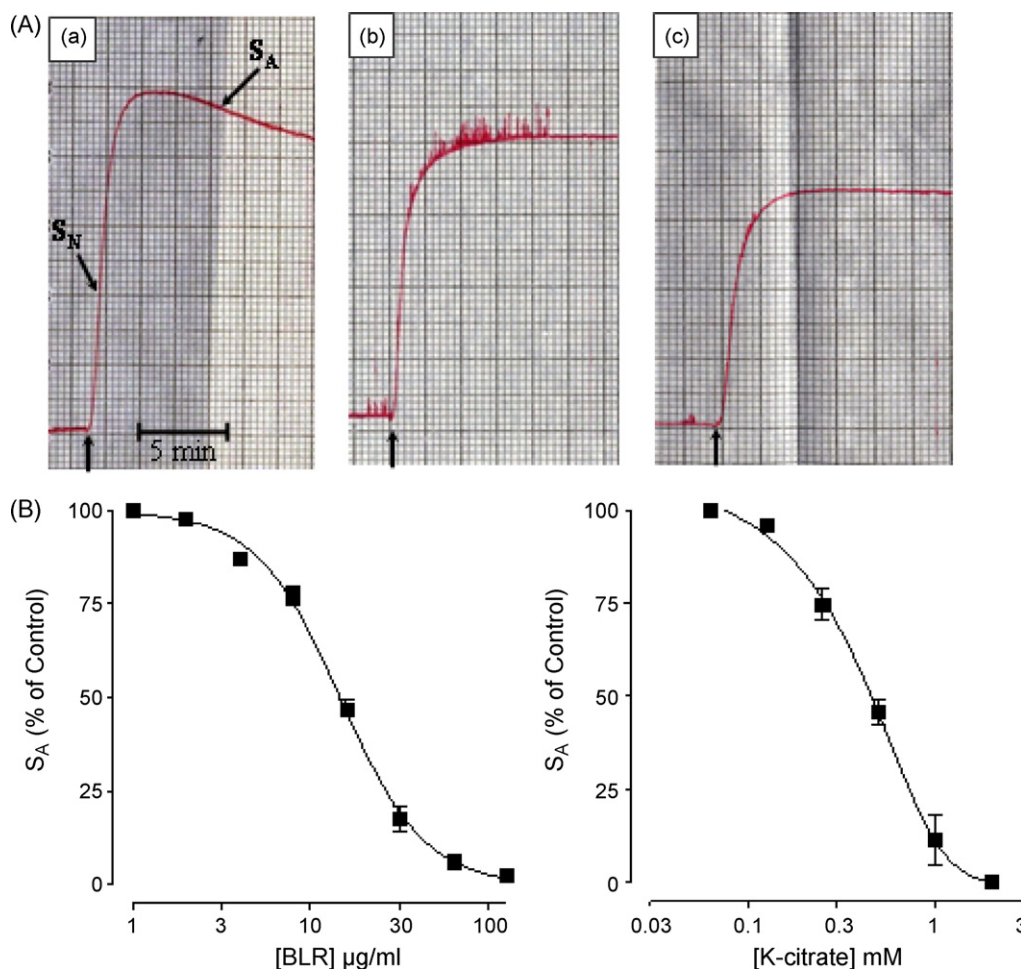
Fig. 1. Effect of *Bergenia ligulata* rhizome extract (BLR) on calcium oxalate crystallization. Panel (A) is typical tracings of time course measurement of turbidity changes in metastable solutions of CaC_2O_4 recorded as (a) control and (b) in the presence of BLR (125 μg/ml) and (c) potassium citrate (1 mM) and panel (B) is concentration–response curves of BLR and potassium citrate on S_A of the turbidity curves. Symbols shown are mean ± S.E.M. ($n = 3$). The arrows (↑) in panel (A) point the addition of $Na_2C_2O_4$ to $CaCl_2$ solutions. S_N, slope of crystal nucleation and S_A, slope of crystal aggregation.

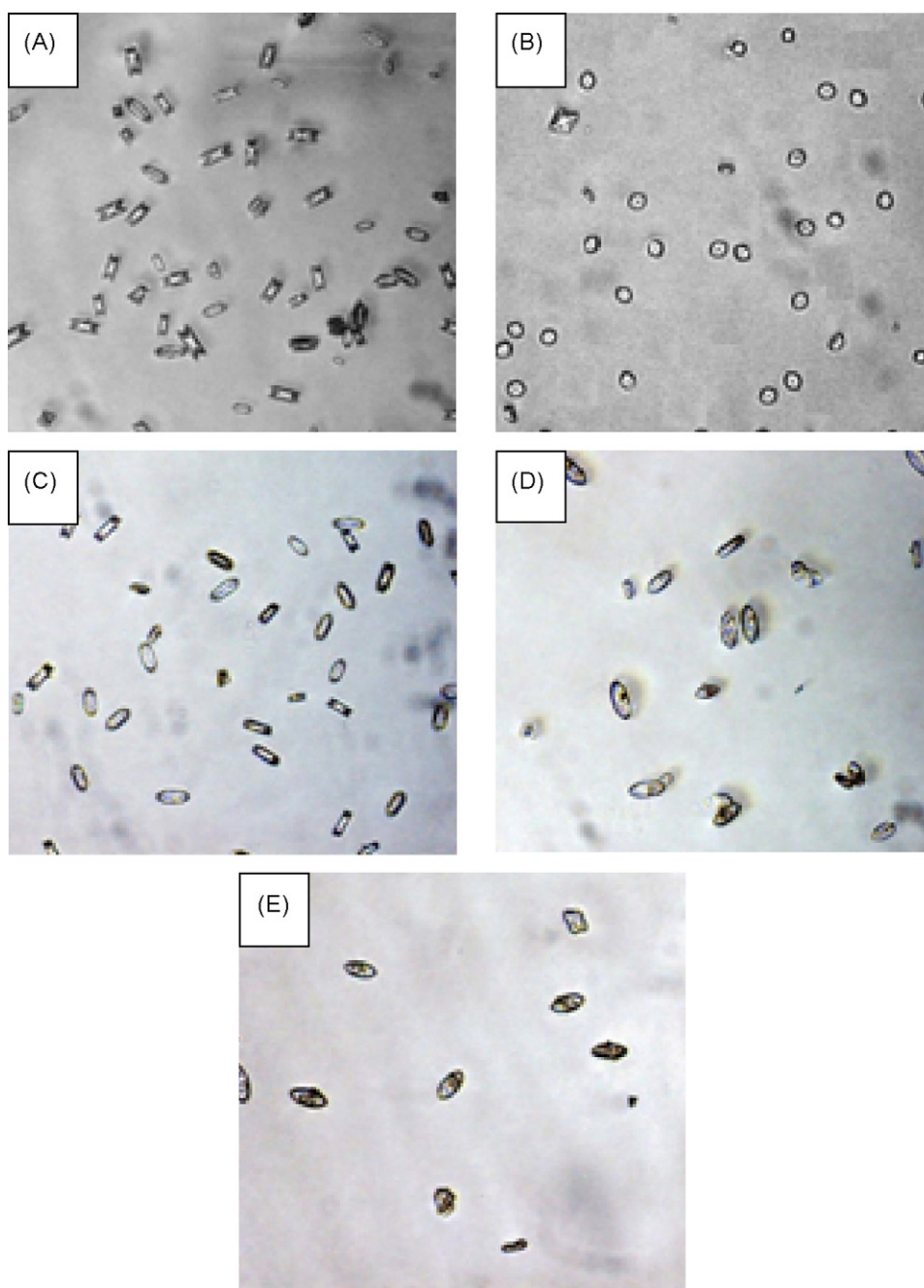**Fig. 2.** The CaC_2O_4 crystals, observed under inverted microscope (200×), formed in the metastable solution of CaC_2O_4 in the absence (A) and the presence of (B) potassium citrate (1 mM) and *Bergenia ligulata* rhizome extract (BLR), (C) 0.125 mg/ml, (D) 0.25 mg/ml and (E) 0.5 mg/ml.

(1.26–3.21) and caused 33.7 ± 4.4 and 73.5 ± 4.6% inhibition of lipid peroxidation at 50 and 150 µg/ml, respectively.

3.2. In vivo studies

3.2.1. Diuretic effect

The effect of various doses of BLR on rat urine volume, pH and Na^+, K^+ and Ca^{2+} excretion is given in Table 1. BLR increased urine volume at the doses of 3, 5 mg/kg ($p < 0.05$) and 10 mg/kg ($p < 0.01$), indicating diuretic effect. At the next higher dose (20 mg/kg), urine volume did not increase above that of saline treated group ($p > 0.05$). The reference diuretic, HCT (10 mg/kg), also increased the urine output ($p < 0.01$). In addition to an increase in the urine output, BLR, and similarly HCT, increased urine excretion of Na^+ and K^+ compare to

the saline treated animals. HCT also caused an increase in the urine pH and decrease in Ca^{2+} excretion ($p < 0.01$), but BLR was found devoid of these effects.

3.2.2. Effect observed in the animal model

Body weights, water intake and urine volume, pH and composition recorded before the start of treatment was not significantly different among the groups (data not shown). The parameters recorded from groups of animals at the end of 21 days of treatment period are given in Table 2.

Only a few CaC_2O_4 crystals were seen in the 3 h morning urine of the vehicle control animals, whereas, the hyperoxaluric treatment induced significant ($p < 0.01$) CaC_2O_4 crystalluria, prevalently dipyramid shaped CaC_2O_4 dihydrate crystals (Fig. 4A and B). At

Table 1
Diuretic effect of *Bergenia ligulata* rhizome extract (BLR).

		Urine vol. (ml)/100 g body weight/6 h	Na$^+$ (mmol)/100 g body weight/6 h	K$^+$ (mmol)/100 g body weight/6 h	Ca^{2+} (μmol)/100 g body weight/6 h	pH
Control		0.68 ± 0.19	0.12 ± 0.02	0.09 ± 0.01	8.12 ± 1.76	5.94 ± 0.05
BLR mg/kg	3	1.1 ± 0.24	0.14 ± 0.01	0.13 ± 0.01	7.48 ± 2.37	6.05 ± 0.07
	5	1.4* ± 0.21	0.17 ± 0.01	0.16 ± 0.03	6.69 ± 1.76	6.56 ± 0.22
	10	1.61** ± 0.11	0.23* ± 0.03	0.18* ± 0.01	6.86 ± 1.33	6.87 ± 0.22
HCT mg/kg	10	2.06** ± 0.27	0.35** ± 0.03	0.19** ± 0.01	1.29* ± 0.49	7.42** ± 0.27

Values given are mean ± S.E.M. ($n = 6$).
* $p < 0.05$.
** $p < 0.01$ vs. control.

5 mg/kg, BLR visibly reduced the crystal size with out significantly decreasing the number but at 10 mg/kg there was a significant decrease in urine crystal count as well as crystal size (Fig. 4C and D). Water intake and 24 h urine volume were high in the untreated group ($p < 0.01$) compare to those of the control animals. Lithogenic treatment also reduced pH of urine in the untreated group as compare to that of the control group, although not to a significant extent. A co-treatment with BLR prevented increase in urine volume and water intake ($p < 0.05$) in a dose-dependent manner, although these parameters remained higher than those of the control animals even at 10 mg/kg ($p < 0.05$). In parallel with crystalluria, there was an increased oxalate and decreased Ca^{2+} concentration ($p < 0.01$) of the

Fig. 3. Upper panel (A) shows free radical scavenging activity and lower panel (B) shows lipid peroxidation inhibitory effect of BLR and the reference drug butylated hydroxytoluene (BHT). Symbols shown are mean ± S.E.M. ($n = 3$).

urine collected from the untreated animals. BLR at 10 mg/kg prevented the change in urinary oxalate ($p < 0.01$) and Ca^{2+} ($p < 0.05$) contents but the protective effect was insignificant at 5 mg/kg. Other changes in the urine composition, induced by the lithogenic treatment which however did not found as statistically significant, were decreased urinary contents of citrate and Mg^{2+} and increased excretion of uric acid and inorganic phosphate. The urine composition of BLR treated group was not markedly different from that of the control, except Mg^{2+}, which rose above both the untreated and control animals and became significantly higher than that of the former group.

Lithogenic treatment caused impairment of renal functions of the untreated rats as evident from the markers of glomerular and tubular damage: raised BUN and serum creatinine, reduced creatinine clearance and increased urinary protein loss ($p < 0.01$), which were dose-dependently prevented in the animals receiving a simultaneous treatment with BLR.

The stone inducing regimen caused a significant loss in the body weight of the untreated group ($p < 0.01$), whereas, there was a net gain in the body weights of the animals receiving BLR, at both the doses tested, becoming comparable to that of the vehicle treated control at 10 mg/kg.

Kidneys excised from untreated group were larger and heavier than the control animals ($p < 0.01$), whereas, in BLR (10 mg/kg) treated group kidneys were not significantly different from those of the control animals but the protection was insignificant at 5 mg/kg. When observed under polarized light microscope, many birefringent crystalline deposits in the histological preparations were seen in tubules of all regions of kidneys: cortex, medulla and papilla, of all the animals in the untreated group (Fig. 5Ba and b). In BLR treated groups, such deposits were found in 5/6 and 1/6 rats, respectively, in groups receiving 5 and 10 mg/kg. Deposits were also visibly small and less abundant compare to those in the untreated kidneys (Fig. 5Bc). The crystals were shown to be CaC$_2$O$_4$ when stained black with Pizzolato's method. The renal tubules were also markedly dilated in the entire kidney of the in untreated rats.

Stone inducing treatment enhanced MDA and carbonyl protein content ($p < 0.01$), decreased GSH level ($p < 0.05$) and activities of the antioxidant enzymes including SOD ($p < 0.01$), GPX ($p < 0.05$) and catalase ($p < 0.01$) in kidneys of the untreated rats as compare to kidneys of the control animals. A simultaneous treatment with BLR protected against the oxidative changes induced by lithogenic treatment in a dose-dependent manner.

4. Discussion

In view of its medicinal use, *Bergenia ligulata* rhizome extract was studied to evaluate its antiurolithic potential using different models.

The effect of BLR on CaC$_2$O$_4$ crystallization kinetics was studied by the time course measurement of turbidity. Tracing of the experiment given in Fig. 1 is a typical of the pattern reported by some
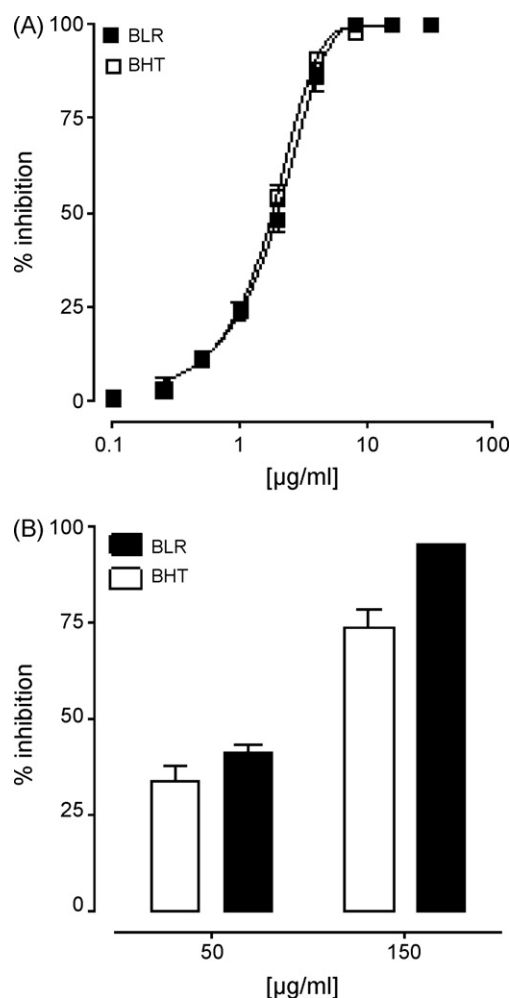
Table 2
Various parameters recorded from groups of rats after 21 days of the treatment.

	Control	Untreated	BLR 5 mg/kg	BLR 10 mg/kg
Change in body weight (%)	12.42 ± 1.67	$-5.06^{**} \pm 1.23$	$2.17^{**,\#\#} \pm 1.58$	$9.17^{\#\#} \pm 2.15$
Crystalluria/mm^3	6.2 ± 4.2	$375 \pm 27^{**}$	382 ± 31	$44 \pm 22^{\#\#}$
Water intake ml/24 h	6.13 ± 0.47	$28^{**} \pm 7.41$	16.0 ± 2.41	$10.3^{\#} \pm 1.17$
24 h urine parameters				
Urine vol. (ml)	6.71 ± 0.45	$27.50^{**} \pm 6.76$	$17.83^{*} \pm 3.24$	$11.33^{*,\#} \pm 1.41$
pH	6.42 ± 0.13	6.20 ± 0.12	6.30 ± 0.08	6.42 ± 0.13
Oxalate (mg)	0.46 ± 0.10	$2.03^{**} \pm 0.25$	$1.48^{**} \pm 0.26$	$0.74^{\#\#} \pm 0.11$
Ca^{2+} (mg)	3.50 ± 0.20	$1.93^{**} \pm 0.24$	$2.54^{*} \pm 0.38$	$3.07^{\#} \pm 0.57$
Mg^{2+} (mg)	3.29 ± 0.32	2.89 ± 0.11	3.48 ± 0.24	$3.68^{\#} \pm 0.31$
Citrate (mg)	21.0 ± 1.36	190 ± 1.90	18.0 ± 2.70	18.0 ± 1.10
IP[a] (mg)	6.50 ± 0.90	7.90 ± 1.20	7.60 ± 0.60	7.40 ± 0.90
UA[b] (mg)	0.69 ± 0.15	1.17 ± 0.40	1.02 ± 0.25	0.88 ± 0.24
TP[c] (mg)	2.66 ± 0.41	$9.85^{*} \pm 2.53$	8.09 ± 1.40	$3.60^{\#} \pm 0.68$
Serum values				
SC[d] (mg/dl)	0.86 ± 0.05	$1.43^{**} \pm 0.12$	1.26 ± 0.16	$0.89^{\#} \pm 0.10$
CC[e] (mg/min)	0.86 ± 0.06	$0.50^{**} \pm 0.06$	0.64 ± 0.10	$0.85^{\#\#} \pm 0.07$
BUN[f] (mg/dl)	19.89 ± 1.40	$57.04^{**} \pm 12.46$	46.3 ± 11.40	$20.5^{\#\#} \pm 1.08$
Kidney parameters				
Weight (g)	0.65 ± 0.03	$1.17^{**} \pm 0.10$	$1.03^{*} \pm 0.14$	$0.79^{\#} \pm 0.09$
CD[g]	0.00	$28.67^{**} \pm 8.56$	19.80 ± 8.30	$4.83^{\#} \pm 3.72$
Markers of oxidation/mg protein				
MDA[h] (nmol)	0.57 ± 0.10	$5.45^{**} \pm 0.85$	2.46 ± 1.10	$1.07^{\#\#} \pm 0.42$
PCC[i] (nmol)	4.98 ± 0.74	$12.50^{**} \pm 1.48$	9.80 ± 1.90	$7.40^{\#} \pm 1.08$
Antioxidant enzymes/mg protein				
GSH[j] (nmol)	18.0 ± 1.00	$12.80^{*} \pm 1.20$	14.4 ± 1.50	$17.10^{\#} \pm 1.60$
SOD[k] (U)	6.06 ± 0.7	$2.80^{**} \pm 0.41$	4.36 ± 0.53	$5.20^{\#} \pm 0.80$
GPX[l] (U)	0.61 ± 0.08	$0.36^{*} \pm 0.02$	0.45 ± 0.06	$0.59^{\#} \pm 0.06$
Cat[m] (μM H$_2$O$_2$/min)	31.82 ± 2.69	$17.65^{**} \pm 1.98$	21.70 ± 4.03	26.0 ± 2.53

Values given are mean ± S.E.M. ($n = 6$).

* $p < 0.05$.
** $p < 0.01$ vs. control group.
\# $p < 0.05$.
\#\# $p < 0.01$ vs. untreated group.
[a] Inorganic phosphate.
[b] Uric acid.
[c] TP.
[d] Serum creatinine.
[e] Creatinine clearance.
[f] Blood urea nitrogen.
[g] Number of crystal deposits/100× field.
[h] Malondialdehyde.
[i] Protein carbonyl content.
[j] Reduced glutathione.
[k] Superoxide dismutase.
[l] Glutathione peroxidase.
[m] Catalase.

previous studies (Hess et al., 1995). The initial positive slope of the turbidity curve is mainly due to an increase in the particle number resulting from crystal nucleation. After a plateau is achieved, a progressive decrease of absorbance (negative slope) despite continuous stirring reflects the decrease in the particle number, due to crystal aggregation (Hess et al., 1995). In this study, BLR inhibited the CaC$_2$O$_4$ crystal aggregation in a concentration-dependent manner, similar to potassium citrate, a well-known inhibitor of CaC$_2$O$_4$ crystallization and in wide clinical use for the management of urolithiasis (Tiselius, 2003). In the incubation study, BLR caused a decrease in crystal count and modified calcium oxalate monohydrate crystal morphology. A similar change in the morphology of calcium oxalate monohydrate crystals has been previously reported with citrate and Mg^{2+} (Guerra et al., 2006). Formation of crystals along urinary tract, driven by urinary supersaturation, is primary requisition for the subsequent stone formation (Hess and Kok, 1996), although crystal formation does not necessarily lead to stone formation (Kok, 1997). Researchers have identified crystal retention as a critical step for the formation of clinically symptomatic stone from a free particle. Crystal agglomeration has

long been recognized as the most important process leading to crystal retentions, also suggested by reduced ability of urine from patients with recurrent calcium stones to inhibit crystal aggregation than non-stone formers (Robertson et al., 1969; Finlayson and Reid, 1978). Various physiological inhibitors of urolithiasis found in urine including citrate have been shown to decrease the saturation of CaC$_2$O$_4$ and inhibit crystal nucleation, growth and aggregation, while reduced crystallization inhibiting capacity of urine can play a role in stone formation (Moro et al., 2005). Interference with crystal growth and aggregation therefore seems a possible therapeutic strategy for the prevention of recurrent stone disease. *Bergenia ligulata* rhizome extract is previously reported to inhibit CaC$_2$O$_4$ crystal precipitation and growth (Garimella et al., 2001; Joshi et al., 2005a,b). These results verify the previous studies and also identify the presence of CaC$_2$O$_4$ crystal aggregation inhibiting constituents in *Bergenia ligulata*.

Animal and cellular studies have shown that exposure to high levels of oxalate and CaC$_2$O$_4$ crystals produce cellular injury mediated by membrane lipid peroxidation through intracellular oxygen free radical generation. It has been demonstrated that epithelial cell
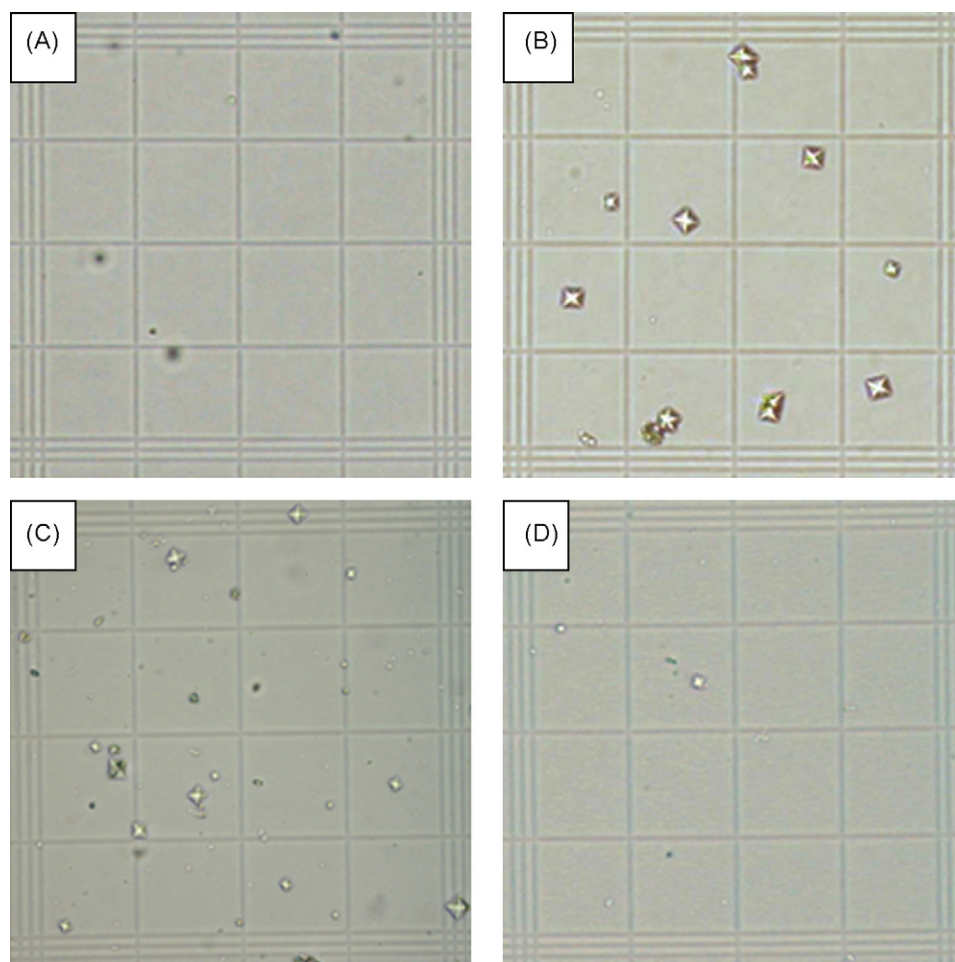
Fig. 4. The CaC_2O_4 crystals, viewed under light microscope (400×), in 3 h morning urine from (A) control animals, (B) the untreated group and the groups receiving *Bergenia ligulata* rhizome extract (BLR), (C) 5 mg/kg and (D) 10 mg/kg.

injury facilitates the events of CaC_2O_4 crystal nucleation, aggregation by lowering concentration at which crystal forms and promotes crystal retention in renal tubules crucial for subsequent stone development (Khan and Hackett, 1991; Khan, 1995; Moro et al., 2005). Recently obtained human data are also suggestive of the development of oxidative stress in kidney stone patients (Huang et al., 2003). Several experimental studies have shown that antioxidants such as vitamin E, catechin and selenium can protect against oxidative injury by oxalate and crystal deposition while some urinary macromolecules are also shown to possess protective effect against oxalate injury (Kumar and Selvam, 2003; Thamilselvan and Menon, 2005; Itoh et al., 2005). Antioxidant potential of BLR was estimated by free radical scavenging and lipid peroxidation inhibitory activity, where BLR caused scavenging of DPPH free radical and inhibited ferrous-ascorbate-induced lipid peroxidation of rat kidney homogenate similar to BHT, a standard antioxidant. BLR is previously shown to exhibit free radical scavenging activity (Bagul et al., 2003). This study confirms the previous findings as well as reports lipid peroxidation inhibitory potential of *Bergenia ligulata.*

When tested for the diuretic effect, BLR increased urine output in a dose-dependent manner (3–10 mg/kg). Interestingly, the administration of next higher dose (20 mg/kg) did not cause further increase in the diuretic effect and the urine volume reduced to a volume comparable to that of saline treated group. A possible explanation of disappearance of the diuretic effect with the dose increment is co-existence of anti-diuretic component in the plant extract. A given plant may exhibit multiple therapeutic activ-

ities probably on account of having a mixture of phytochemicals (Williamson, 2001). In our previous studies, we have seen the presence of combination(s) of activities within a plant extract including both the synergistic and the antagonistic. When there is the co-existence of apparently contrast activities in a plant, a given activity of the combination has been found to appear in a very dose specific manner (Gilani et al., 2005a,b), which is probably meant by nature to offset the effect beyond a certain limit.

The increase in the urine volume by BLR observed in this study was also accompanied by an increase in the Na^+ and K^+ excretion similar to the standard diuretic, HCT, suggesting that an increase in the urine output by BLR is caused by its increased urinary lose of electrolytes. HCT treatment also increased urine pH and decreased urine Ca^{2+} content but these effects were not observed with BLR. Urinary supersaturation with stone forming minerals is primary requisition for crystal precipitation and a major risk factor for the stone development (Hess and Kok, 1996). Thiazide diuretics due to their hypocalciuric and diuretic effects reduce urinary supersaturation of calcium salts and are commonly used to treat calcium stone disease (Goldfarb and Coe, 2005).

Renal CaC_2O_4 deposition induced by EG and ammonium chloride in rats is frequently used to mimic the urinary stone formation in humans (Thamilselvan et al., 1997; Atmani et al., 2003; Tsai et al., 2008). Therefore, we evaluated the medicinal effect of *Bergenia ligulata* rhizome on CaC_2O_4 urolithiasis using this model.

The analysis of crystalluria after 21 days of treatment with CaC_2O_4 stone inducing agents showed that untreated animals excreted abundant and larger crystals than the treated animals.
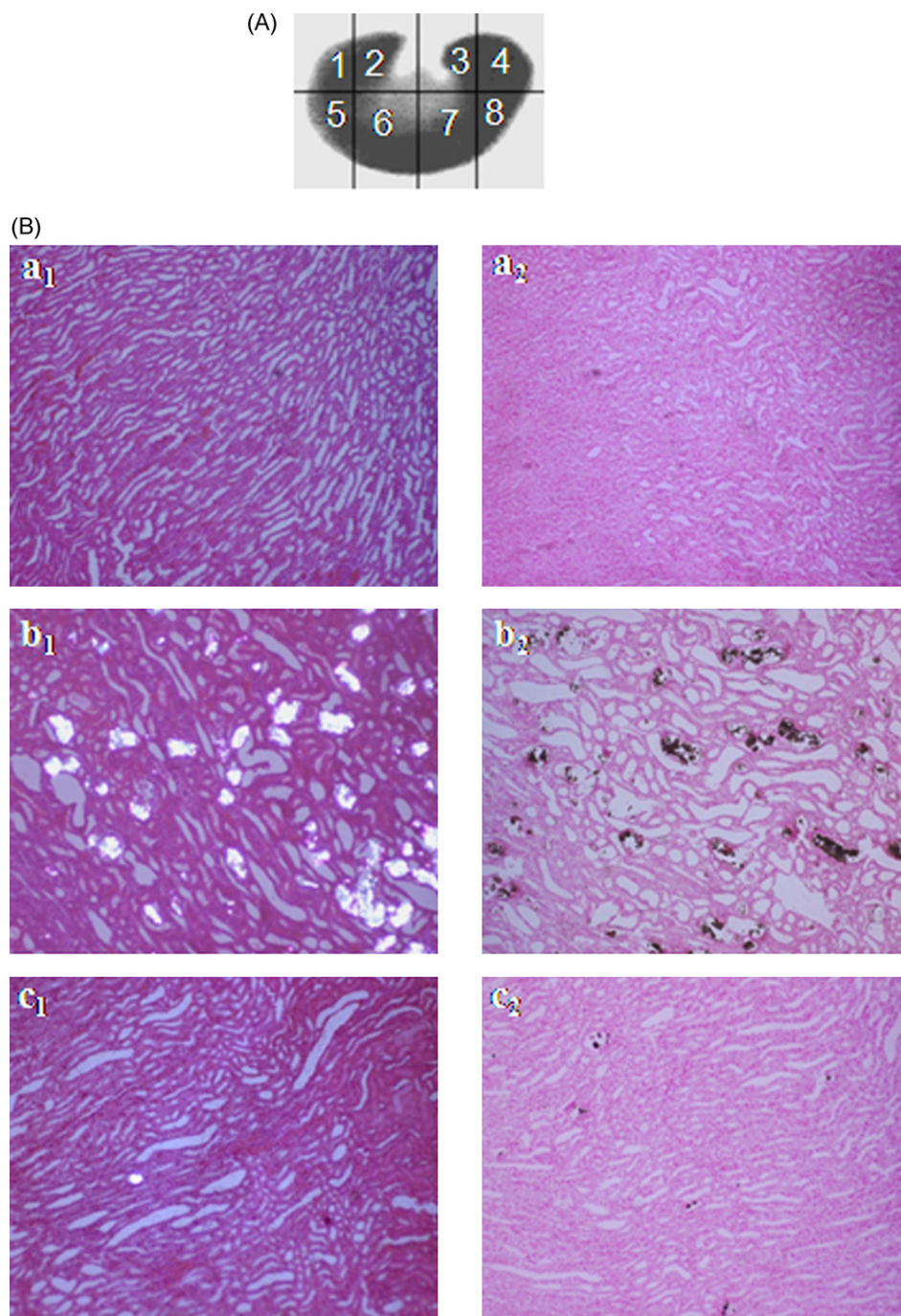
Fig. 5. (A) shows pattern of lines drawn on saggital section of kidney to divide it into 8 equal parts for crystal counting and (B) is representative microscopic images (100×) of kidney sections from (a) control animals, (b) untreated group and (c) group treated with *Bergenia ligulata* rhizome extract (BLR), 10 mg/kg. 1 and 2 in the subscript reflect sections under polarized light microscope after Haematoxylin & Eosin staining and light microscope after Pizzolato's staining, respectively.

Crystalluria could occur similarly in both healthy and stone forming individuals where the latter tend to excrete larger and aggregated particles than the formers (Robertson et al., 1969). CaC_2O_4 crystal agglomerates tend to retain in kidney by trapping in renal tubules and develop into renal stones (Atmani et al., 2003). BLR significantly prevented the polyuria associated with lithogenic treatment (Fan et al., 1999), although urine output remained still higher than that of the control animals which can be ascribed to its intrinsic diuretic activity. Consistent with some previous reports, stone induction by hyperoxaluria caused an increase in oxalate and decrease in Ca^{2+} excretion in the untreated group (Fan et al., 1999; Park et al., 2007), which was prevented by BLR in a dose-dependent manner. There

was also an increase in Mg^{2+} excretion in BLR treated group. Mg^{2+} is a well-known inhibitor of calcium phosphate and CaC_2O_4 crystal growth. The complexing between Mg^{2+} and oxalate increases the solubility product of CaC_2O_4 (Tiselius, 2003). Diets high in Mg^{2+} have been found to protect against deposition of CaC_2O_4 in the kidneys of vitamin B_6 deficient rats (Gershoff and Andrus, 1962). Promising results in preventing recurrence have also been shown in patients treated with potassium magnesium citrate (Ettinger et al., 1997). The crystallization inhibitory potential of BLR, as evident from the *in vitro* crystallization study and examination of the crystalluria, thus could be the result of increased Mg^{2+} content of the urine of treated rats, although the presence of addition inhibitory

constituent(s) and the effect of reduced oxalate excretion in BLR treated rats cannot be ignored.

Stone inducing treatment caused hypertrophy and extensive CaC$_2$O$_4$ crystal deposition in kidneys of untreated rats accompanied by oxidative damage as reflected from increased levels of markers of oxidative injury: MDA and protein carbonyl content, and decreased activities of antioxidant enzymes and GSH levels in kidneys as well as deteriorated renal functions. The renal tubules were markedly dilated in the entire kidney of all untreated rats, and this might have caused by distal obstruction of renal tubular flow by large crystals. BLR significantly prevented from all these effects of lithogenic treatment, thus confirming antiurolithic effect, as well as, antioxidant potential in the *in vivo*. *Bergenia ligulata* rhizome extract has been shown effective in dissolving struvite calculi developed in rat urinary bladder by foreign body insertion (Seth et al., 1974). This study reveals the antiurolithic potential in *Bergenia ligulata* against the most commonly occurring CaC$_2$O$_4$ kidney stones. Hyperoxaluria is a major risk factor for CaC$_2$O$_4$ nephrolithiasis. Several *in vivo* and *in vitro* studies have demonstrated that exposure to high level of oxalate results in greater production of superoxide and hydroxyl free radicals, leading to redox imbalance and has been manifested as antioxidant depletion, peroxidation of lipid and oxidation of protein (Hackett et al., 1994; Thamilselvan et al., 1997), changes in membrane integrity and cell death (Kumar and Selvam, 2003; Thamilselvan and Menon, 2005; Itoh et al., 2005). These changes facilitate CaC$_2$O$_4$ crystal adherence and retention in renal tubules (Khan, 1995). The inhibitory effect of BLR on CaC$_2$O$_4$ crystal retention in renal tubules thus can be attributed to its antioxidant activity.

5. Conclusions

Results of this study indicating the presence of antiurolithic effect in *Bergenia ligulata* rhizome against calcium oxalate stones, mediated possibly through a combination of CaC$_2$O$_4$ crystal inhibitory, diuretic, antioxidant and hypermagneseuric effects, rationalize its medicinal use for urinary stone disease.

Acknowledgements

References

Aebi, H.E., 1987. Enzymes I: oxidoreductases, transferases. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. VCH-Verlagsggesellschaft, Weinheim, pp. 273–285.

Atmani, F., Slimani, Y., Mimouni, M., Hacht, B., 2003. Prophylaxis of calcium oxalate stone by *Herniaria hirsute* on experimentally induced nephrolithiasis in rats. BJU International 92, 137–140.

Bagul, M.S., Ravishankara, M.N., Padh, H., Rajani, M., 2003. Phytochemical evaluation and free radical scavenging properties of rhizome of *Bergenia ciliata* (Haw.) Sternb. forma *ligulata* Yeo. Journal of Natural Remedies 3, 83–89.

Barros, M.E., Lima, R., Mercuri, L.P., Matos, J.R., Schor, N., Boim, M.A., 2006. Effect of extract of *Phyllanthus niruri* on crystal deposition in experimental urolithiasis. Urological Research 34, 351–357.

Baumann, J.M., 1998. Stone prevention: why so little progress? Urological Research 26, 77–81.

Consolini, A.E., Baldini, O.A.N., Amat, A.G., 1999. Pharmacological basis for the empirical use of *Eugenia uniflora* L. (Myrtaceae) as antihypertensive. Journal of Ethnopharmacology 66, 33–39.

Daly, J.A., Ertingshausen, G., 1972. Direct method for determining inorganic phosphate in serum with the "centrifichem". Clinical Chemistry 18, 263–265.

Ebisuno, S., Komura, T., Yamagiwa, K., Ohkawa, T., 1997. Urease induced crystallization of calcium phosphate and magnesium ammonium phosphate in synthetic urine and human urine. Urological Research 25, 263–267.

Ettinger, B., Pak, C.Y.C., Citron, J.T., Thomas, C., Adams-Huet, B., Vangessel, A., 1997. Potassium-magnesium citrate is an effective prophylaxis against recurrent calcium oxalate nephrolithiasis. Journal of Urology 158, 2069–2073.

Fan, J., Glass, M.A., Chandhoke, P.S., 1999. Impact of ammonium chloride administration on rat ethylene glycol urolithiasis model. Scanning Microscopy 13, 299–306.

Finlayson, B., Reid, F., 1978. The expectation of free and fixed particles in urinary stone disease. Investigative Urology 15, 442–448.

Garimella, T.S., Jolly, C.I., Narayanan, S., 2001. In vitro studies on antilithiatic activity of seeds of *Dolichos biflorus* Linn. and rhizomes of *Bergenia ligulata* Wall. Phytotherapy Research 15, 351–355.

Gershoff, S.N., Andrus, S.B., 1962. Effect of vitamin B6 and magnesium on renal deposition of calcium oxalate induced by ethylene glycol administration. Proceedings of the Society for Experimental Biology and Medicine 109, 99–102.

Gilani, A.H., Bashir, S., Janbaz, K.H., Khan, A., 2005a. Pharmacological basis for the use of *Fumaria indica* in constipation and diarrhea. Journal of Ethnopharmacology 96, 585–589.

Gilani, A.H., Bashir, S., Janbaz, K.H., Shah, A.J., 2005b. Presence of cholinergic and calcium channel blocking activities explains the traditional use of *Hibiscus rosasinensis* in constipation and diarrhoea. Journal of Ethnopharmacology 102, 289–294.

Goldfarb, D.S., Coe, F.L., 2005. The medical management of stone disease. In: Davison, A.M., Cameron, J.S., Ritz, E., Grünfeld, J., Winearls, C.G., Ponticelli, C., Ypersele, C.V. (Eds.), Oxford Text Book of Clinical Nephrology. Oxford University Press Inc, New York, pp. 1199–1279.

Guerra, A., Meschi, T., Allegri, F., Schianchi, T., Adorni, G., Novarini, A., Borghi, L., 2004. Calcium oxalate crystallization in untreated urine, centrifuged and filtered and ultrafiltered urine. Clinical Chemistry and Laboratory Medicine 42, 45–50.

Guerra, A., Meschi, T., Allegri, F., Prati, B., Nouvenne, A., Fiaccadori, E., Borghi, L., 2006. Concentrated urine and diluted urine: the effects of citrate and magnesium on the crystallization of calcium oxalate induced in vitro by an oxalate load. Urological Research 34, 359–364.

Hackett, R.L., Shevock, P.N., Khan, S.R., 1994. Madin-Darby canine kidney cells are injured by exposure to oxalate and to calcium oxalate crystals. Urological Research 22, 197–204.

Hess, B., 2003. Pathophysiology, diagnosis and conservative therapy in calcium kidney calculi. Therapeutische Umschau. Revue thérapeutique 60, 79–87.

Hess, B., Kok, D.J., 1996. Nucleation growth and aggregation of crystals. In: Coe, F.L., Favus, M.J., Pak, C.Y., Parks, J.H., Preminger, G.M. (Eds.), Kidney Stones, Medical and Surgical Management. Lippincott-Raven, Philadelphia, PA, USA, pp. 3–32.

Hess, B., Meinhardt, L., Giovanoli, J.P., 1995. Simultaneous measurement of calcium oxalate crystal nucleation and aggregation. Impact of various modifiers. Urological Research 23, 231–238.

Huang, H.S., Ma, M.C., Chen, C.F., Chen, J., 2003. Lipid peroxidation and its correlation with urinary levels of oxalate, citric acid, and osteopontin in patients with renal calcium oxalate stones. Urology 61, 1123–1128.

Huang, D.J., Lin, C.D., Chen, H.J., Lin, Y.H., 2004. Antioxidant and antiproliferative activities of sweet potato (*Ipomea batata* [L.] Lam 'Taniong 57') constituents. Botanical Bulletin of Academia Sinica 45, 179–186.

Kang, D.G., Yun, C.K., Lee, H.S., 2003. Screening and comparison of antioxidant activity of solvent extracts of herbal medicines used in Korea. Journal of Ethnopharmacology 87, 231–236.

Itoh, Y., Yasui, T., Okada, A., Tozawa, K., Hayashi, Y., Kohri, K., 2005. Preventive effects of green tea on renal stone formation and the role of oxidative stress in nephrolithiasis. Journal of Urology 173, 271–275.

Joshi, V.S., Parekh, B.B., Joshi, M.J., Vaidya, A.B., 2005a. Herbal extracts of *Tribulus terrestris* and *Bergenia ligulata* inhibit growth of calcium oxalate monohydrate crystals *in vitro*. Journal of Crystal Growth 275, e1403–e1408.

Joshi, V.S., Parekh, B.B., Joshi, M.J., Vaidya, A.D.B., 2005b. Inhibition of the growth of urinary calcium hydrogen phosphate dihydrate crystals with aqueous extracts of *Tribulus terrestris* and *Bergenia ligulata*. Urological Research 33, 80–86.

Kapoor, L.D., 1990. CRC Hand Book of Ayurvedic Medicinal Plants. CRC Press, Florida, p. 74.

Khan, S.R., 1995. Calcium oxalate crystal interaction with renal tubular epithelium, mechanism of crystal adhesion and its impact on stone development. Urological Research 23, 71–79.

Khan, S.R., Hackett, R.L., 1991. Retention of calcium oxalate crystals in renal tubules. Scanning Microscopy 5, 707–712.

Kok, D.J., 1997. Intratubular crystallization events. World Journal of Urology 15, 219–228.

Kraisintu, K., 2003. The status of medicinal and aromatic plants in Cambodia, Laos, the Philippines, Thailand and Vietnam. In: Vasisht, K., Kumar, V. (Eds.), Medicinal Plants and Their Utilization. United Nations Industrial Development Organization and the International Centre for Science and High Technology, Trieste, pp. 3–54.

Kumar, M.S., Selvam, R., 2003. Supplementation of vitamin E and selenium prevents hyperoxaluria in experimental urolithic rats. Journal of Nutritional Biochemistry 14, 306–313.

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods in Enzymology 186, 464–478.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

Moe, O.W., 2006. Kidney stones: pathophysiology and medical management. Lancet 367, 333–344.

Moro, F.D., Mancini, M., Tavolini, I.M., Marco, V.D., Bassi, P., 2005. Cellular and molecular gateways to urolithiasis: a new insight. Urologia Internatinalis 74, 193–197.

Moron, M.A., Depierre, J.W., Mannervik, B., 1979. Concentrations of glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochimica et Biophysica Acta 582, 67–78.

National Research Council, 1996. Guide for the Care and Use of Laboratory Animals. National Academy Press, Washington, p. 1.

Pak, C.Y.C., 1989. Role of medical prevention. Journal of Urology 141, 798–801.

Park, H.K., Jeong, B.C., Sung, M., Park, M., Choi, E.Y., Kim, B.S., Kim, H.H., Kim, J.I., 2007. Reduction of oxidative stress in cultured renal tubular cells and preventive effects on renal stone formation by the bioflavonoid quercetin. Jounal of Urology 179, 1620–1626.

Pendse, A.K., Ghosh, R., Goyal, A., Singh, P.P., 1984. Effect of indigenous drugs on idiopathic hyperoxaluria in stone formers. Asian Medical Journal 27, 136–140.

Pizzolato, P., 1971. Mercurous nitrate as a histochemical reagent for calcium phosphate in bone and pathological calcification for calcium oxalate. The Histochemical Journal 3, 463.

Robertson, W.G., Peacock, M., Nordin, B.E.C., 1969. Calcium crystalluria in recurrent renal stone formers. Lancet 11, 21–24.

Seth, S.D.S., Prabhakar, M.C., Bapna, B., Arora, R.B., 1974. Studies on the antilithiatic property of *Bergenia ligulata*. Journal of Research in Indian Medicine 9, 1–3.

Thamilselvan, S., Hackett, R.L., Khan, S.R., 1997. Lipid peroxidation in ethylene glycol induced hyperoxaluria and calcium oxalate nephrolithiasis. Journal of Urology 157, 1059–1063.

Thamilselvan, S., Khan, S.R., Menon, M., 2003. Oxalate and calcium oxalate mediated free radical toxicity in renal epithelial cells: effect of antioxidants. Urological Research 31, 3–9.

Thamilselvan, S., Menon, M., 2005. Vitamin E therapy prevents hyperoxaluria-induced calcium oxalate crystal deposition in the kidney by improving renal tissue antioxidant status. BJU International 96, 117–126.

Tiselius, H.G., 2003. Epidemiology and medical management of stone disease. BJU International 91, 758–767.

Tiselius, H.G., Hallin, A., Lindbäck, B., 2001. Crystallization properties in stone forming and normal subject's urine diluted using a standardized procedure to match the composition of urine in the distal part of the distal tubule and the middle part of the collecting duct. Urological Research 29, 75–82.

Tsai, C.H., Chen, Y.C., Chen, L.D., Pan, T.C., Ho, C.Y., Lai, M.T., Tsai, F.J., Chen, W.C., 2008. A traditional Chinese herbal antilithic formula, Wulingsan, effectively prevents the renal deposition of calcium oxalate crystal in ethylene glycol-fed rats. Urological Research 36, 17–24.

Usmanghani, K., Saeed, A., Alam, M.T., 1997. Indusyunic Medicine: Traditional Medicine of Herbal, Animal and Mineral Origin in Pakistan. B.C.C. and T. Press, University of Karachi, Karachi, p. 122.

Williamson, E.M., 2001. Synergy and other interactions in phytomedicines. Phytomedicine 8, 401–409.

Wong, E.H.Y., Knight, J.A., Hopfer, S.M., Zaharia, O., Leach C.N.Jr., Sunderman F.W.Jr., 1987. Lipidperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde–thiobarbituric acid adduct. Clinical Chemistry 33, 214.