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Effect of ethanolic extract of *Phyla nodiflora* (Linn.) Greene against calculi producing diet induced urolithiasis

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Urolithiasis in its different forms is a frequently encountered urological disorder. For many years it has been at the forefront of urology. In the present study ethanolic extract of whole plant of *Phyla nodiflora* (Linn.) Greene was studied for its antiurolithiatic activity against most common type of renal stones i.e. calcium oxalate type. Calcium oxalate urolithiasis was induced by administration of Gentamycin and calculi producing diet (5% ammonium oxalate in standard rat pellet feed). The extract was also assessed for effect on *in vivo* antioxidant parameters like lipid peroxidation, reduced glutathione, catalase in hyperoxaluric kidney and *in vitro* scavenging of nitric oxide and 2-diphenyl-2-picryl hydrazyl free radicals. Ethanolic extract of *P. nodiflora* exhibited significant effect in preventing calcium oxalate stone formation and also in dissolving the pre-formed calcium oxalate stones in the kidney along with significant effect on both *in vitro* and *in vivo* antioxidant parameters. The present study clearly demonstrates the antiurolithiatic activity of *P. nodiflora* supporting the traditional claim.

Keywords: Antiurolithiatic activity, Calcium oxalate, Renal stones, Calculi producing diet, *Phyla nodiflora*, Urolithiasis.

IPC code; Int. cl.[8] — A61K 36/00, A61P 13/04

Introduction

Urolithiasis has plagued humans since antiquity and constitutes a major health problem. Kidney stones develop as a result of complicated interactions of biological events that are most likely triggered by genetic susceptibility coupled with dietary factors and lifestyle[1]. Despite dramatic progress in both medical and surgical areas, still the basic mechanisms of stone formation, identity of indicators of recurrence and its complete prevention remain an enigma. The goal of surgical approach is the removal of existing stones and medical treatment is the prevention of recurrent stone formation. Research should focus on dietary and phytotherapy, as these may play a vital role in preventing recurrence of renal stones.

Phyla nodiflora (Linn.) Greene (Family — Verbenaceae), a perennial herb, grows in maritime areas near rivers throughout the subcontinent, Africa and other tropical and subtropical regions. The plant is an anodyne, cardiotonic, antibacterial, diuretic, antilithic, parasiticide and refrigerant[2]. It is also good for ulcers, wounds, burning sensation, asthma, bronchitis, thirst and loss of consciousness[3]. Previous phytochemical investigations on this plant have resulted in the isolation of several flavone glycosides, including lippiflorin A & B, nodiflorin A & B, nodifloritin A & B, alkaloids, essential oil, resin, stigmasterol, ß-sitosterol, sugars, mono and diflavone sulphates of nepetin, jaceosidin, hispidulin and 6-hydroxyluteolin[4-7].

P. nodiflora showed a mild degree of CNS stimulation, direct myocardial depression in frog's perfused heart and hypotensive action in dog[8]. On smooth muscles it produced slight relaxation and also antagonized the effects of acetylcholine, histamine and barium chloride. *P. nodiflora* showed significant anti-inflammatory [9] and diuretic activity[10].

The present study plans to systematically evaluate *P. nodiflora* to verify the claim made in the Ayurveda, as literature survey showed that no scientific work was carried out to support the antiurolithiatic activity of *P. nodiflora*.

Materials and Methods

Collection of plant material and extraction

Plant material used in this study consisted of the whole plant of *P. nodiflora*, collected in and around

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Tirumala hills, Chittor District, Andhra Pradesh. The plant was authenticated by Dr. Madhavachetti, Department of Botany, Sri Venkateswara University, Tirupati. A Voucher specimen (012/07) was deposited in the Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Tirupati.

The shade dried whole plant was powdered mechanically and passed through the sieve (coarse 10/44). In each step, about 150 g of the dried powder was extracted with 1000 ml of 95% ethanol in a 2 litre round bottom flask by refluxing over a water bath. The extraction was carried out in three batches. The extract was concentrated *in vacuo* till syrupy consistency was obtained and then dried on a water bath (yield-62 g/kg). Since ethanolic extract was water-insoluble, it was suspended in distilled water (0.25 g/ml). A uniform suspension was made for administration to animals without any suspending agent.

Animals

Male Wistar albino rats (150-200 g) were used in the present study. They were housed in polypropylene cages, with standard rat pellet food and water *ad libitum* for several days before the beginning of the experiment with natural light:dark cycle. The experimental protocols were approved by the Institutional Animal Ethical Committee (Approval No: 1016/a/06/CPCSEA).

Drugs

Gentamycin was procured from Ranbaxy, India. All other chemicals used are of analytical grade and obtained from S.D. Fine Ltd., India.

Acute toxicity and gross behavioural changes

Healthy adult albino rats were fasted overnight with free access to drinking water. They were divided into six groups, each containing six animals. Group-I animals served as control, received distilled water (2 ml/kg/orally) and Group-II to Group-VI animals received 0.5, 1, 2, 4 and 8 g/kg/orally of ethanolic extract of *P. nodiflora* (EPN), respectively by gastric intubation using a soft rubber catheter.

The animals were observed continuously for 2 hours and then intermittently at gaps of one hour till six hours for behavioural, neurological and autonomic profiles[11]. At the end of 48 hours, number of deaths was observed to calculate LD_{50} of the extracts[12].

Antiurolithiatic studies - Calcium oxalate stones

Hyperoxaluria and calcium oxalate deposition in the kidney was induced using Gentamycin (40 mg/kg/s.c) and calculi producing diet (CPD). The latter was made from powdered standard rat pellet feed (Gold Mohur Ltd.) mixed with ammonium oxalate (5%), then made into pellets and dried[13].

Study design

For studying the effect of EPN on calcium oxalate stones, rats were divided into seven groups each consisting of 6 animals. Group I received only vehicle (distilled water) orally for 30 days, serves as normal animals. Group II received Gentamycin (40 mg/kg/day, s.c, day 1 to 8); CPD and vehicle (from day 1 to 15). This group served as preventive control. Group III animals received Gentamycin (40 mg/kg/day, s.c, day 1 to 8); CPD and EPN (0.5 g/kg, from 1-15 days). Group IV animals were treated with Gentamycin (40 mg/kg/day, s.c, day 1 to 8); CPD and EPN (1 g/kg, from 1-15 days). Groups III and IV assess the ability of the EPN in preventing the calcium oxalate stone formation. Group V was treated with Gentamycin (40 mg/kg/day, s.c, day 1 to 8); CPD (from day 1 to 15) and vehicle (from day 16 to 30). This group served as curative control. Groups VI received Gentamycin (40 mg/kg/day, s.c, day 1 to 8); CPD (day 1-15) and EPN (0.5 g/kg, from 16-30 days). Groups VII received Gentamycin (40 mg/kg/day, s.c, day 1 to 8); CPD (day 1-15) and EPN (1 g/kg, from 16-30 days). Groups VI and VII study *per se* effect of EPN in dissolving the preformed stones.

Assessment of urinary parameters

The rats were hydrated with distilled water (5 ml/animal), housed in separate metabolic cages and urine samples were collected for 24 hours, at the end of 15 days (II, III and IV groups) and 30 days (I, V, VI and VII groups). The urinary *p*H was determined, samples were centrifuged at 2500 rpm (REMI, R24) for 5 min and the supernatant was estimated for calcium and oxalate[14,15].

Assessment of kidney parameters

At the end of the experimental period, the rats were sacrificed by decapitation. The kidneys were carefully removed, washed in ice cold 0.15 M KCl and their weight was recorded. One kidney from each animal was put in 10% formalin and used for histological studies. The other kidney was sliced into two equal halves and one half was homogenized in 10% HCl. The homogenate was centrifuged at 2500 rpm for 3 minutes and the supernatant was used for the estimation of calcium and oxalate. The other half was stored in ice cold saline and used for estimating the *in vivo* antioxidant parameters.

Estimation of *in vivo* antioxidant parameters

Kidneys stored in ice cold saline (0.9% sodium chloride) were homogenized in chilled potassium chloride (1.17%) using a homogenizer. The homogenates were centrifuged at 800 g for 5 minutes at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 g for 20 minutes at 4°C to get the post mitochondrial supernatant which was used to assay lipid peroxidation (LPO), reduced glutathione (GSH) and catalase activity[16-18].

Histopathological examination

For microscopic evaluation, kidneys were fixed in 10% neutral phosphate buffered formalin solution. Following dehydration in ascending series of ethanol (70, 80, 96, 100%), tissue samples were cleared in xylene and embedded in paraffin. Tissue sections of 5 µm were stained with hematoxylin-eosin. A minimum of 10 fields for each kidney slide were examined for tubular necrosis and presence of calcium oxalate crystals.

In vitro antioxidant parameters

The *in vitro* antioxidant activity of the ethanolic plant extract, EPN was assessed for nitric oxide (NO)[19] and 2-diphenyl-2-picryl hydrazyl (DPPH) free radicals scavenging activities at different concentrations[20].

Statistical analysis

All the data were presented as Mean ± SEM. The software used was Statistica version 6.0. Statistical analysis was performed by ANOVA test for multiple comparisons followed by Tukey-Krammer test. The statistical significance was set at $p < 0.05$.

Results

Acute toxicity and gross behavioural changes

The ethanolic extract *P. nodiflora* (EPN) was found to be safe, since no animal died even at the maximum single dose 8 g/kg, p.o. The EPN did not produce any significant behavioural changes except an increase in urination.

Antiurolithiatic studies - Calcium oxalate stones

Urinary pH

The urinary *p*H in normal animals was between 6.0 and 7.0. On induction of calcium oxalate stones, the *p*H reduced to 5.0-6.0 in both the preventive-control and curative-control groups. After completion of the study, preventive (III and IV) and curative (VI and VII) groups treated with EPN showed an increase in the urinary *p*H (7.0-8.0) when compared to the respective control groups.

Wet kidney weight

A significant increase in kidney weight was observed in the preventive-control and curative-control groups when compared to the normal animals. Treatment with EPN reduced kidney weight significantly in both the preventive and curative groups when compared to their respective control groups (Table 1).

Table 1 — Effect of ethanolic extract of *Phyla nodiflora* (EPN) on kidney weight, deposition of calcium and oxalate in kidney and urinary excretion of calcium and oxalate

Group	Treatment	Kidney weight (g/100 g,b.wt)	Kidney deposition (mg/g)		Urinary excretion (mg/dl)	
			Calcium	Oxalate	Calcium	Oxalate
I	Normal	0.51 ± 0.01	0.85 ± 0.17	0.35 ± 0.15	2.14 ± 0.92	0.99 ± 0.19
II	Preventive Control	0.63 ± 0.01*	1.55 ± 0.43*	1.53 ± 0.62*	6.97 ± 1.85*	3.85 ± 1.04*
III	EPN (0.5 g/kg)	0.52 ± 0.01*	1.25 ± 0.35	1.10 ± 0.33	4.25 ± 1.28*	2.20 ± 0.95*
IV	EPN (1 g/kg)	0.42 ± 0.01*	0.92 ± 0.23*	0.87 ± 0.19*	4.02 ± 1.29*	2.16 ± 0.94*
V	Curative Control	0.71 ± 0.01*	3.32 ± 1.09*	2.17 ± 0.96*	8.10 ± 1.93*	4.28 ± 0.88*
VI	EPN (0.5 g/kg)	0.45 ± 0.01*	2.12 ± 0.94	1.09 ± 0.92	5.04 ± 1.79*	2.21 ± 0.81*
VII	EPN (1 g/kg)	0.40 ± 0.01*	1.64 ± 0.89*	0.92 ± 0.19*	4.26 ± 1.03*	2.16 ± 0.92*

Values are expressed as mean ± SEM of 6 observations; Statistical comparisons are made between Group I vs Group II and V; Group II vs III and IV; Group V vs Group VI and VII (*, p <0.05)

Calcium and oxalate deposition in kidney

On administration of Gentamycin and CPD, an increase in the deposition of calcium and oxalate in the kidney was observed when compared to the normal group animals indicating the formation of calcium oxalate stones (Table 1).

On treatment with EPN for 15 days, higher dose produced a significant decrease in the calcium and oxalate deposition in both the preventive (IV) and curative groups (VII) when compared to their respective-control groups (II and V). These results indicate the efficiency of EPN in preventing the formation also in dissolving pre-formed calcium oxalate calculi in the kidney.

Urinary excretion of calcium and oxalate

A significant increase in the urinary excretion of calcium and oxalate was observed in both the preventive-control (II) and curative-control (V) groups on feeding with CPD when compared to the normal group (I) (Table 1).

A significant decrease in the urinary excretion of calcium and oxalate was observed in both the preventive (III and IV) and curative groups (VI and VII), when compared to their respective controls (II and V), on treatment with EPN for 15 days.

These results of the urinary excretion data clearly support that EPN can reduce supersaturation of urine with calculogenic ions.

Histological studies

Administration of Gentamycin (40 mg/kg, s.c.) for the first eight days produced renal tubular damage and haemorrhages in the kidneys of rats. Feeding with CPD for 15 days caused glomerular destruction, glomerular atrophy, tubular dilatation and deposition of honey coloured calcium oxalate crystals in the inter-tubular spaces in the preventive-control (II) and curative-control group (V) animals (Plate 1).

Treatment with EPN reduced the renal tubular membrane damage, haemorrhages and atrophy when compared to control kidney sections. The extract was not effective in reversing the tubular dilatations but calcium oxalate crystals were not observed in the kidney sections of the animals treated with EPN,

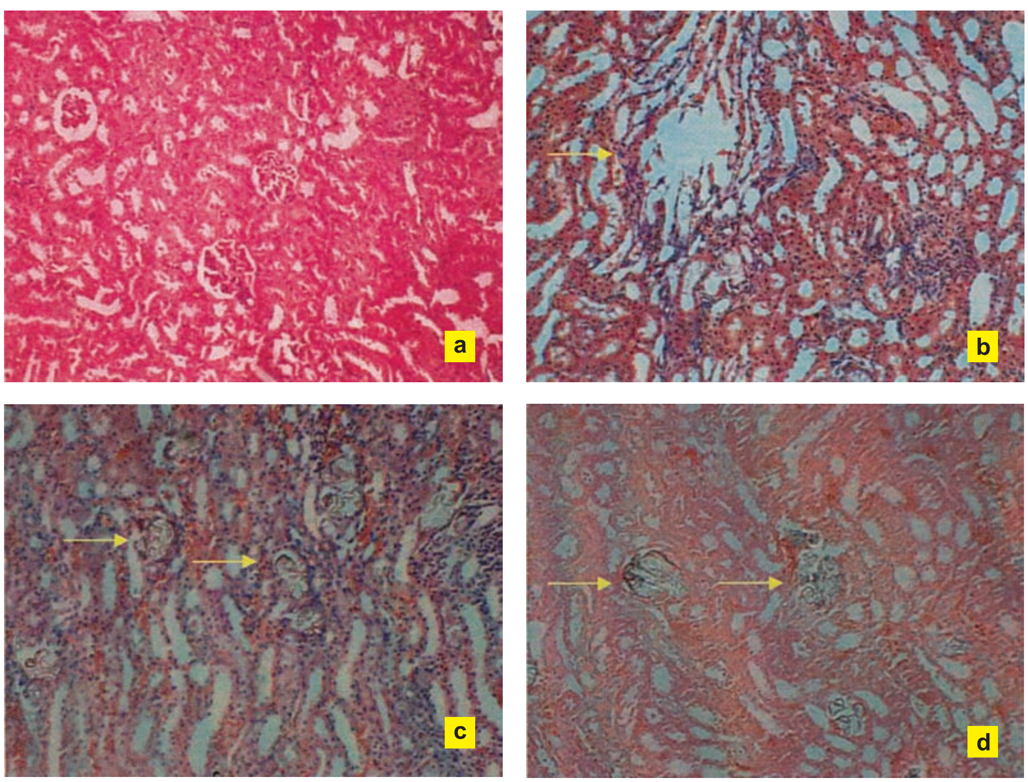

Plate 1 — Histological sections of kidney - Normal, Gentamycin treated and Gentamycin + CPD treated at 40 X, (a) Normal Kidney showing normal glomerular structure; (b) Gentamycin treated – Renal tubular damage and tubular dilatation; (c) Preventive control fed with CPD and Gentamycin (S.C) showing deposition of calcium oxalate crystals along with tubular dilatation; and (d) Curative control fed with CPD and Gentamycin (S.C) showing deposition of calcium oxalate crystals along with tubular dilatation

suggesting it's efficacy as antiurolithiatic agent (Plate 2).

***In vivo* antioxidant parameters**

On feeding with CPD for 15 days, a significant increase in the levels of malondialodehyde (MDA) along with a significant decrease in the levels of antioxidant enzymes, reduced glutathione (GSH) and catalase was observed in the preventive-control (II) and curative-control (V) groups when compared to the normal group (I) indicating increased oxidative stress (Table 2).

On treatment with EPN for 15 days, a significant decrease in the levels of MDA and increase in the levels of GSH and catalase in the kidneys were observed in both the preventive (III and IV) and curative groups (VI and VII) when compared to their respective control groups (II and V).

***In vitro* antioxidant parameters**

Nitric oxide generated from sodium nitroprusside at physiological *p*H was inhibited effectively by the EPN and the IC_{50} values of the plant extract was found to be 97.29 ± 1.12 µg/ml. Ascorbic acid, used as a reference standard has shown nitric oxide scavenging potential with an IC_{50} value of 70.02 ± 1.24 µg/ml.

The EPN had shown a significant (p <0.001) effect of free radical scavenging of DPPH, with IC_{50} values 118.80 ± 3.75 µg/ml (EPN). The reference standard ascorbic acid also showed significant free radical scavenging of DPPH with an IC_{50} value of 74.98 ± 4.32 µg/ml.

Discussion

In spite of intensive research to establish the mechanisms of stone formation, dietary management, evaluation of medicinal plants and other agents in the treatment of urinary stones, till to date there is no standard drug available. The main drawbacks in the development of a standard drug may be different chemical forms of renal stones and different biochemical disorders that lead to urolithiasis.

Hyperoxaluria can provoke calcium oxalate urolithiasis in both humans and rats. Oxalate metabolism is considered to be almost identical between rats and humans. Thus, a rat model of calcium oxalate urolithiasis can be used to investigate

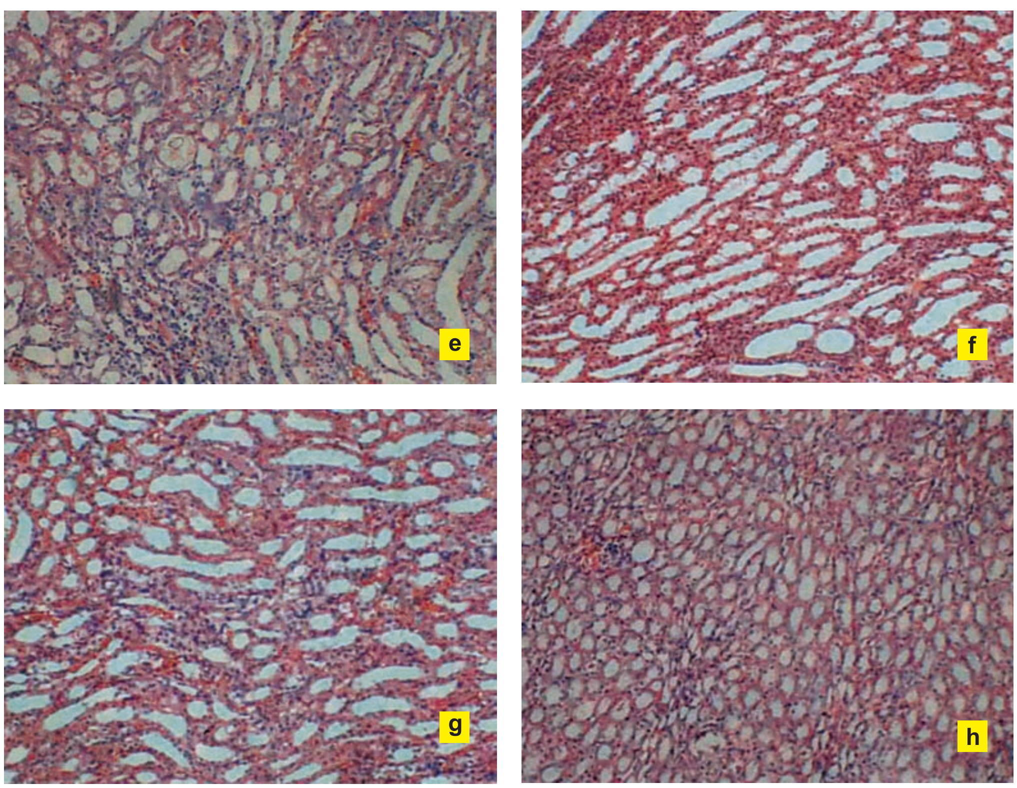

Plate 2 — Histological sections of kidney – EPN treated at 40 X magnification. (e-h) Group-III, IV, VI and VII treated with EPN (calcium oxalate deposition is not observed)

Table 2 — Effect of ethanolic extract of *Phyla nodiflora* (EPN) on *in vivo* antioxidant parameters

Group	Treat-ment	LPO (μm/mg tissue)	GSH (μg/mg tissue)	Catalase (μm of H_2O_2/min/mg tissue)
I	Normal	0.52 ± 0.13	1.67±0.64	0.052±0.003
II	Preventive-control	1.23± 21*	0.72±0.21*	0.021±0.001*
III	EPN (0.5 g/kg)	0.82±0.14*	1.02±0.23*	0.034±0.004*
IV	EPN (1 g/kg)	0.64±0.11*	2.34±0.32*	0.046±0.002*
V	Curative control	1.54±0.32*	0.64±0.11*	0.018±0.002*
VI	EPN (0.5 g/kg)	0.92±0.11*	2.21±0.41*	0.042±0.001*
VII	EPN (1 g/kg)	0.86±0.21*	3.43±0.36*	0.057±0.003*

Values are expressed as mean ± SEM of 6 observations; Statistical comparisons are made between Group I vs Group II and V; Group II vs III and IV; Group V vs Group VI and VII. (*, $p<0.05$)

the mechanisms involved in human kidney stone formation and also for screening new agents with antiurolithiatic activity[21].

Focal necrosis, the loss of border membranes and the occurrence of membrane debris in the tubule lamina are few factors that induce renal stone formation[22]. Treatment with high doses of Gentamycin in rats was reported to induce stone formation by causing such damages. The membranous debris produced by Gentamycin acts as nucleation site for calcium oxalate crystallization. Moreover, Gentamycin has been shown to inhibit calcium reabsorption in proximal tubules[23]. In the present study, 5% ammonium oxalate is used instead of 3% ammonium oxalate as reported by Sanjay Kumar *et al*[13], because the preliminary studies have shown less incidence of calculi deposition with 3% ammonium oxalate. This treatment schedule of Gentamycin and ammonium oxalate increases calcium and oxalate super saturation, renal tubular injury and produce conditions conducive to the formation and growth of calcium oxalate stones[13].

Calcium oxalate stone formation is not a spontaneous phenomenon. The main cause of calcium oxalate stone formation appears to be chronic hyperoxaluria[21]. In the present study, Gentamycin and CPD induced hyperoxaluria not only increased calcium oxalate deposition in the kidney but also caused papillary damage and incrustations, corroborating with the earlier reports of Baumann[24]. A similar elevation of renal stone forming constituents in rats fed with calculi producing diet using glycolic acid has been reported earlier[25]. Administration of EPN significantly reduced calcium and oxalate deposition in the kidneys of both preventive and curative groups.

The super saturation of urine with calcium oxalate, the most common component of kidney stones is an important factor in crystallization, with later steps being nucleation, growth and aggregation[26,27]. If super saturation or later steps in crystallization can be prevented, urolithiasis can be avoided. In this context, the changes in the urinary oxalate levels are relatively much more important than those of calcium[28]. It has been reported that oxalate has about 15-folds greater effect on calcium oxalate crystallization than urinary calcium alone[29].

In the present study, a significant increase in urinary excretion of calcium and oxalate was observed along with the formation of calcium oxalate type of stones, which is similar to the reports of Prasad *et al* [30]. Urinary oxalic acid forms complexes with multiple cationic salts to form oxalate salts, which are soluble when formed with magnesium but when complexed with calcium forms insoluble calcium oxalate thus causing crystalline precipitation of renal calculi of calcium oxalate type[31,32].

Treatment with EPN caused a significant reduction in the urinary excretion of calcium and oxalate, thus reducing super saturation of urine. This might be responsible for preventing and also in dissolving the preformed calcium oxalate type of stones.

The results of the histological studies and wet kidney weight support the results of the deposition in kidney and urinary excretion of calcium and oxalate in the present study. On histological examination, both the preventive and curative control groups showed calcium oxalate crystals in majority of tubules accompanied by inter-tubular hemorrhages and atrophy. These observations support the presence of renal calculi in renal medulla region as observed in human urolithiasis. Treatment with EPN showed very few crystals in the focal region of kidney, indicating the ability of EPN in dissolving the pre-formed calculi.

The type of stones formed in human subjects can be predicted from the *p*H of the fasting urine[33]. Crystalluria is *p*H dependent. Dissolution of calculi can be achieved by alteration in urinary *p*H[34]. If the *p*H is acidic 5.0 or below, the stones likely to form are of uric acid type, if 5.0-6.5 calcium oxalate type and if alkaline (7.2 or above) indicates magnesium ammonium phosphate type. In the present study, a decrease from normal *p*H of 6.0-7.0 to 5.0-6.0 was observed on induction of calcium oxalate type of stones in the CPD model. Treatment with EPN reversed the acidic *p*H to normal. This increase in urinary *p*H might be responsible for dissolving the complexes of calcium and oxalate, which contributes to their significant antiurolithiatic activity.

In the treatment of kidney stones, plants are used as antilithics either to dissolve the stones or to aid their passing to guard against further retention. There are reports in the literature attributing the antilithiatic activity to the diuretic property of the plants[35,36]. In the present study also antiurolithiatic activity of *P. nodiflora* may be due to its diuretic activity which is attributed to the presence of potassium nitrate and tannins[10].

Oxalate and oxidative stress act in a synergy to enhance the risk of urinary stones. Hence, in the present study EPN was assessed for its effect on *in vivo* antioxidant parameters like LPO, GSH and catalase in hyperoxaluric kidney (CPD model) and also for *in vitro* scavenging of nitric oxide and DPPH free radicals at different concentrations.

In urolithiasis, oxalate has been reported to induce lipid peroxidation. Both *in vivo* and *in vitro* studies have revealed that the induction of LPO by oxalate is mediated through the inhibition of catalase[37,38]. Increased LPO enhances oxalate binding activity, which in turn promotes nucleation and aggregation of crystals. Further, depletion of antioxidants (enzymatic or nutritional) adds up to the progression of LPO[39]. In the present study, on induction of calcium oxalate crystals by feeding with CPD, a significant increase in LPO and decrease in GSH and catalase levels were noted. Treatment with EPN restored the levels of catalase and GSH. This might be responsible for the prominent decrease in LPO and also important in restoring the cell membrane damage.

In the *in vitro* antioxidant studies, EPN had showed moderate nitric oxide and good DPPH radical scavenging activity with increasing concentrations of the extract. A number of polyphenolic compounds such as quercetin, rutin, resveratrol, catechin and bioflavonoids have been found to inhibit the nitric oxide synthesis[40,41]. Reports indicate the presence of flavone glycosides lippiflorin A & B, nodiflorin A & B, nodifloritin A & B and luteolin in *P. nodiflora*[4,7,42]. Hence, the antioxidant activity of *P. nodiflora* may also be due to the presence of polyphenols and flavonoids. This reveals that *P. nodiflora* possesses the potential to reduce damage to renal membranes induced by Gentamycin and hyperoxaluria by CPD, thus supporting their significant antiurolithiatic activity against calcium oxalate stones.

Conclusion

The results of the present study have shown that the urinary stones could be dissolved with ethanolic extract of *P. nodiflora*. The recurrence of stones could also be prevented to a greater extent. The antiurolithiatic activity of this plant can be attributed to it's ability to reduce the supersaturation of urine with calculogenic ions, diuretic property and antioxidant potential. Further work is necessary to isolate the active constituents responsible for the antiurolithiatic activity.

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