Original Article

Anti-urolithiatic Activities of *Macrotyloma uniflorum* Mediated through Multiple Pathway

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ABSTRACT

Background: *Macrotyloma uniflorum* Linn. (Fabaceae) seeds are widely used for their diuretic and urolithiatic effects in India. The present study investigated the effect of n-butanol fraction of *M. uniflorum* (nBFMU) on kidney stone using *in vitro* and *in vivo* method. **Materials and Methods:** Nucleation, crystal growth, crystal aggregation and crystal dissolution assays were performed for nBFMU. Two doses of nBFMU (400 and 800 *mg/kg*) were studied for their diuretic activity and sodium oxalate (NaOx) (70 *mg/kg, i.p*) induced urolithasis in male Wistar albino rats. Determination of body and kidney weight, measurement of various biochemical parameters in biological samples and examination of histology of kidney at the end of experiment were also done. **Results:** nBFMU exhibited a concentration dependent inhibitory activity on nucleation and aggregation along with decreased number of crystals of calcium oxalate (CaOX) produced in metastable solutions of CaOX in the *in vitro* experiments. Co-administration of nBFMU with NaOX has significantly ($p<0.001$) increased the urine volume and the level of calculus inhibitors like magnesium, citrate and decreased the level of calculus promoters like oxalate, calcium, urea and uric acid. nBFMU supplement also prevented the pathological changes in kidney and increased the glomerulus activity of the kidney. **Conclusion:** These results indicate that nBFMU showed significant activity in urolithiasis which might be due to its diuretic, CaOX crystal formation inhibitory effects and its ability to increase the levels of inhibitors and decrease the level of promoters of urolithiasis.

Key words: Diuresis, Kidney stone, *Macrotyloma uniflorum*, Sodium oxalate, Urolithiasis.

INTRODUCTION

Urolithiasis, the stone formation in urinary system is a common and painful disease, which was first noted at 4800 BEC in Egyptian mummies. In all over the world, approximately 4-15% of the human populations suffer from urinary stone. As per the survey of National Health and Nutrition Examination in 2012, 7.1% of women and 10.6% of men were affected by kidney stone disease in United States.[1] As per the epidemiological studies, men are more affected as compared to women and are more prevalent between 20-49 ages in both sexes.[2] The recurrence rate at every year is 10-23%, in that 50% occured in 5-10 years and 75% occurred in 20 years. Approximate 12% population of India is suffering with urolithiasis every year with the high incidence states denote as "Stone belt", i.e. Gujarat, Maharashtra, Rajasthan, Delhi, Punjab, Haryana.[3] Since urolithiasis is a multifactorial disease, its etiology is very complex and highly unpredictable.

Management of urolithiasis mostly depends on stones size and its location in urinary system. In most of the cases stone are removed by surgical treatment like extreacorporeal shock wave lithotripsy, percutaneous nephrolithotomy, ureteroscopy but unfortunately stone recurrence was observed about 50% after removal by surgical treatment.[4] Surgical treatments causes side effect such as hypertension, tubular necrosis, hemorrhage and fibrosis

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of the kidney.[5] Stone formation is a continues process and recurrence is very commonly observed, so there is an increased interest in the alternate supporting therapy for the management of urolithiasis using natural products and ayruvedic medicines. In Ayurveda, many traditional plants are reported to be used in the treatment of urolithiasis. Those traditional plants may offer complex spectrum of actions, like diuretic, analgesic, antimicrobial, anti-inflammatory, antispasmodic properties and litholytic and anticalcifying activities, without any major side effects.[6]

Macrotyloma uniflorum [Lam.] Verdc, commonly known as Horsegram is extensively cultivated, in India, Sri Lanka, Burma and Australia. However, the maximum genetic diversity region is considered to be in the Old World Tropics, especially in India and Himalayas.[7] In India, Horsegram is one of the highly nutritious pulse crop with ethno-medicinal values, which is commonly known as *Kulattha* (Sanskrit), *Kollu* (Tamil), *Kurti-kalai* (Bengali), *Muthira* (Malayalam), *Ullavallu* (Telugu), *Gahot* (Kumaon and Garhwal) and etymologically, *Gahot* means" which destroys stone in initial stage".[8,9] In Southern India seed of Horse gram are known as a poor man's pulse. Consumption of cooked horse gram seeds with cooked rice is common in the rural people of India.[10] According to Ayurveda, the seeds are acrid, bitter, acrid, dry, hot and used as anthelmintic, astringent, antipyretic and in the conditions such as uterine stones, asthma, tumors, bronchitis, hiccup, piles, urinary discharges, heart-troubles, intestinal colic and diseases of the brain and eyes, inflammation and liver troubles.[9,11,12] Its decoction is used traditionally in menstrual dysfunctions and leucorrhoea. Furthermore, the cooked liquor of the horse gram seeds with spices is considered to be a potential remedy for the throat infection, common cold and fever.[13] Decocation of *M. uniflorum* seeds with powder of *Tephrosia purpurea* used in kidney stone.[14] Seeds of *M. uniflorum* contain proteins, carbohydrates, lipids, amino acids, falvonoids, phenolic acids, fatty acids, tannins, phytosterols, saponins, anthocyanidins and minerals like calcium, iron and molybdenum. Phenolic acids of *M. uniflorum* seeds are considered to be the most potent antioxidants which act by scavenging ROS and free radicals. The principal phenolic compounds of *M. uniflorum* seeds are flavonoids like quercetin, kaempferol and myricetin, vanillic, ρ-hydroxybenzoic and ferulic acids.[15] Flavonoids like rutin,[16] quercetin[17] have been reported to prevent stone formation, decreased the stone deposition in kidney cell and prevent the oxidative stress. However, the potential bioactive components and the underlying mechanisms associated with treat urolithiasis are still unknown. The results of recent studies have shown that saponin of the plant could inhibit the formation of calcium oxalate (CaOX) stones *in vitro* and *in vivo*, correlating with their antioxidant, anti-inflammatory, diuretic, antibacterial and other protective effects. Thus, the objective of present study is to evaluate the saponin rich fraction of *M. uniflorum* on experimentally induced CaOX crystallization via *in vitro* and *in vivo* methods.

MATERIALS AND METHODS

Materials

All chemicals used in experiment were of analytical grade. Standard drug Cystone (Himalaya Drug Company) purchased from local market of Ahmedabad. Calcium, Uric acid, Urea, Phosphorus, Creatinine, Uric acid (Accucare kits) estimation kits were procured from Lab-Care Diagnostics Pvt Ltd. Ahmedabad, Gujarat, India.

Plant material and preparation of plant extract

Dried seeds of *M. uniflorum* purchased in June 2016 from local market of Ahmedabad, Gujarat, India. Seeds were authenticated by Dr. B. L. Punjani, Ethnobotanist, Head, P.G. Center in Botany, Talod, Gujarat, India. The specimen was submitted to the Pharmacognosy department of Institute of Pharmacy, Nirma University. (Ref No. IPNSAVPMU2015) Dry seeds were grounded into the fine powdered by electric grinder. Powdered drug was stored in the tight container at ambient temperature for further use.

Powder of *M. uniflorum* (100 gm) seeds was refluxed with 500 ml of petroleum ether for 24 hr. Layer of petroleum ether was collected and powder was again refluxed with methanol (500 ml) for 24 hr. The extract was filtered and evaporated using a rotary vacuum evaporator at 50°C, that collected dry extract (7.45 % *w/w*) labelled as methanolic extract. Methanolic extract was solublised in water and again fractionated with dichloromethane, ethyl acetate and n-butanol (1:1 *v/v*) for 2 hr at room temperature using separating funnel. n-butanol fraction was evaporated at room temperature and was labeled as nBFMU (n-butanol fraction of *M. uniflorum*) (1.5% *w/w*)

Phytochemical screening and quantitative estimation of phytoconstituents

Phytochemical screening of nBFMU was carried out to identify the nature of phytoconstituents present in the extract. Total flavonoid content was measured using colorimetric assay of aluminum chloride.[18] Total saponins were measured according to the method described by Obadoni and Ochuko.[19]

In vitro experiments

In vitro evaluation of nBFMU on CaOX crystallization was determined by the method described by Hennequin *et al.*[18] with some modification. Calcium chloride (1-5 *mmol/l*) and sodium oxalate (NaOX) (1-5 *mmol/l*) solutions were prepared in a buffer containing NaCl 0.15 *mol/l* and Tris–HCl 0.05 *mol/l* at *pH* 6.5 for *in vitro* experiments

Nucleation assay

Nucleation assay was performed with some modification in method described by Hennequin *et al.*[20] In this method 9 ml of calcium chloride (5 mmol/l) solution was mixed with 1 ml of nBFMU at different concentration (250, 500, 750, 1000, 1250, 1500 µg/ml) and 9 ml of NaOX at different concentrations of ranging from (1-5 mmol/ml) was added in each beaker. For standard drug (cystone) experiment, 1 ml of cystone at different concentration (250, 500, 750, 1000, 1250, 1500 µg/ml) was added in replace of nBFMU. Optical density of mixed solution was measured at 620nm at room temperature and nucleation rate was estimated by comparing induction time in the presence of nBFMU with that of the control.

Aggregation assay

Aggregation assay has been performed using method described by Atmani and Khan[21] with some modification. CaOX crystal seeds were prepared by mixing NaOX (50 mmol/l) and $CaCl_2$ (50 mmol/l). Solutions were incubated at 60°C for 1 hr in water bath and cooled at room temperature and kept it for overnight. CaOX crystals were harvested by centrifugation and then evaporated at 37°C. CaOX crystals were used at a final concentration of 0.8 mg/ml in buffer solution containing Tris–HCl 0.05 mol/l and NaCl 0.15 mol/l at *pH* 6.5. Assay was performed at room temperature in the presence and absence of nBFMU. The percentage inhibition of aggregation was calculated by comparing the turbidity in the presence of nBFMU and standard drug (cystone) at concentrations ranging from (250, 500, 750, 1000, 1250, 1500 µg/ml) with that obtained in control using formula 1

% Inhibition of aggregation = 100-Rate of aggregation (IR) (1)

where, rate of aggregation is defined using equation 2

Rate of aggregation (IR) = ((Turbidity of sample)/(Turbidity of control)) × 100 (2)

Crystal growth assay

The percentage inhibition of CaOX crystal growth was evaluated in presence of nBFMU and cystone by adapting the procedure described by Chaudhary *et al.*[22] with some modification. 1 ml slurry of CaOX monohydrate crystals was mixed with 10 ml of 2 mM calcium chloride solution. Add immediately 10 ml of NaOX solution in different concentrations (2, 2.5, 3, 3.5 mM). Consumption of oxalate immediately started after addition of NaOX and solution was stirred for 15 min using magnetic stirrer at 800 rpm and absorbance was recorded at 214 nm, with and without nBFMU and standard drug cystone at different concentration (250, 500, 750, 1000, 1250, 1500, 2000 µg/ml). The relative inhibitory activity was calculated using equation 3

% Relative Inhibitory Activity = ((C-S)/C) × 100 (3)

Where '*C*' is the rate of reduction of free oxalate without any extract and '*S*' is the rate of reduction of free oxalate with the extract.

Calcium oxalate dissolution

CaOX crystal dissolution assay was performed as described by Saso *et al.*[23] followed with some minor modification. CaOX seeds were prepared as mentioned in the aggregation method above. A series of nBFMU solution from 250 µg/ml to 2000 µg/ml concentrations were prepared and 1 ml of nBFMU solution was added to the 10 *mg* of CaOX seeds in tubes followed by incubation at room temperature for overnight under mild mixing using vortex mixer. The tubes were centrifuged and seeds were washed, dried and weighted again. The ability of nBFMU to dissolve CaOX seeds was calculated using formula 4.

% Crystal Dissolution = ((Intial Weight-Final Weight)/(Intial Weight)) × 100 (4)

Animal study

Adult Male Wistar albino rats (180-250 *g*) were housed at the animal house of Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India under controlled environmental condition (temperature of 22-25°C, relative humidity (55 ± 5 %) and 12 hr light-dark cycle) and animals have received food pellet and water *ad libitum*. The study protocol was approved by the Institutional Animal Ethics Committee of Institute of Pharmacy. (IP/PCOG/PHD/19/015, dated 28 July 2016)

Acute toxicity study

The acute toxicity study was carried out in wistar male rats as per OECD guideline. Toxicity study was started from lower dose 1000 mg/kg followed by increasing dose depending on mortality to 2000 mg/kg, 4000 mg/kg and 6000 mg/kg. Zero mortality was observed upto extract dose of 6000 mg/kg.

Determination of diuretic activity

The diuretic activity of nBFMU was determined by method described by Lipschitz *et al.*[24] Twenty Four healty rats (200-250 *g*) were selected and randomly divided into 4 groups with 6 animals in each group. All experimental animals were fasted for 18 hr prior to the experiment and allocated only for water during the fasting period. Normal and standard groups were given saline (20 ml/kg) and hydrochlorothiazide (HCT) (10 mg/kg, as standard) respectively, while treated groups received the same ml of saline containing 400 mg/kg and 800 mg/kg of nBFMU, p.o. as a single dose. The rats were kept separately in metabolic cages. The urine was collected in cylinders at an interval of one hr for 6 hr. Total collected urine volume was measured.

Sodium oxalate model of urolithiasis

In NaOX model animals were divided into five groups (normal, disease control, standard and treatment groups with 2 doses of nBFMU) with 6 animals in each group. In normal group (Group I) animals were administered saline (2.5 ml/kg) and 70 mg/kg NaOX in saline was administered intraperitoneally for 7 days to induce urolithiasis in all the animals expect normal group animals.[25] The standard group (Group III) animals were administered Cystone (750 mg/kg, p.o.) for 7 days and Group IV and V (treatment group I and II respectively) were administered with nBFMU (400 mg/kg and 800 mg/kg, p.o.) respectively. Animal Body weights were recorded during the experiment and relative body weights (RBW) of animals were calculated using formula 5 as follow.

$$\text{Relative body weight} = ((\text{Absolute body weight})/(\text{Weight at } 1^{st} \text{ day of treatment})) \times 100 \quad (5)$$

Where, Absolute body weight was measured at one time interval.

Relative organ weight was calculated to compare the changes in the organ weight and calculated according to the equation 6:

$$\text{Relative organ weight} = ((\text{Organ weight})/(\text{Body weight (at the last day)})) \times 100 \quad (6)$$

Urine collection and analysis

On 8th day, animals were kept in individual metabolic cages and 24 hr urine samples were collected. The urinary pH, urine volume and crystalluria were determined. Urine was acidified by addition of drop of concentrated hydrochloric acid and stored at -20°C for determination of urea, calcium, uric acid, magnesium and phosphate using standard kits. The citrate and oxalate were estimated by the method described by Rajagopal[26] and Hodgkinson[27] respectively.

Serum collection and analysis

Blood was collected under light ether anesthetic condition from retro-orbital plexus by capillary method. Blood was centrifuged at 10,000 *g* for 10min and serum was separated for the analysis of uric acid, calcium, magnesium, Blood Urea Nitrogen (BUN) and creatinine using diagnostic kits.

Kidney histopathology and homogenate analysis

The abdomen was incised, opened and both kidneys were removed from each animal of under study. Extraneous tissue was removed from isolated kidneys and kidneys were weighed and rinsed with ice-cold normal saline. One kidney was fixed with neutral formalin solution (10% *v/v)* and after harvesting, horizontally sliced and sent to histology services (Accupath diagnostic laboratory, Ahmedabad, Gujarat, India.) for Hematoxylene and Eosin staining. The section of kidney were observed under microscope for examination of CaOX crystal depositions and the presence of CaOX crystal, tubular casts, glomerular congestion, interstitial edema, blood vessel congestion, epithelial adhesion and inflammatory cells. Another kidney was finely chopped and 20% of homogenate was prepared in Tris–HCl buffer at *pH* 7.4. Kidney homogenate was used for determination of calcium, uric acid, phosphate, oxalate, urea, LDH and catalase.

Statistical analysis

Results data were expressed as mean ± SEM. The results among the groups were analysed by one-way ANOVA followed by Dunnett's test using Graphpad Prism version 6. Results were consider significant when value of $p<0.05$ or $p<0.001$.

RESULTS

Phytochemical screening and quantitative estimation of phytoconstituents

The nBFMU was qualitatively analysised for various phytoconstitutents using chemical tests. The study revealed that presence of flavonoid, saponin, carbohydrates,

phytosterols and phenolic compounds. The total flavonoid content of nBFMU was found to be 7.52 ± 0.40 mg quercetin equivalents/g of extract. Total saponin content of the powdered drug was found to be 30.32 ± 0.62 mg diosgenin equivalent/g of extract.

In vitro study

In Nucleation assay, in control group as the concentration of oxalate was increased the nucleation rate was increased and in treatment group as the concentration of oxalate was increased the nucleation rate was decreased in dose depended manner. In lower concentration of NaOx (2 mM), nBFMU showed the maximum inhibition (55.43 ± 0.55 %) at higher dose (1500 µg/ml) where at lower dose (250 µg/ml) showed inhibition (33.59 ± 0.55 %) and similar effective as compared to cystone, which showed (61.56 ± 0.34 %) inhibition at higher dose (1500 µg/ml) and (37.93 ± 0.44 %) inhibition at lower dose (250 µg/ml), while at higher concentration of NaOx (10 mM), nBFMU showed the (35.43 ± 0.17 %) inhibition at higher dose (1500 µg/ml) and (27.92 ± 0.16 %) inhibition at lower dose (250 µg/ml), as shown in Table 1 and cystone showed (41.48 ± 0.13 %) inhibition at higher dose (1500 µg/ml) and (25.22 ± 0.04 %) inhibition at lower dose (250 µg/ml), as shown in Table 2. After nucleation formation, another step in urolithiasis is crystal growth. As the oxalate concentration was increased, the crystal formation rate was also increased. In present study, prevention of crystal growth was directly related to the concentration of nBFMU, highest inhibition (85.49 ± 0.56 %) was observed at 2000 µg/ml. When we have increased the concentration of NaOx (2-3.5 mmol/ml) then at same dose level inhibition effect was decreased in nBFMU and cystone as shown in Table 3 and Table 4 respectively. In aggregation assay, Figure 1 and Table 5 showed that crystals were less aggregated in nBFMU treated group with 50.60 ± 0.24 inhibition of aggregation at higher dose 1750 µg/ml. In CaOX Dissolution assay, after overnight incubation of overnight CaOx seed were mild-vortexed the weight of CaOx seed was decreased with increase in the concentration of drug. Figure 2 and Table 6 shows that, 2000 µg/ml of nBFMU showed 85.10 % CaOx seeds dissolution as compared to the standard drug cystone which dissolved 69.56 %. IC_{50} of nBFMU for aggregation and CaOX dissolution were 1623.53 µg/ml and 400 µg/ml respectively.

Effect on diuresis

nBFMU has showed a significant diuretic activity at the dose of 800 mg/kg (13.01 ± 0.37 ml/100 gm/6 hr) as compared to normal group (8.51 ± 0.26 ml/100 gm/6 hr), furthermore, the effect of nBFMU at dose of 800 mg/kg was also comparable with the standard diuretic agent, HCT (14.08 ± 0.39 ml/100 gm/6 hr). (Figure 3)

Table 1: Effect of nBFMU on calcium nucleation with the increasing amount of NaOX.

Conc. of Drug (µg/ml)	% Inhibition of Nucleation				
	2 mmol NaOx	4 mmol NaOx	6 mmol NaOx	8 mmol NaOx	10 mmol NaOx
250	33.59±0.55	32.01±0.35	31.41±0.27	30.60±0.18	27.92±0.16
500	38.31±0.66	35.06±0.29	33.18±0.26	31.88±0.19	29.22±0.23
750	41.63±0.56	38.32±0.34	35.10±0.21	33.76±0.15	30.70±0.22
1000	46.74±0.64	41.52±0.23	38.03±0.25	35.08±0.18	32.03±0.13
1250	52.49±0.44	43.71±0.30	39.49±0.30	36.49±0.19	33.83±0.19
1500	55.43±0.55	45.31±0.23	41.06±0.26	37.47±0.22	35.43±0..17

Table 2: Effect of cystone on calcium nucleation with the increasing amount of NaOx.

Conc. of Drug (µg/ml)	% Inhibition of Nucleation				
	2 mmol NaOx	4 mmol NaOx	6 mmol NaOx	8 mmol NaOx	10 mmol NaOx
250	37.93±0.44	35.86±0.17	32.78±0.13	29.70±0.18	25.22±0.04
500	43.93±0.56	42.31±0.22	39.19±0.17	34.91±0.11	30.97±0.13
750	50.96±0.44	47.24±0.17	43.33±0.08	39.61±0.07	34.44±0.10
1000	54.79±0.45	51.96±0.18	46.82±0.17	42.18±0.15	37.86±0.11
1250	58.62±0.40	54.49±0.23	48.63±0.19	44.27±0.11	40.46±0.13
1500	61.56±0.34	56.95±0.17	50.00±0.17	45.59±0.12	41.48±0.13

Table 3: Effect of nBFMU on CaOX crystal growth with the increasing amount of NaOx.				
	% Inhibition of CaOX crystal growth			
Conc. of Drug (µg/ml)	2 mmol NaOx	2.5 mmol NaOx	3 mmol NaOx	3.5 mmol NaOx
250	62.83±0.42	57.71±0.52	34.00±0.25	37.77±0.42
500	65.93±0.49	60.55±0.32	37.65±0.32	39.86±0.48
750	69.60±0.57	63.90±0.45	39.67±0.73	42.77±0.42
1000	71.80±0.49	66.74±0.39	45.71±0.45	45.13±0.30
1250	75.22±0.64	69.72±0.45	49.66±0.38	47.84±0.42
1500	79.38±0.63	72.93±0.38	53.31±0.32	50.41±0.36
1750	83.45±0.57	75.46±0.45	55.40±0.33	52.22±0.30
2000	85.49±0.56	78.59±0.32	57.41±0.45	55.76±0.42

Table 4: Effect of cystone on CaOX crystal growth with the increasing amount of NaOx.				
Conc. of Drug (µg/ml)	% Inhibition of CaOX crystal growth			
	2 mmol NaOx	2.5 mmol NaOx	3 mmol NaOx	3.5 mmol NaOx
250	54.93±0.49	51.15±0.39	31.76±0.38	29.09±0.42
500	58.92±0.42	54.21±0.45	36.53±0.39	31.18±0.40
750	62.83±0.41	58.16±0.46	42.35±0.45	35.13±0.41
1000	67.23±0.40	60.85±0.51	45.11±0.45	38.26±0.48
1250	69.43±0.56	65.02±0.32	49.14±0.58	41.11±0.30
1500	73.10±0.42	68.45±0.22	51.60±0.53	44.23±0.60
1750	77.09±0.49	72.85±0.39	54.51±0.45	46.73±0.25
2000	80.92±0.42	74.34±0.40	58.61±0.38	51.25±0.43

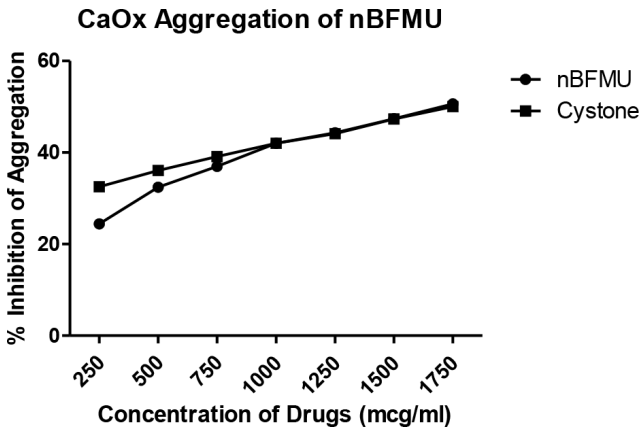

Figure 1: Effect of nBFMU on *in vitro* CaOx crystal aggregation.

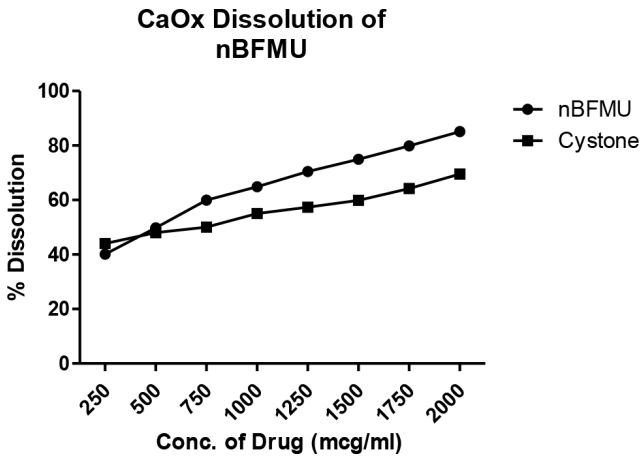

Figure 2: Effect of nBFMU on *in vitro* CaOx crystal dissolution.

Table 5: Effect of nBFMU and cystone on crystal aggregation.		
Conc. of Drug (µg/ml)	% Inhibition of crystal aggregation	
	nBFMU	Cystone
250	24.42±0.28	32.51±0.14
500	32.40±0.23	36.06±0.25
750	36.99±0.24	39.12±0.19
1000	42.02±0.23	42.02±0.23
1250	44.32±0.38	44.10±0.19
1500	47.37±0.18	47.32±0.23
1750	50.60±0.24	50.00±0.19

Effect on various parameter of NaOX model

Effect on relative body and organ weight, urine volume, urinary pH and crystalluria

Administration of NaOX (70 mg/kg, i.p) for 7 days caused significant ($p < 0.001$) reduction in relative body weight and increased relative organ weight in disease group as compared to the normal group. While simultaneous treatment with nBFMU (400 and 800 mg/kg) and cystone (750 mg/kg, std drug) significantly ($p < 0.001$) increased the relative body weight and decreased the

Table 6: Effect of nBFMU and cystone on crystal dissolution.

Conc. of Drug (µg/ml)	% Dissolution of CaOx crystals	
	nBFMU	Cystone
250	40.10±0.78	44.00±0.76
500	49.83±0.61	48.00±0.28
750	59.96±0.49	50.00±0.29
1000	64.83±0.72	55.00±0.86
1250	70.44±0.47	57.40±0.49
1500	74.96±0.66	59.86±0.41
1750	79.83±0.44	64.23±0.43
2000	85.10±0.78	69.56±1.09

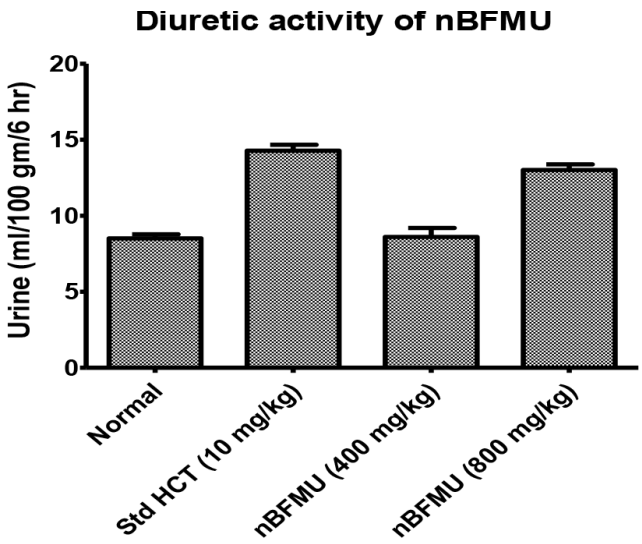

Figure 3: Diuretic effect of nBFMU in rats.

relative organ weight as compared to the control group. Significant ($p < 0.001$) decreased in volume of urine (ml/24hr) was observed in disease control group as compared to normal group, while nBFMU (400 and 800 mg/kg) and cystone (750 mg/kg, std drug) showed significant ($p < 0.001$) improvement in output of urine as compared to disease control group. Furthermore, significant ($p < 0.001$) decrease in urinary *pH* was observed in disease control group, which was significantly ($p < 0.001$) increased in nBFMU (400 and 800 mg/kg) and cystone (750 mg/kg, std drug) group. The data are listed in Table 7. In crystalluria study of urine, CaOX crystals were absent in normal group animals (Figure 4(a)), whereas large size and more number of crystals were observed in disease control group animal urine. (Figure 4(b)). In nBFMU and cystone treated animals urine were showed very less number and small size of CaOX crystals. (Figure 4(d) and 4(e) and 4(c)).

Effect on urinary parameter

Administration of NaOX (70 mg/kg, i.p) for 7 days to Wistar rats resulted in hyperoxyluria. Due to that excretion of various urolithiatic promoters in urine such as oxalate, calcium, urea, uric acid and phosphate levels were significantly ($p < 0.001$) increased in the urine of disease control group and various urolithiatic

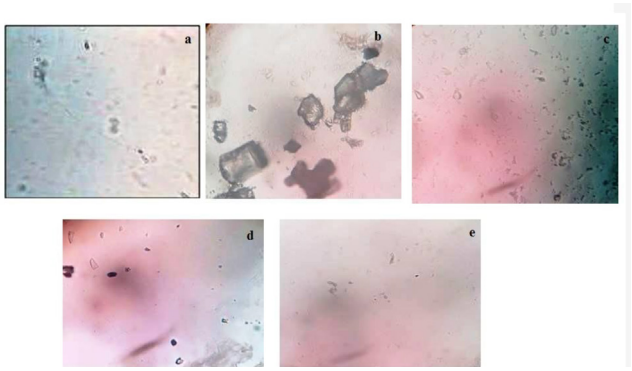

Figure 4: CaOX crystal observed under microscope in 24 hr urine of rat. (a) Normal control group showed absence of crystal (b) Disease control group showed large crystal and (c) Standard group (cystone 750 mg/kg) (d) Treatment group I (400 mg/kg) and (e) Treatment group II (800 mg/kg) showed less number of crystals in urine

Table 7: Effect of nBFMU on body weight and organ weight, urine volume, urinary pH in NaOX induced urolithiasis.

	Group I (Normal control)	Group II (Disease control)	Group II (Standard control)	Group 1V (Treatment Group I)	Group V (Treatment Group II)
Body weight (%)	101.4±0.3	96.16±0.51^{a*}	100.7±0.14^{b*}	103.6±0.38^{b*}	102.5±0.37^{b*}
Relative organ weight (%)	0.49±0.03	0.61±0.03^{a*}	0.52±0.01$^{b\#}$	0.52±0.02^{b*}	0.51±0.01^{b*}
Urine Volume (ml/24 hr)	8.12± 0.42	4.37±0.24^{a*}	11.00±0.21^{b*}	11.55±0.33^{b*}	14.13±0.23^{b*}
Urinary pH	6.76±0.04	5.32±0.04^{a*}	6.45±0.02^{b*}	5.82±0.06^{b*}	6.37±0.06^{b*}

All values are expressed in mean±SEM ($n = 6$), one-way ANOVA followed by Dunnett's test.
* $p < 0.001$ statistically significant
# $p < 0.005$ statistically significant
a compared with normal Group
b compared with disease control group

inhibitors like citrate and magnesium level was found significantly ($p < 0.001$) decreased in the urine and due to renal function impairment, decreased in creatinine clearance in urine of disease control group as compared to normal group. However, supplementation with nBFMU (400 and 800 mg/kg) showed a significant ($p < 0.001$) reduction in urolithiatic promoter and significantly increased urolithiatic inhibitors and creatinine clearance level as compared to the disease control group. Furthermore, treatment groups results were also comparable with the Cystone (750 mg/kg) treated group. The data are listed in Table 8.

Effect on serum parameter

Induction of CaOX stone in renal cause function impairment in renal, resulting in increased glomerular and tubular markers in serum. In the current study, serum calcium, phosphorus, uric acid and urea levels were increased and magnesium level was decreased significantly ($p < 0.001$) in diseased control group when compared with normal group. While simultaneous treatment with nBFMU (400 and 800 mg/kg) showed significantly ($p < 0.001$) decreased calcium, phosphorus, uric acid and urea level where magnesium level was increased when compared to disease control group. Treatment groups results was similar to the standard group (Cystone, 750 mg/kg). The data are listed in Table 8.

Effect on Kidney homogenate parameter

Urolithiatic promoters like oxalate, calcium, phosphate and uric acid level were significantly ($p < 0.001$) increased in kidney of disease control group as

Table 8: Effect of nBFMU on serum, urine and Kidney homogenate parameters in NaOX induced urolithiasis in rats.

	Group I (Normal Control)	Group II (Model Control)	Group II (Standard Control)	Group 1V (Treatment Group I)	Group V (Treatment Group II)
Urine Parameter (mg/24 hr)					
Calcium	2.85±0.19	4.8±0.26^{a*}	3.18±0.10 ^{b*}	3.46±0.13 ^{b*}	2.95±0.07 ^{b*}
Oxalate	4.14±0.17	10.15±0.05 ^{a*}	6.80±0.08 ^{b*}	7.14±0.06^{b*}	5.23±0.23 ^{b*}
Phosphate	4.22±0.11	6.95±0.27 ^{a*}	5.60±0.19 ^{b*}	5.60±0.15 ^{b*}	4.82±0.04 ^{b*}
Uric acid	1.93±0.06	3.89±0.07 ^{a*}	2.16±0.06 ^{b*}	2.31±0.21 ^{b*}	2.01±0.01 ^{b*}
Urea	0.58±0.03	1.36±0.08 ^{a*}	0.80±0.05 ^{b*}	0.86±0.38 ^{b*}	0.70±0.03 ^{b*}
Citrate	21.25±0.92	7.99±0.08 ^{a*}	18.46±0.18 ^{b*}	12.91±0.18 $^{b\#}$	17.32±0.20 ^{b*}
Magnesium	3.10±0.26	1.07±0.07 ^{a*}	2.83±0.11 ^{b*}	1.53±0.04 $^{b\#}$	2.31±0.06 ^{b*}
Creatinine Clearance	36.17±1.51	10.00±2.05 ^{a*}	44.61±2.01 ^{b*}	63.55±3.62 ^{b*}	75.16±5.25 ^{b*}
Serum Parameter (mg/dl)					
Calcium	9.71±0.19	12.26±0.16 ^{a*}	9.97±0.19 ^{b*}	10.77±0.19 $^{b\#}$	10.12±0.25 ^{b*}
Phosphate	4.63±0.51	7.90±0.23 ^{a*}	5.33±0.32 ^{b*}	5.49±0.15 $^{b\#}$	4.85±0.19 ^{b*}
Uric acid	3.68±0.40	6.28±0.18 ^{a*}	4.23±0.25 ^{b*}	3.75±0.32^{b*}	3.05±0.22^{b*}
Urea	12.50±1.03	27.68±0.89 ^{a*}	15.18±0.89 ^{b*}	22.32±0.89 $^{b\#}$	12.50±1.03 ^{b*}
Magnesium	3.30±0.11	1.82±0.08 ^{a*}	2.83±0.11 ^{b*}	2.43±0.06 $^{b\#}$	3.09±0.05 ^{b*}
Kidney Homogenate Parameter					
Calcium (mg/gm tissue)	4.92±0.08	8.14±0.11 ^{a*}	6.03±0.07 ^{b*}	5.68±0.12 ^{b*}	5.1±0.11 ^{b*}
Oxalate (mg/gm tissue)	1.39±0.09	5.91±0.16 ^{a*}	2.41±0.31 ^{b*}	2.49±0.12 ^{b*}	2.15±0.12 ^{b*}
Uric acid (mg/gm tissue)	2.23±0.17	3.90±0.23 ^{a*}	2.59±0.10 ^{b*}	3.61±0.01 $^{b\#}$	3.14±0.03 ^{b*}
Phosphate (mg/gm tissue)	2.95±0.23	5.14±0.31 ^{a*}	3.41±0.13 ^{b*}	3.79±0.16 $^{b\#}$	3.05±0.15 ^{b*}
Catalase (nmoles of H_2O_2 utilized/ min/ mg Protein)	1.69±0.05	0.72±0.05 ^{a*}	1.39±0.11 ^{b*}	1.29±0.05 $^{b\#}$	1.49±0.05 ^{b*}

All values are expressed in mean±SEM ($n = 6$), one-way ANOVA followed by Dunnett's test.
* $p < 0.001$ statistically significant
# $p < 0.005$ statistically significant
a compared with normal Group
b compared with disease control group

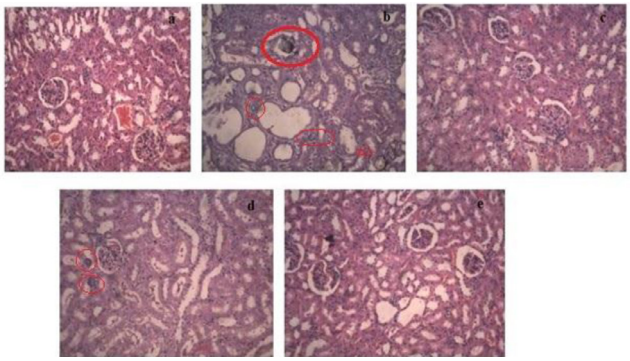

Figure 5: Histology of rat kidney. (a) Normal group (b) Disease control group showed crystal deposition having large size (c) standard group (cystone 750 mg/kg) (d) Treatment group I (400mg/kg) and (e) Treatment group II (800 mg/kg) showed less number of crystal and normal structure of kidney.

compared to normal group. However, those promoters were found to be significantly ($p < 0.001$) decreased in the kidney of treatment groups as compared to the disease control group in dose depended manner. NaOX administration significantly decreased the activity of antioxidant enzyme catalase ($p < 0.001$) in disease control group while in treatment groups significant increase in catalase enzyme activity; provided protection against oxidative changes in the tissue. The data are listed in Table 8.

Effect on Histopathology of Kidney

In normal group, kidney section showed normal architecture of the kidney (Figure 5(a)), while in disease control group due to administration of NaOX agent caused severe damage to the glomeruli, tubules, medulla and interstitial spaces. The presence of CaOX crystals in intratubular space also seen (Figure 5(b)) in disease control group kidney section while in treatment groups sections major damage was recovered and less crystal deposition in intratubular space. (Figure 5(d) and 5(e)). In standard group section crystal deposition was not observed and renal damage was found recovered. (Figure 5(c)).

DISCUSSION

Renal stone formation is a biological process that involves a physicochemical element and crystallisation. An important factor in crystallization is nucleation that leads to crystal growth and crystal aggregation which are responsible for stone formation. Agents that cause inhibition of crystallization and modifiers of these process or decreased oxalate supersaturation are major interest agents for urolithiasis treatment. Various inhibitors can affect crystal nucleation, growth or aggregation.[28] In present study, *in vitro* assay were designed to address the key elements like nucleation, crystal growth and aggregation of the crystal. Nucleation is a first step for the initiation of crystals formation; Park *et al.*[29] reported that severity of stone formation is directly proportional to level of urinary oxalate, as supersaturation levels of ions increase spontaneously crystallize the particles and increased the nucleation formation. As increased in oxalate concentration, nucleation rate was increased. nBFMU inhibited the stone formation by inhibiting the nucleation of CaOX in solution in dose depended manner with respect to the concentration of NaOX. This activity of nBFMU could be its ability to complex with oxalate and calcium ions in solution and decrease supersaturation level. After nucleation, free calcium and oxalate particles which present in fluid, attached to the preformed CaOX crystal and increase in the crystal size. Crystal growth was dependent on the concentration level of oxalate and calcium. Present study showed that nBFMU prevent the crystal growth in dose depended manner, but inhibition rate was found decreased at the same dose of extract with the gradually increase in NaOX concentration. Small crystals were easily excreted in urine but numerous crystals come together and adhere to form large crystal which usually retain in renal tubules and promoting the stone formation. Therefore, the crystal aggregation process is thought be a fundamental step in the renal stone development. nBFMU inhibited the *in vitro* CaOX aggregation in concentration depended manner. Some findings suggested that chemical treatments may be a useful for improving the efficacy of stone treatment via dissolving larger or harder stones. Zhou *et al.*[30] reported that buffered EDTA solvents may be feasible chemical treatment modalities for improving the efficacy of CaOx stone dissolution. In present study, nBFMU was found active in dissolving the CaOX stones in concentration depended manner, which indicate that nBFMU contain some of the components that constitutes to increase the CaOX stone dissolution. Qualitative phytochemical estimation of nBFMU revealed the presence of flavonoids, saponins and phenolic compounds. These phytoconstituents are of ulmost significance for inhibiting kidney stone formation. Saponins possess antilithic properties and are known to disintegrate mucoproteins that are crucial components of stone matrix.[31] Rutin, quercetin, hyperoside and diosmin are known as flavonoids with high antioxidant and anti-lithiatic activities.[32] nBFMU contains 7.52 ± 0.40 mg quercetin equivalents/g flavonoids and 30.32 ± 0.62 mg diosgenin equivalent saponin/g. Therefore, the prevention of nucleation,

crystal growth, crystal aggregationl and crystal dissolving property of nBFMU.

In the *in vivo* animal model, male rats were selected for the induction study because the urinary system of male rates resembles to that of human[33] and earlier studies have also showed that the amount of stone deposition in male rats was significantly higher as compared to the female rats.[34] Lee *et al.*[35] and Iguchi *et al.*[36] have also reported that sex hormone of female has inhibitory effect on renal stone formation in rats. Stones formed in kidneys of rats and humans are identical at the ultra-structural level in nature and composition of their matrix, thus rat models of urolithiasis are helpful experimental tools for exploring the pathphysiology and management of disease. Spontaneous formation of CaOX is very rare in rats, Thus the renal stone is experimentally induced and rats are made hyperoxaluric either by exposure to the toxin ethylene glycol, or by administration of excessive amount of oxalate, or by doing various nutritional manipulations in rat food.[37] Stones with smaller size can easily travel through the urinary system, but larger size stones may lead to the obstruction and pain in renal capillary tube. This could also result in decrease the food consumption. Due to that decrease in the body weight and increased the weight of kidney due to deposition of stone. Present study also revealed a similar pattern with significant reduction in relative body weight and increased the relative organ weight in disease control group. In nBFMU treated rats body weight and organ weights were similar to the normal group animals, that indicate that nBFMU might be prevent the pain.

Many studies showed that administration of NaOX in animals induced the stone formation which caused by hyperoxaluria and subsequent hypercalciuria, which further lead to the increased retention and excretion of oxalate.[38] An increased urinary levels of calcium and oxalate favors the nucleation process and precipitated CaOX attached to renal tubules and create more nucleation centres for new CaOX crystals. Decreased excretion of calcium level from 4.8 ± 0.26 to 2.95 ± 0.07 mg/24 hr and oxalate level from 10.15 ± 0.05 to 5.23 ± 0.23 mg/24 hr upon treatment with nBFMU (800 mg/kg) indicate that nBFMU might be reducing the supersaturation of oxalate and calcium ions in urine and preventing stone formation or growth in kidney.

Urinary pH is the important factor in formation of stone in kidney. At low urinary pH solubility of CaOX stone decrease and promote the CaOX stone formation. In present study urinary pH of disease control group was 5.32 ± 0.04, which indicates that CaOX solubility is minimum and hence urine get supersaturated with oxalate and calcium ions, start the stone formation. nBFMU treated rats showed that significant increase in urinary pH (6.37 ± 0.06, $p < 0.001$), which indicate that nBFMU increase the solubility of CaOX stone and decrease the supersaturation level of ions in urine.

Decrease in the urine volume increases the saturation level of oxalate and start the events of CaOX crystal formation. In nBFMU treated rats urine volume was found significantly increased (14.13 ± 0.23 mg/24 hr, $p < 0.001$) as compared to disease control group (4.37 ± 0.24 mg/24 hr) which decreased the saturation level of ions like oxalate, calcium and prevents the crystal formation. Diuresis effect of nBFMU could be reponsible for fluse out the excessive amount of ions and helped in mechanical expulsion of stone.

Increased excretion of uric acid and inorganic phosphate has been reported in stone formers and also observed in hyperoxaluric rats. High levels of urinary phosphate along with oxalate provides an appropriate environment for formation of calcium phosphate crystals, which further induces CaOX deposition in renal and may be responsible for aggregation of CaOX.[34] Uric acid which interferes CaOX solubility, induces the heterogeneous nucleation of CaOX stone and reduces the natural inhibitory activity of glycosaminoglycans in urine.[39] Glycosaminoglycans blocks the growth site of crystals and preventing or delayed the further development of crystal growth and aggregation.[40] There was significant increase in excretion of inorganic phosphate (6.95 ± 0.27 mg/24 hr) and uric acid (3.89 ± 0.07 mg/24 hr) in urine in the disease control group than normal group (4.22 ± 0.11 and 1.93 ± 0.06 mg/24 hr respectively), that level was significantly decreased in nBFMU treated groups (4.82 ± 0.04 and 2.01 ± 0.01 mg/24 hr respectively, $p < 0.001$), which indicate that nBFMU may prevent the deposition of crystal in renal by preventing calcium phosphate crystals formation and increasing the solubility of CaOX crystals and increased the activity of glycosaminoglycans in the kidney.

Magnesium is considered as urolithiatic inhibitor. It makes complexes with oxalate as well as calcium and reduces the supersaturation of CaOX and as a consequence, the CaOX crystals growth and rate of nucleation were also reduced.[41] In disease control group levels of magnesium was decreased (1.07 ± 0.07 mg/24 hr, $p < 0.001$) but in standard group and nBFMU treated groups magnesium level was increased (2.83 ± 0.11 and 2.31 ± 0.06 mg/24 hr respectively, $p < 0.001$) in urinary samples as compared to disease control group. It indicates that nBFMU increased level of magnesium which makes complexes with oxalate as well as calcium and

reduced the supersaturation of CaOX and prevent the crystallization.

Hypocitraturia is the metabolic abnormality in renal stones patients. Citrate has stone inhibiting action in urine.[34] Citrate binds with the calcium in urine to form a soluble complex and this increases the urine pH.[42] Low urinary pH favored CaOX stone formation. In present study, it was found that, nBFMU treatment significantly increased (17.32 ±0.20 mg/24 hr, $p < 0.001$) the level of citrate as compared to disease control group (7.99 ± 0.08 mg/24 hr) and prevented the risk of stone formation.

In renal stone patients there was decreased in urinary output due to decreases glomerular filtration rate, this leads to the accumulation of waste products in the blood like nitrogenous substance such as urea, creatinine and decrease in creatinine clearance.[43] The administration of nBFMU significantly lowered the serum urea (12.50 ± 1.03 mg/dl, $p < 0.001$) and enhanced creatinine clearance (75.16 ± 5.25 mg/dl, $p < 0.001$) as compared to disease control group (27.68 ± 0.89 and 10.00 ± 2.05 mg/dl, respectively). This effect can be attribute due to diuresis effect of nBFMU.

Histopathology of kidney in calculi induced rats showed irregular shaped crystal of CaOX were deposition inside the tubules which caused the proximal renal tubules dilation along with interstitial inflammation that might be attributed due to excessive amount of oxalate in kidney. Oxalate is the precursor molecule for lipid peroxidation, which reacts with polyunsaturated fatty acids in the cell membrane and damage the renal tissue.[44] Co-treatment with nBFMU decreased the size and number of CaOX crystal deposition in renal tubules and also prevented damages in the calyxes and tubules.

Such rapid and acute diuretic activity of nBFMU may be due to presence of active phytochemicals such as flavonoids, steroids, terpenoids and phenolic compound. Diuretic action is a main part in treatment of renal stone patients, as an increase in the urine volume in urinary system will help to dissolve the CaOX stones and prevent the further retention in renal tissues and flush out the deposits crystals. The lithotriptic effect of nBFMU may be due to presence of saponin and flavonoids which identified by phytoconstituents analysis in the present study. Earlier studies reported that saponin rich plants play very essential role in prevention of stone formation in kidney against agents induced urolithiasis in rats.[45]

CONCLUSION

On the basis of above results and discussion, it can be concluded that, nBFMU has antiurolithiatic activity in NaOX induced urolithiasis by promoting various urolithiatic inhibitors like magnesium and citrate and suppressing various urolithiatic promoters like calcium, oxalate, phosphate in 24 hr urine, serum and renal tissue. In addition, diuretic activity of nBFMU helps to flush out stone promoters in urine and decreased supersaturation level of ions in urine and this prevents new CaOX nuclei formation. Diuretic activity also smooths the surface of CaOX due to that decrease in renal tissue damage and also increased the CaOX stone dissolution. Thus, the current result highlight that the seeds of *M. uniflorum* have potential and beneficial effect in the prevention of CaOX stone formation in kidney. But, for find out to effective chemical constituents and detail mechanism(s) of that component of nBFMU, further studies are necessary.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

BUN: Blood urea nitrogen; **CaOX:** Calcium oxalate; **HCT:** Hydrochlorothiazide; **NaCl:** Sodium chloride; **NaOX:** Sodium oxalate; **nBFMU:** n-butanol fraction of *M. uniflorum*; **LDH:** Lactase dehydrogenase; **ROS:** Reactive oxygen species.

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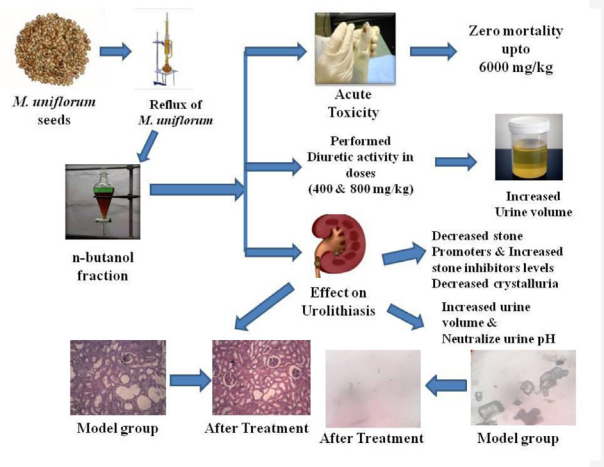
PICTORIAL ABSTRACT



SUMMARY

The investigations indicate that nBFMU fraction rich in saponin content. *In vitro* studies indicate that nBFMU prevent crystal formation and increase the dissolution of CaOX stone. *In vivo* model indicate that nBFMU has diuretic activity, prevent supersaturation of ions in urine and prevent CaOX crystal formation and deposition in kidney, increase CaOx inhibitors level and decrease promoter level in body. All above action indicate that nBFMU having antiurolithiatic activity through multiple pathways.

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