ORIGINAL ARTICLE

Anti-urolithiatic activity of standardized extract of *Biophytum sensitivum* against zinc disc implantation induced urolithiasis in rats

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ABSTRACT

Biophytum sensitivum (L.) DC (family: *Oxalidaceae*) has been used in the Indian indigenous system of medicine, Ayurveda, for the treatment of various health aliments including renal calculi. The present study was undertaken to investigate the anti-urolithiatic activity of standardized methanolic extract of whole plant of *B. sensitivum* (MBS) in rats. Urolithiasis was induced by surgical implantations of zinc disc in the urinary bladders of rats. Upon postsurgical recovery, different doses of MBS (viz., 100, 200, and 400 mg/kg body weight) were administered to zinc disc-implanted rats for the period of 7 days by the oral route. Anti-urolithiatic activity was evaluated by measuring various dimensions of stones and estimating levels of various biomarkers in serum and urine samples. A significant decrease in urinary output was observed in the disc-implanted animals, which was prevented by the MBS treatment. Supplementation with MBS caused significant improvement in glomerular filtration rate and protein excretion. The elevated levels of serum creatinine, uric acid, and blood urea nitrogen were also prevented by the MBS treatment. The MBS treatment showed reduced formation of deposition around the implanted zinc disc. The higher dose of MBS (400 mg/kg) found more effective. These results indicate that the administration of MBS significantly prevents the growth of urinary stones. The possible mechanism underlying this effect is mediated collectively through diuretic, antioxidant and anti-inflammatory effects of the plant. The results concluded that the methanolic extract of whole plant of *B. sensitivum* possessed significant anti-urolithiatic activity.

Key words: Calculi, cystone, high-performance thin layer chromatography analysis

INTRODUCTION

Urolithiasis is the third prevalent disorder of the urinary system that is approximately 2–3% in the general population. Urinary calculi, if untreated, may cause serious medical consequences such as extreme obstruction, hydronephrosis, infection, and hemorrhage in the urinary tract system.[1] Surgical operation, lithotripsy, and local calculus disruption using high-power laser are commonly used techniques to remove the calculi. However, these procedures are associated with the risk of acute renal injury leading to decrease in renal function. Moreover, an increase in stone recurrence is also observed.[2] The recurrence rate without preventive treatment is approximately 10% at

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1st year, 33% at 5th year, and 50% at 10th year indicating the need to develop suitable alternative therapy.[3]

Medicinal plants are always remained important source of drugs. Some medicinal plants and proprietary composite herbal preparations are reported to be effective in the treatment as well as prevention of recurrence of renal calculi with minimal side effects.[4] In Indian indigenous system of medicine, several plants including *Biophytum sensitivum* are claimed to be useful for the renal calculi that need scientific documentation.[5,6] *B. sensitivum* (L.) DC (family: *Oxalidaceae*), commonly known as "life plant," is a mesophytic under-shrub growing in slightly moist places. The plant been extensively studied for its various biological activities and therapeutic potentials such as analgesic, anti-pyretic, anti-inflammatory, immunomodulatory, antitumor, antidiabetic, antioxidant, antibacterial, antihypertensive, chemoprotective, radioprotective, antifertility, etc.[7] However, the rationale behind its usefulness as an anti-urolithiatic has not been established yet through the systematic pharmacological study. Therefore, the present study was planned to evaluate the anti-urolithiatic activity of methanolic extract of *B. sensitivum* against zinc disc implantation induced urolithiasis in rats.

MATERIALS AND METHODS

Animals

Male Wistar albino rats weighing between 150 and 200 g procured from National Institute of Biosciences, Pune, India were used for this study. The animals were acclimatized for 10 days under standard conditions in the animal house approved by Committee for the Purpose of Control and Supervision of Experiments on Animals, India. The animals fed with standard diet supplied by Nutrivet Life Sciences, Pune, India. The study protocol was approved by the Institutional Animal Ethics Committee (Ref. No.: MIP/IAEC/2012-13/M1/Appr/002).

Chemicals and apparatus

Amentoflavone (Sigma-Aldrich, Germany), sodium oxalate (Qualigens Fine Chemicals, India), ketamine (Injection Aneket, Neon Laboratories Ltd., India), diazepam (Injection Calmpose, Ranbaxy Laboratories Ltd., India), ibuprofen (Tablet Brufen, Abbott India Ltd., India), and cystone (Tablet Cystone, Himalaya Drug Company, India) were used for this study. Apparatus such as the metabolic cages (New Neeta Chemicals, India), cold centrifuge (BioEra, India), precoated silica gel aluminum high-performance thin layer chromatography (HPTLC) plate $60F_{254}$ (E. Merck, Darmstadt, Germany), HPTLC system (Camag, Muttenz, Switzerland), and UV-spectrophotometer (LabIndia, India) were used in the study.

Collection of plant material and preparation of extract

The whole plant material of *B. sensitivum* was collected from the local region of Pune, India. It was authenticated by Dr. J. Jayanthi, Botanical Survey of India, Pune, India (Ref. No: BSI/WRC/Tech./2012/446; Date: 16/10/2012). The plant material was washed thoroughly in water and shade dried at room temperature. The dried whole plant of *B. sensitivum* was coarsely powdered, packed into soxhlet column and extracted with 70% v/v methanol in water at 65–70°C for 22 h. The obtained methanolic extract of *B. sensitivum* (MBS) was evaporated at 45°C and then dried in oven. The dried extract was stored in airtight container.

Standardization of methanolic extract of *Biophytum sensitivum* by high-performance thin layer chromatography

The MBS was standardized for the content of marker compound, amentoflavone using HPTLC method. Amentoflavone and MBS were dissolved in methanol for the analysis. The sample solutions were applied on prewashed and activated precoated silica gel aluminum HPTLC plate $60F_{254}$ (20 cm × 10 cm with 250 µm thickness) in the form of band of 6 mm width with a 100 µl syringe (Camag, Muttenz, Switzerland) using a Camag Linomat V sample applicator (Camag, Muttenz, Switzerland). HPTLC plates were then developed with 20 ml mobile phase consisting of toluene: ethyl formate: formic acid (5:4:1, v/v/v). Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber saturated with the solvent system. The chamber saturation time for the solvent system was 15 min at room temperature of 25°C ± 2°C and relative humidity of 60% ± 5%. After chromatography, plates were dried in an air current. Densitometric scanning was performed using Camag TLC scanner III with winCATS software version 1.4.4 (Camag, Mutttenz, Switzerland) at 366 nm.[8]

Preliminary phytochemical analysis

The MBS was subjected to qualitative analysis for various phytoconstituents using well-established procedures.[9]

Anti-urolithiatic activity of methanolic extract of *Biophytum sensitivum*

The three dose levels (100, 200 and 400 mg/kg) were used for the evaluation of anti-urolithiatic effect of MBS in this study.[10]

Surgical implantation of zinc disc in bladders of rats

The implantations of zinc disc in the urinary bladders of rats were carried out by earlier reported method.[11] In brief, rats were fasted for 10 h before the surgical procedure. However, they were orally administered with 4 ml of water on 15–20 min prior to anesthesia to dilate their urinary bladder. The rats were operated in sterile conditions under ketamine (80 mg/kg, i.p.) and diazepam (4 mg/kg, i.m.) anesthesia. The urinary bladders were exposed through a suprapubic incision, and a small cut was taken to open

the lumen of the bladder. The zinc disc of average weight 20 ± 2 mg was inserted into the bladder and the incision was closed by maximum 2 stitches of absorbable sterile surgical sutures. The urinary bladder was pushed back into its earlier position. The muscular layer of the abdomen was separately sutured using sterile absorbable sutures. The skin incision was then stitched with sterile silk sutures. All operated rats were administered with ibuprofen (30 mg/kg, p.o.) on the day of surgery immediately after recovery from anesthesia, and this dose was repeated after 24 h. In addition, the rats were treated topically with antibiotic dusting powder and allowed to recover for minimum next 3 days. Based on recovery, rats were then subjected to further treatment.

Experimental design

The completely recovered rats were randomly divided into six groups containing six animals in each. Group-I served as sham-operated group (underwent surgical procedure but without implantation of zinc disc) and received 5% w/v gum acacia solution (5 ml/kg, p.o.) for next 7 days. Group-II was zinc disc-implanted group and received 5% w/v gum acacia solution (5 ml/kg, p.o.) for next 7 days. Group-III was zinc disc-implanted and standard drug-treated group, received cystone (500 mg/kg) for next 7 days. Group-IV, V and VI were zinc disc-implanted and MBS-treated groups, received 100, 200, and 400 mg/kg of MBS, respectively for next 7 days. Cystone and MBS were suspended in 5% w/v gum acacia solution and given once daily by oral route (5 ml/kg) for 7 days.

Serum analysis

One hour after the last dose of treatment, blood was collected from retro-orbital sinus under ether anesthesia. Serum was separated by centrifugation at 10,000 ×*g* for 10 min and analyzed for creatinine, uric acid, blood urea nitrogen (BUN) spectrophotometrically using diagnostic kits (Beacon Diagnostic Pvt. Ltd., India).

Urine analysis

After blood collection, all animals were kept in individual metabolic cages for the collection of 5 h urine samples. The collected urine samples were measured for volume, total protein and creatinine levels. The total protein and creatinine levels were estimated using diagnostic kits (Beacon Diagnostic Pvt. Ltd., India).

Stone estimations

After urine collection period, all animals were sacrificed by cervical dislocation. The urinary bladders were exposed, zinc disc along with adhered crystals was removed and the weight of calculi was noted. The dimensions of zinc disc with adhered crystals were also determined using Vernier caliper.

Statistical analysis

All the results were expressed as mean ± standard error of mean. The statistical significance was calculated using one-way analysis of variance followed by Dunnett's comparison test, and $P < 0.05$ was considered as statistically significant.

RESULTS

Extraction and high-performance thin layer chromatographic standardization of methanolic extract of *Biophytum sensitivum*

The yield of MBS was found to be 25% w/w. The HPTLC desitogram of MBS showed twelve peaks and peak number 7 corresponding to Rf - 0.42 was identified as amentoflavone by comparing with HPTLC densitogram of reference standard of amentoflavone showing peak at Rf - 0.48. Based on peak area, percent content of amentoflavone in MBS was found to be 0.07% w/w [Figure 1].

Preliminary phytochemical analysis

It revealed prominent presence of carbohydrates, proteins and amino acids, flavonoids, tannins, saponins, and phenolic compounds.

Effect on urine parameters

The mean urine volume of rats of sham-operated group was 2.48 ± 0.17 ml/5 h, which was significantly ($P < 0.001$) reduced to 1.27 ± 0.11 ml/5 h in the zinc disc-implanted group [Table 1]. This reduced urine volume was significantly ($P < 0.05$) increased by the treatment with MBS in dose-dependent manner. The highest dose of MBS (400 mg/kg) was found to be equipotent ($P < 0.001$) to the reference standard cystone (500 mg/kg).

The mean urinary creatinine level of sham-operated rats was 1.78 ± 0.08 mg/dl, which was significantly ($P < 0.001$) increased to 2.41 ± 0.13 mg/dl in the zinc disc-implanted rats [Table 1]. This increased urinary creatinine level was significantly ($P < 0.05$) decreased by treatment with MBS in dose-dependent manner. The highest dose of MBS (400 mg/kg) was found to be equipotent ($P < 0.001$) to the reference standard cystone (500 mg/kg).

The mean urinary total protein level of rats of sham-operated group was 1.07 ± 0.11 mg/5 h. This urinary total protein level was significantly ($P < 0.001$) increased to 3.10 ± 0.21 mg/5 h in the zinc disc-implanted group [Table 1]. This increased urinary total protein level was significantly ($P < 0.001$) reduced by treatment with cystone (500 mg/kg) and with MBS in dose-dependent manner. All three doses of MBS (100, 200, and 400 mg/kg) were found equipotent ($P < 0.001$) to the reference standard cystone (500 mg/kg).

Effect on serum parameters

The mean serum creatinine level of sham-operated rats was 0.71 ± 0.02 mg/dl, which was significantly ($P < 0.001$) increased to 1.88 ± 0.06 mg/dl in the zinc disc-implanted rats [Table 2]. This increased serum creatinine level was

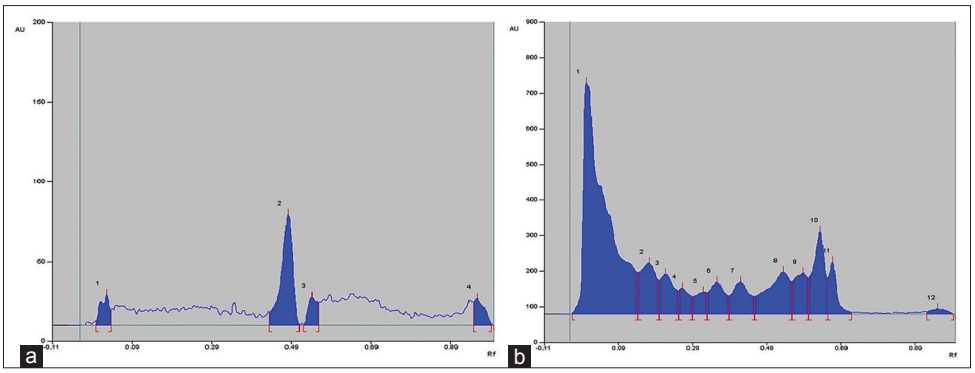

Figure 1: High-performance thin layer chromatography densitogram of (a) Amentoflavone (peak number 2) (b) Methanolic extract of *Biophytum sensitivum* standardized to amentoflavone (peak number 7) scanned at 366 nm

Table 1: Effect of MBS on urine parameters in urolithiasis-induced rats

Groups	Group details	Urine volume (ml/5 h)	Creatinine (mg/dl)	Total protein (mg/5 h)
Group-I	Sham-operated	2.48±0.17	1.78±0.08	1.07±0.11
Group-II	Zinc disc-implanted	1.27±0.11[c]	2.41±0.13[c]	3.10±0.21[c]
Group-III	Zinc disc-implanted and cystone-treated (500 mg/kg)	2.28±0.16[c]	1.80±0.10[c]	1.25±0.13[c]
Group-IV	Zinc disc-implanted and MBS-treated (100 mg/kg)	1.92±0.14[a]	1.98±0.10[a]	1.85±0.16[c]
Group-V	Zinc disc-implanted and MBS-treated (200 mg/kg)	2.02±0.15[b]	1.90±0.10[b]	1.65±0.13[c]
Group-VI	Zinc disc-implanted and MBS-treated (400 mg/kg)	2.20±0.16[c]	1.81±0.08[c]	1.40±0.11[c]

Values are expressed as mean±SEM, number of animals (n)=6, [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$, values of Group-II were compared with Group-I and those of Group-III, IV, V, and VI with Group-II. SEM: Standard error of mean, MBS: Methanolic extract of Biophytum sensitivum

Table 2: Effect of MBS on serum parameters in urolithiasis-induced rats

Groups	Group details	Creatinine (mg/dl)	Uric acid (mg/dl)	BUN (mg/dl)
Group-I	Sham-operated	0.71±0.02	1.24±0.04	37.95±0.74
Group-II	Zinc disc-implanted	1.88±0.06[c]	3.29±0.10[c]	51.13±1.36[c]
Group-III	Zinc disc-implanted and cystone-treated (500 mg/kg)	0.83±0.02[c]	1.34±0.04[c]	39.12±0.81[c]
Group-IV	Zinc disc-implanted and MBS-treated (100 mg/kg)	1.25±0.03[c]	2.25±0.07[c]	46.20±1.02[a]
Group-V	Zinc disc-implanted and MBS-treated (200 mg/kg)	0.98±0.03[c]	1.86±0.06[c]	44.29±0.98[c]
Group-VI	Zinc disc-implanted and MBS-treated (400 mg/kg)	0.85±0.02[c]	1.41±0.05[c]	42.66±0.96[c]

Values are expressed as mean±SEM, number of animals (n)=6, [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$, values of Group-II were compared with Group-I and those of Group-III, IV, V, and VI with Group-II. SEM: Standard error of mean, MBS: Methanolic extract of *Biophytum sensitivum*, BUN: Blood urea nitrogen

significantly ($P < 0.001$) decreased by the treatment with cystone (500 mg/kg) and with MBS in dose-dependent manner. All three doses of MBS (100, 200 and 400 mg/kg) were found equipotent ($P < 0.001$) to the reference standard cystone (500 mg/kg).

Zinc disc implantation caused significant ($P < 0.001$) increase in the serum uric acid level from 1.24 ± 0.04 mg/dl to 3.29 ± 0.10 mg/dl [Table 2]. This increased serum uric acid level was significantly ($P < 0.001$) decreased by the treatment with cystone (500 mg/kg) and with MBS in dose-dependent manner. All three doses of MBS (100, 200, and 400 mg/kg) were found equipotent ($P < 0.001$) to the reference standard cystone (500 mg/kg).

The mean serum BUN level of sham-operated rats was 37.95 ± 0.74 mg/dl, which was significantly increased to 51.13 ± 1.36 mg/dl in the zinc disc-implanted rats [Table 2]. This increased serum BUN level was significantly ($P < 0.05$) decreased by the treatment with cystone (500 mg/kg) and with MBS in dose-dependent manner. The intermediate and highest doses of MBS (200 and 400 mg/kg) were found to be equipotent ($P < 0.001$) to the reference standard cystone (500 mg/kg).

Effect on creatinine clearance

Zinc disc implantation caused significant ($P < 0.001$) decrease in the creatinine clearance, i.e. glomerular filtration rate (GFR), from 1.23 ± 0.06 ml/h to 0.32 ± 0.03 ml/h [Figure 2]. This decreased creatinine clearance was significantly ($P < 0.05$) increased by the treatment with cystone (500 mg/kg) and with MBS in dose-dependent manner. The intermediate and highest doses of MBS (200 and 400 mg/kg) were found to be equipotent ($P < 0.001$) to the reference standard cystone (500 mg/kg).

Effect on weight and dimensions of calculi

The amount of calculi deposited around the implanted zinc disc was found to be 78.32 ± 5.65 mg. Animals treated with cystone (500 mg/kg) and all doses of MBS (100, 200, and 400 mg/kg) showed significant ($P < 0.001$) reduction in the deposition of calculi as compared to zinc disc-implanted group. This reduced deposition of calculi was also confirmed from the significant ($P < 0.01$) decrease in the diameter and thickness of the calculi in the animals treated with cystone (500 mg/kg) and all doses of MBS (100, 200, and 400 mg/kg) as compared to zinc disc-implanted group [Table 3].

DISCUSSION

Renal calculi can be experimentally induced by either oral administration of various chemicals such as ethylene glycol to rats or surgical implantation of foreign material like zinc disc in the urinary bladder of rats. The chemically-induced calculi model has their own limitations like high incidence of nephrotoxicity, metabolic acidosis and occurrence of calculi in renal cortex that is situation opposite as found in human urolithiasis.[12] However, zinc disc model induces renal calculi with minimal renal damage and mimic the etiology of urinary stone formation in humans.[13] Therefore, we evaluated the anti-urolithiatic potential of *B. sensitivum* using zinc disc implantation induced urolithiasis in rats. In zinc disc implantation induced lithiasis, the implanted zinc disc acts as a nidus that subsequently leads to deposition of a urolith composed of magnesium ammonium phosphate around the disc.[14]

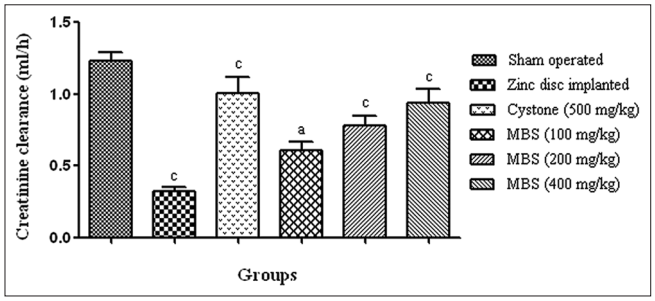

Figure 2: Effect of methanolic extract of *Biophytum sensitivum* on creatinine clearance in urolithiasis-induced rats. Values are expressed as mean ± standard error of mean, *P* values: $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$, values of zinc disc-implanted group were compared with sham-operated group whereas those of treated groups with zinc disc-implanted group

Consistent with previous reports, urine output was decreased in disc-implanted group as compared to sham-operated group.[15] This suggests obstruction of urinary bladder due to formation of large urinary calculi. In the present study, the MBS treatment increased urine output. This may be due to prevention of stone formation or direct diuretic effect of MBS which reduced supersaturation of urine with precipitating substances and thereby stone formation. The exact cause may be revealed upon overall results; however, irrespective of mechanism this enhanced urine output is beneficial to restore normal condition.

As reported in some previous reports, significant increase in the urinary total protein excretion was observed in disc-implanted animals.[16] Supersaturation of urine with precipitating substances results in precipitation of crystal initiation particle which when trapped acts as a nidus leading to subsequent crystal growth. This is associated with proteinuria that reflects proximal tubular dysfunction.[17] Treatment of MBS showed significant reduction in the protein excretion and thus might have prevented the nidus formation of crystal formation. The proteinuria, due to adversely altered function of the urinary system, is a critical point that starts major complications and hence any ideal therapy would prevent it. The MBS produced initial effect of enhancing urinary output, further shown reduction in protein excretion that is indirect indication of either recovery of urinary dysfunction or prevention of crystal-induced damage. This action of MBS is considered as most fruitful outcome because MBS not only provide symptomatic action but also has its direct action at membrane level.

Nucleation and subsequent growth of renal calculi are next step that involves lipid peroxidation and cause renal damage by reacting with polyunsaturated fatty acids in cell membrane.[18] This renal damage is indicated by the elevated serum levels of creatinine, uric acid, and BUN, which are markers of glomerular and tubular damage. In the present study, there was a significant increase in the serum levels of creatinine, uric acid, and BUN of disc-implanted animals,

Table 3: Effect of MBS on weight and dimensions of calculi in urolithiasis-induced rats

Groups	Group details	Weight (mg)	Dimensions (mm)	
			Diameter	Thickness
Group-I	Sham-operated	-	-	-
Group-II	Zinc disc-implanted	78.32±5.65	4.92±0.35	5.85±0.23
Group-III	Zinc disc-implanted and cystone-treated (500 mg/kg)	15.66±1.20^{c}	1.70±0.30^{c}	1.97±0.14^{c}
Group-IV	Zinc disc-implanted and MBS-treated (100 mg/kg)	45.64±3.58^{c}	3.20±0.33^{b}	3.83±0.18^{c}
Group-V	Zinc disc-implanted and MBS-treated (200 mg/kg)	28.34±1.67^{c}	3.08±0.18^{c}	2.82±0.15^{c}
Group-VI	Zinc disc-implanted and MBS-treated (400 mg/kg)	17.44±1.27^{c}	1.55±0.31^{c}	1.73±0.14^{c}

Values are expressed as mean±SEM, number of animals (n)=6, $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$, values of Group-III, IV, V, and VI were compared with Group-II.
SEM: Standard error of mean, MBS: Methanolic extract of Biophytum sensitivum

whereas MBS treatment showed to prevent the elevation of serum levels of these markers. This indicates that MBS act by inhibiting the lipid peroxidation and thereby reduces the extent of tubular dysfunction. The glomerular and tubular damage mediated by lipid peroxidation is another important step in the progress of pathology. If this is left untreated, condition becomes more worsens through initiation of series of complications. The MBS has shown significant restoration indicating better control on progress of pathology which can be termed as important characteristics to label it as ideal therapy.

In addition, a significant decrease in the GFR was also observed in disc-implanted animals. This indicates that the animals were suffering from compromised renal functions due to increased size of urinary calculi which is in accordance with earlier published reports.[16] The MBS treatment showed to increase the GFR, which may be due to its collective effects of control on growth of calculi and its diuretic effect. This dual action makes MBS as an ideal therapy for urolithiasis expecting faster recovery leading to minimization or prevention of the time-dependent complications. These observed actions at every step from enhanced urine output to improved GFR may be due to effects of various phytoconstituents that interact to give synergistic effect with putative mechanism of action.

Consistent with previous reports, implantation of zinc disc in the bladder of rats caused accumulation of calculi around the disc.[16] The weight of disc is direct method to evaluate anti-urolithiatic activity wherein MBS has shown significant reduction that is in accordance with the other results of this study further supporting that MBS has anti-urolithiatic potential.

Earlier study reported a potent antioxidant[19] and anti-inflammatory[10,20] capacity of extract of *B. sensitivum*. Therefore, MBS may prevent calculi formation by antioxidant, anti-inflammatory and diuretic constituents of the plant.

CONCLUSION

The study concluded that the administration of methanolic extract of whole plant of *B. sensitivum* showed significant anti-urolithiatic activity as indicated by improvement in disc weight and related biochemical parameters. The exact mechanism of action is not established but attributed to putative mechanism mediated by various phytochemicals interacting toward synergism. The possible mechanism underlying this effect is mediated collectively through diuretic, antioxidant and anti-inflammatory effects of the plant. The further phytochemical exploration is required to establish the exact mechanism of action.

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Conflicts of interest
There are no conflicts of interest.

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