Journal of Pharmaceutical Research Vol. 9, No. 2, April 2010 : 70-75.

EVALUATION OF *Ruellia tuberosa* L. FOR ANTIUROLITHIATIC AND ANTIOXIDANT ACTIVITIES IN RATS

Sailaja B, Bharathi K and Prasad KVSRG*
Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam (Women's University), TIRUPATI-517 502, India.

Received on : 26.08.2009 Revised : 20.03.10 Accepted : 22.03.10

ABSTRACT

The roots of *Ruellia tuberosa* L. are recommended for kidney stone disorders in the Indian traditional system of medicine. Ethanolic extract of *R. tuberosa* roots was evaluated for antiurolithiatic activity against 0.75% v/v ethylene glycol and 2% w/v ammonium chloride induced calcium oxalate urolithiasis and for antioxidant activity against hyperoxaluria induced oxidative stress in male albino rats. Ethylene glycol and ammonium chloride administration increased the deposition of calcium and oxalate in the kidneys, urinary excretion of calcium, oxalate and creatinine in the preventive and curative control rats. In these groups, increased levels of malondialdehyde and depleted levels of antioxidant enzymes, reduced glutathione and catalase were observed. On treatment with the extract, a significant reduction in the deposition of calcium, oxalate and also urinary excretion of calcium, oxalate and creatinine was observed, indicating its antiurolithiatic effect. The extract administration also decreased the extent of lipid peroxidation and enhanced the levels of antioxidant enzymes in the kidneys of urolithic rats, reflecting its antioxidant efficacy against hyperoxaluria induced renal oxidative stress. Results of the present study support the traditional claim of *R. tuberosa* roots in treating renal calculi.

Keywords: *Ruellia tuberosa*; *Calcium oxalate urolithiasis; Ethylene glycol/Ammonium chloride; Oxidative stress; Antiurolithiatic and Antioxidant activities.*

INTRODUCTION

Kidney stones affect up to 5% of the world population[1]. In normal conditions, Oxalate (Ox), an end product of metabolism is excreted in urine. However, under certain pathological conditions, Ox interacts with calcium within the renal tubular lumen to form calcium oxalate (CaOx)[2]. In addition, studies show that renal cells on exposure to Ox and/or CaOx crystals generate reactive oxygen species (ROS), develop oxidative stress (OS) and associated cellular injury[3].

In spite of advances in the present practice of medicine, the formation and growth of calculi continues to trouble mankind as there is no satisfactory drug to treat kidney stones. In India, many indigenous drugs are in use for the treatment of urinary calculus disease. The plant *Ruellia tuberosa* L. (Acanthaceae), commonly known as Minnier root is widely used in the Indian traditional system of medicine. The roots are reported to be useful in the treatment of urinary stones[4]. The plant is also used as a diuretic, antidiabetic and antipyretic[5]. Hence, the present study was planned to evaluate the roots of *R. tuberosa* for antiurolithiatic and antioxidant activities as a preventive and curative against ethylene glycol and ammonium chloride induced CaOx kidney stones and hyperoxaluria induced renal OS.

MATERIALS AND METHODS

Plant material

R. tuberosa plant was collected from Thummalagunta, Tirupati during August-September of 2007. The plant was authenticated by Dr. K. Madhavachetty, Department of Botany, Sri Venkateswara University, Tirupati. The voucher specimen was preserved at Institute of Pharmaceutical Technology, Sri Padmavathi Mahila Visvavidyalayam, Tirupati.

Preparation of the extract

About 500 g of the shade dried and coarsely powdered *R. tuberosa* roots was extracted with 2 liters of 95% ethanol by cold maceration for 12 h followed by refluxing over a water bath for 4 h at 70°C. The extract thus obtained was filtered and the procedure was repeated thrice. The combined filtrates were concentrated to a semisolid consistency under reduced pressure (yield 15.5% w/w) and the resultant semisolid extract was stored in a refrigerator (8°C) until further use. A 10 % w/v aqueous suspension was prepared from the extract daily before administration to rats. The extract was subjected to preliminary phytochemical testing.

Experimental animals

Healthy adult male albino rats of Wistar strain weighing 150-200g were used in the present study. The rats were

***Correspondence :** kvsrgprasad@yahoo.co.in

housed in polypropylene cages under hygienic conditions and maintained on standard pellet diet (Gold Mohur, Bangalore) and water *ad libitum*. The animals were acclimatized and maintained at 25±2°C in a well ventilated room under natural photoperiodic conditions throughout the study. Animal Ethical norms were followed during all experimental procedures and all experimental protocols were approved by Institutional Animal Ethical Committee.

Acute toxicity and gross behavioral changes study

Rats were divided into five groups of six animals each and were fasted overnight with free access to drinking water. Group I animals served as normal and received distilled water (10 ml/kg, orally). Group II to V animals received 0.5, 1.0, 2.0 and 4.0 g/kg of ethanolic extract of *R. tuberosa* (ERT) respectively, orally by gastric intubation using a soft catheter. After administration of the extract, the animals were observed continuously for 2 h and then intermittently at one hour interval up to 4 h and at the end of 48 h the number of deaths was recorded to calculate LD_{50}[6]. The animals were also observed for gross behavioral changes during acute toxicity studies[7].

Antiurolithiatic activity

Induction of urolithiasis

In the present study, CaOx urolithiasis was induced in rats by free access to drinking water containing 0.75% (v/v) ethylene glycol (EG) and 2% (w/v) ammonium chloride (AC) for 15 days[8].

Study protocol

The rats were divided into seven groups consisting of six per group and were put on the following treatment.

Group I: **Normal** – Received distilled water 10 ml/kg, orally and drinking water *ad libitum* from 1-15th day.

Group II: **Preventive control** - Received distilled water 10 ml/kg, orally and drinking water containing EG and AC *ad libitum* from 1- 15th day.

Group III: **Preventive treated** - Received ERT 0.5 g/kg, orally and drinking water containing EG and AC *ad libitum* from 1-15th day.

Group IV: **Preventive treated** - Received ERT 1.0 g/kg, orally and drinking water containing EG and AC *ad libitum* from 1-15th day.

Group V: **Curative control** - Received drinking water containing EG and AC *ad libitum* from 1- 15th day and distilled water 10 ml/kg, orally from 16-30th day.

Group VI: **Curative treated** - Received drinking water containing EG and AC *ad libitum* from 1-15th day and ERT 0.5 g/kg, orally from 16-30th day.

Group VII: **Curative treated** - Received drinking water containing EG and AC *ad libitum* from 1-15th day and ERT 1.0 g/kg, orally from 16-30th day.

Evaluation of urinary parameters

Rats were hydrated with 5 ml of distilled water orally, placed in separate metabolic cages and 24 h urine samples were collected from overnight fasted rats on day 15 from normal, preventive control and preventive treated groups. Whereas, from curative control and curative treated groups, urine samples were collected on day 30. The samples were centrifuged at 2,500 rpm at 30±2°C for 5 min. The supernatant was used to determine pH and quantitative estimation of calcium[9], oxalate[10] and creatinine[11].

Kidney homogenate analysis

The rats were sacrificed by cervical decapitation after respective treatment schedules. Kidneys were perfused with ice-cold saline (0.9% w/v sodium chloride) and carefully isolated. One kidney from each animal was washed in ice-cold 0.15 M KCl and weighed. Then a 10% w/v of the kidney homogenate was prepared in 10 N HCl. The homogenate was centrifuged at 2,500 rpm at 30±2°C for 3 min and the supernatant was used to estimate calcium[9] and oxalate[10].

Assessment of oxidative stress (OS)

The other kidney was washed with chilled normal saline and weighed. A 10% w/v homogenate was prepared in ice cold phosphate buffer (pH 7.4) using a glass homogenizer. The homogenate was centrifuged at 800 rpm at 4°C for 5 min to separate nuclear debris. The resultant supernatant was centrifuged at 10,000 rpm at 4°C for 20 min to get post mitochondrial supernatant (PMS), which was used to estimate lipid peroxidation as malondialdehyde (MDA)[12], and antioxidant enzymes, reduced glutathione (GSH)[13] and catalase[14].

***In vitro* antioxidant studies**

DPPH free radical scavenging

The free radical scavenging activity of the extract was examined *in vitro* using 1,1- diphenyl-2-picrylhydrazyl (DPPH) stable free radical[15]. Solutions of the extract at different concentrations 100, 200, 400, 800 and 1000 µg/ml were added to 100µM of DPPH in ethanol, the tubes were kept at ambient temperature for 20 min and the absorbance was measured at 517 nm. The difference in absorbance between the control and the test was taken and expressed as percentage scavenging of the DPPH by the extract. Ascorbic acid was used as the standard. Results were expressed as means of triplicates. IC_{50} was also determined for the extract.

Nitric oxide free radical scavenging

Sodium nitroprusside (10mM) in phosphate buffer (pH 7.7) was incubated with 100, 200, 400, 800 and 1000 µg/ml of the extract dissolved in ethanol in triplicate, and the tubes were incubated at 25ºC for 120 min. After incubation, 0.5 ml of the reaction mixture was diluted with 0.5 ml of Griess reagent (2% *o*-phosphoric acid, 1% sulphanilamide, 0.1% N-napthylethylenediamine). Ascorbic acid was used as the standard. The absorbance of the pink chromophore formed during diazotization of nitrite with sulphanilamide and

subsequent coupling with N-napthylethylenediamine was measured at 546 nm against the corresponding blank solution. The degree of free radical scavenging in the presence and absence of different concentrations of the extract was measured. The difference in absorbance between the control and the test was taken and expressed as percentage free radical scavenging of the NO by the extract[16]. IC_{50} was also determined for the extract.

Statistical analysis

The results were expressed as mean ± SEM. The inter-group variation was measured by one way analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons and statistical significance was considered at *P*d" 0.01.

RESULTS

Preliminary phytochemical studies

On preliminary phytochemical screening, the extract showed the presence of flavonoids, tannins, saponins, steroids and carbohydrates.

Acute toxicity and gross behavioral changes

R. tuberosa extract was found to be safe, as no animal died up to 4.0 g/kg orally and there were no gross behavioral changes except for an increase in urination.

Antiurolithiatic activity

Kidney weight

On EG/AC administration, a significant (*P*<0.001) increase in kidney weight was observed in both the preventive and curative control groups, when compared to the normal group. On treatment with ERT for 15 days, a dose dependent significant reduction in the kidney weight was observed in both the preventive and curative treated groups, when compared to their respective controls (Table 1).

Table 1. *Effect of ERT on kidney weight, deposition of calcium and oxalate in the kidney*

Group	Treatment	Kidney weight (g/100g)	Calcium (mg/g kidney tissue)	Oxalate (mg/g kidney tissue)
I	Normal	0.38 ± 0.02	0.32 ± 0.02	0.95 ± 0.09
II	Preventive control	0.48 ± 0.02[a]	0.95 ± 0.02[a]	3.74 ± 0.18[a]
III	ERT 0.5g/kg	0.37 ± 0.02[c]	0.51 ± 0.02[c]	2.04 ± 0.04[c]
IV	ERT 1.0g/kg	0.36 ± 0.02[c]	0.42 ± 0.02[c]	1.65 ± 0.06[c]
V	Curative control	0.44 ± 0.02[b]	0.93 ± 0.02[b]	3.65 ± 0.17[b]
VI	ERT 0.5g/kg	0.35 ± 0.02[d]	0.79 ± 0.03[d]	2.21 ± 0.05[d]
VII	ERT 1.0g/kg	0.34 ± 0.02[d]	0.62 ± 0.02[d]	1.91 ± 0.05[d]

Values are expressed as mean±SEM, n=6
a-P<0.001, b-P<0.01 compared to normal group I
c-P<0.01, d-P<0.001 compared to preventive- control group II
e-P<0.01, f-P<0.001 compared to curative -control group V
ERT- Ethanolic extract of R. tuberosa

Urine pH

In normal rats, urine pH was 7.0 to 7.5. In the preventive and curative control groups a sharp decline in urinary pH to 5.0 – 6.0 was observed. ERT administration increased the urine pH to 6.5 – 7.0 in both the preventive and curative treated groups.

Deposition of calcium and oxalate in the kidney

In the preventive and curative control groups, free access to drinking water containing EG and AC, resulted in a significant enhancement in the kidney calcium and oxalate deposition. Treatment with ERT at 0.5 and 1.0 g/kg, produced a significant dose dependent reduction in kidney calcium and oxalate deposition in the preventive and curative treated groups (Table 1).

Urinary excretion of calcium, oxalate and creatinine

Urinary calcium, oxalate and creatinine excretion was enhanced significantly (*P*<0.001) in the preventive and curative control groups on EG/AC administration. A significant (*P*<0.001), dose dependent reduction in urinary calcium, oxalate and creatinine excretion was observed in the preventive and curative treated groups, on treatment with ERT at 0.5 and 1.0 g/kg orally, when compared to their respective control groups (Table 2).

Table 2. *Effect of ERT on urinary creatinine, calcium and oxalate excretion*

Group	Treatment	(mg/dl)		
		Creatinine	Calcium	Oxalate
I	Normal	7.83 ± 0.48	8.56 ± 0.19	1.70 ± 0.14
II	Preventive control	28.83 ± 0.40[a]	21.75 ± 0.66[a]	9.12 ± 0.23[a]
III	ERT 0.5g/kg	17.17 ± 0.48[c]	14.92 ± 0.09[c]	4.12 ± 0.12[c]
IV	ERT 1.0g/kg	16.00 ± 0.37[c]	13.15 ± 0.08[c]	2.69 ± 0.13[c]
V	Curative control	26.17 ± 0.54[b]	20.66 ± 0.17[b]	8.47 ± 0.29[b]
VI	ERT 0.5g/kg	22.17 ± 0.40[d]	17.43 ± 0.18[d]	6.09 ± 0.28[d]
VII	ERT 1.0g/kg	20.00 ± 0.45[d]	15.47 ± 0.07[d]	4.42 ± 0.19[d]

Values are expressed as mean±SEM, n=6
a-P<0.001 when compared to normal group I
b-P<0.001 when compared to preventive- control group II
c-P<0.001 when compared to curative-control group V
ERT- Ethanolic extract of R. tuberosa

Oxidative stress

***In vivo* LPO**

In group II and V animals, EG/AC induced lithogenesis produced a significant enhancement in the renal MDA levels (119.30±3.02) and (93.20±4.03) respectively, when compared to the normal group (67.00±4.3). After treatment with ERT (0.5 and 1.0 g/kg, a dose dependent significant (*P*<0.001) reduction in the kidney MDA levels was observed in the preventive and curative treated groups, when compared to their respective control groups (Table 3).

Table 3. *Effect of ERT on in vivo lipid peroxidation and antioxidant parameters*

Group	Treatment	MDA (nM/mg tissue)	GSH (nM/mg tissue)	Catalase (µM/mg tissue)
I	Normal	67.00 ± 4.28	74.99 ± 4.14	365.70 ± 14.15
II	Preventive control	119.30 ± 3.02[a]	24.50 ± 1.64[a]	81.80 ± 4.34[a]
III	ERT 0.5g/kg	41.19 ± 0.76[c]	48.52 ± 2.92[c]	212.50 ± 12.35[c]
IV	ERT 1.0g/kg	32.22 ± 0.98[c]	89.70 ± 7.43[c]	272.90 ± 9.60[c]
V	Curative control	93.20 ± 4.03[b]	26.95 ± 2.57[b]	109.80 ± 2.66[b]
VI	ERT 0.5g/kg	53.97 ± 1.13[d]	46.52 ± 2.79[d]	165.10 ± 8..37[d]
VII	ERT 1.0g/kg	43.54 ± 0.66[d]	62.70 ± 5.25[d]	217.90 ± 12.41[d]

Values are expressed as mean±SEM, n=6
a-P<0.001 when compared to normal group I
b-P<0.001, c-P<0.01 when compared to preventive- control group II
d-P<0.001, e-P<0.01 when compared to curative -control V
ERT- Ethanolic extract of R. tuberosa

***In vivo* antioxidant enzymes**

GSH and catalase levels of the kidney were significantly decreased in the preventive and curative control groups on EG/AC administration for 15 days, when compared to the normal group. On treatment with ERT, a significant rise in the renal GSH and catalase levels was observed in the preventive and curative treated groups (Table 3).

***In vitro* antioxidant parameters**

DPPH scavenging

The extract exhibited a concentration dependent DPPH free radical scavenging effect. IC_{50} of ERT is 578.63 µg/ml, comparable with IC_{50} of ascorbic acid (58.45µg/ml).

Nitric oxide free radical scavenging

ERT showed a concentration dependent inhibition of NO induced free radicals. (IC_{50}- 789.37µg/ml), compared to ascorbic acid (IC_{50} -7.04µg/ml).

DISCUSSION

Urinary stone disease is a common, painful and expensive medical condition[17]. Though extracorporeal shock wave lithotripsy has facilitated the stone removal and reduced the morbidity associated with urinary stones, recurrence is common[18]. Several experimental and clinical studies on some of the plants used in the Indian traditional system of medicine proved their efficacy in the management of renal stone disease. Therefore, it is advisable to evaluate plants used in the traditional medicine to treat kidney stone disease for antiurolithiatic activity, which might be also useful in reducing stone recurrence rate.

Rats are commonly used to study the pathogenesis of human CaOx kidney stone disease, as Ox metabolism is regarded almost similar in rats and humans[19]. Ingestion of EG/AC has been found to be a reliable inducer of Ox lithiasis in rats[20]. EG is converted to endogenous oxalic acid by the liver enzyme glycolate oxidase[21] and AC induces urinary acidification, is supposed to upset the enzyme sorting mechanism in the tubular cells in the kidney[20], thus favors adhesion and retention of CaOx particles within the renal tubules[22]. Hence, in the present study, EG/AC in drinking water was employed to induce hyperoxaluria in rats.

Urinary supersaturation in relation to stone forming constituents, mainly urinary oxalate is important in renal calculi formation[23], as urinary oxalic acid complexes with calcium and forms insoluble CaOx crystals[24]. Enhanced deposition and urinary excretion of calcium and oxalate in the preventive and curative control group animals indicate that administration of EG/AC induced hyperoxaluria. These results are in consistent with the earlier reports of Touhami *et al* (2007)[22]. An increase in the kidney weight and enhanced urinary creatinine excretion in the control group animals also substantiated these results.

On administration of ERT, the dose-dependent reduction in calcium and oxalate deposition in the kidneys and their urinary excretion in both the preventive and curative treated groups implies the potential of *R. tuberosa* in both preventing the formation and dissolving the preformed CaOx stones. On treatment with the extract, the significant reduction in the elevated urinary creatinine in both the treated groups reflects the improvement in hyperoxaluria induced renal impairment.

Dissolution of calculi can be achieved by alteration in urinary pH[25]. If the pH is 5.0 or below, the stones likely to form are of uric acid type, if 5.0-6.5, calcium oxalate type and if above 7 indicates crystals of magnesium ammonium phosphate. In the present study, a decrease in the normal urine pH of 7.0-7.5 to 5.5-6.0 in both the control groups, indicates hyperoxaluria induced CaOx stone formation. In the preventive and curative treated groups, ERT administration restored the pH to 6.5-7.5, supporting the decrease in the deposition and excretion of calcium and oxalate.

Mucoproteins have significant affinity for CaOx surface and promote the growth of crystals and cement them[26]. Grases, *et al*[27] have reported that saponins act by disintegrating the mucoprotiens, thereby prevent calcium and oxalate deposition and excretion. Saponin rich fraction of *H. hirsuta* was found to decrease CaOx crystal adhesion to renal epithelial cells by precoating the crystals[28]. Earlier chemical studies on *R. tuberosa*, revealed the presence of lupane type pentacyclic triterpene saponin derivatives, lupeol and betulin[29,30]. In the present study also, preliminary phytochemical screening of ERT revealed the presence of saponins. Thus, in the ERT treated groups, saponins might have reduced calcium and oxalate deposition by pre-coating CaOx crystals and disintegrating the mucoprotiens.

The stone forming effects of EG are also ascribed to its hyperoxaluria induced oxidative damage[22]. Oxalate has been reported to induce LPO and to cause renal tissue damage[31]. As kidney is rich in polyunsaturated fatty acids, is susceptible to ROS attack[32]. Excessive generation of ROS and/or a reduction in cellular antioxidant levels results in the development of OS. Moreover, low concentration of renal cellular glutathione favors LPO and subsequent retention of calcium and oxalate in the kidneys[33].

MDA is one of the most common byproducts of ROS induced OS[3]. In the present study, increased levels of MDA, diminished levels of GSH and catalase in the preventive and curative control groups indicate that EG/AC administration promoted extensive generation of ROS. The resultant ROS, may have consumed GSH and catalase excessively and impaired antioxidant protection. Thus, the unquenched ROS may have provoked cellular damage and resulted in enhanced OS, which might have further favored the accumulation and retention of oxalate and subsequent deposition of CaOx.

Studies show that treatment with antioxidants prevents CaOx deposition in the kidney and reduce Ox excretion[34]. Curhan *et al* (1998) have reported that daily consumption of tea reduced the risk of kidney stone formation in women by 8%[35]. Health benefits of tea are due to its antioxidant properties of flavonoids[36] which act by quenching ROS and also by chelating metal ions like iron and copper[37]. Lupeol and betulin were proposed to act by scavenging oxalate promoted free radicals and enhancing body antioxidant status, thus reducing oxalate induced renal peroxidative tissue damage[38].

In the present study, lowered levels of MDA and enhanced levels of antioxidant enzymes, GSH and catalase in the kidneys of the preventive and curative treated animals indicate attenuation of hyperoxaluria induced LPO and oxidative damage. Results of the present study are in agreement with the previous reports of Curhan *et al* (1998)[35] on tea. Flavonoids may have minimised ROS by free radical scavenging and prevented further generation, by metal chelating property, as flavonoids like cirsimaritin, cirsimarin, cirsiliol 4'-glucoside, sorbifolin and pedalitin, were reported in *R.tuberosa*[30]. Lupeol and betulin might have decreased LPO and enhanced GSH and catalase by scavenging oxalate promoted free radicals and by enhancing body antioxidant status. *In vitro* antioxidant studies also supported the hyperoxaluria induced free radical scavenging effect of ERT. Thus, the saponin and flavonoid principles of *R. tuberosa* together might have been responsible for the inhibition of CaOx crystal aggregation and stone formation.

The results support the use of *R. tuberosa* roots as an effective alternative in treating CaOx urolithiasis. Disintegration of the mucoproteins and pre-coating of CaOx crystals by saponins and the antioxidant effect of lupeol, betulin and flavonoid principles may be responsible for the possible antiurolithiatic activity of *R. tuberosa*. Further studies are necessary to find out the chemical components responsible for the antiurolithiatic activity of *R. tuberosa.*

REFERENCES

1. Asplin JR, Favus MJ, Coe FL. In Brenner and Rector's the kidney. 5th edn. Brenner BM, ed. Philadelphia: Saunders, 1996, p 1893.
2. Otnes B. Scand J Urol Nephrol. 1983; 17: 93-9.
3. Khan SR. Urol Res. 2005; 33:349-57.
4. Thammanna, Narayana Rao K. In Medicinal Plants of Tirumala. Prasad MVS, Publisher. TTD, Tirupati: 1990, p 55.
5. Chiu NY, Chang KH. In The illustrated medicinal plants of Taiwan (2), Mingtong Medical J. 1995; 226:1.
6. Crossland J. In Lewis Pharmacology. New York: Churchill Livingstone, 1980, p 137.
7. Sheth UK, Dandkar NK, Usha GK. In Selected topics in experimental pharmacology. 1st edn. 1972, p 124.
8. Khan SR, Glenton P. J Urol. 1995; 153:811-17.
9. Lorentz K. Clin Chim Acta. 1982; 126:327- 33.
10. Hodgkinson A, Williams A. Clin Chim Acta. 1972; 36:127-32.
11. Browers LD. Clin Chem. 1980; 26:551-655.
12. Nichans WG, Sannelson D. Eur J Biochem. 1968; 6:126-30.
13. Jollow D, Mitchel L, Zampaglione N, Gilete J. Pharmacol. 1974; 1:151-69.
14. Aebi H. In Meth Enzymol. Colowick SP, Kaplane NO (eds). 1984; 05:121-6.
15. Blois MS. Nature. 1958; 181:1199-1200.
16. Marcocci L, Maguire JJ, Droy-Lefaix MT, Parker L. Biochem Biophys Res Commun. 1994; 201:748-55.
17. Sierakowski R, Finlayson B, Landes RR, Finlayson CD, Sierakowski N. Invest Urol. 1978; 15:438-41.
18. Ljunghall S, Dadanielson BG. B J U. 1984; 56:122-4.
19. Atmani F, Slimani Y, Mimouni M, Aziz M, Hacht B, Ziyyat A. J Ethnopharmacol. 2004; 95: 87-93.
20. Khan SR, Hackett RL. Scan Electr Microsc. 1985; 11:759.
21. De Bruijn WC, Boeve ER, Van Run PRWA, Van Miert PPMC, Romijn JC, Verkoelen CF, Cao LC, Schroder FH. Scanning microscopy. 1994; 8:541-50.
22. Touhami M, Laroubi A, Elhabazi K, Louna F, Zrara I, Grases F, Chait A. BMC Urol. 2007; 7:18.
23. Robertson WG, Peacock M. Nephron. 1980; 26:105-10.
24. Pak CYC. In Renal stone disease. Boston Martinus Nijhoff Publishing. 1987, p 120.
25. King Jr JS. J Urol. 1967; 97: 583-91.
26. Leal JJ, Finlayson B. Invest Urol. 1977; 14:278.
27. Grases F, March JG, Ramis M, Costa-Bauzá A. Phytotherapy Res. 1993; 7:146-9.
28. Atmani F, Farell G, Lieske JC. J Urol. 2004; 172:1510-14.

29. Rastogi RP, Mehrotra BN. In Compendium of Indian Medicinal Plants. A CDRI series, PIB, New Delhi: 1993, vol (3), p 558.

30. Lin CF, Haung YL, Cheng LY, Sheu SJ, Chen CC. J Chin Med. 2006; 17: 103-9.

31. Karadi RV, Gadge NB, Alagawadi KR, Savadi RV. J Ethnopharmacol. 2006; 105:306-11.

32. Devinder S, Rajnendrapal K, Vikas C, Kanwaljit C. J Med Food. 2006; 9:443–50.

33. Toblli JE, Ferder L, Stella I, De Cavanagh MVE, Angerosa M, Inserra F. J Urol. 2002; 168:1550.

34. Selvam R. Urol Res. 2002; 30:35-47.

35. Curhan G, Willet W, Speizer F, Stampfer M. Am Intern Med. 1998; 128:534-40.

36. Vinson J, Dabbagh Y, Serry M, Jang J. J Agri Food Chem. 1995; 43:2800-2.

37. Miller N, Castelluccio C, Tijburg L, Rice-Evans C. FEBS Letters. 1996; 392:404.

38. Malini MM, Lenin M, Varalakshmi P. Pharmacol Res. 2000; 41:413-8.