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ORIGINAL ARTICLE

Antiurolithic and Antioxidant Activity of Ethanol Extract of Whole-plant *Biophytum sensitivum* (Linn.) DC in Ethylene-Glycol-induced Urolithiasis in Rats

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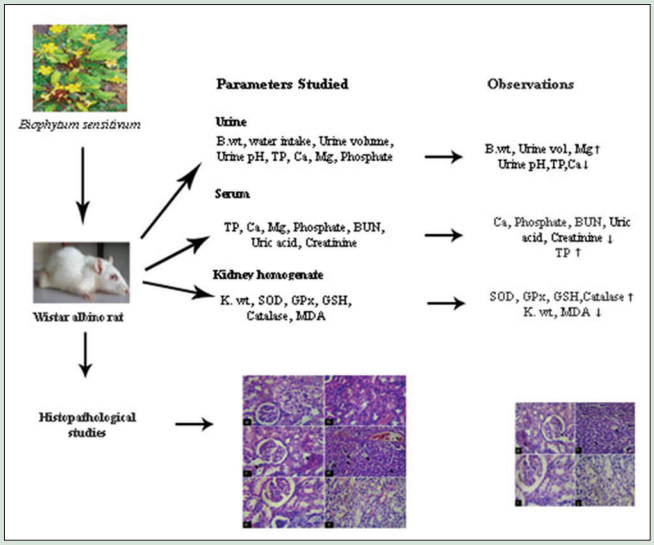
ABSTRACT

Background: *Biophytum sensitivum* Linn. DC is considered as one of the ten sacred plants called as "Dasapushpam" of Kerala state in India. Grounded leaves of this plant have been used conventionally as antiurolithic and diuretic. 'However, enough scientific evidences were not available about the effect of this plant as nephroprotective and antiurolithic. **Objective:** The present study was undertaken to investigate antiurolithic and antioxidant activity of ethanol extract of whole plant *B. sensitivum* Linn. DC (EEBS) on ethylene-glycol (EG)-induced urolithiasis in Wistar albino rats. **Materials and Methods:** EG 0.75% v/v in drinking water was fed to all groups, except the control group for 28 days to induce urolithiasis in rats. Groups I, II, and III served as control, toxic control, and standard Cystone groups, respectively. Animals in Group IV were administered with EEBS from 15th day to 28th day, while Group V animals were administered with EEBS from 1st day to 28th day. Several renal functional and injury markers in urine and serum were determined. Antioxidant enzyme activities were also recorded. **Results:** Co-administration with EEBS exhibited protective effect against EG-induced proteinuria, hypercalciuria, hypomagnesuria hypercalcemia, and hyperphosphatemia. Serum protein levels were significantly increased, whereas blood urea nitrogen, creatinine, and uric acid levels were significantly lowered. EEBS-treated rats significantly attenuated the aberrations in the antioxidant enzyme activities, body weight, kidney weight, urine output, and urine pH compared to toxic control animals. **Conclusion:** Hence, this study confirmed the usefulness of *B. sensitivum* as an antiurolithic and antioxidant agent.

Key words: Antioxidant, antiurolithic, *Biophytum sensitivum*, Cystone, ethylene-glycol

SUMMARY

Antiurolithic and antioxidant activity of ethanol extract of whole-plant *B. sensitivum* Linn. DC (EEBS) on ethylene-glycol-induced urolithiasis in Wistar albino rats were evaluated. Phytochemical screening indicated the presence of carbohydrates, alkaloids, steroids, saponins, proteins, aminoacids, flavonoids, fixed oils, tannins, and phenolic compounds. Based on the reports of previous research work, it can be confirmed that flavonoids and saponins present in this plant might be responsible for its nephroprotective and antiurolithic activities.



Abbreviations Used: EEBS: Ethanol extract of *Biophytum sensitivum*, EG: Ethylene-glycol, B.wt: Body weight, TP: Total protein, Ca: Calcium, Mg: Magnesium, BUN: Blood urea nitrogen, K.wt: Kidney weight, SOD: Superoxide dismutase, GPx: Glutathione peroxidase, GSH: Reduced glutathione, MDA: Malondialdehyde.

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DOI: 10.4103/pr.pr_83_17

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INTRODUCTION

Urolithiasis is a worldwide problem and is estimated that 12% of the world population experiences renal stone disease with a recurrence rate of 70%–80% in male and 47%–60% in female.[1] Kidney stone formation is the third most common affliction of the urinary tract, which is exceeded by the urinary tract infections and prostate diseases.[2] At present, no satisfactory drugs are available in the market for the treatment, prevention, or recurrence of kidney stone formation.[3] Synthetic drug for the treatment of urolithiasis is associated with higher incidence of adverse drug reactions. Invasive procedures including extracorporeal shock wave lithotripsy, ureteroscopy, and nephrolithotomy are considered to be effective, but they are costly, may reduce renal functions, and cause acute renal injury, infections, and recurrence of kidney stone formation.[4]

As various etiological factors are involved in stone formation,[5] the treatment is aimed at multiple targets such as antispasmodic, anti-inflammatory, diuretics, antibiotics, muscle relaxants, analgesics, and antioxidant activities. Preclinical studies have demonstrated that calcium oxalate crystals directly induce renal epithelial cell injury

Cite this article as: Abhirama BR, ShanmugaSundaram R. Antiurolithic and antioxidant activity of ethanol extract of whole-plant *Biophytum sensitivum* (Linn.) DC in Ethylene-Glycol-induced urolithiasis in rats. Phcog Res 2018;10:181-7.

mediated through lipid peroxidation (LPO) and oxygen-free radical generation.[6] Recent clinical data are also supporting such findings that formation of urinary stones leads to oxidative stress in patients.[7] Multiple chemical constituents present in medicinal plants aimed at multiple targets and may offer effective, inexpensive, and safe remedy for the treatment of urolithiasis. Ureteric calculus disappeared within 55 days of treatment with "Cystone" a herbomineral composition, by inducing diuresis.[8]

Biophytum sensitivum Linn. DC (common names: *Nilaccurunki*, *Tintaanaalee* in Tamil; *Mukkutti* in Malayalam; *Lajalu*, *Lajjaalu*, *Lakshmana* in Hindi) belongs to family *Oxalidaceae*.[9] Phytochemical investigation of *B. sensitivum* had revealed the presence of large amount of phenolic and polyphenolic compounds, saponins, polysaccharides, pectin, and essential oils. Main bioactive constituents are bioflavonoids such as amentoflavone with trace amounts of cupressoflavone, luteolin, isoorientin, and isovitexin.[10,11] Whole-plant *B. sensitivum* has been used conventionally in the treatment of urinary calculi.[12] Recent pharmacological studies showed that it has antioxidant,[13] antibacterial,[14] antidiabetic,[15] antitumor,[16,17] cardioprotective,[18] immunomodulation, radioprotective, anti-inflammatory activites and many more.[19] The search for antilithiatic drugs possessing significant antioxidant activities from natural sources has gained great potential, and therefore, the present study was aimed to investigate *in vivo* antiurolithic and antioxidant activity of ethanol extract of whole-plant *B. sensitivum* (EEBS) on ethylene-glycol (EG)-induced urolithiasis in Wistar albino rats.

MATERIALS AND METHODS

Animals

Adult male albino rats of Wistar strain weighing between 150 and 200 g were housed in clean polypropylene cages with stainless steel filter tops under the standard laboratory condition of 12:12 dark and light cycle, 50% humidity, and temperature 25°C ± 2°C. They were allowed free access to standard commercial rat feed pellets (SAI Animal Feed Ltd., Bangalore, India) and were given water *ad libitum*. The animals were acclimatized to the laboratory condition for 7 days before the commencement of experiments. The experiments were performed based on Animal Ethics Guidelines of Institutional Animals Ethics Committee (KMCRET/Ph. D/12/2015-16). The rats received human care according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals.

Drugs and chemicals

EG was obtained from Qualigens Fine Chemicals, Mumbai, India, and Cystone was procured from Himalaya Health Care, Bangalore, India. All other chemicals and reagents used in this study were of analytical grade.

Animal grouping

EG-induced urolithiasis model was used to assess the antiurolithic and antioxidant activity in Wistar albino rats.[20,21] Animals were divided into five groups, containing six animals each, designated as Groups I, II, III, IV, and V. Group I served as normal control and received regular rat food and drinking water *ad libitum*. EG (0.75% v/v) in drinking water was fed to Group II to Group V for the induction of renal calculi, for a period of 28 days. Group II served as lithiatic control and received vehicle (1% tween 80). Group III received standard antiurolithic drug, Cystone (750 mg/kg p.o.) from 15th day to 28th day.[22] Group IV served as curative regimen received EEBS (250 mg/kg) from 15th day to 28th day. Group V received EEBS (500 mg/kg) from 1st day to 28th day, served as preventive regimen. Extracts were given once daily p.o.

Plant source and identification

The whole-plant *B. sensitivum* was collected from Shevaroy Hills, Salem District, Tamil Nadu, and was taxonomically identified and authenticated by Dr. A. Balasubramanian, Executive Director, ABS Botanical Conservation, Research and Training Centre, Karipatti, Salem (Dt.), Tamil Nadu (Ref. No-AUT/JKK/095).

Preparation of plant extract

The whole plant was washed and dried in shade for about 3 weeks. Dried plant was coarsely powdered, sieved (mesh size = 40), and stored in an air-tight container at room temperature. Powdered plant material (500 g) was sequentially extracted with petroleum ether (60°C–80°C) for defatting the drug and then with 70% ethanol using soxhlation method. The obtained solvent extract was filtered and evaporated to dryness at 45°C under reduced pressure using a rotary evaporator. The dried extract was stored in the air-tight container.[23]

Phytochemical investigation

The EEBS was tested for the presence of alkaloids, flavonoids, phenolic compounds, tannins, glycosides, proteins, saponins, terpenoids, steroids, carbohydrates and starch using the standard procedures.[24]

Acute toxicity studies

Acute oral toxicity studies were not performed as studies of ethanolic extracet of whole plant *B.sensitivum* have already been reported. Previous studies by Anidya *et al*.,[25] used 5000 mg/kg dose of EEBS in albino rats. Hence, one-tenth of this dose, 500 mg/kg, and lower dose 250 mg/kg have been selected for the present study.

Analysis of urine

At the end of treatment, the animals were housed in individual metabolic cages and 24-h urine samples were collected on the 28th day. Change in body weight, water intake, urine volume, and pH were recorded after completing the experimental period. Total urine volume was measured using the measuring cylinder and reported in mL. The acidity of the urine was tested using a pH meter. A drop of concentrated HCl was added to the collected urine and stored at 4°C. Urine was then analyzed for total protein, calcium, phosphate, and magnesium.

Analysis of blood

After collecting the urine, animals were then anesthetized with ketamine hydrochloride and the blood was collected from tail vein under mild anesthetic condition. Serum was separated by centrifugation at 10,000 rpm for 20 min and analyzed for total protein, calcium, magnesium, phosphate, blood urea nitrogen (BUN), uric acid, and creatinine.

Kidney homogenate analysis

The rats were sacrificed after the administration of last dose of toxicant by euthanasia method. Both kidneys from each animal were isolated, washed, with ice-cold physiological solution. The left kidney was homogenized with a motor-driven Teflon-coated homogenizer with 0.1 M Tris-HCl buffer (pH 7.4) to get 10% w/v homogenate. The homogenate was then centrifuged for 10,000 rpm at 4°C for 10 min. The clear supernatant was collected and used for the estimation of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), reduced glutathione (GSH), and malondialdehyde (MDA) levels.

Determination of total protein content

Total protein content was determined by the method of Lowry *et al.*[26] 4.5 mL of alkaline (6 mmol/L) $CUSO_4$ reagent and 0.5 mL of Folin's phenol reagent were added to test tubes containing working standard (bovine serum albumin, 100 mg/mL); 0.2 mL of test solution. Distilled water of 1 mL served as blank. Mixed well and the blue color developed was observed at 640 nm after 15 min.

Determination of calcium level

The estimation was followed by the instructions according to the Clinichem calcium kit manufacturer, based on the method of Lorentz.[27] 1 mL working reagent was added to test tubes containing 20 µL standard solution, 20 µL sample used as test solution, and 20 µL distilled water used as blank. Mixed and incubated the reaction mixtures at room temperature for 5 min, and the absorbance was measured at 570 nm.

Determination of phosphate level

Phosphate level was determined by the method of Fiske and Subbarow.[28] 1 mL of 2.5% molybdic acid reagent was mixed with 5 mL protein-free filtrate/urine and 5 mL standard solution. Mixed all the solutions well and added 0.4 mL of 0.25% amino naphtha sulfonic acid and made up to 10 mL mark with distilled water. Kept for 5 min, and the absorbance was measured at 680 nm.

Determination of magnesium level

Urinary and serum levels of magnesium were determined according to the method of Heaton.[29] 1 mL serum/urine was mixed with 6 mL trichloroacetic acid (10%). The tubes were shaken, allowed to stand for at least 10 min, and centrifuged. 2 mL of supernatant was then mixed with 0.5 mL polyvinyl alcohol (0.2%), 2.5 mL titan yellow (15 mg%), and 1 mL sodium hydroxide (20%) and allowed to stand for 10–20 min. For standard, 1 mL magnesium sulphate solution was used. 1 mL distilled water was used as blank. The absorbance was measured at 540 nm.

Determination of blood urea nitrogen level

The BUN was estimated by Berthelot method (Fawcett and Scott)[30] using the commercially available kit (Kamineni Life Sciences Pvt. Ltd. Hyderabad, India). 1000 µL of working reagent-I containing urease reagent and a mixture of salicylate, hypochlorite, and nitroprusside were added to 10 µL of serum, 10 µL of standard urea (40 mg/dL), and 10 mL of purified water to prepare test, standard, and blank, respectively. Mixed well and incubated at 37°C for 5 min. Then, 1000 µL of reagent-II containing alkaline buffer was added to all the test tubes, which were incubated at 37°C for 5 min. The intensity of blue-green color produced is directly proportional to the concentration of urea in the sample and absorbance was measured spectrophotometrically at 578 nm.

Determination of uric acid level

Uric acid content was estimated by following the method of Caraway.[31] To 2 mL of supernatant of serum after centrifugation or standard uric acid solution were mixed with 0.6 mL of 10% phosphotungstic acid and 0.6 mL of 10% sodium carbonate. A blank was set up with 3.0 mL of water. After 10 min, the color developed was read at 640 nm.

Determination of creatinine level

The serum creatinine concentration was estimated using the commercially available kit (Cresent Biosystems, Goa, India) by alkaline picrate method (Bonsnes and Taussky).[32] 2.0 mL of picric acid reagent was added to 0.2 mL of serum, mixed well, and centrifuged at 3000 rpm to obtain a clear supernatant. 100 µL of buffer reagent was added to 1.1 mL of supernatant, 0.1 mL of standard creatinine, and 0.1 mL of distilled water to prepare test, standard, and blank, respectively. 1.0 mL of picric acid reagent was added to blank and standard. The intensity of orange color formed was read at 520 nm spectrophotometrically.

Determination of superoxide dismutase

SOD was estimated according to the procedure described by Kakkar *et al.*[33] 0] 0.5 mL of kidney homogenate was diluted with distilled water (0.5 mL) and treated with 0.25 mL of ethanol and 0.15 mL of chloroform. Supernatant of kidney homogenate was removed after centrifuging the mixture for 1 min. 1.5 mL of buffer was added to 0.5 mL of kidney homogenate and the reaction was started by adding 0.4 mL of epinephrine. Change in optical density per minute was determined at 480 nm in double beam UV-VIS spectrophotometer. Activity of SOD was expressed as unit/min/mg of protein.

Determination of catalase

The catalase activity was assayed by the method of Sinha.[34] Kidney homogenate (0.1 mL) was mixed with 1.0 mL of phosphate buffer and 0.5 mL of hydrogen peroxide. 1 mL of the reaction mixture was withdrawn and 0.2 mL dichromate/acetic acid reagent was blown at the interval of 1 minute to arrest the reaction. The standard hydrogen peroxide in the range of 4 to 20 µL were taken and treated similarly. The tubes were heated in a boiling water bath for 10 min. The green color developed was read at 570 nm and the activity of catalase was expressed as µmol of hydrogen peroxide consumed/min/mg protein.

Determination of glutathione peroxidase

The glutathione peroxidase activity was measured according to the method of Rotruck *et al.*[35] 0.2 mL each of EDTA, sodium azide, reduced glutathione, hydrogen peroxide, 0.4 mL of buffer and 0.1 mL of enzyme (kidney homogenate) were mixed and incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 mL of tricarboxylic acid and the tubes were centrifuged. To 0.5 mL of supernatant, 3mL of sodium hydrogen phosphate and 1 mL of 5,5′-dithiobis(2-nitrobenzoic acid) were added and the color developed was read at 412 nm. GPx activity is expressed as µ moles of glutathione oxidized/min/mg protein.

Determination of reduced glutathione

Reduced glutathione was estimated by Ellman's procedure.[36] Tissue homogenate (250 mL) was treated with 1 mL of 5% tricarboxylic acid in a 2 mL Eppendorf tube and precipitate was removed by centrifugation at 3000 rpm for 10 min at room temperature. To 250 mL of the above supernatant, 1.5 mL of 0.2 M phosphate buffer was added and mixed well. 250 mL of 0.6 mM of Ellman's reagent [5,5′-Dithiobis(2-nitrobenzoic acid) solution] was added to the above mixture and the absorbance was measured at 412 nm within 10 min. Amount of glutathione expressed as µg/mg of protein.

Determination of lipid peroxidation

LPO was estimated by the method of Okhawa *et al.*[37] 1 mL of kidney homogenate was mixed with 0.2 mL 4% (w/v) sodium dodecyl sulfate, 1.5 mL 20% acetic acid in 0.27 M hydrochloric acid (pH 3.5), and 15 mL of 0.8% thiobarbituric acid (TBA, pH 7.4). The mixture was heated in a hot water bath at 85°C for 1 h. The intensity of the pink color developed was read against a reagent blank at 532 nm. The concentration was expressed as *n* moles of MDA per mg of protein.

Histopathological studies

The right kidney was fixed in 10% neutral-buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin for histopathological examination.[38]

Statistical analysis

The results were expressed as mean ± SEM. Statistical significance between means was analyzed by one-way analysis of variance followed by "Dunnett's test." $P < 0.05$ was considered statistically significant.

RESULTS

The percentage yield of petroleum ether extract of *B. sensitivum* and EEBS was 4.92% w/w and 12.54% w/w, respectively. Preliminary phytochemical investigation indicated that EEBS showed the presence of carbohydrates, steroids, flavonoids, alkaloids, fixed oils, tannins, saponins, protein, aminoacid and phenolic compounds.

Effect of ethanol extract of *Biophytum sensitivum* on general and urinary parameters of control and experimental animals

Table 1 depicts the effect of EEBS on general and urinary parameters of control and experimental animals that were obtained at the end of the experiment in each group. Body weight was measured before starting the experiment. Parameters recorded on the 28th day during the experimental period revealed that there was significant ($P < 0.001$) gain in body weight in animals co-treated with 250 mg/kg and 500 mg/kg of EEBS and Cystone (750 mg/kg) compared to calculi-induced group [Table 1, Groups III, IV, and V]. Significant ($P < 0.001$) weight reduction was observed in Group II animals compared to control group [Table 1, Group II]. Similarly, urine volume was significantly ($P < 0.01$) higher in Cystone and both EEBS-treated groups [Table 1, Group III, IV, V], compared to animals in Group II. Changes in urine pH with respect to the doses were found to be significant (250 mg/kg: 7.1 ± 0.05 and 500 mg/kg: 6.9 ± 0.10). The urine pH was reduced significantly ($P < 0.01$) in extract and Cystone-treated rats compared with animals in Group II. In this study, total protein, calcium, and phosphate content in urine increased significantly ($P < 0.001$) in EG-induced group compared to control, whereas p.o. administration of 250 and 500 mg/kg of EEBS attenuated total protein and calcium levels ($p<0.01$). Total protein ($p<0.001$), calcium and phosphate levels ($p<0.01$) were significantly lowered in animals co-treated with Cystone. Magnesium excretion was significantly ($P < 0.05$) reduced in EG-treated group compared to control group. Treatment with Cystone at the dose of 750 mg/kg ($P < 0.01$) and EEBS 500 mg/kg ($P < 0.001$) significantly increased magnesium level compared to lithiatic group [Table 1, Groups III, V].

Effect of ethanol extract of *Biophytum sensitivum* on serum biochemistry of control and experimental animals

Table 2 illustrates the effect of EEBS on serum parameters of control and experimental animals in each group. The serum levels of calcium, phosphate, creatinine, uric acid ($P < 0.01$), BUN ($P < 0.001$), and magnesium ($P < 0.05$) were significantly elevated, whereas total protein levels ($P < 0.001$) were lowered in calculi-induced animals compared to control group, indicating impaired renal function [Table 2, Group II]. Co-treatment with Cystone ($P <0.01$) and EEBS at 250 mg/kg (curative regimen) and 500 mg/kg (preventive regimen) significantly ($P < 0.001$) increased serum total protein, whereas BUN, creatinine, uric acid, calcium, and phosphate levels were were significantly reduced, compared to calculi-induced group [Table 2, Groups III, IV, V].

Table I: Effect of EEBS on general and urinary parameters of control and experimental animals

Parameters studied (Unit)	Group I normal control	Group II EG 0.75% v/v	GroupIII EG + Cystone 750 mg/kg	Group IV EG + EEBS 250 mg/kg	Group V EG + EEBS 500 mg/kg
General					
Change in body wt (g)	5.50±1.02	2.60±0.87***a	5.25±2.94***b	4.81±1.26***b	4.90±1.92***b
Water intake (mL/24 h)	17.67±0.02	17.22±0.01**a	18.62±0.16**b	17.45±0.04ns	17.50±0.01ns
Urine					
Urine volume (mL/24 h)	17.83±0.00	10.83±0.08**a	16.40±0.00**b	17.98±0.00**b	21.85±0.08***b
Urine pH	6.8±0.05	8.2±0.11**a	6.86±0.11**b	7.10±0.05**b	6.90±0.10**b
Total protein (g/dL)	2.02±0.00	5.13±0.08***a	2.45±0.01***b	3.38±0.00**b	3.03±0.00**b
Calcium (mg/dL)	8.02±0.00	14.68±0.03***a	12.02±0.07**b	11.93±0.06**b	10.23±0.03**b
Phosphate (mg/dL)	3.23±0.01	5.46±0.01**a	4.12±0.07**b	5.40±0.03ns	5.39±0.01ns
Magnesium (mg/dL)	2.17±0.01	2.04±0.01*a	2.43±0.01**b	2.06±0.05ns	2.66±0.06***b

Values are expressed in mean±SEM (n=6), *P<0.05, **P<0.01, ***P<0.001; [a]significant compared with control group, [b]significant compared with calculi-induced group, [ns]not significant.

Table 2 : Effect of EEBS on serum biochemistry of control and experimental animals

Parameters Studied (Unit)	Group I normal control	Group II EG 0.75% v/v	GroupIII EG + Cystone 750 mg/kg	Group IV EG + EEBS 250 mg/kg	Group V EG + EEBS 500 mg/kg
Total protein (g/dL)	7.46±0.02	5.76±0.03***a	7.20±0.01**b	7.43±0.01***b	7.86±0.07***b
Calcium (mg/dL)	9.29±0.07	11.16±0.01**a	9.39±0.02**b	10.42±0.00**b	9.15±0.01**b
Phosphate (mg/dL)	6.58±0.06	8.06±0.01**a	7.18±0.01**b	8.02±0.01ns	8.00±0.01*b
Magnesium (mg/dL)	2.30±0.00	2.33±0.00*a	2.51±0.00**b	2.31±0.00ns	2.32±0.00ns
BUN (mg/dL)	36.09±0.01	58.53±0.01***a	34.51±0.00***b	41.75±0.01***b	39.87±0.03***b
Creatinine (mg/dL)	0.57±0.00	0.88±0.00**a	0.60±0.01**b	0.49±0.00***b	0.46±0.00***b
Uric acid (mg/dL)	2.75±0.01	5.91±0.00**a	3.34±0.01**b	5.66±0.27**b	4.91±0.01**b

Values are expressed in mean±SEM (n=6), *P<0.05, **P<0.01, ***P<0.001; [a]significant compared with control group, [b]significant compared with calculi-induced group, [ns]not significant

Effect of ethanol extract of *Biophytum sensitivum* on markers of oxidation in control and experimental animals

The effect of EEBS on LPO and antioxidant status in rats exposed to EG-induced urolithiasis showed a significant ($P < 0.01$) decrease in SOD, CAT, GSH, and GPx level compared to Group I normal animals [Table 3, Group II]. Compared to animals in group II, co-administration of EEBS significantly increased GPx and SOD levels. A significant ($P < 0.01$) increase in production of MDA was observed in Group II toxic control animals compared to Group I normal animals. Co-administration of Cystone EEBS to Group IV and Group V animals significantly ($P < 0.01$) decreased MDA levels compared to Group II animals [Table 3] (Groups III, IV, V).

Results of histopathological studies

Histopathological analysis revealed severe glomeruli injury and hematuria indicated by RBCs deposition, interstitial inflammation, and dilation of proximal tubules along with crystal deposits, in calculi-induced rats [Figure 1c and d]. There were no abnormalities or calcium oxalate deposits inside tubules of animals in normal control group [Figure 1a and b]. Group IV animals treated with EEBS at a dose of 250 mg /kg showed normal glomerulus, tubular dilation with crystal deposits, normal tubular vacuolation and epitelial loss. [Fiure 1e and f]. Co-administration with EEBS at a dose of 500 mg/kg in Group V animals [Figure 1i and j] markedly decreased crystal deposition in renal tubules compared to animals treated with EG in group II.

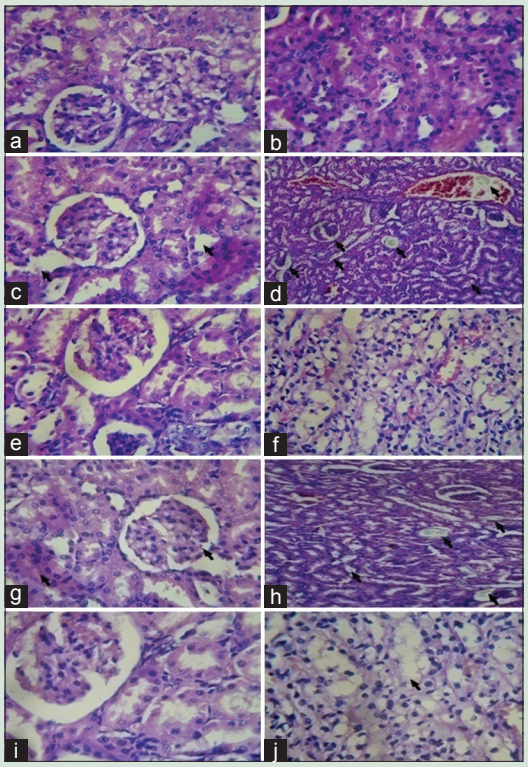

Figure 1: Light microscopic histology and calcium oxalate deposits in the section of kidney. Left side showing kidney section of glomerular region and right side showing tubular region. (a and b) Normal control rat. (c and d) Ethylene-glycol-induced urolithiasis rat. (e and f) Cystone-treated rat. (g and h) Ethanol extract of *Biophytum sensitivum* (250 mg/kg)-treated rat. (i and j) Ethanol extract of *Biophytum sensitivum* (500 mg/kg)-treated rat (40× for all images)

DISCUSSION

Agents such as EG, sodium oxalate, ammonium oxalate, and glycolic acid are commonly used for inducing urolithiasis.[39] In this study, EG was used to induce renal stone formation in male rats. Urinary system of male rats resembles that of human[40] and *in vivo* accumulation of glycolic acid, a metabolite of EG in the body is responsible for nephrotoxicity.[41]

A significant decrease in body weight and water intake was observed in calculi-induced group compared to animals in control group. Decreased in the quantity and frequency of food intake was found to be the major factor of weight loss. Physiological imbalance and/or mental stress may be the other factors influencing weight reduction.[42] Administration of EEBS results in increased intake of diet (frequency and quantity), water intake, and subsequent increase in body weight. Decreased urine output plays a major role in inducing kidney stones.[43] Both curative and preventive doses of EEBS increased urine output in a dose-dependent manner along with normalization of pH. These results indicated that the diuretic activity of EEBS promotes flushing out of deposits, thereby inhibiting saturation and precipitation of stone forming constituents. Nucleation and aggregation of crystals in urine is pH dependent. Uric acid stones are predominant in highly acidic urine, whereas calcium oxalate and calcium phosphate stones occur in highly alkaline urine.[44,45] In this study, pH of urine was found to be 8.2 ± 0.11 in calculi-induced animals in Group II, indicating a favorable environment for the formation of kidney stones. Treatment with plant extracts (EEBS 500 mg/kg) significantly reduced urine pH to 6.9 ± 0.10. In response to 28 days of EG (0.75% v/v) treatment, the risk of formation of renal calculi increased by raising the level of stone forming constituents in urine. Excess level of stone forming chemicals in the urine results in toxic effects on the renal epithelial cells, leading to alteration in membrane integrity, generation of reactive oxygen species, development of oxidative stress, and depleted source of antioxidant enzymes and LPO.[46] Renal epithelial injury further promotes retention as epithelial injury exposed to a variety of crystal adhesion molecules. Increased concentration of urinary calcium is a factor favoring the nucleation, aggregation, or retention of calcium oxalate or calcium phosphate.[47] Promising results in preventing recurrence have been shown in patients treated with potassium magnesium citrate. Magnesium deficiency promotes the nucleation and retention of calcium in renal tubule. Magnesium binds to oxalate to form a soluble complex and reduces the concentration available for calcium oxalate precipitation.[48] Increased urinary excretion of protein reflects proximal tubular dysfunction. Increased urinary excretion of calcium and phosphate observed in calculi-induced group (Group II) provides an environment appropriate for calcium phosphate crystals formation. Supplementation with EEBS (500 mg/kg) served as a preventive regimen and the curative regimen receiving EEBS at a dose of 250 mg/kg significantly restored the alteration in the level of urinary calcium. Total protein level was significantly reduced and magnesium level was increased in animal group treated with EEBS, which clearly indicating antiurolithic activity of EEBS.

Glomerular filtration rate decreases due to the obstruction to the outflow of urine by stones in the urinary system or due to the damage of renal parenchyma. This promotes accumulation of the waste products, particularly nitrogenous substances such as urea, creatinine, and uric acid in the blood.[49] This study showed a significant increase in serum uric acid, creatinine, BUN, calcium, magnesium, and phosphate level in the EG-treated group compared to normal control. These serum biochemicals except magnesium decreased following treatment with

Table 3 : Effect of EEBS on markers of oxidation in control and experimental animals

Parameters analyzed in Kidney homogenate	Group I normal control	Group II EG 0.75% v/v	Group III EG + Cystone 750 mg/kg	Group IV EG + EEBS 250 mg/kg	Group V EG + EEBS 500 mg/kg
Kidney weight	0.63±0.01	1.06±0.01^{**a}	0.71±0.00^{**a}	0.82±0.00^{**a}	0.69±0.02^{***a}
SOD	1.60±0.02	0.88±0.01^{**a}	1.20±0.01^{**a}	1.26±0.00^{**a}	1.28±0.08^{**a}
Catalase	1.36±0.00	1.19±0.00^{**a}	1.37±0.00^{**a}	1.12±0.00^{ns}	1.21±0.01^{ns}
GPx	0.95±0.01	0.53±0.00^{**a}	0.75±0.00^{**a}	0.59±0.01^{ns}	0.75±0.02^{**a}
GSH	1.23±0.01	1.07±0.00^{**a}	1.27±0.00^{**a}	1.09±0.01^{ns}	1.12±0.01ns
LPO	0.63±0.01	0.85±0.09^{**a}	0. 65±0.02^{**a}	0.74±0.00^{**a}	0.70±0.01^{**a}

Units: Kidney weight (g); SOD (unit/min/mg protein); CAT (µmole of H_2O_2 consumed/min/mg protein); GPx (µmole of glutathione oxidized/min/mg protein); GSH (µg/mg protein); LPO (nmoles of MDA formed/mg protein). Values are expressed in mean±SEM (n=6), *P<0.05, **P<0.01, ***P<0.001; [a]significant compared with control group, [b]significant compared with calculi-induced group

EEBS in dose-dependent manner. Serum total protein level was found to be elevated in Group III, Group IV, and Group V animals, indicating inhibition of urolithiasis.

Oxidative stress is a condition associated with reduction in endogenous antioxidative enzyme level, which catalyze neutralization of many types of free radicals.[50] These enzymes play a significant role in destroying the peroxides and promote antioxidant defenses to an organism. Lowering of their activities resulted in the accumulation of lipid peroxides and increased oxidative stress.[51] In this study, it was observed that EEBS treatment, serving both as preventive and curative regimens, significantly decreased MDA levels and increased activity of antioxidant enzymes in the kidneys, thereby inhibiting the changes associated with oxidative stress.

Histopathological examination of the kidneys substantiated the results obtained from *in vivo* evaluation of antiurolithic and antioxidant activity in animals. Dilation of renal tubule, hematuria, and glomerular injury might be due to the presence of crystals which causes obstruction in tubular blood flow. Kidney weight was significantly increased in calculi-induced group (P < 0.001) compared to normal control group. Significant weight reduction of the kidney was observed in animals treated with EEBS which might be due to the increased excretion of stone-forming chemicals. Previous studies have confirmed and reported that 28 days of EG (0.75%, v/v) administration in rats resulted in the formation of renal calculi.[52] Recent clinical data also suggested the use of extract of various plants as antioxidant and antiurolithic agent.[53,54]

CONCLUSION

Our findings demonstrated that administration of EEBS mediated its effect through multiple pathways including significant antioxidant activity, diuretic activity along with normalization of urine pH, protective effect against drug-induced proteinuria, hypercalciuria, hypercalcemia, and hyperphosphatemia. EEBS also restored the elevated serum level of BUN, uric acid, and creatinine and diminished total protein level. Improvement in the body weight of animals and lowering of kidney weight were also observed. Hypermagnesuric effect observed in the EG + EEBS-treated animals, clearly indicating its antiurolithic activity. Hence, our findings provide necessary evidence for the ethnomedicinal usage of this plant as nephroprotective and antiurolithic agent. Antiurolithic activity may be attributed mainly due to the presence of saponins, as it possesses anticrystallization properties.[55] Significant antioxidant property of EEBS is due to the presence of bioactive phytoconstituents such as amentoflavone, a bioflavonoid with trace amounts of cupressoflavone, luteolin, isoorientin, and isovitexin. Hence, supplementation of EEBS along with drugs inducing urolithiasis may exhibit excellent antiurolithic and antioxidant activity.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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