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Antiurolithiatic efficacy of combination preparations of *Dolichos biflorus* and *Crataeva nurvala*: folk medicines used in Indian traditional medicine

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Abstract

Background: In spite of advances in the modern allopathic medicines, there is no satisfactory treatment of kidney stones, so formation and growth of calculi continues to trouble mankind. In India, many herbal formulations are in use for the treatment of urolithiasis. The purpose of the present study was to investigate the antiurolithiatic efficacy of combined extract of plants *Dolichos biflorus* (*D.b*) (hydroalcoholic seed extract) and *Crataeva nurvala* (*C.n*) (aqueous bark extract) in ethylene glycol-induced urolithiasis in Wistar rats. Rats were divided into 4 groups. Ethylene glycol (0.75% v/v, p.o.) was administered for 35 days. Different drug treatments were given from the 21st to 35th day of the study. On the last day, rats were sacrificed, and different samples were taken for further analysis.

Results: Both the combination drug treatments were found to be effective in treating urolithiasis. More significant protection was observed on treatment with the fraction ratio of *D.b* + *C.n* (3:1). Histopathology analysis showed degenerated glomeruli and inflammatory cells in urolithiasis control. The same were regenerated on treatment with combined extract of the two plants.

Conclusion: Administration of the combined plant extracts in a ratio of *D.b* + *C.n* (3:1) possesses better efficacy against ethylene glycol-induced urolithiasis in rats which may be evaluated further for mechanistic pathway elucidation in vivo.

Keywords: Urolithiasis, Ethylene glycol, *Crataeva nurvala*, *Dolichos biflorus*, Herbal formulation

Background

Use of plants has been there in India since the ancient times. These are used normally for treatment of a number of diseases. Nowadays, people are going back to the old treatment strategies [1]. The traditional Ayurvedic approach to health is comprehensive, effective, and promising. One of the most noteworthy contributions of Ayurveda is the science of herbal combinations where traditional medical practitioners prescribe a combination of herbal products for better effect [2, 3]. So, this approach is used in the current study to treat urolithiasis. Worldwide prevalence varies among specific areas with the highest percentage of 7–13% for North America, 5–9% for Europe, and 1–5% for Asian countries [4]. The incidence of urinary stones has been rising over the previous years because of change in lifestyle and food intake habits [5].

The etiology of this disease is multi-factorial and is powerfully related to nutritional lifestyle habits or practices [6]. Although there are few recent reports of beneficial effects of medical treatments in enhancing clearance of calculi in the distal ureters [7], no

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promising antiurolithiatic agent has been reported, especially for the prevention of the recurrence of stones. In this regard, many plants have been conventionally used to treat kidney calculi and have been shown to be effective. In Ayurveda and folk medicine, many herbs are used in management of urolithiasis. Many researchers throughout the world are working on ascertaining the potential of herbs in treatment of this disorder. Some of the Indian medicinal plants having antiurolithiatic potential are *Sesbania grandiflora* L. Pers [8], *Aerva lanata* L. Juss. Ex. Schult. [9], *Moringa oleifera* Lam. [10], *Asparagus racemosus* Willd. [11], *Rotula aquatica* Lour. [12], *Cyclea peltata* (Lam.) Hook F. & Thoms [11], *Tribulus terrestris* L. [13], *Musa sapienta* L. [14], *Ammannia baccifera* L. [15], *Mimosa pudica* L. [16], *Crataeva nurvala* Buch-Ham. [17], etc.

Crataeva nurvala (Capparidaceae) is an evergreen tree indigenous to India [18–20]. It is prevalently found grown in other countries namely Bangladesh, Pakistan, Philippines, South America, China, and Africa. It is a leafy, moderate-sized deciduous tree with soft wood and fragrant whitish to milky white colored, polygamous flowers. The fruit of this medicinal tree is berry with globe shape and woody rind embedding seeds in yellow pulp. The outer surface of bark is wrinkled and grey white in color, covered with large number of lenticels. The flowering and fruiting season of this tree is December–May and June–August. Traditionally, its bark is used as demulcent, tonic, stomachic, laxative, diuretic, antipyretic, and rubefacient whereas roots are lithotropic and laxative [21].

Dolichos biflorus (Fabaceae) is a slender, trailing or sub-erect, branched, and downing herb, native to India and is found at an altitude of up to 1000 m. It is mainly cultivated in Andhra Pradesh, Tamil Nadu, and Karnataka [22]. This plant has alternate, stipulate and trifoliate leaves, membranous leaflets, and axillary, papilionaceous usually yellow (or white) flowers. The flowers may be more than one together but without a common peduncle. The pods are 3.7–5.0 cm by 0.6–0.8 cm, recurved, tipped with a persistent style. The number of seeds is 5–6 per pod. Traditionally, young plant is used mainly for kidney disorders, dysuria, sores, and tumors. Seeds of the plant are used as diuretic, spasmolytic, for treatment of urinary trouble, kidney stones, piles, pain, constipation, wounds, urinary calculi, cough, edema, and asthma. The soup of seeds is beneficial in enlarged liver and spleen and menstrual complaints whereas aqueous extract of the seeds is given to woman after child birth [23].

The current study was initiated with the aim of evaluating the efficacy of combined extract of *Dolichos biflorus* and *Crataeva nurvala* on kidney functioning using ethylene glycol-induced urolithiasis in experimental animals. The main aim of the study is to provide a better efficacious drug which is having lesser or no side-effects and a better treatment option for patients suffering from urolithiasis.

The current study highlighted the efficacy of traditionally used medicinal herbs' use in treatment of urolithiasis which may prove beneficial as very less number of effective medicines are there, and no proven drug therapy is in use currently. Besides, the study may be further extended for the drugs' mechanistic pathway delineation and determine proper functioning of the drug after administration in vivo.

Methods

Reagents and chemicals

S. No.	Chemicals	Source
1.	Ammonia	Rankem Pvt. Ltd.
2.	Ammonium chloride	Renkem Pvt Ltd.
3.	Benedict's reagent	Himedia Co. Ltd.
4.	Benzene	SD Fine Chemicals Pvt. Ltd.
5.	Chloroform	SD Fine Chemicals Pvt. Ltd.
6.	Conc. H_2SO_4	SD Fine Chemicals Pvt. Ltd.
7.	Copper sulphate	Himedia Co. Ltd.
8.	Ethanol	SD Fine Chemicals Pvt. Ltd.
9.	Ethylene glycol	SD Fine Chemicals Pvt. Ltd.
10.	Fehling's solution A and B	Himedia Co. Ltd.
11.	Ferric chloride	Himedia Co. Ltd.
12.	Formalin solution	SD Fine Chemicals Pvt. Ltd.
13.	Hydrochloric acid	Renkem Pvt Ltd.
14.	Lead acetate	Renkem Pvt Ltd.
15.	Mayer's reagent	Renkem Pvt Ltd.
16.	Ninhydrin solution	Renkem Pvt Ltd.
17.	Nitric Acid	Renkem Pvt Ltd.
18.	Normal Saline	SD Fine Chemicals Pvt. Ltd.
19.	α-naphthol	Renkem Pvt Ltd.
20.	Potassium Permanganate	Himedia Co. Ltd.
21.	Saturated $CaSO_4$	Himedia Co. Ltd.
22.	Sodium chloride	Himedia Co. Ltd.
23.	Sodium glyoxalate	SD Fine Chemicals Pvt. Ltd.
24.	Sodium hydroxide	SD Fine Chemicals Pvt. Ltd.
25.	Tween 80	SD Fine Chemicals Pvt. Ltd.

Raw materials and extraction

The dried stem bark of *Crataeva nurvala* and seeds of *Dolichos biflorus* were collected from local market of Kurukshetra. Botanical authentication of the plant parts was carried out at NISCAIR, New Delhi, by Dr. H. B. Singh where voucher specimens of plants have been

deposited in the Herbarium & Museum, NISCAIR, (National Institute of Science Communication and Information Resources), New Delhi (NISCAIR/RHMD/Consult/-2011-12/1926/226). The powdered material was passed through sieve no. 40 and then extracted with hydro-alcohol (30:70) for *D. biflorus* seeds (700 g) and distilled water for *C. nurvala* stem bark (800 g) using Soxhlet-apparatus (according to the solubility characteristics of individual material). The extracts were then subjected to solvent evaporation under reduced pressure using rotary evaporator for complete drying. Two ratios of the extract dose (500 mg/kg) were taken for the experiment, i.e., *D.b* + *C.n* (1:1 and 3:1).

Preliminary phytochemical screening of extracts

Both plant extracts were subjected to qualitative chemical analysis in order to detect the presence of various classes of phytoconstituents as per reported methods [22, 23]. Different phytochemicals evaluated were proteins, alkaloids, glycosides, carbohydrates, terpenoids, saponins, and flavonoids.

Selection of animals

Wistar rats of either sex (200–250 g) were obtained from central animal house of the university. They were housed in institutional animal house providing 12 h light and dark cycle at 28 °C ± 2 °C. The Institutional Animal Ethics Committee (IAEC) approved (Letter no. 340A/19) the experimental protocol, and care of laboratory animals was taken as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Govt. of India. After the acclimatization period of 1 week, all the animals were maintained on standard laboratory diet and tap water (with glycolated water) ad libitum.

Methodology

Twenty four Wistar rats were divided into 4 groups ($n = 6$). For the first 3 days of the experiment, ammonium chloride (1%) was administered intra-abdominally along with ethylene glycol (0.75% v/v, p.o.) to hasten the stone formation process [24].

Group I [urolithiasis-control] received normal diet and ethylene glycol (0.75%) only (without extract or standard drug) dissolved in water for 35 days. The animals received tween-80 (5%) from the 21st to 35th day. Group II [cystone (750 mg/kg) (standard)] received normal diet and ethylene glycol dissolved in water from the 1st to 20th day. Cystone (750 mg/kg) was administered orally to rats from the 21st to 35th day along with ethylene glycol administration in water. Group III [*D.b* + *C.n*: 1:1] and group IV [*D.b* + *C.n*: 3:1] received normal diet and ethylene glycol dissolved in water from the 1st to 35th days and respective ratio of extracts from the 21st to 35th day along with ethylene glycol.

Urine analysis

All animals were kept in metabolic cages, and urine samples were collected on the 1st, 7th, 14th, 21st, 27th, and 35th day to analyze the changes in the urinary variables like urinary output and urinary oxalate concentration. Animals had free access to drinking water during the urine collection period. One drop of concentrated hydrochloric acid was added to the urine before being stored at 40 °C [24]. On the 35th day, all the rats were euthanized using pentobarbitone sodium (80 mg/kg), and blood was withdrawn. Serum was separated by centrifugation at 10,000 × *g* for 10 min and analyzed for creatinine and calcium. Serum creatinine was estimated by the methods of Bonsnes and Taussky [25].

Histopathological analysis

The kidney tissues were cut longitudinally and processed for hematoxylin and eosin staining. Briefly, the longitudinal sections were fixed by neutral-buffered formalin (10%) and subsequently embedded in paraffin. Then, the kidney tissue sections (7-μm thick) were stained by hematoxylin and eosin dyes to study the morphological changes in the kidney tissues.

Statistical analysis

All the data was presented as mean ± SEM. The data was analyzed by one-way ANOVA followed by Dunett's *t* test. $p \leq 0.05$ was considered statistically significant.

Results

Preliminary phytochemical screening

The results of phytochemical screening showed the presence of a number of secondary metabolites including carbohydrates, alkaloids, proteins, and flavonoids (Table 1). Highest concentration of terpenoids; slightly less alkaloid, flavonoids, and

Table 1 Phytochemical analysis of *Dolichos biflorus* seeds and *Crataeva nurvala* stem bark

Phytoconstituents	Plants	
	Dolichos biflorus	*Crataeva nurvala*
Alkaloids	-	++
Glycosides	+	--
Flavonoids	++	++
Carbohydrates	+	++
Proteins	++	
Saponins	-	+
Terpenoids	-	+++

Table 2 Effect of combinations of *Dolichos biflorus and Crataeva nurvala* (*D.b* + *C.n*) in urinary output in ml/24 h

S. No	Groups	Urine output (ml)					
		0 days	7 days	14 days	21 days	28 days	35 days
1.	**Urolithiasis control**	10.2 ± 0.45	11.25 ± 0.27	8.89 ± 0.31	7.49 ± 0.38	6.55 ± 0.30	4.67 ± 0.56
2.	**Cystone (750 mg/kg)**	12.36 ± 0.32	11.71 ± 0.31	10.36 ± 0.24*	9.16 ± 0.12	12.71 ± 0.24**	12.32 ± 0.17**
3.	***D.b* + *C.n* (1:1)**	11.17 ± 0.17	10.69 ± 0.24	9.02 ± 0.56	8.05 ± 0.37	9.69 ± 0.32**	10.71 ± 0.45**
4.	***D.b* + *C.n* (3:1)**	11.71 ± 0.52*	10.89 ± 1.05	9.12 ± 0.21	8.39 ± 1.56	9.82 ± 0.76**	11.39 ± 0.33**

Values are expressed as mean ± S.E.M for six animals in each group. One-way ANOVA followed by Dunnett's *t* test, where $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ as compared to urolithiasis control

carbohydrate content; and very less saponin-content were found in *Crataeva nurvala* extract. No glycosides were found in the extract of the tree bark.

In case of *Dolichos biflorus* seeds, moderate concentrations of proteins and flavonoids were found to be present whereas little glycosides and carbohydrate content were also found in the pulse whereas no traces of terpenoids and saponins were found on evaluation of the seed extract (Table 1).

Urine output

Urinary output is decreased during the induction period of the experimental study, i.e., 1–20 days in all the groups (Table 1). In urolithiasis-control group (10.2 ± 0.45 to 7.49 ± 0.38 ml on the 21st day and 4.67 ± 0.56 ml on the last day of the study), the urinary flow reduced due to the formation of crystals in the kidneys whereas the urinary output of standard group was found to be increased in the last 2 weeks of the study (9.16 ± 0.12 ml on the 21st day to 12.32 ± 0.17 ml on last day of the study). In both the test groups, urinary output was increased significantly as compared with the urolithiasis-control group (10.71 ± 0.45 ml and 11.39 ± 0.33 ml for *D.b* + *C.n* (1:1) and *D.b* + *C.n* (3:1) respectively at the end of the study). From the interpretation of above results, it was found that the treatment groups significantly normalized the urinary output as compared to the urolithiatic control group comparable to the standard drug treatment (Table 2).

Oxalate concentration

Oxalate concentration was found to be increased in all the groups during the induction period (Table 3). In the urolithiatic control group, it rose throughout the study (terminal day concentration = 0.98 ± 0.28 mg/dl). When combined extract treated rats' urinary oxalate levels were evaluated, it was found that there was no significant change when compared to the disease control group values, although the concentration of oxalate was found to be reduced with combination treatment in ratio 1:1 with a mean oxalate concentration of 0.83 ± 0.17 mg/dl as compared to the combined-extract ratio 3:1 obtaining a mean concentration value of urinary oxalate as 0.85 ± 0.52 mg/dl.

Effect on creatinine level

The results of serum creatinine level evaluated in experimental animals have been mentioned in Table 4. In urolithiasis control, increased creatinine level was found whereas in the standard and test groups it was increased in the starting first 2 weeks but decreased in the last 2 weeks of the experiment, but results were insignificant. So, it may be suggested that for a change in concentration of the oxalate levels of urine, a longer duration of treatment with the test drug combination will be needed in order for it to be effective and normalize the oxalate levels.

Effect on calcium level

Serum calcium level was also determined to check the concentration of calcium in rat blood (Table 5). The concentration of calcium increased in all the animals during the induction period, and after drug treatment, there was a decrease in the levels of serum calcium as compared to the urolithiasis control (13.24

Table 3 Effect of combinations of *Dolichos biflorus and Crataeva nurvala* (*D.b* + *C.n*) on urinary oxalate level

S. No.	Groups	Urinary oxalate (mg/dl)					
		0 days	7 days	14 days	21 days	28 days	35 days
1.	**Urolithiasis control**	0.63 ± 0.33	0.68 ± 0.27	0.95 ± 0.21	0.95 ± 0.38	0.96 ± 0.30	0.98 ± 0.28
2.	**Cystone (750 mg/kg)**	0.61 ± 0.17	0.69 ± 0.25	0.95 ± 0.32	0.97 ± 0.37	0.83 ± 0.34	0.82 ± 0.27
3.	***D.b* + *C.n* (1:1)**	0.64 ± 0.21	0.67 ± 0.56	0.90 ± 0.28	0.96 ± 0.33	0.87 ± 0.26	0.83 ± 0.17
4.	***D.b* + *C.n* (3:1)**	0.60 ± 0.33	0.63 ± 0.26	0.89 ± 0.34	0.97 ± 0.41	0.88 ± 0.27	0.85 ± 0.52

Values are expressed as mean ± S.E.M for six animals in each group. One-way ANOVA followed by Dunnett's *t* test

Table 4 Effect of combinations of *Dolichos biflorus and Crataeva nurvala* (*D.b* + *C.n*) on serum creatinine level (mg/dl)

S. No.	Groups	Serum creatinine (mg/dl)					
		0 days	7 days	14 days	21 days	28 days	35 days
1.	**Urolithiasis control**	0.24 ± 0.01	0.23 ± 0.06	0.25 ± 0.05	0.28 ± 0.04	0.26 ± 0.98	0.26 ± 0.71
2.	**Cystone (750 mg/kg)**	0.23 ± 0.06	0.2 ± .040	0.23 ± 0.08	0.22 ± 0.08	0.2 ± 0.06	0.20 ± 0.01
3.	***D.b* + *C.n* (1:1)**	0.24 ± 0.79	0.22 ± 0.08	0.24 ± 0.02	0.28 ± 0.01	0.25 ± 0.21	0.24 ± 0.69
4.	***D.b* + *C.n* (3:1)**	0.23 ± 0.01	0.21 ± 0.02	0.23 ± 0.04	0.26 ± 0.07	0.22 ± 0.89	0.22 ± 0.21

Values are expressed as mean ± S.E.M for six animals in each group. One-way ANOVA followed by Dunnett's *t* test

± 0.02 mg/dl for disease control whereas 8.12 ± 0.05 mg/dl, 11.94 ± 0.08 mg/dl, and 9.2 ± 0.03 mg/dl for standard and both the test drug combinations respectively at the end of the study). All the three treatments were found to reduce the serum calcium levels significantly. Ratio 3:1 of *D.b* + *C.n* (9.2 ± 0.03 mg/dl) showed better results than ratio 1:1 (11.94 ± 0.08 mg/dl) and were comparable to the standard group (8.12 ± 0.05 mg/dl). These results indicate that the combination of the two test drugs shows more effective protection when the concentration of *D.b* is more, i.e., in the ratio of *D.b* + *C.n* (3:1) against ethylene glycol-induced urolithiasis.

Histopathological assessment

Histopathological studies were done to confirm the efficacy of the combined extracts (Fig. 1) in urolithiasis control group, the tubules show high degree of degeneration, and the glomeruli were seen to be disarranged and dilated. Tissue damage was more in the disease control group kidney tissues as compared to the treatment group. In standard group, very less crystal-deposition was observed, and tissue damage was also reduced. There found to be regeneration of the tubular tissues in group III rats receiving ratio (1:1) of *D.b* + *C.n*. Some tissue damage was there, and the glomeruli were also not properly regenerated. In group IV rats receiving ratio 3:1, the glomeruli were found to have intact shape of the glomerulus, and there seem to be less amount of injury to the tubules and other parts of the tissue.

Discussion

Formation of calculi is associated with supersaturation of urine with stone forming constituents. The environment of urinary system becomes more susceptible for formation of calcium stones when there is abundance of oxalate and calcium in the body [6, 26]. Researchers have proved that repeated administration of ethylene glycol (0.75% v/v) causes generation of kidney stone probably due to the presence of calcium oxalate [25]. Increase in the urinary oxalate and calcium concentration (chronic calciuria) is considered as one of the main reason responsible for formation of calculi [25, 26]. Oxalate metabolism is disturbed by ethylene glycol as it increases the substrate availability, which raises the activity of oxalate-synthesizing enzymes. Catalysis of oxidation by glycolic acid oxidase and glyoxalate reduction result in the formation of glycolate and oxalate [27]. In present study, it was observed that the combination of *D.b* + *C.n* at both the ratios 1:1 and 3:1 decreased the oxalate level in urine at the selected dose and also increased the urinary output though the urinary oxalate levels were not so significantly reduced or normalized. Hence, it provides protection against urolithiasis by decreasing levels of causative factor for calculi in kidney. Decrease in glomerulus filtration due to obstruction in kidney causes amassing of waste products in blood; thus, levels of waste components like creatinine increase in blood [27, 28]. In the present investigation, it was observed that levels of serum creatinine in test group taking the combined extract in a ratio 3:1 were comparable to that of standard group. The levels of serum calcium were also significantly reduced in case of drug treatment group (ratio 3:1). The test drug combination of *D.b* + *C.n* at a ratio 3:1 was found to be

Table 5 Effect of combinations of *Dolichos biflorus and Crataeva nurvala* (*D.b* + *C.n*) on serum calcium level (mg/dl)

S. No.	Groups	Serum calcium (mg/dl)					
		0 days	7 days	14 days	21 days	28 days	35 days
1.	**Urolithiasis control**	10.96 ± 0.04	11.60 ± 0.14	12.21 ± 0.05	12.63 ± 0.02	12.26 ± 0.01	13.24 ± 0.02
2.	**Cystone (750 mg/kg)**	10.01 ± 0.02	10.08 ± 0.12**	9.94 ± 0.17**	9.64 ± 0.11**	8.78 ± 0.14**	8.12 ± 0.05**
3.	***D.b* + *C.n* (1:1)**	10.82 ± 0.14	11.10 ± 0.153	11.41 ± 0.09	12.21 ± 0.03*	12.01 ± 0.11	11.94 ± 0.08**
4.	***D.b* + *C.n* (3:1)**	10.26 ± 0.01	10.84 ± 0.11**	10.98 ± 0.14**	10.56 ± 0.11**	10.21 ± 0.08**	9.2 ± 0.03**

Values are expressed as mean ± S.E.M for six animals in each group. One-way ANOVA followed by Dunnett's *t* test, where *$p \leq 0.05$ and **$p \leq 0.01$ as compared to control

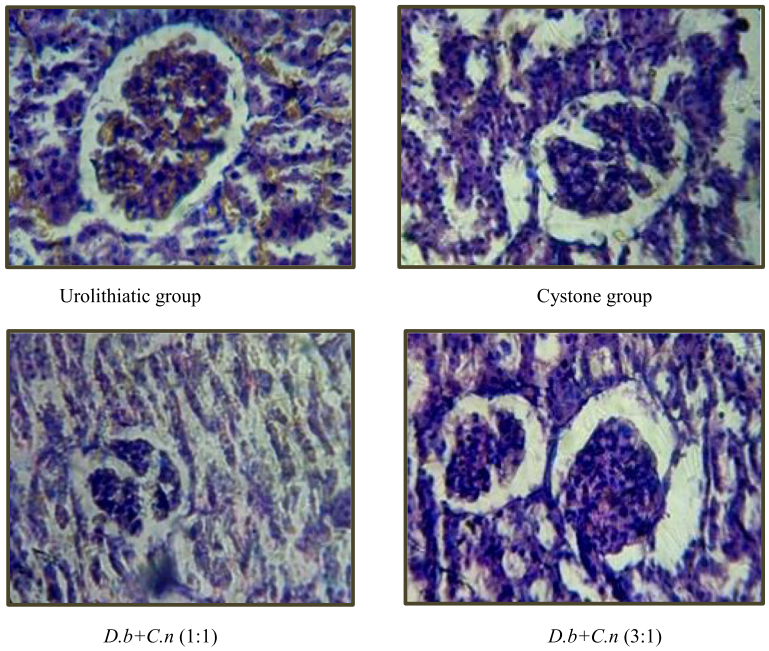

Fig. 1 Histopathological analysis of kidneys of rats in ethylene glycol-induced urolithiasis

more effective in normalizing the tested parameters which have direct or indirect effect on urolithiasis. There are several phytochemicals like flavonoids, terpenoids, and saponins, which could be responsible for the antiurolithiatic effect of the drugs under study [8, 9, 29]. The preliminary phytochemical analysis of the extracts of the plants has shown the presence of flavonoids, saponins, and terpenoids; hence, antiurolithiatic activity of combination preparations could be due to the presence of these constituents, which may act either individually or in combination. The histopathological changes reveal the protective effect of the combined extract with regeneration of tissues and normalization of glomeruli in the treated groups. On the basis of above results, it may be implicated that both of these drugs have potential value in treatment of urolithiasis, and these can be studied further for a longer duration to evaluate the changes in oxalate and calcium levels in the body and also for mechanistic study of the protective pathways underlying the protective effect of these extracts against urolithiasis.

Conclusion

From the present study, it is concluded that the ratio 3:1 of combined formulation of hydroalcoholic extract of *Dolichos biflorus* and aqueous extract of *Crataeva nurvala* possess better potency against urolithiasis. This report may offer therapeutic alternative for the treatment of urolithiasis. The observed antiurolithiasis effects probably involve the synergistic or additive action of phytoconstituents of combined plants extracts.

Abbreviations

C.n: *Crataeva nurvala*; *D.b*: *Dolichos biflorus*; IAEC: Institutional Animal Ethics Committee; CPCSEA: Committee for the Purpose of Control and Supervision on Experiments on Animals; v/v: Volume by volume; p.o.: Per oral; SEM: Standard error of mean; ANOVA: Analysis of variance

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Plant authentication

Botanical authentication of the plant parts was carried out at NISCAIR, New Delhi, by Dr. H. B. Singh where voucher specimens of plants have been deposited in the Herbarium & Museum, NISCAIR, (National Institute of Science Communication and Information Resources), New Delhi (NISCAIR/RHMD/Consult/-2011-12/1926/226).

Authors' contributions

SK drafted the work, revised it, and analyzed the results. MC designed the study and done substantial contribution in analysis of the data. SR made substantial contribution in acquisition of raw material and revision of the study. All the authors have read and approved the manuscript.

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Availability of data and materials

All data and material are available upon request.

Ethics approval and consent to participate

Laboratory animals were obtained from the Institutional Central Animal Facility (Letter no. 340A/19), Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra, Haryana, as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) Govt. of India. The animal experiments were duly approved from IAEC (Institutional Animal Ethical Committee).

Consent for publication
NA

Competing interests
No competing interests to declare.

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