Microcrystallization prevention with *Phyllanthus niruri* (Chanca Piedra)

The following studies have observed the effects of *P. niruri* on metabolic or physicochemical factors that reduce crystal nucleation, growth, or aggregation in urine.

Urol Res (2004) 32: 362–366
DOI 10.1007/s00240-004-0432-8

ORIGINAL PAPER

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Phyllanthus niruri normalizes elevated urinary calcium levels in calcium stone forming (CSF) patients

Received: 24 June 2003 / Accepted: 26 April 2004 / Published online: 19 June 2004

Abstract *Phyllanthus niruri* is a plant used for years in Brazil to treat urinary calculi. We prospectively evaluated the effect of *P. niruri* intake on 24 h urinary biochemical parameters in an attempt to assess its in vivo effect in calcium stone forming (CSF) patients. A total of 69 CSF patients (39 males and 30 females, 38 ± 8 years old) were randomized to take either *P. niruri* ($n = 33$) (450 mg capsules, td) or placebo ($n = 36$) for 3 months. Blood calcium, uric acid, citrate, magnesium, oxalate, sodium and potassium were determined at baseline and at the end of the study. A subset analysis was made in patients classified according to the presence of metabolic abnormalities (hypercalciuria, hyperuricosuria, hyperoxaluria, hypocitraturia and hypomagnesiuria). Overall, there were no significant differences in the mean values of urinary parameters between the urine samples before and after *P. niruri* intake, except for a slight reduction in mean urinary magnesium after *P. niruri*, which was within the normal range. However, in the subset analysis, we observed that *P. niruri* induced a significant reduction in the mean urinary calcium in hypercalciuric patients (4.8 ± 1.0 vs 3.4 ± 1.1 mg/kg/24 h, $P < 0.05$). In this short-term follow-up, no significant differences in calculi voiding and/or pain relief between the groups taking *P. niruri* or the placebo were detected. Our data suggest that *P. niruri* intake reduces urinary calcium based on the analysis of a subset of patients presenting with hypercalciuria. Larger trials including primary hypercalciuric stone formers should be performed in order to confirm these findings and to determine the possible clinical consequences of urinary calcium reduction during *P. niruri* administration.

Keywords *Phyllanthus niruri* · Natural products · Nephrolithiasis · Hypercalciuria · Calcium stone formers · Urinary calculi

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Introduction

In Brazil, a tea made from the plant *Phyllanthus niruri* (stone breaker or "quebra pedra") has been used in folk medicine to treat urinary calculi, among other conditions [1].

According to Calixto et al. [1], alkaloids from plants of the genus *Phyllanthus* present an antispasmodic activity leading to smooth muscle relaxation, mostly evidenced in the urinary tract, which would facilitate the elimination of urinary calculi. The analgesic activity of *P. niruri* has also been demonstrated by other investigators in Brazil [2, 3].

Previous studies by our laboratory have shown that the aqueous extract of *P. niruri* has an inhibitory effect on calcium oxalate (CaOx) crystal growth and aggregation in an in vitro model of crystallization of human urine [4]. In addition, the whole plant aqueous extract prevented an increase in the size of matrix bladder calculi as well as in the size and number of formed satellite crystals in a rat model [5]. Campos and Schor [6] also demonstrated that the aqueous extract of *P. niruri* reduced calcium oxalate crystal uptake by canine distal tubular cells, without evidence of cytotoxicity or biochemical alterations of the culture medium.

Taken together, these findings are consistent with the notion that the active constituents of *P. niruri* may have a beneficial effect in the treatment of urinary calculi.

Our laboratory also demonstrated that human volunteers who received large oral doses of *P. niruri* (20 g/day, in the form of tea) presented no detectable clinical or biochemical adverse effects, with excellent tolerability

[1]. Nevertheless, there is no report to date on the effects of *P. niruri* on the promoters or inhibitors of lithogenesis in humans.

Therefore, based on the promising experimental data, and also on an adequate safety profile, the aim of the present study was to evaluate, in a controlled manner, the effect of *P. niruri* on the urinary excretion of promoters and inhibitors of lithogenesis in CSF patients.

Patients and methods

A total of 69 CSF patients (39 males/30 females, 38 ± 8 years old) were randomly assigned to take a 450 mg capsule (three times a day) of lyophilized 2% aqueous extract of *P. niruri* ($n = 33$) or placebo (*Chicorium sativum*, $n = 36$) for a period of 3 months. As safety was one of our main concerns and no clinical or laboratory endpoint was available to define the *P. niruri* therapeutic range, we arbitrarily chose a total daily dose of *P. niruri* approximately 15 times lower than that employed in toxicological studies previously conducted in our laboratory (unpublished data). *C. sativum* was considered a good alternative for a placebo because its color and taste are similar to those of *P. niruri*. It is a green vegetable commonly consumed by our population and there has been no report of its having any toxic effect, even when consumed in quantities far higher than those administered to our patients. The protocol was submitted to the University Ethics Committee and approved.

All patients included presented with at least one renal stone. Diagnosis was based on the presence of stone(s) in both renal ultrasonography (US) and plain abdominal x-ray (thus, calcium-containing stones). Patients with diseases that might have led to secondary calcium stones, such as primary hyperparathyroidism, distal renal tubular acidosis, urinary tract infections, primary hyperoxaluria, etc., were not included in the study.

Both the *P. niruri* and placebo capsules were manufactured in the same way. *P. niruri* was grown at the experimental center of the Universidade Estadual de Campinas, CPQBA, Paulínia, São Paulo, Brazil. A voucher specimen (ref. 481) has been deposited in the herbarium of the same institution. A *P. niruri* crude extract was obtained from the whole plant, as is done in folk medicine. Plant samples were cut and dried at 50°C for 2 months in a ventilated room. After drying, the plants were ground in a mechanical mill and used for tea preparation. The tea was stirred for 30 min at 72°C and then vacuum filtered, concentrated, lyophilized and encapsulated.

A phytochemical analysis (fingerprint) of *P. niruri* was performed by high performance liquid chromatography (HPLC) assay (Fig. 1). The samples were prepared by dissolving the capsules in 20% ethanol. The resultant solution was partitioned with methylene chloride three times and the aqueous phase filtered through a 0.22 μm filter (Millipore, USA) and injected into the HPLC analyzer.

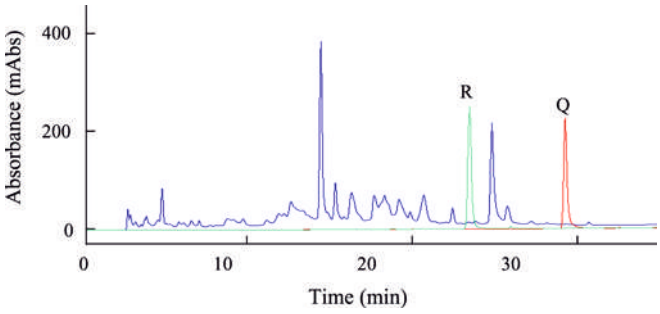

Fig. 1 Typical chromatogram from a *Phyllanthus niruri* phytochemical analysis. The tracing is representative of three experiments. Rutin (*R*) and quercetin (*Q*) (Sigma Chemical, USA) were used as reference compounds

Blood calcium, uric acid, sodium, potassium and creatinine, and 24 h urinary calcium, uric acid, citrate, magnesium, oxalate, sodium and potassium were determined at baseline and at the end of the study.

Urinary oxalate was measured with the Sigma Oxalate Diagnostic kit (Sigma, St Louis, Mo., USA). Calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer Atomic Spectrophotometer 290B). Creatinine was measured by the alkaline picrate Jaffe reaction [7] and uric acid by the uricase method [8]. Urinary citrate was determined by the citrate-lyase enzymatic reaction [9]. Sodium and potassium were measured by flame emission spectrophotometry.

The criteria for metabolic disturbances, based on the literature [10], were defined as: hypercalciuria (urinary calcium ≥4 mg/kg/day), hyperoxaluria (urinary oxalate ≥45 mg/day), hyperuricosuria [urinary uric acid ≥750 mg/day (female) or ≥800 mg/day (male)], hypocitraturia (urinary citrate ≤ 320 mg/day) and hypomagnesiuria (urinary magnesium ≤ 60 mg/day).

A subset analysis was made in patients classified according to the presence of metabolic abnormalities (hypercalciuria, hyperuricosuria, hyperoxaluria, hypocitraturia and hypomagnesiuria).

Urinary tract US was performed before and at the end of the study in order to detect the number and size of calculi.

The non-parametric Mann-Whitney U-test was used to compare the results between groups. The Wilcoxon test was used to compare the results obtained for the same group before and after *P. niruri* administration. Statistical significance was defined as $P < 0.05$.

Results

As shown in Table 1, the groups were similar in terms of gender distribution, age and body weight. The relative distribution of metabolic abnormalities, such as hypercalciuria, hyperuricosuria, hypocitraturia, hyperoxaluria and hypomagnesiuria, was also similar between groups. Some patients presented more than one metabolic abnormality.

Table 1 Number and percentage (%) of patients with metabolic diagnoses in both groups. [a] mean ± SD

	Placebo ($n=36$)	*Phyllanthus niruri* ($n=33$)
Gender	22 male/14 female	17 male/16 female
Age (years)[a]	37 ± 8	39 ± 9
Body weight (kg)[a]	70 ± 11	68 ± 13
Hypercalciuria	12 (33%)	8 (24%)
Hyperuricosuria	3 (8%)	1 (3%)
Hypocitraturia	10 (28%)	15 (45%)
Hyperoxaluria	6 (17%)	6 (18%)
Hypomagnesiuria	7 (19%)	3 (9%)

Serum biochemical parameters were not significantly different following *P. niruri* or placebo administration (Table 2). The mean values of urinary promoters or inhibitors of lithogenesis are shown in Table 3. There was no significant difference in mean urinary volume, calcium, uric acid, creatinine, sodium, potassium, citrate or oxalate after *P. niruri* or placebo administration compared to the baseline. There was a slight but significant decrease in mean urinary magnesium after *P. niruri* administration (82 ± 15 vs 74 ± 19 mg/24 hs), which occurred within normal limits.

Despite of the lack of statistical differences between the mean parameters induced by *P.niruri*, when patients were classified according to their individual metabolic abnormality(ies), as shown in Table 4, a significant difference was found among the patients with hypercalciuria, who exhibited lower urinary calcium levels after *P. niruri* (4.8 ± 1.1 vs 3.4 ± 1.1 mg/kg/24 h, $P < 0.05$). Urinary calcium did not change in either the non-hypercalciuric patients or in those subjects who received the placebo. Individual values of urinary excretion of calcium (mg/kg/24h) in hypercalciuric and non-hypercalciuric patients are shown in Fig. 2. A separate analysis was made according to gender for metabolic disturbances, such as hypercalciuria and hyperuricosuria, the definitions criteria of which are different for males and females. When analyzing urinary calcium levels according to gender, we observed that both female and male hypercalciuric patients presented a significant reduction in mean urinary calcium after *P. niruri* intake (5.1 ± 1.3 vs 3.9 ± 1.4 and 4.5 ± 0.6 vs 3.0 ± 0.6 mg/kg/24h, respectively) but not after placebo intake (4.6 ± 0.1 vs 5.3 ± 2.0 and 5.0 ± 0.7 vs 4.2 ± 1.8 mg/kg/24h, respectively). Among the normocalciuric males, there were no differences in urinary calcium after *P. niruri* or the placebo. The normocalciuric females presented a slight but significant increase in mean urinary calcium, after administration of both *P. niruri* and the placebo (2.3 ± 0.7 vs 3.0 ± 1.4 and 2.2 ± 0.5 vs 2.9 ± 1.1 mg/kg/24h, respectively), but this increase occurred within the normal limits. Urinary uric acid levels were not different when separately analyzed for females or males with normo- or hyperuricosuria.

Finally, the number of calculi as well as calculus size observed by US were not modified by *P. niruri* or the placebo (Table 5). Four of 33 patients receiving *P. niruri* and five of 36 patients receiving the placebo passed calculi during the 3 months of the study (data not shown).

Table 2 Serum parameters pre- and post-placebo or *P. niruri* administration. Values are means ± SD

	Placebo		*P. niruri*	
	Pre	Post	Pre	Post
Calcium (mg/dl)	9.5 ± 0.6	9.5 ± 0.4	9.1 ± 1.4	9.5 ± 0.4
Uric acid (mg/dl)	4.9 ± 1.7	4.9 ± 1.6	5.2 ± 1.6	4.9 ± 1.6
Creatinine (mg/dl)	0.9 ± 0.1	0.8 ± 0.3	0.8 ± 0.1	0.8 ± 0.2
Sodium (mEq/l)	140 ± 2.5	139 ± 1.3	139 ± 2	138 ± 1.6
Potassium (mEq/l)	4.4 ± 0.4	4.3 ± 0.4	4.3 ± 0.3	4.3 ± 0.3

Discussion

Urolithiasis is a disease highly prevalent throughout the world, carrying significant morbidity and consequent costs. Although considerable efforts have been made to identify effective treatments for the disease, this is a goal yet to be achieved.

Herbal medicines have been used for a long time for the treatment of many different diseases, with variable and sometimes negative consequences. Thus, carefully conducted studies are essential to evaluate the potential use of a given plant or compound for treating a particular illness. Despite the widespread use of *P. niruri* as a folk remedy for urinary calculi in Brazil, no controlled study evaluating its effect on lithogenesis has been published.

The present study was designed to evaluate the potential therapeutic role of *P. niruri* in urolithiasis. In our clinical practice, there have been anecdotal reports of higher calculus voiding among patients who consumed *P. niruri* preparations. In the present series, however, neither the number nor the size of the calculi observed by US was modified after *P. niruri* administration. In addition, no effect of *P. niruri* was detected on painful crisis frequency or magnitude. As this study was not intended to identify the differences in clinical characteristics, we believe that the short-term nature of our observations and the relatively small number of patients might have accounted for these negative findings. Although we did not find any significant effect on calculus elimination following the short-term administration of *P. niruri*, a potentially important effect related to urolithiasis, namely control of hypercalciuria, was disclosed. Regardless of gender, patients presenting with hypercalciuria, one of the most prevalent biochemical abnormalities found in urolithiasis, had their urinary calcium levels significantly reduced following a 3 month period of *P. niruri* administration. Although this conclusion is drawn from a subgroup analysis, all patients were randomized to receive *P. niruri* or a placebo and treated in a double-blind fashion. While all hypercalciuric patients receiving *P. niruri* had their calcium levels normalized, the response of hypercalciuric patients receiving the

Table 3 Urinary parameters pre- and post-placebo or *P. niruri* administration. Values are means ± SD. * $P < 0.05$ pre vs post

	Placebo		*P. niruri*	
	Pre	Post	Pre	Post
Volume (ml)	1,965 ± 702	2,048 ± 951	1,786 ± 700	1,839 ± 874
Calcium (mg/24 h)	231 ± 108	230 ± 109	200 ± 86	206 ± 97
Uric acid (mg/24 h	555 ± 196	539 ± 267	504 ± 174	490 ± 157
Creatinine (mg/24 h)	1,497 ± 456	1,481 ± 565	1,385 ± 380	1,379 ± 452
Sodium (mEq/24 h)	243 ± 111	228 ± 103	219 ± 99	186 ± 72
Potassium (mEq/24 h)	53 ± 24	57 ± 23	48 ± 18	53 ± 16
Citrate (mg/24 h)	497 ± 266	477 ± 289	366 ± 215	427 ± 389
Magnesium (mg/24 h)	88 ± 30	81 ± 25	82 ± 15	74 ± 19*
Oxalate (mg/24 h)	31 ± 13	33 ± 15	31 ± 18	33 ± 13

Table 4 Urinary parameters pre- and post-placebo or *P. niruri* administration according to the metabolic disturbance. Values are means ± SD. * $P < 0.05$ pre vs post

	Placebo			*P. niruri*		
	n	Pre	Post	*n*	Pre	Post
Calcium (mg/kg/24 h)						
Hypercalciurics	12	4.9 ± 0.6	4.5 ± 1.8	8	4.8 ± 1.0	3.4 ± 1.1*
Non-hypercalciurics	24	2.4 ± 0.6	2.8 ± 1.0	25	2.4 ± 0.8	2.9 ± 1.5
Uric acid (mg/24 h)						
Hyperuricosurics	3	1,023 ± 193	1,179 ± 467	2	815 ± 52	732 ± 168
Non-hyperuricosurics	33	513 ± 132	481 ± 151	31	484 ± 159	474 ± 145
Citrate (mg/24 h)						
Hypocitraturics	10	242 ± 52	268 ± 124	16	223 ± 53	246 ± 62
Non-hypocitraturics	26	554 ± 177	447 ± 187	17	554 ± 177	447 ± 187
Oxalate (mg/24 h)						
Hyperoxalurics	6	53 ± 8	44 ± 23	6	61 ± 17	48 ± 12
Non-hyperoxalurics	30	27 ± 9	31 ± 12	27	25 ± 9	29 ± 11
Magnesium (mg/24 h)						
Hypomagnesiurics	7	42 ± 9	57 ± 14	3	53 ± 6	69 ± 6
Non-hypomagnesiurics	29	99 ± 21	87 ± 24	30	85 ± 13	75 ± 20

placebo was not significantly different from the baseline pre-treatment levels. In addition, urinary calcium levels in non-hypercalciuric patients remained unaffected by *P. niruri* administration.

We recognize that our results are preliminary and that we do not have the necessary elements to explain the intriguing effects of *P. niruri* or to conclude that it will be beneficial for the long-term treatment of urolithiasis. In analyzing our results, some points must be taken into consideration. We did not use a purified compound but rather a liophylized extract (containing a mixture of substances, including alkaloids, flavonoids, terpenes, lignans, tannins and coumarins). It has been reported that the aqueous extract of *P. niruri* contains more than 50 different chemical compounds. The phytochemical and pharmacological properties of the genus *P. niruri* have been accounted for by the action of different substances. Rutin, β amylin, β-sitosterol and caffeic acid posses anti-inflammatory and/or analgesic activities, geranin inhibits angiotensin-converting enzyme, quercetin inhibits phosphodiesterase, niruside inhibits HIV reverse transcriptase, and repandusinic acid A may act as a hepatoprotector[1]. To date, none of these compounds has been shown to have an effect on calciuria. Moreover, literature on well-conducted clinical trials with natural products is scarce, and only a handful of papers on *P. niruri* are available. Only a single clinical study has addressed the effects of *P niruri* on the urinary system [11], and experimental reports from our group focusing on the relationship between *P. niruri* and lithogenesis are available [4, 5, 6]. Thus, attempts to clearly interpret the present findings may lead to inaccuracies and would be speculative. In order to provide mechanistic insights into the effects of *P. niruri* on urinary calcium, additional studies should be carried out.

In summary, we have identified a potential beneficial effect of *P. niruri* on hypercalciuria, an important risk factor for stone formation. These findings will have to be confirmed in a larger trial involving only hypercalciuric patients. In addition, longer-term studies are necessary to define whether these biochemical modifications can be translated into clinical benefit.

Acknowledgements This research was supported by CEME (Central de Medicamentos), FOR (Fundação Oswaldo Ramos), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). We thank Dr. Antonio José Lapa and Mirtes Midori Tanae for providing the aqueous extract of *Phyllanthus niruri*.

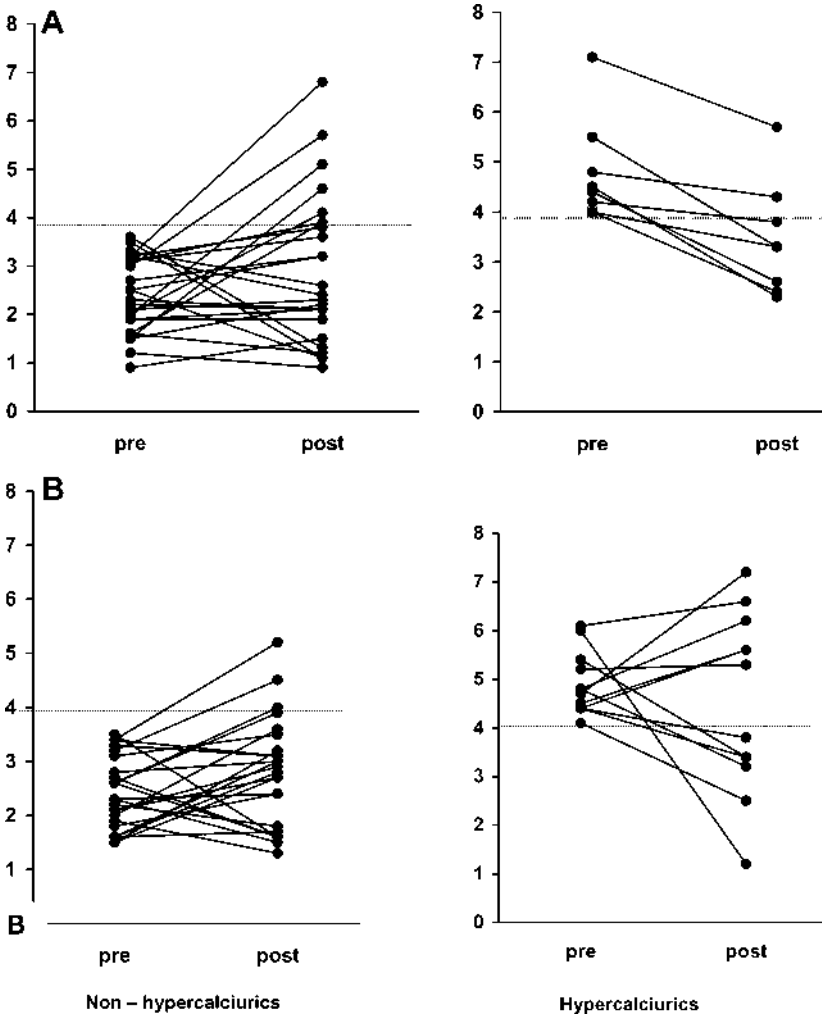

Fig. 2 Urinary calcium (mg/24h) pre and post *P. niruri* (**A**) or placebo (**B**) administration in hypercalciuric and non-hypercalciuric patients

Table 5 Number of calculi and calculus size pre- and post-placebo or *P. niruri* administration detected by ultrasonography. Values are means ± SD

	Placebo		*P. niruri*	
	Pre	Post	Pre	Post
Number of calculi	2.0 ± 1.2	1.6 ± 1.4	1.8 ± 0.9	1.5 ± 1.4
Calculi size (cm)	0.6 ± 0.2	0.6 ± 0.3	0.6 ± 0.2	0.6 ± 0.2

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ORIGINAL ARTICLE

Vol. 44 (4): 758-764, July - August, 2018
doi: 10.1590/S1677-5538.IBJU.2017.0521

Effect of *phyllanthus niruri* on metabolic parameters of patients with kidney stone: a perspective for disease prevention

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ABSTRACT

*Phyllanthus niruri (P.niruri) o*r stone breaker is a plant commonly used to reduce stone risk, however, clinical studies on this issue are lacking.
Objective: To prospectively evaluate the effect of *P. niruri* on the urinary metabolic parameters of patients with urinary lithiasis.
Materials and Methods: We studied 56 patients with kidney stones <10mm. Clinical, metabolic, and ultrasonography assessment was conducted before (baseline) the use of *P. niruri* infusion for 12-weeks *(P. niruri)* and after a 12-week (wash out) Statistical analysis included ANOVA for repeated measures and Tukey's/McNemar´s test for categorical variables. Significance was set at 5%.
Results: Mean age was 44±9.2 and BMI was 27.2±4.4kg/m2. Thirty-six patients (64%) were women. There were no significant changes in all periods for anthropometric and several serum measurements, including total blood count, creatinine, uric acid, sodium, potassium, calcium, urine volume and pH; a significant increase in urinary potassium from 50.5±20.4 to 56.2±21.8 mg/24-hour (p=0.017); magnesium/creatinine ratio 58±22.5 to 69.1±28.6mg/gCr24-hour (p=0.013) and potassium/creatinine ratio 39.3±15.1 to 51.3±34.7mg/gCr24-hour (p=0.008) from baseline to wash out. The kidney stones decreased from 3.2±2 to 2.0±2per patient (p<0.001). In hyperoxaluria patients, urinary oxalate reduced from 59.0±11.7 to 28.8±16.0mg/24-hour (p=0.0002), and in hyperuricosuria there was a decrease in urinary uric acid from 0.77±0.22 to 0.54±0.07mg/24-hour (p=0.0057).
Conclusions: P.niruri intake is safe and does not cause significant adverse effects on serum metabolic parameters. It increases urinary excretion of magnesium and potassium caused a significant decrease in urinary oxalate and uric acid in patients with hyperoxaluria and hyperuricosuria. The consumption of *P.niruri* contributed to the elimination of urinary calculi.

ARTICLE INFO

Keywords:
Kidney Calculi; Disease; Urolithiasis

Int Braz J Urol. 2018; 44: 758-64

Submitted for publication:
September 24, 2017

Accepted after revision:
February 13, 2018

Published as Ahead of Print:
March 10, 2018

INTRODUCTION

Risk factors for urolithiasis include congenital, genetic, environmental, dietary and metabolic aspects; chronic diseases including obesity, hypertension and diabetes are also associated with urinary calculus formation (1). Overall, urinary

calculi derive from a combination of some of the factors involved in its pathophysiology (1). The worldwide prevalence of urinary calculi is 8.8% and is more frequent in Caucasians, obese individuals, and those with a low income (1). The recurrence rate of urinary calculi is 50% within 10 years of the first episode (2). In addition to the conditions stated above, metabolic disorders such as hypercalciuria and hypocitraturia are typically involved in the genesis of urinary calculi (3).

Knowledge of the pathophysiological mechanisms involved and their risk factors, such as low urinary volume and high intake of calories, sodium and protein are important for modifying the natural history of the disease (2). Indeed, a reduction in the recurrence of urinary calculi of at least 50% can be achieved with dietary guidelines, lifestyle changes and the use of specific medications (4, 5).

In addition to conventional treatment for lithiasis, medicinal plants have long been used worldwide (6). *Phyllanthus niruri* or "stone breaker tea" is one such natural alternative that is inexpensive, easy to obtain and has a low incidence of adverse effects (7). To date, anti-inflammatory (8), anti-hyperuricemic (9), and diuretic properties have been described for this plant (10). Although many studies have shown the beneficial effects of *P. niruri* and its potential to inhibit the formation of kidney stones, clinical studies remain scarce (11, 12).

Thus, the aim of this study was to evaluate the effects of *P. niruri* on metabolic parameters in patients with urolithiasis.

This protocol was submitted to and approved by the Ethics Committee for Research Project Analysis of the Clinical Hospital, University of São Paulo, Medical School under number 0304/11. It was also approved and sponsored by the Foundation of Research Support of São Paulo (FAPESP) under number 12/50031-7.

MATERIALS AND METHODS

The study was developed at the Urologic Division of the Clinical Hospital, University of São Paulo, Medical School. All patients included presented one or multiple stones smaller than 10mm. Diagnosis was based on ultrasonography or computed tomography (CT). The age of the patients ranged between 18 and 60 years.

Exclusion criteria included patients with a serum creatinine level >2.0mg/dL, urinary tract infection, non-controlled diabetes, chronic liver disease, cancer or pregnant women.

The study was divided into three stages: baseline, *P. niruri* and washout. The baseline stage was that prior to intervention. The *P. niruri* stage consisted of 12 weeks of ingestion of an infusion tea prepared with the dry extract of *P. niruri*, according to literature recommendations (13), a week of rest was included without using the plant after each week of consumption (2 weeks). The final step, called washout, involved 12 weeks without ingestion of *P. niruri*. The patients themselves were the controls, and each patient was followed for 26 weeks. The patients were subjected to clinical, anthropometric, serum and urinary metabolic analyses at all stages of the study.

Demographic data included age, race, sex, family history and medication use. Clinical data comprised systolic and diastolic blood pressure and anthropometric evaluation (weight, height, body mass index (BMJ). Serum biochemical and urinary analyses and renal ultrasonography were performed at baseline, immediately after *P. niruri* use and at the end of the washout period. Image evaluation was performed for all patients by the same radiology physicians who were blinded to the group composition.

Serum biochemical analysis was performed during the baseline, *P. niruri* and washout periods and included a complete blood count and assessment of urea, creatinine, sodium, potassium, glucose (fasting), uric acid, total and ionized calcium, beta human chorionic gonadotropin (HCG; females only), total cholesterol and fractions, triglycerides, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, amylase and bilirubin levels. Serum samples were collected from all participants after fasting for 12 hours.

Twenty-four-hour urinary measurements included calcium, oxalate, citrate, uric acid, magnesium, sodium, potassium, creatinine, urea and phosphorus levels. Urinalysis with urinary pH

measurement and a urine culture was performed using spontaneous voided urine.

The criteria for evaluation of metabolic abnormalities in 24-hour urine samples (mg/g creatinine) were hypercalciuria (calcium >240/>200mg/24-hour), hyperoxaluria (oxalate>40mg/24-hour), hypocitraturia (citrate<320/<290mg/24-hour), hypernatriuria (sodium >150mg/24-hour) hyperuricosuria (uric acid >0.75/>0.6g/24-hour), abnormal pH (<5.8 and >6.2) and low urine volume (<2L/day) for men and women, respectively.(4).

After receiving clinical, laboratory and abdominal ultrasound evaluations, the patients included in the study received a monthly visit and 60 sachets of *P. niruri* as a dry extract containing 4.5g of the herb. The patients were instructed to prepare an infusion containing 250mL of boiling water for each 4.5g herb sachet and to drink two sachets/day.

Extracts of *P. niruri* (family Euphorbiaceae) were obtained from qualified manufacturers and from medicinal plant laboratories, and we obtained physicochemical and microbiological analysis reports for all lots used in this study. The plant was produced in Brazil, subjected to a shade drying method, stored at a temperature of 15-35°C in a dry place and conserved with gamma irradiation to avoid contamination by fungi or bacteria. The minimum tannin content was 1.5%, and the moisture content was 17%. Indications for use in disorders of the urinary tract include renal lithiasis, cramps, cystitis and nephritis. The plant is known to have analgesic, anti-inflammatory and hepatoprotective properties.

The use of medications for hypertension or other diseases was not discontinued for the study or considered in the analysis. Only patients using potassium citrate were instructed to discontinue its use two months prior to initiation of the study to avoid interference with the results of 24-hour urine citrate dosing.

Statistical analysis was performed with SAS-Statistical Analysis Software Version 9. Continuous variables are presented as the mean and standard deviation, and categorical variables are presented as frequency percentages. We used analysis of variance (ANOVA) for repeated measures, which takes into account patients being evaluated at three different times (repeated measures) for comparing the results. Significant differences between periods or stages of evaluation were compared with Tukey's test and the McNemar test. The significance level was set at 5%.

RESULTS

In total, 430 patients were enrolled and initially screened, and 75 patients were considered eligible according to the inclusion criteria. Fifty-six patients completed the study after a few dropped out due to work or personal problems, surgeries or other treatments for non-urological (breast carcinoma, biliary calculus) diseases.

Of the 56 patients who remained, 36 (64.3%) were women, and 52 (92.8%) were Caucasians. The mean age was 44±9.2 years (22-58 years), and the baseline BMI was 27.2±4.4kg/m^2. Thirty patients (53.6%) had a family history of calculi, 53 (94.6%) were sedentary, 27 (48.2%) had hypertension, and 26 (46.4%) had metabolic syndrome.

Of the studied cohort, 26 patients (46.4%) were using antihypertensive drugs; one patient (1.8%) was under hypoglycaemic medication, and four patients (7.1%) were using antidepressants, among other medications. Diastolic blood pressure showed a slight increase, though not significant, during the *P. niruri* period, reaching 76.0±10.5mmHg and then decreasing to 72.5±10.5mmHg (p=0.02) during the washout period.

Abdominal pain was reported by 37 (66.1%) patients during the *P. niruri* period; dysuria occurred in 11 (19.6%) cases, haematuria in eight (14.3%) cases, and nausea and epigastric pain in six cases each (10.7%). However, these symptoms did not lead to discontinuation of infusion use in any case.

No significant changes in serum measurements, except for a significant decrease in alkaline phosphatase after the use of *P. niruri* when compared to the baseline period (67.7±22.2x63.5±20.6mg/dL; p=0.017), were found.

The 24-hour urine analysis revealed only a significant increase in potassium levels between the basal and washout periods, from 47.3±16.7mg/24-

-hour to 56.2±21.8mg/24-hour (p=0.017). However, a trend towards increased urinary potassium was noted during the use of the tea.

Analysis of electrolytes adjusted for 24-hour urine creatinine (Cr) revealed an increase in potassium and magnesium levels. This increase reached statistical significance in the washout period, as summarized in Table-1.

In the baseline stage, hypernatriuria was the most frequent urinary disturbance, occurring in 34 patients (60.7%). Hypocitraturia and hypercalciuria were observed in 24 (42.8%) cases, hyperuricosuria in six (10.7%), hyperoxaluria in five (8.9%), and low urine volume in 31 (55.3%). Furthermore, the pH value changed in 21 (37.5%) patients.

Evaluation of patients with urinary abnormalities at the baseline stage showed a tendency towards an increase in urinary citrate among the hypocitraturic patients (211.8±123.7 to 322.3±145.8mg/24-hour, p=0.2193). In addition, oxalate was significantly reduced, from 59.0±11.7 to 28.8±16.0mg/24-hour (p=0.0002), among those with hyperoxaluria, and uric acid was significantly decreased, from 0.77±0.22 to 0.54±0.07mg/24--hour (p=0.0057), among hyperuricosuric patients (Table-2).

Ultrasonography evaluation performed in the three periods of the study revealed that the total number of stones decreased in 38 (67.8%) patients, as summarized in Table-3 (from 3.2±2.02 to 2.0±2.07) and size. In 10 patients (17.8%), no alterations in the number of calculi were noted, and in eight patients (14.3%), an increase in the number of upper urinary stones was observed after the *P. niruri* period. Some patients reported spontaneous stone passage between the 21st and 70th day of the *P. niruri* period: four patients eliminated six stones, and another five individuals reported the presence of sandy fragments in the urine during the *P. niruri* period.

DISCUSSION

The formation of urinary calculi is associated with different risk factors. Its prevalence is

Table 1 - Urinary parameters in patients treated with *P. niruri*.

Measure	MEAN (SD)			ANOVA P	TUKEY		
	Baseline	mg/vol.24-hour *P. niruri*	Wash out		Baseline x *P. niruri*	Baseline x Wash out	*P. niruri* x Wash out
Calcium	202.7±116.1	211.8±96.2	190.3±92.6	0.401	0.849	0.675	0.371
Uric acid	0.5±0.2	0.5±0.2	0.5±0.2	0.875	0.870	0.990	0.934
Sodium	171.6±74.0	166.1±78.9	182.7±62.2	0.368	0.881	0.610	0.344
Urea	20.1±6.0	18.6±7.7	20.9±8.4	0.070	0.278	0.679	0.063
Potassium	**47.3±16.7**	**50.5±20.4**	**56.2±21.8**	**0.023**	0.536	**0.017**	0.192
Citrate	379.9±191.1	398.1±219.0	412.8±175.8	0.515	0.922	0.488	0.739
Oxalate	24.4±15.5	23.9±14.5	22.3±9.7	0.639	0.952	0.619	0.795
Creatinine	1.3±0.5	1.3±0.5	1.2±0.5	0.672	0.682	0.773	0.989
Phosphorus	755.3±308.8	792.4±324.6	684.9±281.1	0.090	0.817	0,265	0.085
Magnesium	73.8±35.4	85.6±37.6	82.3±37.6	0.114	0.113	0.299	0.864
***Mg/Cr**	**58.0±22.5**	**66.1±23.4**	**69.1±28.6**	**0.012**	0.081	**0.012**	0.736
***K/Cr**	**39.3±15.1**	**42.7±19.3**	**51.3±34.7**	**0.009**	0.587	**0.008**	0.095
pH	6.1±0.9	6.0±0.9	6.1±0.9	0.6698	0.7916	0.9705	0.6663
Volume	1927±614.5	2028.8±790.6	2014.7±656.6	0.4361	0.4044	0.8567	0.7481

SD = standard deviation; **K** = potassium; **Mg** = magnesium; **Cr** = creatinine

Table 2 - Metabolic alterations in 24-hour period urine at baseline and when taking *P. niruri* and Wash out.

Metabolic alteration	Measure Urine 24- hour	Reference	Baseline	MEAN (SD) *P. niruri* mg/ vol.24-hour	Wash out	ANOVA P
Hypercalciuria (n=24)	Calcium	<240/200*	300.3 ± 106.6	237.2 ± 70.7	227.5 ± 79,3	0.2619
Hyperuricosuria (n=6)	**Uric acid**	**<0.75/0.6***	**0.77 ± 0.2**	**0.54 ± 0.1**	**0.56 ± 0.12**	**0.0057**
Hypocitraturia (n=24)	Citrate	>290/320*	211.8 ± 123.7	322.3 ± 147.3	345.3 ± 147.3	**0.2193**
Hyperoxaluria (n=5)	**Oxalate**	**<40**	**59.0 ± 11.7**	**28.8 ± 16.0**	**33.0 ± 4.4**	**0.0002**
Hypernatriuria (n=34)	Sodium	<150	211.6 ± 62.8	183.5 ± 92.0	200.6 ± 59.7	0.1770

***** = value reference for men/women; **SD** =standard deviation

Table 3 - Number and size of upper urinary calculi in patients treated with *P. niruri*.

Measure	MEAN (SD)			ANOVA P	TUKEY		
	Baseline	*P. niruri*	Wash out		Baseline x *P. niruri*	Baseline x Wash out	*P. niruri* x Wash out
Total calculi (n)	3.2±2.0	2.0±2.1	2.2±2.2	**0.0005**	**0.0005**	**0.015**	0.672
Right kidney (n)	1.6±1.4	1.1±1.2	1.2±1.4	**0.0176**	**0.0176**	**0.201**	0.587
Left kidney (n)	1.6±1.4	0.9±1.1	1.0±1.1	**0.0003**	**0.0003**	**0.003**	0.938
Size (mm)	15.6±10.6	9.4±8.9	11.2±11.1	**0.0002**	**<0.0001**	**0.045**	0.206

SD = standard deviation; **N** = 56

high worldwide with an increase in morbidity and health costs in a number of countries. However, fully effective treatment and prevention have yet to be established, and the use of medicinal plants may be helpful as coadjuvants. The use of *P. niruri*, a very common plant found in different countries, has been shown to be a viable alternative, though more clinical studies are necessary.

In the present study, we sought to evaluate the use of *P. niruri* in patients with small urinary stones. When we analysed urinary variables, we found no significant changes in urinary volume for a 24-hour period with the use of an infusion of the plant. The urine volume was close to the minimum recommended in the literature, which is 2L/day (3, 4). The patients in this study exhibited average values of 1927mL, 2029mL, and 2015mL daily for the baseline, *P. niruri* and washout periods, respectively. It is possible that the patients were aware of the importance of fluid intake to prevent the formation of new calculi and were thus already consuming the quantity of liquid recommended at the beginning of the study; this would have resulted in no significant change in urine volume. The diuretic effects of *P. niruri* have been described in experimental studies (10, 15), though Nishiura et al. (11) reported different results in a clinical study.

The increase in the 24-hour urine electrolytes found in this study may be related to the decrease in the number of calculi during the imaging evaluation. Increases in urinary potassium and magnesium levels lead to alkalinisation of the urine and consequently to an increase in urinary citrate, a potent inhibitor of calcium stone formation (5).

Potassium can moderate the concentration of sodium in urine and promote the elevation of citrate, which acts to correct urinary pH and acidity, possibly contributing to an increase in calcium solubility (5). These changes may interfere with some stages of crystallization in urine, such as a reduction in the nucleation, growth and aggregation of calcium oxalate crystals (5, 14). We

observed a significant increase in potassium in 24-hour period urine samples between the baseline and washout periods, which was also noted in the change in the potassium/g Cr 24-hour ratio and in the change in the magnesium/g Cr 24-hour ratio. The increased potassium and magnesium levels observed in the study patients may help to explain the normalization of metabolic changes observed following the use of *P. niruri*.

Urine pH in the sample urinalysis did not change throughout the study and remained at a mean value between 6.0 and 6.1.

Diastolic blood pressure showed a significant reduction between the *P. niruri* and washout periods (16). A report of a hypotensive effect of *P. niruri* can be found in the literature (8).

The calculus imaging evaluation performed showed a reduction in the number of calculi after the *P. niruri* stage, as shown in Table-3, considering that the number of stones shown on ultrasound was lower due to the fact the some patients probably passed some of them during the study.

Although the same method of evaluation was utilized in the study performed by Nishiura et al., those authors did not show a change in the number and size of calculi before and after the use of a *P. niruri* extract in individuals with urinary lithiasis (11). In contrast, it has been reported that *P. niruri* promoted a reduction in the number of calculi and their appearance in relation to the most fragile aspect of the calculi structure (7, 17-19).

Although ultrasonography is not considered a gold standard for the evaluation of small calculi, we performed this evaluation for the purpose of clinical monitoring of the patients because repeated CT scans in a short period of time can result in undue radioactivity exposure, which is not recommended (20).

Some patients experienced haematuria and abdominal pain. The exact cause of these events not was clear and continuous monitoring of patients at monthly visits was implemented as a precaution during the study. The haematuria and abdominal pain may be related to small calculi eliminated during that period, as patients observed the presence of sand fragments in the urine. Regardless, we cannot attribute the pain reported to the consumption of *P. niruri* because this symptom is very frequent in patients with lithiasis.

In this study, there were no changes in serum levels of liver enzymes, urea and serum creatinine. In previous experimental studies, no adverse acute or chronic toxic effects, such as kidney, heart, liver or neurological effects, were reported with the use of *P. niruri* (17, 18). In a human study, Wang et al. (21) found the normalization of liver enzymes when the plant was used in patients with chronic liver disease. However, Nishiura et al. (11) reported no change in serum and urinary parameters when analysing 69 patients and a control group with and without the use of *P. niruri* (11).

Most studies to date have focused on the hepatoprotective effects of *P. niruri*, and the genus Phyllanthus has been utilized in the treatment of various liver disorders (8). The reduction in alkaline phosphatase levels observed in this study was beneficial. In addition to showing that there was no toxicity with the use of the plant extract, this decrease may have contributed to the reduction in the number of calculi at the end of the study, because alkaline phosphatase is a biochemical marker of bone metabolism and may be involved in the mechanism of calculus formation in hypercalciuria (22).

According to our evaluation of patients with metabolic alterations at the baseline and *P. niruri* periods, a significant normalization of urinary uric acid and oxalate values occurred in those with hyperuricosuria and hyperoxaluria, respectively. Citrate levels tended to norma lize in the same cohort, though without statistical significance, likely due to the small sample size. Moreover, no significant change was noted for patients with hypercalciuria or hypernatriuria at the basal stage.

Overall, clinical studies with more patients are needed to validate the use of *P. niruri* in daily practice, particularly in patients with baseline urinary metabolic disorders. *P. niruri* is an abundant natural resource available in many countries, and it can reduce the health system expenses associated with conventional drugs, which are often inaccessible to the majority of the population for long-term treatment.

CONCLUSIONS

P. niruri intake is safe and does not cause significant adverse effects or significant serum

metabolic changes. The use of the tea of this plant increases urinary excretion of magnesium and potassium. Patients with specific urinary metabolic changes such as hyperuricosuria and hyperoxaluria may benefit from ingestion of this tea.

CONFLICT OF INTEREST

None declared.

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Actas Urológicas Españolas 49 (2025) 501791

Actas Urológicas Españolas

www.elsevier.es/actasuro

REVIEW ARTICLE

Phyllanthus niruri in the management of nephrolithiasis: A systematic review of the literature

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Received 22 January 2025; accepted 5 April 2025
Available online 4 June 2025

KEYWORDS
Phyllanthus;
Nephrolithiasis;
Lithotripsy

Abstract
Introduction and objectives: Nephrolithiasis is one of the most prevalent urological pathologies worldwide, with an increasing incidence and multifactorial etiology, particularly influenced by diet. Surgical interventions or extracorporeal shock wave lithotripsy (ESWL) are the cornerstone treatments. However, as emphasized by the EAU and AUA guidelines, post-surgical medical management is recommended to reduce recurrence risk. *Phyllanthus niruri* (PN), widely used in traditional medicine, has been extensively researched, yielding mixed results and presenting an opportunity to explore its role further. This review aims to evaluate PN's potential in enhancing treatment efficacy and reducing stone recurrence.
Materials and methods: A systematic literature review was conducted, encompassing articles published from January 1994 to September 2022 in English and Spanish. The review included studies on humans and rats accessible through the authors' institutional affiliations. Titles and abstracts were screened, and relevant studies were selected for in-depth analysis.
Results: Out of the 16 selected studies, various mechanisms of action for PN were identified, such as promoting glycosaminoglycan (GAG) aggregation, inhibiting nucleation processes, and altering stone density to favor a stone-free state (SFR). Evidence consistently supports PN's long-term safety, confirmed by serial measurements of serum electrolytes and liver function. Novel applications, such as PN as an adjuvant to ESWL, show benefits for lower renal pole stones.

DOI of original article: https://doi.org/10.1016/j.acuro.2025.501791
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https://doi.org/10.1016/j.acuroe.2025.501791

Conclusions: Growing evidence suggests that PN, when used alongside traditional interventions, is safe, without significant adverse effects, and may improve SFR outcomes after ESWL.

PALABRAS CLAVE
Phyllanthus;
Litiasis;
Litotricia

Phyllanthus niruri en el manejo de las litiasis urinarias: revisión sistemática de la literatura

Resumen

Introducción y objetivos: La nefrolitiasis es una de las patologías urológicas más prevalentes, con una incidencia en aumento y una etiología multifactorial muy influenciada por hábitos dietéticos. La cirugía y la litotricia extracorpórea por ondas de choque (LEOC) son la piedra angular del tratamiento, siendo el tratamiento médico postquirúrgico recomendado por las guías de la EAU y AUA para reducir el riesgo de recurrencia. *Phyllanthus niruri* (PN), usado en la medicina tradicional, ha sido ampliamente estudiado con resultados diversos. Por ello nuestro objetivo en este trabajo es evaluar el potencial de PN a la hora de reducir la recurrencia de litiasis.

Material y métodos: Realizamos una revisión sistemática de la literatura, incluyendo artículos en inglés y español publicados entre enero 1994 y septiembre 2022. Esta revisión incluye estudios tanto en humanos como en ratas accesibles a través de las instituciones de los autores. Se realizó un primer cribado con títulos y resúmenes, y los estudios relevantes se seleccionaron para su análisis en profundidad.

Resultados: De los 16 estudios seleccionados, se identificaron varios mecanismos de acción como promover la agregación de glucosaminoglucanos, inhibir la cristalización y modificando la densidad de las litiasis favoreciendo una mayor tasa libre de litiasis. La literatura apoya la PN como un tratamiento seguro a largo plazo, gracias a mediciones sobre la función renal y los electrolitos de los pacientes tratados. Además, indicaciones más novedosas como la PN antes de la LEOC, parece ofrecer beneficios en el tratamiento de las litiasis en grupo calicial inferior.

Conclusiones: Las litiasis renales suponen una carga significativa para los sistemas sanitarios. La evidencia creciente sugiere que la PN, cuando se combina con los tratamientos estándar, es segura y no tiene efectos adversos significativos, ayudando además a mejorar la tasa libre de litiasis y los resultados después de la LEOC.

Introduction

Nephrolithiasis is the third most common urinary tract condition, with a global prevalence of 10–12% in developed countries. It is more common in males and is influenced by genetic, environmental, metabolic, and dietary factors. Clinically, nephrolithiasis may present asymptomatically or as severe, colicky flank pain radiating to the ipsilateral groin. It is often accompanied by hematuria and autonomic symptoms such as nausea and headache, rendering it a debilitating disease.[1,2]

International guidelines highlight surgical options as primary treatment options if necessary, including percutaneous nephrolithotomy, ureteroscopy, and extracorporeal shock wave lithotripsy (ESWL), as primary treatments. Nonetheless, urologists must address the recurrence risk after treatment.[3] With nephrolithiasis carrying a high recurrence risk, pharmacological interventions are limited, exemplified by thiazides for hypercalciuric patients with calcium oxalate stones or potassium citrate for those with hypocitraturia. However, adherence remains low, primarily due to side effects and limited efficacy in preventing recurrence.[4]

Phyllanthus niruri (PN), has been traditionally used for kidney stone management.[1,2] This review explores PN's non-conventional role in managing nephrolithiasis and its potential as a complementary treatment.

Methods

Search strategy and data sources

A systematic literature review was performed following the PRISMA guidelines. Searches were conducted in PubMed, Cochrane CENTRAL, Embase, Scopus, and LILACS. Ongoing studies were also identified through ClinicalTrials.gov, the European Clinical Trials Registry, and the WHO ICTRP. Additional resources included Opengray, conference abstracts, books, and the reference lists of selected articles. Studies published between January 1994 and September 2022 were considered. MeSH/DeCS terms included 'phytotherapy,' 'nephrolithiasis,' 'lithotripsy,' 'urinary stone,' 'herbal

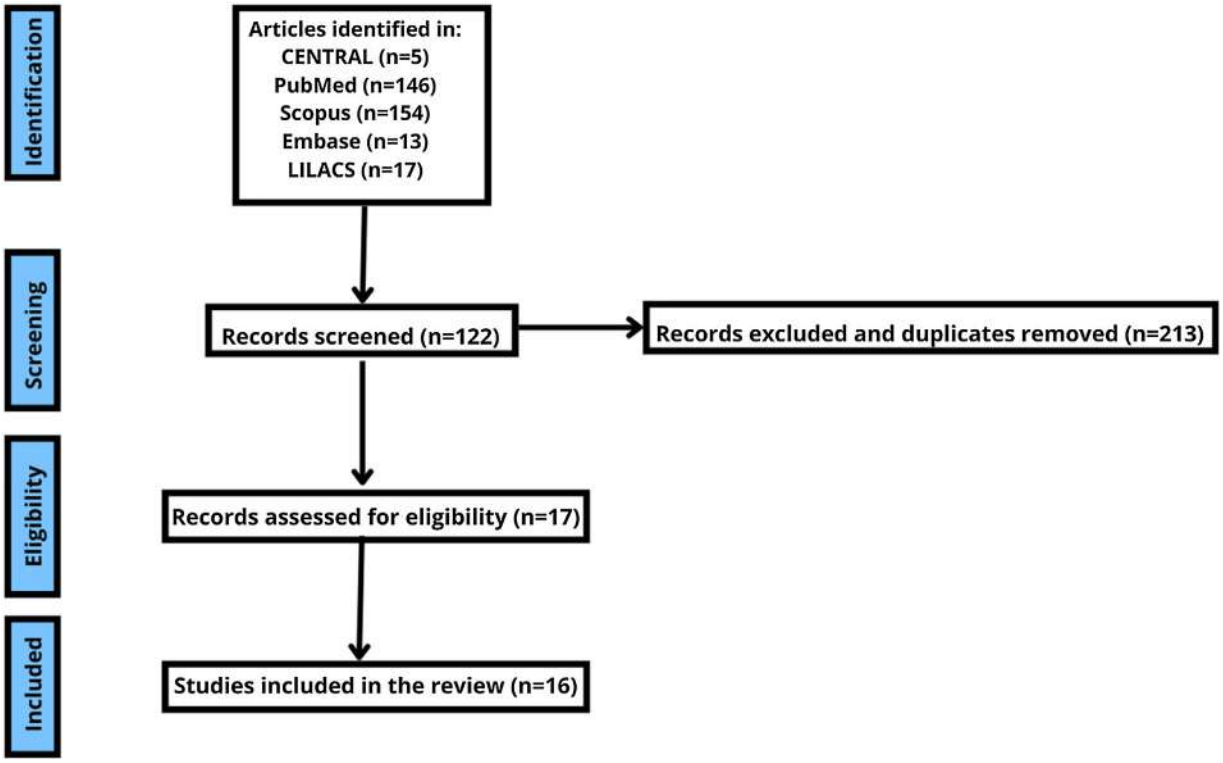

Figure 1 PRISMA flow diagram.

medicine,' and 'Phyllanthus.' The review included findings involving humans and animals, limited to English and Spanish publications, with access through institutional affiliations.

Study selection and data extraction

All authors independently screened titles and abstracts and selected eligible studies. Full texts were then evaluated for inclusion based on predefined criteria. A PICOST format was used for data extraction, encompassing study design, sample size, variables, interventions, comparisons, and outcomes. Data accuracy was verified twice to minimize bias.

Risk of bias assessment

The Cochrane Risk of Bias 2 (RoB 2) tool was employed, considering domains such as random sequence generation, allocation concealment, blinding, selective reporting, and other sources of bias.[5] Several studies showed high risk due to unclear randomization methods and selective reporting. Figs. 2 and 3 summarize the methodological quality of the included studies.

Results

Study selection and characteristics

Following the initial screening of titles and abstracts, duplicates, editor letters, and publications not focused on *Phyllanthus niruri* (PN) were excluded, along with articles outside the specified language and publication period. A total of 16 studies were included in this review. Fig. 1 shows the PRISMA flow diagram.

Mechanisms of action of *Phyllanthus niruri*

The selected studies revealed multiple mechanisms by which PN influences nephrolithiasis:

1 Glycosaminoglycan (GAG) Aggregation: PN has been shown to promote the aggregation of GAGs, which play a protective role against crystal formation.[6]
2 Inhibition of Nucleation: In vitro studies consistently demonstrated PN's ability to inhibit the nucleation of calcium oxalate crystals[7–9]
3 Modification of Residual Stone Density: PN alters stone density, making them more susceptible to the passage and increasing stone-free rate (SFR).[10,11]

In vitro and animal studies

In 2014, Khare et al. published an in vitro study to evaluate the anti-urolithiasis effect of PN extracts, using two standard polyherbal formulations (Neeri and Cystone) for stone dissolution as the control group. This study showed that PN leaf extract had a dissolving potential comparable to that of the drug, dissolving 56.8% of the crystals, vs 58.4% in the Cystone group with ($P > 0.05$) ($F(P < 0.01) = 126.47$, $df = 30$, $SE(d) \pm = 0.002$, $LSD(p = 0.061)$) and 64.8% of Neeri. Additionally, the aqueous extract of PN leafe reduced turbidity

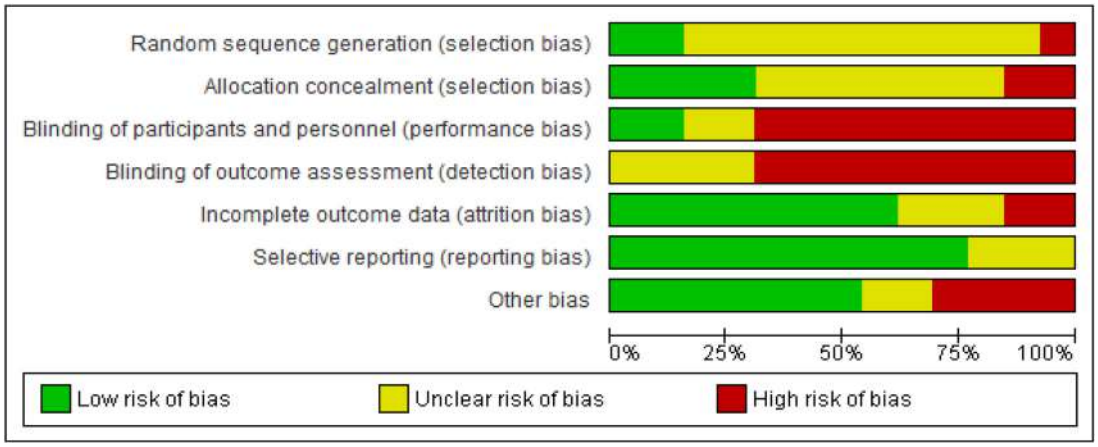

Figure 2 Methodological Quality Chart.

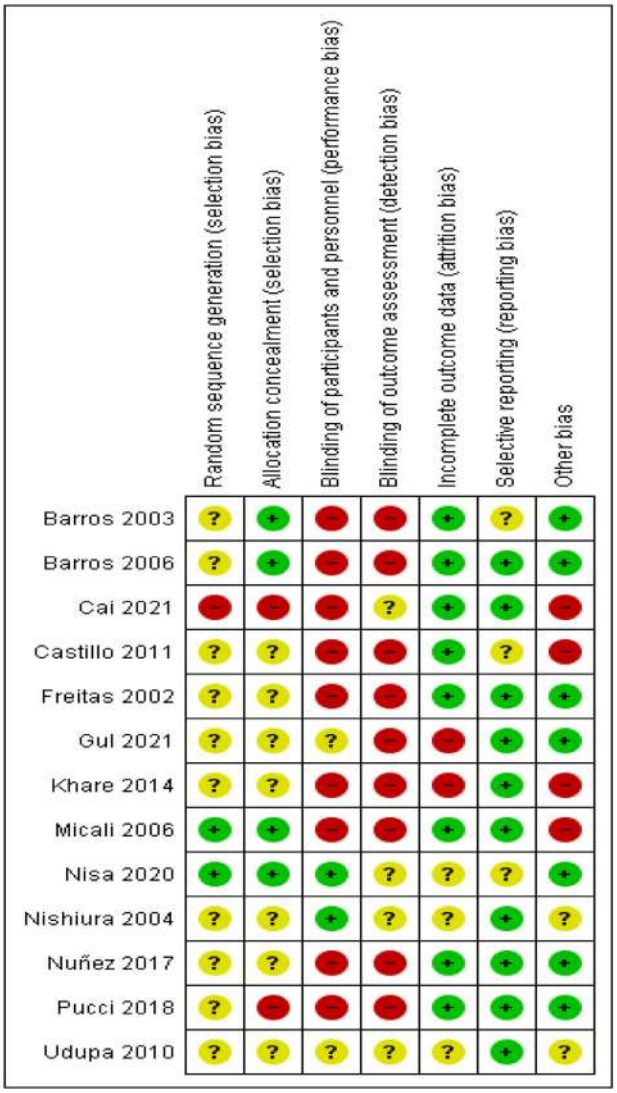

Figure 3 Summary of Methodological Quality.

by up to 53.09% after 180 s of chemical reaction, compared to the 64.8% of the standard drug ($F(P < 0.01) = 106.3$, df = 30, SE(d) ± = 0.39, $LSD(P < 0.05) = 6.28$), thus demonstrating the presence of some anti-lithogenic active compounds in PN.[7]

In 2021, Gul et al., using Cystone as the control group, demonstrated an in vitro inhibition of 66.67% ± 1.61 against calcium oxalate crystal aggregation and a dissolution rate of 63.33% ± 1.44 with aqueous PN extracts. However, differences were observed between the types of PN extracts, depending on the solvent used for extraction.[12]

In 2002, Freitas et al. conducted a study in rats to evaluate the role of aqueous PN extracts in the urinary excretion of endogenous lithogenesis inhibitors, such as GAGs. The study demonstrated that treatment with PN strongly inhibited stone growth compared to the control group. Additionally, it showed that GAG content in the stones was higher in the rats treated with calcium oxalate + PN, compared to the control group (48 g/g of stone versus 16.6 g/g of stone), highlighting the potential role of PN in preventing crystal growth by increasing the incorporation of GAGs into the stones.[6]

Clinical studies in humans

In 2006, Micali et al. conducted a prospective study of 150 patients with calcium oxalate kidney stones who underwent ESWL. One group was given a PN extract for at least 3 months after the procedure, whereas the other group served as control. The SFR at 180 days was 93.5% for patients treated with PN extract, compared to 83.3% in the control group ($p = 0.48$). A benefit was also observed in the subgroup of patients with lower calyx stones, with an SFR of 93.7% vs. 70.8% in the control group ($p = 0.01$). The retreatment rate was 39.7% versus 43.3% in the control group ($p = 0.2$), with no reported side effects.[13]

In 2018, Pucci et al. conducted a prospective study on 56 patients with kidney stones smaller than 10 mm, treated with a PN infusion. This was followed by an evaluation

of plasma metabolic parameters and 24-h urine analysis. A decrease in kidney stones was observed, from 3.2 ± 2 to 2 ± 2 stones per patient ($p < 0.001$). Furthermore, in patients with hyperoxaluria, urinary oxalate decreased from 59 ± 11.7–28.8 ± 16 mg/24 h ($p = 0.0002$), and in patients with hyperuricosuria, urinary uric acid decreased from 0.77 ± 0.22 to 0.54 ± 0.07 mg/24 h ($p = 0.0057$), with no significant changes in serum metabolic parameters.[14]

In 2021, Cai et al. published a prospective study with 82 patients undergoing treatment with a dietary supplement containing PN and *Chrysanthellum americanum* combined with potassium and magnesium citrate for 6 months. It was observed that 60.9% of the patients had a significant reduction in stone size by -6.7 ± 3 mm ($p = 0.001$), and 59.7% had no symptomatic episodes, with an improvement in quality of life compared to baseline ($+0.4 \pm 1$, $p < 0.001$). At the end of the follow-up period, 32.9% of the patients were stone-free.[15] In Table 1 we have summarized the main characteristics of the studies analyzed.

Discussion

Clinical implications of *Phyllanthus niruri*

Considering the high prevalence of nephrolithiasis in the global population, along with the high likelihood of recurrence of kidney stones after treatment, it is crucial to adopt therapeutic approaches that not only improve short-term outcomes but also optimize long-term therapeutic success rates. The management of kidney stones requires effective interventions that, in addition to ensuring the elimination of the stones, minimize the risk of new formations and reduce the costs associated with retreatments. In this context, optimizing SFR is a priority, as a higher SFR implies a reduced need for additional interventions, thereby lowering the overall healthcare costs.

Regarding strategies to improve SFR, the potential of adjunctive therapies such as PN could play a relevant role. PN presents attractive characteristics from a clinical perspective: its safety profile is favorable, with few reported side effects, and it has a positive impact on quality of life measurements, making it a viable option for many patients.[13,15] Additionally, its low cost and the ease with which it can be obtained make this medicinal plant an option in healthcare systems with economic limitations or in areas with limited access to more expensive treatments.[2,14]

The use of PN as a complementary therapy in the treatment of kidney stones becomes particularly interesting when considering the variable of the SFR in ESWL (Extracorporeal Shock Wave Lithotripsy). Although ESWL is one of the most commonly used modalities for treating kidney stones, its effectiveness is not always optimal, especially for harder stones or those located in the lower renal calyces. This highlights the need to explore complementary treatments that could enhance the effectiveness of ESWL and, consequently, improve success rates in stone elimination, as evidenced by Micali et al.[13] This could be due to the antispasmodic activity of the alkaloids extracted from PN, which induce relaxation of the smooth muscle of the urinary tract.[2]

Mechanisms of action

Formation of urinary stones is a process that results from a series of physicochemical events, including supersaturation, nucleation, and growth of the stone, aggregation of the nucleus, and impaction in the excretory system. In this process, calcium oxalate crystals adhere to the plasma membrane of the urothelial cell, causing their endocytosis, which ultimately leads to cell death. These cellular remnants detach from the basal membrane and adhere to other crystals, forming another nucleus of stones. As a result, several authors have proposed therapeutic schemes for the prevention of lithogenesis, specifically influencing the cellular pathways involved in the endocytosis and aggregation of calcium oxalate crystals.[2,16]

More than 50 compounds have been discovered in PN, including alkaloids, lignans, flavonoids, and triterpenes, which inhibit the cytotoxicity induced by calcium oxalate, reduce the excretion of lithogenic promoters, and the markers of crystal deposition in the kidneys.[2]

The role of phytotherapy generates some degree of skepticism in urological practice due to the limited high-quality evidence reported in clinical guidelines. However, both in vitro and in vivo studies have demonstrated the anti-lithogenic role of PN; it inhibits crystal nucleation and growth, increases urinary calcium oxalate excretion, and promotes a less favorable environment for lithogenesis.[4,17] Freitas et al. suggests that this mechanism may be due to the incorporation of GAGs into the stone, independent of urinary excretion of magnesium or citrate, which prevents the formation and aggregation of stones through different potential mechanisms: decreasing cation deposits by neutralizing the negative charges of GAGs, and it would also affect the Tamm-Horsfall protein, modulating the formation and aggregation of crystals, which could potentially prevent the growth of residual lithiasis after ESWL.[2,6]

Barros et al. observed that treatment with PN after stone formation induced changes in their shape and texture, which became more homogeneous and had more compact surfaces, unlike the untreated animals. This suggests that PN interferes with the mineralization process by promoting a different interaction between the crystal and the macromolecules of the organic matrix, which could contribute to improving the SFR or to a less painful stone spontaneous passage.[17]

Limitations and future directions

It is important to note that most of the available studies focused on patients with moderate or small-sized kidney stones.[14] This is relevant because the size of stones can significantly influence the therapeutic approach and the treatment response, limiting the generalization of the results obtained to cases with larger stones, whether in the kidney or in the ureter. Larger stones present additional challenges, such as difficulty in fragmentation or spontaneous elimination, suggesting that the treatments used in

Table 1 In vitro, animal and human clinical studies with PN.

Study	Study	*P. niruri*	Control	Objectives and results
Khare et al.,[7] 2014	In vitro	Water extract with 500 mg of PN	Poly herbal formulation (Cystone and Neeri)	• Disolving potential: Water extract 56.8%, Cystone 58.4%, Neeri 64.8% ($P > 0.05$). • Turbidity reduction: Water extract 53.09% after 180 s of chemical reaction, Neeri 81.23%, Cystone 76.54% ($P > 0.05$).
Gul et al.,[12] 2021	In vitro	100 mg PN	100 mg Cystone	• CaOx inhibition rate: PN dissolved in methanol 66.67% vs. Cystone 92.28%. • Dissolution rate: PN aqueus extract 63.33% vs. PN dissolved in methanol 55% vs. PN dissolved in ethyl acetate 53.33% vs. PN dissolved in n-hexane extract 48.33%.
Freitas et al.,[6] 2002	Animals (rats)	1.25 mg/mL/day PN 42 days	• Sham control • Control + PN • CaOx + water • CaOx + PN	• Calculi formation: 12 in CaOx + water group vs. 3 in CaOx + PN, with a significantly lower final weight of the calculi in PN group (48 g/g vs. 16.6 g/g of stone).
Micali et al.,[13] 2006	Prospective study in humans	2mg/day Uriston® 90 days after ESWL	No treatment	• Stone-free condition with residual fragments less than 3mm: 93.5% in PN group 1 vs. 83.3% in control group ($P = 0.48$). • Stone-free condition without residual fragments: 88.5% in PN group vs. 76.4% in control group ($P = 0.08$).
Pucci et al.,[14] 2018	Prospective study in humans	Infusion tea prepared with 4.5 g of dry extract of *P. niruri*, 2/day during 12 weeks in patients with ≥1 stones <10mm	No control	• Serum measurements: no changes except for a significant decrease in alkaline phosphatase ($P = 0.017$). • Patients with urinary abnormalities at the baseline: – Increase in urinary citrate among the hypocitraturic patients ($P = 0.2193$). – Reduction of oxalate among patients with hyperoxaluria ($P = 0.0002$). – Reduction of uric acid among hyperuricosuric patients ($P = 0.0057$). • Number of stones: decreased in 38 (67.8%) patients, no changes in 10 (17.8%) and increase of upper urinary stones in 8 (14.3%).
Cai et al.,[15] 2021	Prospective study in humans	1 capsule/day of d.e. 15% mg *Phyllanthus niruri*, d.e. ¼ 55 mg Chrysanhellum americanum, 220 mg Tannins, 244 mg K+ and 735 mg Mg + citrates during 6 months in patients with ≥1 stones ≤15 mm	No control	• Stone size: 27 (32.9%) patients had no evidence of stone at the CT scan, 50 (60.9%) lower stones dimension. • Significant reduction in stone size ($P < 0.001$). • Asymptomatic bacteriuria (ABU): reduction of patients with ABU ($P < 0.001$) and correlation between ABU reduction and • stones number and dimension reduction ($P < 0.001$)

the studies mentioned may not be equally effective in these more complex cases.

It is also worth noting that in some of these studies, ultrasonography was used as an imaging control rather than abdominal and pelvic computed tomography (CT) to reduce patient exposure. However, this could have affected diagnostic accuracy and follow-up in some cases, especially in smaller kidney stones.[13]

The available evidence supports the use of additional treatment with *Phyllanthus niruri* (PN) in post-ESWL patients; however, it is still limited for patients who have undergone PCNL and RIRS. While some studies have shown that certain additional therapies may have a positive impact on reintervention or stone elimination rates, most of the available evidence is preliminary, non-standardized, and comes from small-scale studies. This lack of robust data difficults the implementation of recommendation protocols that can guide clinicians on when and how to incorporate adjunctive PN treatment in postoperative management.

To fill this gap, it is essential that controlled, randomized clinical trials are conducted to assess the efficacy of PN in preventing recurrence after intervention. These studies should include laboratory tests to evaluate urinary supersaturation and crystal formation, as well as clinical studies that assess the long-term occurrence of new episodes of urinary stone disease with standardized imaging. Additionally, the appropriate dosage, long-term safety, and potential interactions with other medications should be investigated.

Conclusions

The results of this review highlight PN's potential as a complementary strategy for managing nephrolithiasis, especially in improving ESWL success rates. This herbal remedy stands out for its favorable safety profile, low cost, and accessibility, making it an attractive option in healthcare systems with economic limitations. At the molecular level, PN acts by inhibiting the nucleation and growth of calcium oxalate crystals, which could contribute to preventing the formation and aggregation of kidney stones. However, while the available studies suggest benefits in patients with small or moderate-sized stones, further controlled and randomized research is needed to validate its efficacy, safety, and mechanisms of action, especially in patients with larger stones or those undergoing other treatments like PCNL or/and RIRS. The current evidence, though promising, is still limited and not sufficiently robust to establish standardized clinical protocols.

CRediT authorship contribution statement

La concepción y el diseño del estudio, la adquisición de datos y la interpretación de los datos (V.R.O, J.I.P, CM.A.S, AD.L.E, D.D.V, LM.S.P), borrador del artículo y revisión crítica del contenido intelectual (V.R.O, J.I.P, CM.A.S, AD.L.E, D.D.V, LM.S.P), aprobación definitiva (L.D.A, E.E).

Funding

La presente investigación no ha recibido ayudas específicas provenientes de agencias del sector público, sector comercial o entidades sin ánimo de lucro.

Declaration of competing interest

The authors had no conflict of interest to declare.

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Available online on 25.08.2019 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

Open Access to Pharmaceutical and Medical Research

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Formulation and evaluation of Antiurolithiatic herbal tablet

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ABSTRACT

Phytomedicine based on principles of Ayurveda are need of the hour and is more feasible than allopathic drugs which is not only more expensive in terms of "leads" but is also associated with many unwanted effects. Ethnopharmacological usage and the literature review revealed that the *Abutilon indicum*, *Zea mays, Tribulus terrestris, Phyllanthus niruri* have significant antiurolithiatic activity. After the detailed study of powder of ethanolic extract of seeds of *abutilon indicum*, tassel of *zea mays,fruits of Tribulus terrestris and leaves of Phyllanthus niruri* a formulation using the plant materials was prepared. The formulation was evaluated and standardized as per the Pharmacopoeial standards. The results of preformulation studies revealed that all the values were within acceptable limit. Formulation showed appreciable hardness characteristics (4.35 kg/cm^2), which facilitates its fast disintegration. The friability (0.8%) of formulation indicated that the tablets were mechanically stable. As the average weight of tablets was 505 mg, the acceptable weight variation range is ±7%. Hence the entire formulated tablet passed the weight variation test. The disintegration time of formulations was more than 1 minute. Thus the claims made by the traditional Indian systems of medicine regarding the use of this plant in the treatment of antiurolithiatic activity confirmed. The final conclusion drawn from the above mentioned data is that the possible use of these economical and relatively nontoxic, non-hazardous natural remedies of plant origin may further be explored as they are devoid of major side effects associated with synthetic agents.

Keywords: *Abutilon indicum, Zea mays , Tribulus terrestris, Phyllanthus niruri* , Disintegration, Preformulation

Article Info: Received 11 June 2019; Review Completed 14 Aug 2019; Accepted 20 Aug 2019; Available online 25 August 2019

Cite this article as:

Gupta D, Dubey PK, Formulation and evaluation of Antiurolithiatic herbal tablet, Journal of Drug Delivery and Therapeutics. 2019; 9(4-s):908-913 **http://dx.doi.org/10.22270/jddt.v9i4-s.3613**

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1. INTRODUCTION:-

Urinary stone disease continues to reside in an important place in daily urological practice. The average life time risk of stone formation has been reported in the range of 5-10 %. A predominance of men over women can be observed with an incidence peak between the fourth and fifth decade of life. **(Yadav R. D., 2011).** *Abutilon indicum* species has been widely used as medicine in Ayurvedic system of medicine. *Abutilon indicum* (Malvaceae), commonly known as "Thuthi" is distributed throughout the hotter parts of India. *Abutilon indicum* commonly known as "Atibala" in Sanskrit gives excessive tonic strength. Phytoconstituents like β-Sitosterol (0.2%), tocopherol oil (0.3%) were isolated. **(Dashputre N. L.,2010)**

The word *zea mays* come from two languages. Zea comes from ancient greek and is a generic name for cereal and grains. In traditional medicine, corn is used for relieving diarrhea, dysentery, urinary tract disorder, prostatitis, lithiasis, angina, hypertension and tumor. **(Parle M., 2013)** ***Tribulus terrestris*** is commonly known as puncture vine, caltrop, yellow vine, goat head and devil's horn. It is is a member of the Zygophyllaceae family and is widely distributed in both tropical and mild temperate regions. *T. terrestris* is native to warm temperate and tropical regions of southern Europe, southern and western Asia, throughout Africa, and Australia. *Phyllanthus niruri* is an important medicinal plant. The plant is widely used for the treatment of hepatic disease, oedema, dropsical condition, and urinary troubles.

2. MATERIALS AND METHODS

2.1 Description of plant

Abutilon indicum:- The botanical name of atibala is *Abutilon indicum* and it belongs to family Malvaceae.The plant grows throughout India and in Sri Lanka, at about an elevation of 1000-1, 500 meters. The perennial shrub grows 1.25-2 meters in height. Plant covered with minute hairs. Leaves are alternate, cordate and acute. The leaves are oblong, opposite, toothed, smooth and covered with fine white hair. The flowers are yellow, 2.5 cm in diameter.

***Zea mays*:-** The word zea mays comes from two languages. Zea comes from ancient greek and is a generic name for cereal and grains. In traditional medicine, corn is used for relieving diarrhea, dysentery, urinary tract disorder, prostatitis, lithiasis, angina, hypertension and tumor.

Tribulus terrestris is commonly known as puncture vine, caltrop, yellow vine, goat head and devil's horn. It is is a member of the Zygophyllaceae family and is widely distributed in both tropical and mild temperate regions *T. terrestris* is native to warm temperate and tropical regions of southern Europe, southern and western Asia, throughout Africa, and Australia.

Phyllanthus niruri originated in India, usually occurring as a winter weed throughout the hotter parts. The *Phyllanthus* genus contains over 600 species of shrubs, trees and annual or biennial herbs distributed throughout the tropical and subtropical areas. *Phyllanthus niruri* is a herb of Euphorbiaceae family that grows upto 60 cm. *Phyllanthus niruri* is an annual herb which grows in the wild after first showers of monsoon in Jharkhand, Bihar, Chhattisgarh, etc. states of India. Extracts of this herb have been proven to have therapeutic effects in many clinical studies. *Phyllanthus niruri* is an important medicinal plant. The plant is widely used for the treatment of hepatic disease, oedema, dropsical condition, and urinary troubles.

Extraction

The dried parts of crude drugs were powdered, weighed (500g) and filled in soxhlet apparatus for solvent extraction. The powdered drug was defatted with petroleum ether. (60-80º C).Defatted drug was then dried and again filled in soxhlet apparatus for solvent extraction with solvent ethanol. Solvents were evaporated to get the dried residue of extract. Percentage yield were calculated for each extract. **(Kokate, C. K. 2000)**

2. 2. Formulation

2.2.1 Powdering of extracts: - Colloidal silicon dioxide (Aerosil 200) was added to the extract in a percentage of 2% of adjuvant to dry residue.

2.2.2 Preformulation Studies

The following Preformulation studies were performed:

- Organoleptic studies
- Solubility testing
- Angle of repose
- Loss on drying:
- Total Ash value determination
- Bulk density
- Tapped density
- % Compressibility
- Hausner ratio
- pH
- Particle size
- Extract Excipient Compatibility studies

2.2.2.1 Organoleptic studies: In these studies the organollepic features like colour, odour and physical appearance were observed and recorded.

2.2.2.2 Solubility testing

The solubility's were checked in water pH (7.0), 0.1 N hydrochloric acid solution, absolute alcohol, ethyl acetate, and hexane.

2.2.2.3 Angle of repose

Angle of repose is an important parameter to study the Flow property analysis of any powdered formulation with respect to their frictional forces. Angle of repose is defined as the maximum angel between the surface of the pile of powdered sample and the horizontal plane. Mathematically angle of repose is calculated by height of the pile (H) divided by radius of the pile(R). **(Lachman, L.et al. 1991,Aulton, et al. 1999, Martin et al. 2005)**

Angle of repose (Tan θ) = height of the pile (H)/ radius of the pile(R)

Table 1 Relationship between Angle of repose and powder Flow property

s.no.	Angle of repose	Flow property
1.	<25	Excellent
2.	20-30	Good
3.	30-40	Passable
4.	>40	Very poor

2.2.2.4 Loss on drying

Weighing bottle was dried a in a oven at 105ºC.and weight (w_1) was taken. 3 g of the drug was placed in it. The drug was dried in oven at 100-105 º C for 3 hrs. Drug was then allowed to cool in desiccators. And weigh it again (w_2).

% **Loss on drying (LOD) = {(**$w_1 - w_2$) / w_1} x 100

2.2.2.5 Total ash

Crucible was heated to redness for 30 min and allowed to cool in a desiccators and weighed (w_1). 3 gram of the powdered drug was carefully weighed in the above crucible. The gross weight of the crucible with the contents was noted (w_2). Sample was evenly distributed and dried at 100-105ºC for 1 hour. Ignited to a constant mass .Allowing the crucible to cool in desiccators after ignition. Than crucible was cooled in desiccators and weighed. (w_3). Total ash was calculated in % w/w by the following formula.

Total ash in % w/w= {(w_3-w_1)/ (w_2-w_1)} X 100

Where, w_1-Weight of the empty crucible in grams

w_2- Weight of the crucible + sample in grams

w_3- Weight of the crucible + ash obtained in grams

2.2.2.6 Bulk density

Bulk density is defined as the mass of powder divided by bulk volume.

Bulk density (D_b) = mass of powder (M) / bulk volume (V_b)

Bulk density was determined by measuring the amount of sample required to fill 3/4th volume of a 10ml. capacity graduated measuring cylinder via a funnel and measuring the volume occupied and weighed. **(Lachman, L.et al. 1987, Aulton et al. 1988, Martin et al. 2005)**

2.2.2.7 Tapped density

Tapped density is defined as the mass of powder divided by tapped volume.

Tapped density (D_t) = mass of powder (M) /tapped volume (V_t)

Tapped density was determined by tapping the graduated 10ml. measuring cylinder 100 times from a height of about 1.5 inch. **(Lachman, L.et al. 1987,Aulton et al. 1999, Martin et al. 2005)**

2.2.2.7 % Compressibility

% Compressibility was determined by the following formula

$$\% \text{ Compressibility} = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100$$

2.2.2.8 Hausner's ratio

Hausner's ratio was determined by the following formula.

Tapped density / Bulk density

2.2.2.9 pH

pH was determined by pH meter.

2.2.2.10 Particle size

These studies were carried out by sieve analysis. Sieve no. 60, 80 and 120 were used. Accurately weighed sample was placed (2 g) on topmost /coarsest sieve. Sieves were arranged in the ascending order from top. Agitated the nest of sieves for 5 minutes. Stopped the sieve shaker. Then carefully removed each sieve from the nest, without any loss of material. Reweighed each sieve and determined the weight of material on each one Determined the mass of material in the collecting pan. Reassembled the nest of sieves and agitated for another 5 min. This was done repeatedly for three times .After three times of agitation the end point criterion was achieved (when the change in mass of any of the test sieve is not more or less than 5% of the previous mass on that sieve).

2.2.2.11 Extract excipients interaction study

Powdered extracts excipients interaction study was performed by determination of total flavonoid in mixture of powder of *Abutilon indicum*, *Zea mays*, *Tribulus terrestris* , *Phyllanthus niruri* and excipients at initial, 15 and after 30 days.

2.2.3 Formulation of Tablets

The dried powder extract and other ingredients were mixed uniformly and granules were prepared by wet granulation technique. The lubricated granules were compressed into tablets in an 8-station machine with 500 mg die cavity.

Table 2 formula of Tablets

Ingredient	Quantity per tablet (mg)
Abutilon indicum extract	150
Zea mays extract	100
Tribulus terrestris extract	50
Phyllanthus niruri extract	50
Lactose	90
Talc	20
Starch paste	3 %
Starch dry	25

2.3. Evaluation of Tablets (Indian pharmacopoeia 2007)

Tablets were evaluated for their physical characteristics

2.3.1. Organoleptic properties

Size (thickness), shape, color, taste were determined.

2.3.3. Weight Variation Test

Weight variation test was done by weighing 20 tablets individually calculating the average weight and comparing the individual tablet to the average .The tablet given below shows the weight variation tolerance for uncoated tablets **(Lachman ,L et al.,1991)**

Table 3 Weight variation tolerance for uncoated tablet

Average weight of Tablet (mg)	Maximum % deviation allowed
130 mg or less	10 %
130 mg to 324 mg	7.5 %
More than 324 mg	5.0 %

2.3.2. Tablet hardness

The strength of tablet was expressed as tensile strength (Kg/cm^2). The tablet crush load, in which the force required to break a tablet into halves by compression. It is measured using a tablet hardness tester (Monsanto hardness tester). The test was performed with five tablets. The mean value and the standard deviation were calculated **(Lachman , L et al.,1991)**

2.3.4 Friability

Friability test is performed to assess the effect of friction and shocks. Which may often cause tablet to chip ,cap or break .Roche friabilator was used for the purpose .This device subjects a number of tablets to the combined effect of abrasion and shock by utilizing a plastic chamber that revolves at 25 rpm dropping the tablet at distance of 6 inches with each revolution. Preweight sample of tablets were placed in the friabilator, which were then operated for 100 revolutions. Tablets were dusted and reweigh. Compressed tablets should not loss more than 1% of their weight **(Lachman, L et al., 1991).**

2.3.5 Disintegration Time

One tablet was placed in each of six tubes of DT apparatus .Disintegration test was performed at 37 $\pm 2^0$C Disintegration time defined as time required to disintegrate and pass all fragments through the sieve (# 10) **. (Lachman ,L et al.,1991).**

2.4 Antiurolithiatic activity of formulation (Mousa al Reza et al 2007, F. Atmani et al 2004, R. Vargas S. 1999)

2.4.1 Experimental design

Animal 4 groups and each group having 5 albino rats weighing 120-180 gm. were selected and housed under standard laboratory condition for a period of 14 days prior to the experiment. Experimental protocols were approved by our Institutional animal ethical committee, which follows guidelines of CPCSEA/ IAEC (Committee for the purpose of Control and Supervision of Experiments on Animals/Institutional Animal Ethics Committee), Reg. 1839/PO/Ere/S/15/CPCSEA

Model Ethylene glycol induced model

Standard drug Cystone tablet 500 mg/kg body weight

Method 0. 75% ethylene glycol induced kidney stone

2.4.2 Experimental group

Four groups contain 5 animals in each group were subjected to 0.75% ethylene glycol into drinking water for four weeks.

GROUP I : Control group received only drinking water.

GROUP II: Model control group received drinking water + 0.75% ethylene glycol

GROUP III: received drinking water +0.75% ethylene glycol + Formulated tablet 500 mg/kg

GROUP V: received drinking water +0.75 % ethylene glycol + Standard drug 500 mg/kg

2.4.3 Statistical analysis

Standard evaluation was done using one-way analysis of variance (ANOVA) Statistical significance was set at *P< 0.0001*. Results are presented as mean ± standard errors (S .E.). **(Mousa al reza Hadizadeh et al 2007)**

2.4.4 Parameters

Total urinary volume

Animal were placed in separate metabolic cages 24 hours before the surgery. And total urinary volume was measured, by using measuring cylinder, and reported in ml **(C.Barbas, A. Garcia 2002)**.

Test for acidity

Uric acid crystals were found to deposit most frequently in the concentrated acid urine. Thus the acidity of the urine was tested using pH meter **(C.Barbas, A.Garcia 2002)**

Biochemical parameter of urine

Urinary concentration of calcium, oxalate and Creatinine were measured **(C.Barbas, A.Garcia 2002, Hodgkinson 1970)**.

3.1.1 Preformulation study of powders of extracts

Preformulation study of powder shows that % compressibility of were found poor so they are unable to process as directly compressible formulation to give sufficient compressibility to the powder they should process through wet granulation.**(Table 13,14)** . Angle of repose concluded that powders having poor flow so sufficient lubricant should be added, to improve flow of powder.

3.1.2 Determination of total flavonoid content

Total flavonoid content of ethanolic extract of Abutilon *indicum* was found to be 0.765 (QE mg/100mg of extract), ethanolic extract of *Zea mays* was found to be 0.733 (QE mg/100mg of extract), ethanolic extract of *Tribulus terrestris* was found to be 0.744 (QE mg/100mg of extract) and ethanolic extract of *Phyllanthus niruri* was found to be 0.757 (QE mg/100mg of extract). (**Table 15)**

3.1.3 Drug - excipient interaction

Drug -excipient interaction was performed by determination of total flavonoid content in mixture of powder extract of *Abutilon indicum,Zea mays, Tribulus terrestris, Phyllanthus niruri* and excipients at initial days and after 15, 30 days. Total flavonoid content were found 0.755 (QE mg/100mg of mixture) at initial day, 0.755 (QE mg/100mg of mixture) after 15 days, 0.755 (QE mg/100mg of mixture) after 30 days. (**Table 16)**

3.1.4 Evaluation of formulation (Tablets)

The prepared tablets were subjected to post compression parameters i.e. thickness, weight variation, hardness, friability, Disintegration time, and total flavonoid content. Prepared tablet obey limit for all the physical parameter. (**Table 17)**

3.1.5 Antiurolithiatic activity of formulation

The changes in the urine parameters in the experiment animals during the study are presented **(in Table 18, 19, 20)**.The urine concentration of oxalate, calcium and Creatinine were increased significantly in animals administered with 0.75 percentage ethylene glycol (group II). Four weeks treatment with formulated tablet significantly decreased urine concentration of oxalate (3.78 ± 0.07), calcium (2.82±0.13) and creatinine (4.51 ± 0.09) as compared to model control (oxalate – 11.29 ± 0.22, calcium – 7.85± 0.19, Creatinine – 7.75 ± 0.17) (**Table 20).**Moreover the group treated with formulated tablets (150 mg *Abutilon indicum* + 100 mg *Zea mays+ 50 mg Tribulus terrestris+ 50 mg Phyllanthus niruri*) was found to be most significant from the entire group. The percentage reduction of all parameters of urine were found more in group III (150 mg *Abutilon indicum* + 100 mg *Zea mays+ 50 mg Tribulus terrestris+ 50 mg Phyllanthus niruri*) and in group IV (standard).

Urinary volume significantly decreased in the animals treated with the 0.75 % of ethylene glycol. Urinary volume were increased by 240 percentage in formulated tablet 246 percentage standard drug with compared to model control group.**(Table 18)** Urinary pH significantly increased in the animals treated with the 0.75 % of ethylene glycol. Urinary pH were decreased by 25.95 percentage in formulated tablet and 26.96 percentage standard drug with compared to model control group.**(Table 19)** From the above results it was noted that the formulated tablets were found significant.

4.1.10 Stability study of formulation

The prepared tablets were subjected to post compression parameters i.e. thickness, weight variation, hardness, friability Disintegration time, and total flavonoid content after 3 month at room temperature and accelerated condition. Prepared tablets obey limit for all the physical parameter. The Prepared tablets were found stable after 3 month. **(Table 21)**

DISCUSSION

Urorolithiasis is caused by several biochemical mechanisms. It is a generalized increase in the calcium content of the kidneys. The major causes include those associated with an increase in the urinary levels of calcium crystals precipitation. Kidney stones usually arise because of an imbalance between the kidney's need to conserve fluid and the need to extrude waste products of low solubility. This imbalance often is precipitated by alternations in diet, fluid intake, climate and extent of physical activity.The majority of patients with calcium containing stones excretes excessive amounts of urinary calcium and often has urine that is supersaturated solution of calcium and oxalate salts**. (Sarkisian M.R 2001)** Animal model of kidney stone provide important tools for testing the pathogenesis of kidney stone and for studying the efficacy of potential therapies and their mechanism of action. In the recent years, important advances have been made in the diagnosis and treatment of kidney stones. Standard screening tests employed for the kidney stone study is the 0.75% ethylene glycol induced kidney stone method. It is an efficient method as kidney stone can be induces in animal in a very short duration of time by simultaneously testing the effect of test drug on the kidney stone in rats.

In the present study of kidney stone, after the administration of ethanolic extract of *Abutilon indicum*,ethanolic extract of *Zea mays,* ethanolic extract of *Tribulus terrestris and* ethanolic extract of *Phyllanthus niruri* and their combination to the group of rats urine analysis shows that the occurrence of stone was decreased when compared to the kidney stone control group and combination (ethanolic extract of *Abutilon indicum*,ethanolic extract of *Zea mays,* ethanolic extract of *Tribulus terrestris and* ethanolic extract of *Phyllanthus niruri)* as effective as the standard group.

Under formulation development the components were examined for incompatibility, with dried extracts being as therapeutically active ingredient. The developed formulation was evaluated for various pharmaceutical parameters including stability studies at different environmental conditions. There was no significant physical change observed out in three month's storage. Formulation showed marked antiurolithiatic activity on albino rats, it was concluded that the developed formulation show significant reduction in urine concentration oxalate, calcium and Creatinine.

Table 4 Preformulation study of powders

S.No.	Parameter	*Abutilon indicum*	*Zea mays*	*Tribulus terrestris*	*Phyllanthus niruri*
1	Description	Light Brown Powder with characteristic odour	Light green Powder with characteristic odour	Light Brown Powder with characteristic odour	Dark green Powder with characteristic odour
2	Solubility				
	Alcohol	Soluble	Soluble	Soluble	Soluble
	Water (pH 7)	Slightly soluble	Slightly soluble	Slightly soluble	Slightly soluble
	0.1 N HCl solution	Soluble	Soluble	Soluble	Soluble
	Ethyl acetate	Insoluble	Insoluble	Soluble	Insoluble
	Hexane	Insoluble	Insoluble	Insoluble	Insoluble

Table 5 Preformulation study of powders

S.No	Parameter	*Abutilon indicum*	*Zea mays*	*Tribulus terrestris*	*Phyllanthus niruri*
1	Angle of repose	32	30	31	31
2	Loss on drying	12.4%	10.9%	9.8%	11.2%
3	Ash value	3.98%	3.85%	4.1%	3.98%
4	Bulk density	0.66 g/ml	0.69 g/ml	0.67g/ml	0.65g/ml
5	Tapped density	0.88g/ml	0.94g/ml	0.91g/ml	0.87g/ml
6	% compressibility	17.92%	18.49%	17.88%	18.22%
7	Hausner ratio	1.19	1.21	1.17	1.23
8	pH	6.3	6.5	6.4	6.5
9	Particle size	50-150 µ	50-150 µ	50-150 µ	50-150 µ

Table 6 Determination of total flavonoid content

S.No.	Extract	Total flavonoid Content (QE mg/100mg of extract)
1	Ethanolic extract of *Abutilon indicum*	0.765
2	Ethanolic extract of *Zea mays*	0.733
3	Ethanolic extract of *Tribulus terrestris*	0.744
4	Ethanolic extract of *Phyllanthus niruri*	0.757

Table 7 Drug - excipient interaction

S.No.	Day	Determination of total flavonoid content (QE mg/100mg)
1	initial	0.755
2	After 15 days	0.755
3	After 30 days	0.755

QE- Quercetin equivalents

Powder of *Abutilon indicum* extract + Powder of *Zea mays* extract+ Powder of *Tribulus terrestris* + Powder of *Phyllanthus niruri* + Excipient

Table 8 Evaluation of Tablet

S.No.	Evaluation parameter	Observation
1	Size (Thickness)	4.7± 0.15 mm
2	Shape	Round
3	Color	Light brown
4	Taste	Characteristic
5	Weight variation	Upper limit 1.3% Lower limit 1.7%
6	Hardness	4.35 kg/cm2
7	% Friability	0.8%
8	Disintegration time	10 min.

Antiurolithiatic activity of Formulation

Table 9 Total urinary volume

S.No.	Groups	Total urinary volume (ml) Mean± SE
1.	Normal control	2.44 ±0.14
2.	Model control	1.57 ± 0.10
3	Formulated tablet	5.35 ± 0.17 ***
4	Standard drug 500 mg/kg	5.44 ± 0.15 ***

P<0.001, the treated groups are compared with groups I and II. Values are expressed in Mean±SEM, Statistics: one way ANOVA followed by Dunnet's test, *** highly significant, ** significant and * less significant

Table 10 Determination of urinary pH

S.No.	Groups	pH of urine
1.	Normal control	7.25 ± 0.17
2.	Model control	9.94 ± 0.23
4	Formulated tablet	7.36 ± 0.15**
5	Standard drug 500 mg/kg	7.26 ± 0.19***

P<0.001, the treated groups are compared with groups I and II. Values are expressed in Mean±SEM, Statistics: one way ANOVA followed by Dunnet's test, *** highly significant, ** significant and * less significant

Table 11 Biochemical parameter of Urine

S. no.	Group	Urine parameter (mg/ dl)		
		Oxalate	Calcium	Creatinine
1	Normal control	3.65 ± 0.08	2.75±0.10	4.35 ± 0.16
2	Model control	11.29 ± 0.22	7.85± 0.19	7.75 ± 0.17
3	Formulation(500 mg/kg)	3.78 ± 0.07***	2.82±0.13**	4.51 ± 0.09**
4	Standard (500mg/kg)	3.81 ± 0.06 ***	2.77±0.09***	4.45 ± 0.12***

Table 12 Stability study

Parameters	Formulation at Different Time Interval		
	0th Day	After 3 month	
		Accelerated Condition	At Room Temperature
Color	Light brown	Light brown	Light brown
Shape	Round	Round	Round
Thickness (mm)	4.7±0.15	4.7±0.15	4.7±0.15
Hardness (Kg/cm2)	4.35	4.11	4.35
Friability (%)	0.80	0.72	0.80
Weight variation (Avg. Weight-502.7 mg)	Within The ±5 Limit	Within The ±5 Limit	Within The ±5 Limit
Disintegration Time (min)	10	10	10
Total flavonoid content	0.755	0.720	0.750

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BJU International (2002), **89**, 829–834

The effect of *Phyllanthus niruri* on urinary inhibitors of calcium oxalate crystallization and other factors associated with renal stone formation

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Objective To evaluate the effect of an aqueous extract of *Phyllanthus niruri* (Pn), a plant used in folk medicine to treat lithiasis, on the urinary excretion of endogenous inhibitors of lithogenesis, citrate, magnesium and glycosaminoglycans (GAGs).

Materials and methods The effect of chronic (42 days) administration of Pn (1.25 mg/mL/day, orally) was evaluated in a rat model of urolithiasis induced by the introduction of a calcium oxalate (CaOx) seed into the bladder of adult male Wistar rats. The animals were divided into four groups: a sham control (16 rats); a control + Pn (six); CaOx + water instead of Pn (14); and CaOx + Pn (22). Plasma and urine were collected after 42 days of treatment for biochemical analysis and the determination of urinary excretion of citrate, magnesium and GAGs. The animals were then killed and the calculi analysed.

Results The creatinine clearance or urinary and plasma concentrations of Na^+, K^+, Ca^{2+}, oxalate, phosphate and uric acid were unaffected by Pn or the induction of lithiasis. Treatment with Pn strongly inhibited the growth of the matrix calculus and reduced the number of stone satellites compared with the group receiving water. The calculi were eliminated or dissolved in some treated animals (three of 22). The urinary excretion of citrate and magnesium was unaffected by Pn treatment. However, the mean (SD) urinary concentration of GAGs was significantly lower in rats treated with CaOx + Pn, at 5.64 (0.86) mg/g creatinine, than when treated with CaOx + water, at 11.78 (2.21) mg/g creatinine. In contrast, the content of GAGs in the calculi was higher in the CaOx + Pn rats, at 48.0 (10.4) g/g calculus, than in the CaOx + water group, at 16.6 (9.6) g/g calculus.

Conclusion These results show that Pn has an inhibitory effect on crystal growth, which is independent of changes in the urinary excretion of citrate and Mg, but might be related to the higher incorporation of GAGs into the calculi.

Keywords urolithiasis, renal stone, *Phyllanthus niruri*, calcium oxalate, glycosaminoglycans, magnesium, citrate

Introduction

Phyllanthus niruri (Pn) is a plant belonging to the Euphorbiaceae family, with a worldwide distribution; it is used in Brazilian folk medicine by patients with urolithiasis. Previous reports showed that administering an infusion of Pn to patients with renal calculi was effective in promoting stone elimination and had an inhibitory effect on the formation of stones in an experimental model of calcium oxalate (CaOx) lithiasis in rats [1]. Moreover, even at higher doses of Pn, neither rats nor humans had any acute or chronic toxicity, supporting the therapeutic potential of Pn.

Many factors are involved in the pathogenesis of urolithiasis. Although the presence of a supersaturated milieu is necessary for precipitating CaOx (present in most calculi) acting as a promoter of crystal formation, this is not enough to form a stone, as urine is normally a supersaturated solution and only some individuals are prone to this disease. One reason for this is the presence of inhibitors of lithogenesis in urine, including macromolecules, proteins, citrate and magnesium [2]. Thus, an imbalance between the promoter and inhibitors may represent a potential factor in lithogenesis. The present study was undertaken to determine if the protective effect of Pn might be mediated by its influence on the urinary excretion of some endogenous inhibitors of lithogenesis, including glycosaminoglycans (GAGs), citrate and magnesium, in a model of experimental lithiasis in rats.

Accepted for publication 19 February 2002

Materials and methods

Urolithiasis was induced by introducing a CaOx seed into the bladder of adult male Wistar rats [3]. Briefly, rats under ether anaesthesia had their bladder exposed

through a suprapubic incision and a CaOx crystal (seed) of ≈3 mm in diameter was introduced into the bladder. After suturing the bladder, muscle and skin, the animals were maintained in individual cages for 24 h for observation. Sham-operated animals underwent the same protocol but the CaOx seed was not placed into the bladder. All animals were allowed free access to regular rat chow and tap water.

To prepare the CaOx crystal, small disks of CaOx were obtained by a supersaturation reaction, as described previously [4]. Briefly, 100 mL of calcium chloride (0.4 mol/L) and 100 mL of potassium oxalate (0.4 mol/L) were mixed together by constant drop-wise addition to 300 mL of distilled water for 2 h with shaking at 75°C. The mixture was maintained under agitation at 75°C for an additional 5 h. Crystals were washed and maintained in a stove at 37°C for 2 weeks to allow aggregation and growth of the seed. The resultant material was transferred to a template containing cylinders of 3 mm diameter to obtain small disks of CaOx. Disks were weighed and sterilized before use.

To prepare the Pn aqueous extract, the plant was grown at an experimental centre. Samples of whole plant were dried at 50°C for 2 months in a ventilated room. After drying, the samples were ground in a mechanical mill and used to prepare a tea mixture (5% tea, w/v). The infusion was stirred for 30 min at 72°C and then vacuum-filtered, concentrated and lyophilized.

The aqueous extract of Pn was fed to the rats starting on the first day after introducing the CaOx seed into the bladder. Lyophilized Pn was resuspended in distilled water and administered orally at 1.25 mg/mL/day for 42 days. The rats were divided into four groups: 16 control sham-operated rats that received tap water (0.5 mL, orally) for 42 days; control + Pn (six), normal rats that received Pn for 42 days; CaOx + Pn, 22 animals with a CaOx crystal introduced into the bladder that received Pn for 42 days; and CaOx + water, 14 animals with a CaOx crystal that received 0.5 mL of water orally instead of Pn.

Two days before and 42 days after surgery, all animals were weighed and the mean arterial tail pressure (MAP) measured using the tail-cuff method [5]. They were then placed in metabolic cages (Nalgene, NalgeNunc Int., USA) with no food but free access to tap water, to collect a 24-h urine sample. Urine was collected and conserved in 5% HCl, except for uric acid determination, when it was placed in sodium bicarbonate. Urinary biochemistry and general variables (body weight, MAP, 24 h urinary volume, volume of water ingested and pH) were determined. After urine collection at 42 days, blood samples were obtained from the aorta and the animals killed. The bladder was exposed, and the matrix and satellite calculi removed and analysed. Biochemical determinations included urinary and plasma concentrations of sodium, potassium (photometry, Pegasus II, Tecnow, Brazil), calcium (atomic spectrophotometry), uric acid, magnesium, creatinine (Labtest Diagnostics, Brazil), and oxalate (Sigma Diagnostics, USA). Urinary citrate excretion was determined using an enzymatic method (spectrophotometry). Urinary GAGs, including heparan, dermatan and chondroitin sulphate, were determined in pooled urine (10 mL) from each group, as previously described [6]. Samples were dialysed against distilled water for 5 h at 4°C, the dialysed samples vacuum dried and resuspended in 50 μL of water, and 5–10 μL aliquots submitted to agarose gel electrophoresis. The GAGs were quantified by densitometry after toluidine blue staining.

The calculi were weighed and the content of GAGs assessed [7]. Matrix and satellite calculi were solubilized with 4–5 mL of 9% EDTA and dialysed against distilled water for 24 h at 4°C. The solution obtained was vacuum-dried and resuspended in 150–200 μL of water. GAGs were identified and quantified as described above.

The results are presented as the median (SD), with groups compared using ANOVA followed by the Scheffé multiple-comparison test. For comparing two groups, the nonparametric Mann–Whitney *U*-test was used, with $P < 0.05$ considered to indicate significant differences in all tests.

Results

Table 1 presents the general variables obtained before surgery to introduce the CaOx crystal. Creatinine clearance was determined at 42 days. All groups had a similar increase in body weight after 42 days. The MAP was significantly greater ($P < 0.05$) in the CaOx + water group than in the other groups, including the CaOx + Pn group. There were no differences in the 24-h volume of water ingested, urinary volume or urinary pH among the groups, or between values before and after treatment. Creatinine clearance was not significantly different among the groups, although there was a tendency to greater clearance in groups treated with Pn.

The mean (SEM) number of calculi, including the matrix crystal and the satellite calculi taken from the CaOx + water group, was 12 (1) per animal, while in CaOx + Pn it was significantly less, at 3 (1) per animal. Indeed, some animals (three of 22) had no calculi and had even eliminated the CaOx crystal initially introduced into the bladder, suggesting that Pn induced considerable protection against calculus growth (matrix and satellite) compared with the untreated group. Moreover, as shown in Table 2, the final weight of the calculi (matrix + satellite) was significantly lower in the treated

Table 1 General variables measured before and 42 days after surgery

Median (range)	*Group, before/after surgery*			
Variable	*Sham*	*CaOx + water*	*CaOx + Pn*	*Control + Pn*
Number	16	14	22	6
Body weight, g	314 (383–252)	298 (366–250)	299 (360–252)	285 (312–273)
	337 (415–289)†	327 (389–279)†	316 (415–279)†	341 (403–332)†
MAP, mmHg	118 (135–100)	110 (120–100)	112 (125–100)	122 (135–110)
	127 (140–110)	135 (150–120)*	125 (140–100)	125 (130–120)
24-h water intake, mL	10.7 (20–5)	12.8 (17–5)	15.0 (25–10)	10.0 (15–5)
	14.0 (20–5)	12.7 (25–5)	15.0 (20–5)	12.5 (30–5)
24-h diuresis, mL	12.0 (21–8)	13.5 (18–5)	13.0 (25–3)	13.0 (15–10)
	12.8 (25–7)	11.8 (24–5)	14.3 (25–6)	11.3 (18–8)
pH	6.37 (6.9–6.1)	6.32 (6.6–5.7)	6.52 (8.0–5.7)	6.37 (6.6–6.1)
	6.42 (7.7–6.1)	6.35 (6.5–6.0)	6.49 (7.1–5.8)	6.53 (6.7–6.4)
Creatinine clearance, mL/min	1.13 (2.0–0.6)	1.02 (2.2–0.8)	1.34 (2.5–0.6)	1.32 (1.8–1.0)
Urinary analysis				
Na^+, mmol/L	1.36 (2.8–0.6)	1.70 (2.9–0.5)	1.53 (2.2–0.9)	1.51 (1.9–1.3)
	1.40 (2.9–0.9)	1.98 (2.7–1.3)	1.28 (1.9–0.8	1.39 (1.7–1.2)
K^+, mmol/L	3.65 (5.3–2.3)	3.25 (5.0–2.4)	3.45 (5.3–2.2)	3.35 (4.3–2.7)
	3.40 (4.1–2.2)	3.20 (3.7–2.4)	3.15 (5.0–2.1)	3.50 (4.7–3.2)
Ca^{2+}, mg/24 h	0.47 (0.6–0.3)	0.50 (0.8–0.4)	0.51 (1.2–0.3)	0.51 (0.6–0.5)
	0.53 (1.1–0.3)	0.55 (1.2–0.4)	0.55 (1.3–0.3)	0.63 (0.9–0.5)
Uric acid, mg/24 h	1.66 (3.1–0.5)	1.73 (2.7–0.7)	1.71 (2.8–1.0)	1.76 (2.2–1.5)
	1.89 (3.1–1.3)	1.83 (2.9–1.0)	1.69 (3.0–0.5)	1.89 (2.2–1.6)
Oxalate, mg/24 h	0.52 (0.6–0.4)	0.54 (0.6–0.4)	0.48 (0.6–0.3)	0.51 (0.6–0.4)
	0.48 (0.6–0.3)	0.55 (0.7–0.4)	0.53 (0.6–0.4)	0.56 (0.6–0.5)
Plasma analysis				
Na^+, mmol/L	141 (144–136)	141 (144–136)	140 (143–135)	141 (143–136)
K^+, mmol/L	4.6 (5.4–3.9)	4.8 (5.4–4.0)	4.8 (5.5–4.0)	4.6 (5.1–4.2)
Ca^{2+}, mg/L	10.5 (13.5–8.0)	10.6 (11.8–9.5)	9.7 (11.6–6.6)‡	8.8 (9.8–8.0)‡
Mg^{2+}, mg/L	2.07 (2.4–1.9)	2.11 (2.7–1.7)	2.07 (2.4–1.7)	2.05 (2.4–1.7)
Uric acid, mg/L	1.81 (3.2–0.8)	1.45 (2.7–0.8)	1.85 (2.8–0.4)	1.73 (2.1–1.3)
Oxalate, mg/L	1.91 (2.4–1.2)	1.94 (2.3–1.3)	1.75 (2.4–0.7)	1.79 (2.1–1.7)
Urinary excretion of lithogenesis inhibitors				
Mg^{2+}, mg/24 h	6.90 (8.8–4.8)	8.24 (9.3–6.5)	7.10 (8.8–4.6)	6.95 (7.8–5.8)
	6.85 (8.7–4.6)	7.60 (9.2–5.6)	6.90 (8.9–4.6)	6.85 (8.2–5.0)
Citrate, mg/24 h	23.5 (39.3–12.9)	24.2 (36.6–14.3)	32.9 (50.8–25.8)	27.7 (49.3–15.6)
	28.4 (40.7–16.4)	28.7 (51.8–9.7)	35.2 (53.9–22.8)	23.4 (35.1–16.2)
GAGs, mg/g creatinine				
Heparan sulphate	1.76 (2.0–0.3)	5.60 (15.2–2.3)¶	2.30 (5.5–1.0)	2.51 (7.4–1.0)
Chondroitin sulphate	1.97 (6.3–0.6)	3.87 (10.1–1.1)¶	2.31 (5.2–0.3)	3.58 (7.5–0.3)
Total	3.64 (8.1–1.9)	10.65 (24.2–3.4)¶	6.52 (9.7–1.5)	6.00 (14.6–1.5)

†$P < 0.05$ vs baseline or *sham; ‡$P < 0.05$ vs sham and CaOx + water; ¶$P < 0.05$ vs sham, CaOx + water and control + Pn.

Table 2 Calculus weight and GAG content in the groups with stone growth

Median (range)		
Variable	*CaOx + water*	*CaOx + Pn*
Weight, g		
Initial (matrix)	0.016 (0.0232–0.0007)	0.015 (0.0217–0.0127)
Final (matrix + satellite)	0.174 (0.352–0.019)†	0.028 (0.033–0)*
% increase (SEM)	983 (101)	87 (47)
GAG content		
Total, μg	2.29 (5.11–1.15)	2.56 (6.01–0.98)
μg/g	12.8 (24.5–0.9)	39.6 (70.6–8.5)*

†$P < 0.05$ vs initial or vs *CaOx + water.

group than in the untreated group ($P<0.05$). Figure 1 shows a representative example of calculi from an untreated (panel A) and a Pn-treated animal (panel B).

Urinary Na^+, K^+, Ca^{2+}, uric acid and oxalate excretion were similar among the groups both before and 42 days after surgery (Table 1); the plasma concentrations of Na^+, K^+, Mg^{2+}, uric acid and oxalate were also similar among the groups, but the concentration of Ca^{2+} was lower in groups receiving Pn ($P<0.05$) than in the untreated sham or CaOx + water groups.

The urinary excretion of the inhibitors magnesium and citrate was unaffected by inducing lithiasis or Pn treatment (Table 1), but the concentration of GAGs in urine was surprisingly greater in the CaOx + water group ($P<0.05$) than in the other groups, because there was more of both heparan and chondroitin sulphate. In contrast, the apparently protected CaOx + Pn group had concentrations of GAGs close to those of the control groups (sham and control + Pn). Analysis of total GAG content incorporated into the calculi showed similar quantities for both lithiasis groups, but when these values were corrected for stone weight the relative amount of GAGs was significantly higher ($P<0.05$) in calculi from the CaOx + Pn group than from the CaOx + H_2O group (Table 2).

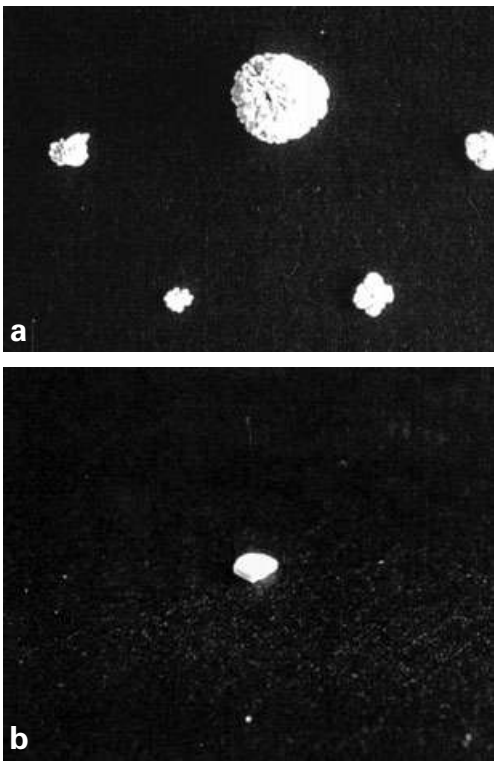

Fig. 1. Calculi (matrix and satellites) taken from an untreated animal (**a**) and a Pn-treated animal (**b**).

Discussion

Phytotherapy is common in folk medicine as an alternative to primary healthcare in many countries. Pn is a widely distributed plant [8] used in Brazilian folk medicine to treat kidney stones. The efficacy of Pn in treating urolithiasis has been evaluated in previous studies, with the results showing that ingesting Pn tea increased the spontaneous elimination of calculi in patients with lithiasis and decreased stone growth in a model of experimental lithiasis in rats [1]. These effects occurred independently of any relevant modification in the urinary excretion of elements known to promote crystallization and stone formation, including calcium, oxalate, uric acid, pH, etc., but they could be masked by the excessive diuresis consequent to the high volume ingested by the animals, as in that protocol the animals had free access to Pn tea. Thus, in the present study we evaluated whether the beneficial effect of Pn would occur even in the absence of any change in fluid volume ingested, and mainly whether this effect of Pn was mediated by changes in the inhibitors of stone formation, including citrate, magnesium and GAGs.

The chronic administration of a small volume of aqueous Pn extract induced a significant reduction in calculus growth and in some animals (three of 22) even the CaOx seed was not found, suggesting that these animals eliminated the CaOx matrix in the absence of any modification in diuresis rate, as the urinary volume was not modified by Pn treatment. A diuretic activity has been attributed to Pn [9], although it was not apparent in the present rats, or in previous studies on humans [10]. This discrepancy might be attributable to differences in Pn dose, which was much lower in the present study than used previously [9]. However, Pn and other plants belonging to the genus *Phyllanthus* have antispasmodic and relaxant effects on many contractile tissues, including trachea, ileum, uterus and aorta [11–13]. These effects might also occur in urethral smooth muscle and contribute to the elimination of smaller calculi.

There was a significant increase in MAP in the lithiasis group with no Pn treatment than in the other groups, including the CaOx + Pn group, possibly associated with pain, mainly during the manipulation of the animals, generating discomfort from the presence of larger calculi than those found in the CaOx + Pn group. Despite its relaxing properties, no *in vivo* hypotensive effect has been reported for Pn. Analgesic properties have also been attributed to Pn [14,15], which may have contributed to reducing animal stress during manipulation.

The administration of Pn did not modify the urinary excretion of any elements that act as calculus promoters, including calcium and oxalate, suggesting that Pn did

not interfere with the tubular transport of these substances. Thus the inhibition of calculus growth was independent of alterations in the urinary concentration of these lithogenic elements. Also, Pn did not interfere with urinary excretion of the protective elements citrate and magnesium, indicating that the anti-lithogenic effect of Pn was not primarily mediated by these inhibitors of lithogenesis.

Surprisingly, untreated lithiasic animals had a significantly higher urinary excretion of GAGs than animals treated with Pn, in which GAG levels were similar to those of the control groups (sham and control + Pn). These data suggest a dissociation between the presence of these macromolecules in the urine and their potential inhibitory effect on calculus growth, as higher levels of GAGs were associated with larger calculi. In addition, the response of urothelium to irritation caused by the larger amount of crystalline material in the bladder could be responsible for the increased urinary excretion of GAGs, as they are integral components of the urothelium [16] and have an important role in the urothelial defence against insults, including bacterial and carcinogenic [17]. Thus, the increased level of urinary GAGs in untreated CaOx animals, as a consequence of stones, should be considered. However, the calculi from the Pn-treated group had higher contents of GAGs, suggesting that Pn reduced the deposition of crystalline particles but did not interfere with GAG adsorption in the calculus, producing calculi apparently with a predominant intracrystalline amorphous organic matrix. Indeed, the calculi from treated animals were much easier to dissolve (data not shown) than those from untreated animals.

The involvement of GAGs in urolithiasis has been extensively evaluated over the last 30 years, but their role as inhibitors of crystallization and/or nucleation of calcium oxalate remains controversial [18–22]. In a model similar to that used in the present study, we previously showed that the exogenous administration of progressive doses of chondroitin sulphate was associated with a progressive increase in calculus size, followed by a proportional increase in the content of chondroitin sulphate inside the calculi [7]; this suggests that *in vivo* chondroitin sulphate promotes the growth of pre-existing crystal by its adsorption to the growth sites of CaOx crystals. The deposition of these polyanionic molecules in the calculus would increase the electrostatic negative force attracting cations, particularly calcium, and promoting crystal growth. The results obtained in the present study suggest that Pn prevented the aggregation of CaOx to the pre-existing crystal without interfering with the aggregation of GAGs. Why Pn was able to prevent the growth of the crystalline part of the calculus with no interference with GAG adsorption (protein matrix) is unknown, but some possible hypotheses are: (i) neutralization of negative charges of GAGs reducing the negative pole for progressive deposition of cations; (ii) active components of Pn could chelate and/or compete with calcium for binding sites on the crystal surface; (iii) Pn could interfere with crystal adhesion to the epithelium. Accordingly, Campos and Schor [23] recently reported that Pn had a potent inhibitory effect on CaOx crystal adhesion and/or endocytosis by renal tubular cells; (iv) an effect of Pn on other proteins, e.g. Tamm-Horsfall protein, nephrocalcin, osteopontin, prothrombin fragment 1, with the potential to modulate crystallization, aggregation and growth [24] of calculi, should be considered.

All these hypotheses, either as individual or simultaneous events, are important in urolithiasis research, as the protective property of this natural product has been reported in several experiments. This might provide the possibility of developing a nontoxic and low-cost alternative for treating and/or preventing this disease.

Acknowledgements

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo e Fundação Oswaldo Ramos. We thank Dr Neil Ferreira and Dr Yara Juliano for statistical analysis assistance and Dr Antonio José Lapa for supplying aqueous extract of the plant.

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Abbreviations: **Pn**, *Phyllanthus niruri*; **CaOx**, calcium oxalate; **GAGs**, glycosaminoglycans; **MAP**, mean arterial tail pressure.

Pak. j. sci. ind. res. Ser. B: biol. sci. 2021 **64B**(1) 81-86

Evaluation of *Phyllanthus niruri* L. from Malaysia for *In-vitro* Anti-Urolithiatic Properties by Different Solvent Extraction

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(received January 29, 2019; revised February 21st, 2019; accepted February 26, 2019)

Abstract. The *Phyllanthus niruri* is traditionally used for curing of kidney disorders and urinary stones in Malaysia. Hence the current work was aimed to evaluate the effect of different solvents extract (n-hexane, ethyl acetate, methanol and water) of *P. niruri* for *in vitro* anti-urolithiatic properties in terms of inhibition activity on CaOx by using the rate of CaOx aggregation assay and dissolution of calcium oxalate (CaOx) crystal by using titrimetry method. Cystone was used as positive control. The effects of cystone on slope of nucleation and aggregation as well as growth of CaOx were evaluated spectrophotometrically. The highest yield percentage of *P.niruri* was occupied by methanol (5.74 %). The maximum inhibition against aggregation of CaOx crystals was also occupied by methanol (66.67 % ± 1.61) and was comprised with alkaloid, steroid, terpenoid and tannin. Dissolution effect on calcium oxalate crystals indicates that the aqueous extracts of *P. niruri* was found to be more effective in dissolution of CaOx with 63.33 % ± 1.44. *P. niruri* significantly ($P < 0.05$) inhibited the slope of nucleation and aggregation of CaOx crystallization, and reduced the crystal density. The results of the present study confirmed that *P. niruri* leaves can be used as remedial mediator for urolithiasis. However, further studies are required for isolation and identification of active constituents and their *in-vivo* confirmation.

Keyword: crystallization, dissolution, nucleation, *P. niruri,* anti-urolithiatic.

Introduction

Urolithiasis (from Greek oûron, " urine" and " stone") is a condition in which urinary calculus is formed or located anywhere in the urinary system or stones are formed in the kidney, bladder or ureters (Sharma *et al*., 2016). Different phytochemical events begins when the formation of kidney stone occurs like crystal nucleation, aggregation and end with retention within the urinary tract. Among the several types of kidney stones, the most common are calcium oxalate representing up to 80% of the analyzed stones. Calcium containing stones may be in the form of pure calcium oxalate (50%) or calcium phosphate (5%) and a mixture of both (45%) followed by magnesium phosphate (15-20%), uric acid (10%) and cystine (1%) (Singanallur *et al*., 2017). There are numerous methods had been reported to reduced or break the kidney stone. Traditional method of treatment is being reported from plants which are the most effective. Plants based on traditional knowledge can lead to the discovery of new drug and development of pharmacologically important products for human health care (Pauzi *et al*., 2018; Subramoniam, 2014). Almost, 80% of the world's population depends on the conventional medicine to cure most of their diseases (Gul *et al*., 2019; Kennedy, 2005). There is a number of plants which show promising anti-urolithiatic activity (Ram *et al*., 2015). Nowadays, these conventional remedies have become more popular because they are very efficient, have low side effects and reduce the reformation of stone. Usually, the decoction and infusion

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methods are used for extraction that is good way for extracting compounds of various plants.

Although water decoction method is still using, even this method abundantly used huge volume of water. Moreover, there are some dis-advantages associated with water such as water that gives an excellent growth for microbes and this condition leads to microbial contamination to the samples. Moreover, it will promote hydrolysis and enzymatic degradation in the plant sample (Azmir *et al*., 2013). In addition, water also attract to an extract along with polar compounds which could obstruct in the identification and quantification (Bandar *et al*., 2013). Furthermore, the large volume of hot water usually means that the plant sample will exposure to unpleasant taste for longer period (Bone and Mills, 2013).

The *Phyllanthus niruri* is the member of the family Euphorbiaceae, and because of its speciality commonly known as as "stone-breaker" (Kieley *et al*., 2008). The habitat of the plant is moist, shady places, rock and some time epiphytes. The morphology of the plants states that leaf blade rounded to complement the elongated egg and green fruit. It can grow up to 60 cm. furthermore, the tastes of *P. niruri* is bitter, cool, and as an astringent (Dalimartha, 2008).

Wang *et al*. (1995) identified the bioactive compounds like alkaloids, coumarins flavonoids, lignans, polyphenols, saponins tannins and terpenoids from different parts of *P. niruri*. Furthermore, Bagalkotkar *et al*. (2006) stated that 50 different bio-active compounds were identified from the *P. niruri*, including flavonoids, alkaloids, triterpenes and lignans. This is the proved that this plant diverse photochemical contents in different experimental studies. Alkaloid and triterpenes reported by many research as inhibited the cytotoxicity activated by calcium oxalate (Malini *et al*., 2000). Therefore, the current study will focus on the analytical methodologies, which include the extraction and its application as anti-urolithiatic activity.

Materials and Methods

Sample collection. The grinded leaves of *P. niruri* were purchased from Seri Subah Agrofarm, Negeri Semblian, Malaysia.

Sample preparation. The grinded plant samples were kept in the room temperature and dry place to maintain them in dry condition. The moisture content of the samples were measured and maintained at consistently about not more than 10 % (Azwanida, 2015). Cystone was used as positive control while, distilled water was used as negative control.

Extraction process. The extraction method was followed by Fermeglia (2008) with slight modification. The plant samples were extracted by unlike non-polar solvents to polar solvents that are *n*-hexane, ethyl acetate, methanol and water. The extraction method was maceration using. The experiment was carried out in three replicates. The following equation used to calculate extraction yield:

$$\text{Total extract yield, Y (\%)} = \frac{\text{Total mass of extraction}}{\text{Total mass of sample}} \text{ x } 100$$

Phytochemicals analysis of the plant samples. Phyto-chemical analysis was performed by standard method followed by Tiwari *et al*. (2011). All extracts used in these assays were 1 mg/mL in concentration.

Evaluation of anti-urolithiatic properties (*in-vitro*). ***Inhibition activity of plant extracts against calcium oxalate (CaOx) crystal by aggregation assay.*** The aggregation assay was done followed by Hess *et al*. (2000) with slight modifications. In addition, the inhibition rates of CaOx aggregation by the extracts were compared with the standard drugs, Cystone. CaOx crystals solution was prepared by using 10 mM calcium chloride dihydrate and 1.0 mM sodium oxalate, containing 200 mM NaCl and 10 mM sodium acetate trihydrate. All tests were conducted at 37 °C and 5.7 pH. For crystallization of CaOx, 25 mL of calcium oxalate solution was shifted to a beaker and placed in a constantly stirring hot plate magnetic stirrer. Next to it added 1 mL of plant extract (1 mg/mL)/ Cystone (1 mg/mL)/distilled water. The formation of the turbidity results immediately after the addition of 25 mL of sodium oxalate solution. The measurement of turbidity formed in terms of absorbance at 620 nm in UV-Vis spectrophotometer. It was started continuously for ten minutes after the mixing of the chemicals. In fact, the turbidity of solution increased indicates the nucleation process, and then decreased after some time which indicates the aggregation process. This experiment was done in three replications. The percentage inhibition rate of CaOx aggregation was calculated according (Sharma *et al*., 2016).

$$\text{Inhibition \%} = [1- (Si/Sc)] \text{ x } 100$$

where;

Sc = slope of aggregation without inhibitor (negative control); Si = slope of aggregation in the presence of inhibitor (positive control/ plant extracts)

Estimation of calcium oxalate by titrimetry method. Calcium oxalate (10 mg) and plant extract or Cystone (100 mg) was weighed respectively, and packed together in the semi-permeable membrane and carefully sutured. Then, it was allowed to suspend in a conical flask containing 100 mL of 0.1M TRIS buffer. The conical flasks were kept at room temperature for seven to eight hours. The remaining contents in the semi-permeable membrane is transferred into a beaker. Next, 1N sulphuric acid (2mL) was added and titrated with $KMnO_4$ until a light pink colour appeared (Dwivedi, 2016). Consequently, 1 mL of 0.9494 N $KMnO_4$ equivalents to 0.1898 mg of calcium.

% dissolved of calcium = [(C-T)/C] x 100

where;

C = precipitate of calcium oxalate remained in control (mg); T = precipitate of calcium oxalate remained when test solution was used (mg).

Statistical analysis. All the experiments were conducted in triplicate and the data were presented as mean values and standard deviation. One way ANOVA applied on data using IBM SPSS Statistics software (Version 20.0, USA) with the level of significant $P< 0.05$.

Results and Discussion

Yields of extraction. As shown in Table 1, the effect of different solvents were studied in terms of the extraction yield. The solvents were selected based on their polarities. Polarity of a solvent plays a considerable role in the extraction process (Ahmad *et al*., 2017).

Based on the result, the highest yield percentage of P.niruri was occupied by methanol (5.74 %) followed by water (2.15%), ethyl acetate (1.46 %), and lastly n-hexane (0.98 %).

Table 1. The percentage yield of herbal plant extracts

Herbal plant	Type of solvent	Mass of sample (g)	Mass of extract (g)	Yields (%)
Phyllanthus niruri	*n*-hexane	50	0.49	0.98
	Ethyl acetate	50	0.73	1.46
	Methanol	50	2.87	5.74
	Aqueous	50	1.08	2.16

Consequently, different solvents exhibited different yield percentage for each plant samples. The results revealed that solvents yield wide range of extraction (0.98 -5.74%). Among all of the solvent used, methanol exhibited the highest percentage of yields at the maximum percentage of 5.74%. This result was similar to Kotze *et al*. (2002) which reported that methanol shown to be the best extraction solvent for Combretum erythrophyllum as compared with other extraction solvents. Similar findings have been observed in other studies done by Suleiman *et al*. (2010) which reported that hexane extract was found to be the lowest amount of extract yielded from Kirkia wilmsii.

Phytochemical associated with anti-urolithiatic properties of plant extracts. The results of phyto-chemical screening in Table 2 revealed that the presence of alkaloid, steroid, terpenoid, tannin, and saponin in plant extracts. However, based on the result obtained, the amount of detectable phytochemicals in every solvent extract is different from each other. This might be due to the different polarity of solvents could selectively extracts different type of phytochemicals (Dailey and Vuong, 2015; KV *et al*., 2014; Chavan *et al*., 2013; Rebey *et al*., 2012). Different type of phytochemicals that are present in each extract might have some positive contribution to anti-urolithiatic effect against calcium oxalate crystals either in term of inhibition or dissolution properties.

Evaluation of anti-urolithiatic properties (*in-vitro*). ***Inhibiting effect of P. niruri on calcium oxalate crystals.*** The inhibition percentage of *P.niruri* extracts was shown in Table 3. The highest inhibition percentage of *P. niruri* extract against aggregation of CaOx crystals was occupied by methanol with percentage of 66.67 % ±

Table 2. The amount of detectable phytochemical of *P. niruri* extract

Type of solvent	Alkaloid	Steroid	Terpenoid	Tannin	Saponin
n-Hexane	++	++	+	-	-
Ethyl acetate	-	++	-	-	++
Methanol	+	+++	+	+	-
Aqueous	-	+	-	+	++

+ = indicates present; '-' = indicates absent; +++ = indicates phytochemicals in high amount; ++ = indicates phytochemicals in good amount; + = indicates phytochemicals in trace but detectable amount.

1.61 and was comprised with alkaloid, steroid, terpenoid and tannin. The studies regarding the phytochemicals in *P.niruri* were proven by Calixto *et al*. (1998) and Narendra *et al*. (2012) which reported that many bio-active compound from this plant have been identified which includes alkaloids, tannin, steroids and triterpenes.

Meanwhile, the second highest percentage of inhibition was hexane extract with 53.68 % ± 2.11 which also contain the same phytochemical with methanol extract but differ in detectable amount. Moreover, aqueous and ethyl acetate extract of *P.niruri* showed quite low inhibition activity compared to methanol (29.12 % ± 1.22) and *n*-hexane (18.95 % ± 1.06). The significant different (P>0.05) between these two values was probably due to the absence of alkaloid and terpenoid in both extracts.

Dissolution of calcium oxalate crystals by titrimetry assay. This study evaluates the anti-urolithiatic activity by dissolving the artificial CaOx packed in semi permeable egg with the help of different solvent extracts of *P. niruri*. The work was performed by using *in-vitro* anti-urolithiatic model for calculating percentage dissolution of CaOx crystals. The amount of CaOx dissolved was nominated as the indicator to evaluate anti-urolithiatic activity. The results for the dissolution percentage of CaOx by plant extracts and standard are shown in Table 4. The amount of CaOx dissolved with standard drug was 73.33 % ± 3.82 which is the highest percentage as compared to plant extracts. Consequently, all extracts showed their ability to dissolve the amount of CaOx inthe range from 65.83 % to 36.67 %.

Based on the phytochemical screening, the ability of dissolving activity of plant extract on CaOx crystals could be carried out effectively with only minimum amount as compared to inhibiting activity (Fig. 1). This is in agreement with similar finding reported by Dwivedi *et al*. (2016), conclusively revealed that Colocasia leaves show good anti-urolithiatic activity by dissolving the CaOx crystals even at low amount of phytochemicals.

Table 3. The percentage of inhibition on rate of CaOx aggregation by plant extract and standard drug, cystone.

Herbal plant/ Standard drug	Type of solvent	Inhibition percentage (%) (Mean ±Standard Deviation)
Cystone	-	92.28 ± 0.61a
Phyllanthus niruri	*n*-hexane	53.68 ± 2.11d
	Ethyl acetate	18.95 ± 1.06h,i
	Methanol	66.67 ± 1.61c
	Aqueous	29.12 ± 1.22g

[a, b, c,,,] Values designated with different alphabets are significantly different from each other.

Table 4. The percentage of dissolution on CaOx crystals by plant extract and standard drugs, cystone.

Herbal plant/ Standard drug	Type of solvent	Dissolution percentage (%) (Mean ±Standard Deviation)
Cystone	-	73.33 ± 3.82a
Phyllanthus niruri	*n*-hexane	48.33 ± 3.82f,g
	Ethyl acetate	53.33 ± 5.20d,e,f,g
	Methanol	55.00 ± 0.00c,d,e,f
	Aqueous	63.33 ± 1.44b,c

[a, b, c,,,] Values designated with different alphabets are significantly different from each other.

Dissolution effect of *P. niruri* on calcium oxalate crystals. The result shows in Fig. 2. indicates that aqueous extracts of *P. niruri* was found to be more effective in dissolution of CaOx with the percentage of 63.33 % ± 1.44. This result was followed by methanol (55.00 %), ethyl acetate (53.33 % ± 5.20) and lastly *n*-hexane extract (48.33 % ± 3.82).

Similar to previous studies, aqueous extract of *Melia azedarach* was studied in male albino Wistar rats against ethylene glycol-induced nephro-lithiasis and this extract has been shown to reduce urinary calcium, oxalate, phosphate and urinary magnesium levels and urinary

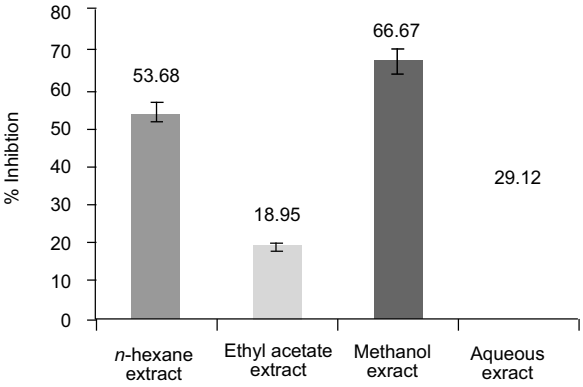

Fig. 1. CaOx inhibition activity of four solvent extracts of *P.niruri*.

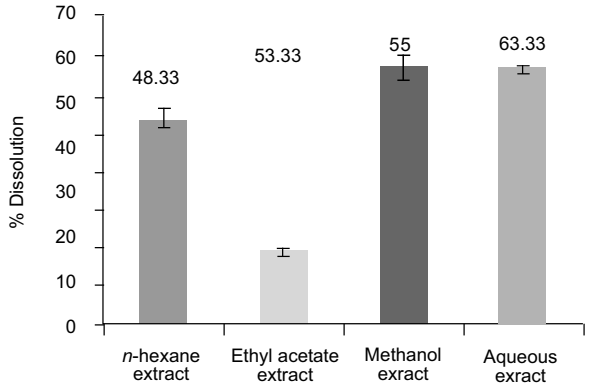

Fig. 2. CaOx dissolution activity of four solvent extracts of *P. niruri*

volume (Garimella *et al*., 2001). Moreover, the aqueous extract of C. Spiralis used at a daily dose of 0.25 and 0.5 g / Kg for 4 weeks reduced the growth of calcium oxalate calculus in the urinary bladder of rats significantly (Viel *et al*., 1999). This indicates that the aqueous type of solvent was capable of extracting various plants effectively and can positively act as anti-urolithiatic agent.

Conclusion

Based on result of extraction yield of all extracts, it has been found that the highest percentage was demonstrated of *P. niruri* extract which obtained by using methanol while the lowest yield percentage was obtained by using n-hexane as the extraction solvent. *P. niruri* extract contains different type of phytochemicals depending on the polarity of the solvent used. According to overall result of phytochemical screening, alkaloids are found to be abundant in hexane extracts while most saponins are contained in water extracts. This result might be affected by the polarity of phyto-chemicals and solvents used. Therefore, the ability of all extracts of *P. niruri* to inhibit and dissolve CaOx crystal might be beneficial in the treatment of urolithiasis in the future. However, there is a need of further scientific investigation and experimental proofs to support these preliminary findings. Besides, an additional work can also be carried out to isolate, purify and characterize bioactive compounds and to identify their possible mechanism of action.

Acknowledgement

The research is funded by the Ministry of Education Malaysia under Fundamental Research Grant Scheme (FRGS) Vot No. 1646 and partially sponsored by Universiti Tun Hussein Onn Malaysia.

Conflict of Interest. The authors declare no conflict of interest.

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Urol Res (2006) 34:351–357
DOI 10.1007/s00240-006-0065-1

ORIGINAL PAPER

Effect of extract of *Phyllanthus niruri* on crystal deposition in experimental urolithiasis

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Received: 4 May 2006 / Accepted: 6 July 2006 / Published online: 1 August 2006

Abstract *Phyllanthus niruri* (Pn) is a plant that has been shown to interfere in the growth and aggregation of calcium oxalate (CaOx) crystals. In the present study we evaluated the effect of Pn on the preformed calculus induced by introduction of a CaOx seed into the bladder of male Wistar rats. Pn treatment (5 mg/rat/day) was initiated immediately or 30 days after CaOx seeding and thus in the presence of a preformed calculus. Animals were sacrificed 50 or 70 days after surgery. The resulting calculi were weighed and analyzed by X-ray diffraction, stereomicroscopy and scanning electronic microscopy. Precocious Pn treatment reduced the number (75%, $P < 0.05$) and the weight (65%, $P < 0.05$) of calculi that frequently exhibited a matrix-like material on its surface, compared to the untreated CaOx group. In contrast, Pn treatment in the presence of a preformed calculus did not prevent further calculus growth; rather, it caused an impressive modification in its appearance and texture. Calculi from Pn-treated animals had a smoother, homogeneous surface compared to the spicule shape of calculi found in the untreated CaOx group. XRD analysis revealed the precipitation of struvite crystals over the CaOx seed and Pn did not change the crystalline composition of the calculi. This suggests that Pn interfered with the arrangement of the precipitating crystals, probably by modifying the crystal–crystal and/or crystal–matrix interactions. Results suggest that Pn may have a therapeutic potential, since it was able to modify the shape and texture of calculi to a smoother and probably more fragile form, which could contribute to elimination and/or dissolution of calculi.

Keywords *Phyllanthus niruri* · Calcium oxalate · Struvite · Urolithiasis · Rats

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Introduction

In recent years there has been a resurgence of interest in medicinal plants that are effective, safe and culturally acceptable as an alternative treatment for many human diseases [1]. About 25% of the drugs prescribed worldwide come from plants [2]. *Phyllanthus niruri* (*P. niruri*) is a plant belonging to the Euphorbiaceae family, which has a worldwide distribution. It is used in Brazilian folk medicine by patients with urolithiasis [3, 4]. Previous clinical studies have demonstrated that *P. niruri* has no acute or chronic toxicity, and preliminary data suggest effects, which promote stone elimination in stone-forming patients, as well as the normalization of calcium levels in hypercalciuric patients [5]. Experimental studies have shown that *P. niruri* reduced the uptake of calcium oxalate crystals by MDCK cells, without evidence of cytoxicity or biochemical alterations of the

culture medium [6]. Moreover, it prevented the growth of calculi in a model of CaOx-induced urolithiasis in rats [7]. Finally it interfered with the CaOx crystallization process in vitro by reducing crystal growth and aggregation and favored the formation of a less adherent dihydrate CaOx crystalline structure [8]. Therefore, all these effects are related to the preventive potential of *P. niruri*. In the present study, we evaluated the effect of *P. niruri* treatment simultaneously or 30 days after the introduction of a CaOx seed into the bladder [9]. We also evaluated the mineral constitution and chemical composition of the stone formed with and without *P. niruri* treatment.

Materials and methods

An aqueous extract, as used in the popular medicine of *P. niruri*, was obtained from the whole plant, which was grown at the Experimental Center of the Estadual University of Campinas, São Paulo. Plant samples were cut and dried at 50°C for 2 months in a ventilated room. After drying, plants were ground in a mechanical mill and used for infusion preparation (5% w/v). The infusion was stirred for 30 min at 72°C and then vacuum filtered, concentrated, and lyophilized. *P. niruri* was administered (20 μg/g body weight/day) by gavage, diluted in 1 ml of distilled water.

CaOx seed

To prepare the CaOx seed, small disks of CaOx were obtained by a supersaturation reaction, as described previously [10]. Briefly, 100 ml of calcium chloride (0.4 mol/l) and 100 ml of potassium oxalate (0.4 mol/l) were mixed together by constant drop-wise addition to 300 ml of distilled water for 2 h with shaking at 75°C. The mixture was maintained under agitation at 75°C for an additional 5 h. Crystals were washed with distilled water to eliminate the excess of chloride and potassium, and the formed crystals were maintained in an oven at 37°C for 2 weeks to allow aggregation and to form a compact CaOx seed. The resultant material was transferred to a template containing cylinders of 4 mm diameter to obtain small disks of CaOx. Disks were weighed and sterilized before use.

Urolithiasis model

Urolithiasis was induced by introducing a CaOx disk (seed) into the bladders of adult male Wistar rats. Briefly, the bladder was exposed through a suprapubic incision under ether anesthesia and a CaOx seed was then introduced into the bladder. After suturing the bladder wall, muscle, and skin, the animals were maintained in individual cages for 24 h for observation. All animals were allowed free access to regular rat chow and tap water. The animals were then housed in cages (four animals/cage), in their respective groups (experimentals/control). The ethics committee of the São Paulo Federal University (no. 0957/02) approved the experimental protocol.

Experimental protocol

Animals were divided into six groups: C ($n = 8$): control animals, without treatment; CPn ($n = 8$): control animals treated with *P. niruri* (Pn); CaOx ($n = 5$): animals with CaOx seed with no treatment. Animals were sacrificed 50 days after the seed implantation: CaOxPn ($n = 4$): animals with CaOx seed receiving Pn treatment during 50 days starting on the day of surgery (early treatment); CaOxPn20 ($n = 5$): Pn treatment was initiated 30 days after surgery (late treatment), and lasted 20 days; CaOxPn40 ($n = 5$): Pn treatment was initiated 30 days after surgery and lasted 40 days. It was previously observed, in a pilot study, that a period of 30 days was sufficient for the development of calculi: thus, this period was adopted to start the *P. niruri* treatment. At the end of the protocol, animals were placed in metabolic cages (Nalgene, NalgeNunc Int., USA), to collect 24 h urine samples. After urine collection, animals were ether anesthetized for blood sampling (obtained from the aorta) and then killed by air injection. The bladder was exposed and the matrix calculus and satellites were removed, washed with distilled water, weighed, and stored. Biochemical determinations included urinary and plasma concentrations of sodium, potassium (photometry, Pegasus II, Tecnow, Brazil), calcium (atomic spectrophotometry), uric acid, magnesium, and creatinine (Labtest Diagnostics, Brazil).

The calculi were photographed by stereomicroscopy (Carl Zeiss, model Stemi SV11), and further analyzed by scanning electron microscopy (SEM). The compositions of calculi were obtained by X-ray diffraction (XRD) using a Siemens/Brucker model D5000 diffractometer (Germany) with Cu-Kα radiation ($\lambda = 0.15437$ nm) at 40 kW and 40 μA (step size: 0.05°θ: time per step: 1 s), and analyzed by computer program (Evaluation Diffract Plus, 2001, France).

Data are expressed as means ± standard error. Statistical analysis was carried out using ANOVA (SigmaStat for Windows version 2.0), followed by the Tukey or Dunn's tests when necessary. Parametric Student *t*-test was used when appropriate. Differences between the data were considered significant at $P < 0.05$.

Results

Biochemical analysis of blood and urine did not reveal significant alterations among groups (data not shown), thus the presence of calculus or *P. niruri* treatment did not induce detectable alterations in the animal metabolism or urinary excretions.

Figure 1 shows representative photographs of the calculi removed from the different groups. For comparison, panel A shows a CaOx seed before implantation into the bladder. The calculi removed from the untreated CaOx group consisted of a main calculus and many satellites, which were rigid and had spicule-shaped aspects (Fig. 1b). In contrast, the material (calculi) obtained from a CaOx animal treated early with *P. niruri* consisted of the CaOx seed covered by a soft material, probably an organic matrix (Fig. 1c). The animals of group CaOxPn20 (Fig. 1d) had a larger number of larger sized satellites. However, there was a clear difference in the shape of the calculi, they had a more regular surface compared with those in the untreated CaOx group.

Table 1 summarizes the data concerning number of calculi and mass. The mean mass of CaOx disks introduced into the bladder was similar among groups. As previously reported for this experimental model, the CaOx seed served as a nidus where organic and inorganic material precipitated, allowing the growth of a main calculus, and satellites. Fifty days after the introduction of CaOx seeds, the resulting calculi were, on average, 25-fold larger in mass than the original seed (0.3283 ± 0.1350 vs. 0.0131 ± 0.0004 g, $P < 0.05$). CaOx animals treated early with *P. niruri* exhibited a significant reduction in both calculus mass and satellite number compared with untreated CaOx animals. However, *P. niruri* treatment initiated after growth of calculi induced a further increase both in calculus mass (Fig. 2) and in the number of satellites.

We were surprised to find that *P. niruri* treatment initiated in the presence of preformed calculi did not minimize the growth of calculi. However, *P. niruri* clearly induced critical changes in calculus shape. These changes varied from a flattening of the edges of spicules to a complete smoothening of calculi surfaces. To observe better this effect, we next analyzed the calculi by stereomicroscopy and SEM, which allowed us to evaluate the calculi in more detail. Figure 3a shows a typical calculus and satellites grown in CaOx animals without treatment, all of them exhibited an irregular surface and a spicule shape. Group CaOxPn (Fig. 3b)

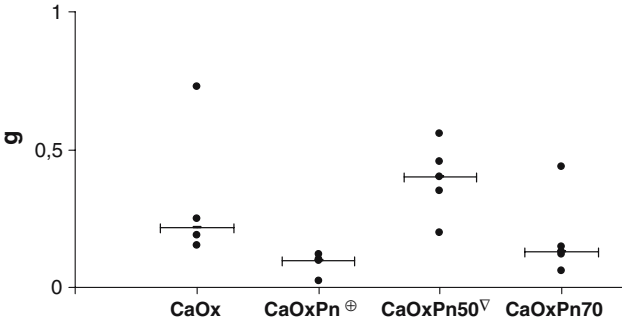

Fig. 2 Weight of calculi. Values represent the sum of the main calculus and the satellites. *Horizontal bars* indicate the median. $P < 0.05$: ⊗versus CaOx: ∇versus CaOxPn

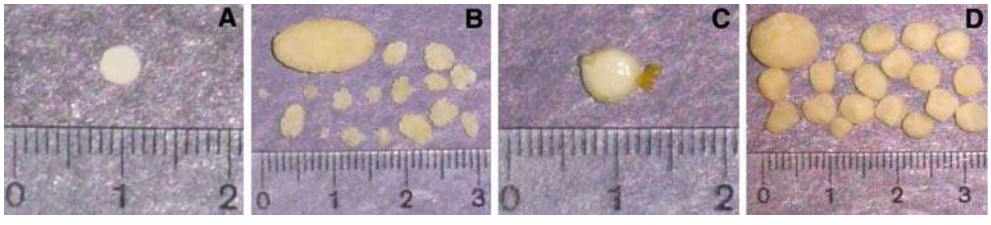

Fig. 1 Optical microscopy of the calculi. **a** CaOx seed. **b** Main calculus and satellites obtained from untreated CaOx animals. **c** Main calculus coated with an organic material from a *P. niruri* treated CaOx animal. **d** Main calculus and satellites from a CaOx animal treated with *P. niruri* in the presence of preformed calculi. The difference in calculus shape between untreated CaOx and *P. niruri* treated animals is clear

Table 1 Analyses of calculi

	CaOx seed mass (g)	Calculus mass (g)	Numbers of satellites
CaOx	0.0131 ± 0.0004	0.3283 ± 0.1350*	9.5 ± 1.6
CaOxPn	0.0131 ± 0.0002	0.0850 ± 0.0206*,***	2.5 ± 0.3**
CaOxPn20	0.0136 ± 0.0006	0.3924 ± 0.0595*	15.3 ± 1.7**,***
CaOxPn40	0.0131 ± 0.0004	0.1793 ± 0.0660*	13.0 ± 2.6***

Values represent the mean ± SE. Calculus mass corresponds to the sum of the wet main calculus and the satellites

$P < 0.05$: *versus seed weight: **versus CaOx: ***versus CaOxPn

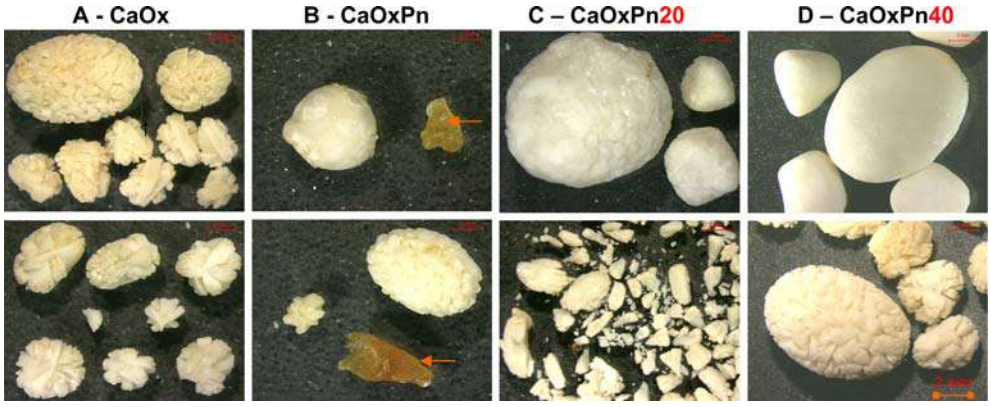

Fig. 3 Stereomicroscopy of the calculi taken from two representative animals (upper and lower panels) of each group. **a** Calculi with a homogeneous spicular shape from untreated CaOx animals. **b** Calculi from CaOx animals treated with *P. niruri* from the first day after CaOx seed implantation showing smaller calculi and very few satellites, mostly composed of an amorphous material (*arrows*). CaOx animals treated with *P. niruri* after growth of calculi for 20 (**c**) or 40 days (**d**). The variability in the response to *P. niruri* treatment is clear in (**c**). The upper panel shows calculi with a smooth surface and the lower panel shows multiple fragments of a probably more fragile calculus

exhibited almost no satellites, when present, however, they had an appearance of a predominantly organic, non-crystalline material. The calculi, when found in this group, were smaller, and had a less irregular surface compared to those found in the CaOx group with no treatment. *P. niruri* treatment initiated in the presence of a preformed calculus (groups CaOxPn20 and CaOxPn40, Fig. 3c, d, respectively) induced a clear difference in calculus surface observed with more detail under SEM (Fig. 4b, c) compared to the CaOx group (Fig. 4a). The spicule flattening or the smoother surface of calculi observed after *P. niruri* treatment on the preformed calculi can be clearly observed in Fig. 4c and d.

A summary of our XRD results is presented in Table 2. Main calculi exhibited the presence of both COM (nidus) and struvite (precipitated material) and the satellites presented only struvite.

Figure 5 shows a main calculus from an untreated CaOx animal, which was fragmented. A different material (struvite) was deposited on the CaOx seed, which acted as a central nidus.

Discussion

The experimental model of urolithiasis used in the present study was induced by a vesical foreign body (CaOx seed) implantation method that was obtained with no significant metabolic or systemic alterations. The vesical CaOx seed acts as a supporting surface, allowing organic, and inorganic material to precipitate over the central nidus, thereby mimicking a spontaneous calculus growth. Furthermore, the functional parameters of renal function, urinary pH, and urinary solute excretions were not significantly modified in this model.

The present urolithiasis model revealed that a main calculus grew over the CaOx seed together with a variable number of satellites. Since this model utilized rats, the deposited inorganic material consisted of magnesium ammonium phosphate, resulting in struvite calculi. However, the central nidus consisted of pure CaOx crystals. This result was expected since the alkaline nature of rat urine propitiates the precipitation of struvite and not CaOx crystals [11].

Results obtained with X-ray diffraction analysis confirmed the presence of both CaOX and struvite in the main calculi, and only struvite in the satellites, this suggests that the satellites probably grew on small fragments released from the main stone and acted as struvite seeds. Moreover, struvite was the main crystalline chemical composition found in all groups, both treated and untreated with *P. niruri*, indicating that the effects of *P. niruri* on the growth of calculi were not related to alterations in the inorganic elements of the calculi.

Two important effects of *P. niruri* were found in the present study. First was its ability to prevent the growth of calculi when the treatment was initiated soon after CaOx seed implantation. Second was the drastic modification in the shape and texture of the preformed calculus and after 20 or 40 days of *P. niruri* treatment the resultant stones exhibited different features. The early *P. niruri* treatment, initiated on the first day after seed implantation, caused a significant inhibition in the growth of calculi, indicating a potential prophylactic effect of *P. niruri*. This result confirmed previous data

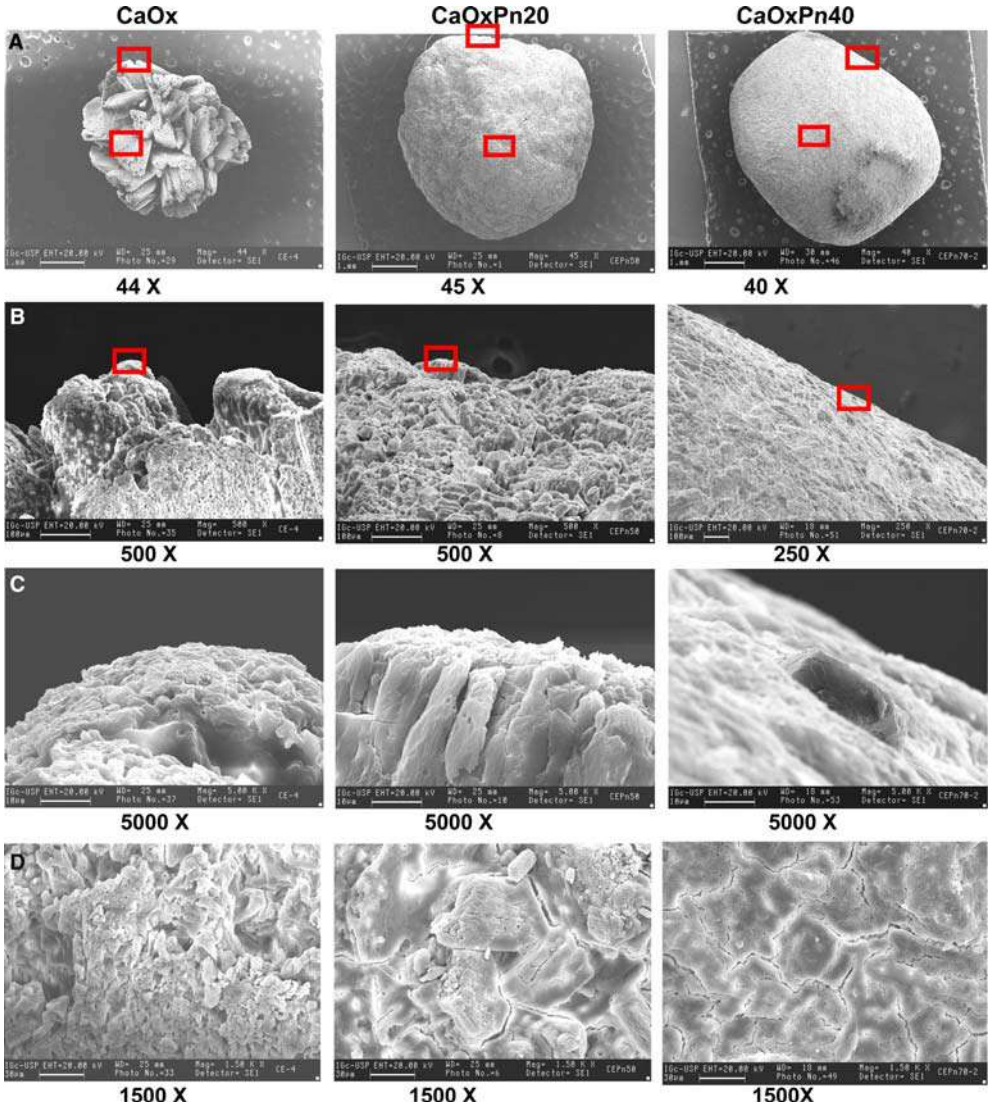

Fig. 4 Scanning electronic microscopy (SEM) of the calculi taken from CaOx untreated and late treated animals during 20 days (CaOXPn20) or 40 days (CaOxPn40). **a** Pictures show a general visualization of the calculi. *P. niruri* treated animals exhibited calculi with more regular shapes. **b** Images show a panoramic view of the calculus surface. **c** Presents the lateral view of the calculus surface in greater detail. It can be seen that the spicules are more flattened and have a smoother surface in groups treated with *P. niruri*. **d** Shows a frontal view of the calculus surface. The differential crystal deposition in calculi from *P. niruri* treated compared to untreated animals is clear

Table 2 Inorganic composition of the calculi analyzed by XRD

	CaOx	CaOxPn	CaOxPn20 satellites
COM	+	+	–
ST	+	+	+
NMM	–	+	–

ST struvite, *COM* calcium oxalate monohydrate, *NMM* non-mineral material

from our laboratory using the same animal model and experimental protocol [7] and suggests that *P. niruri* may have a potential inhibitory effect on the development of urinary calculi, probably by hindering the deposition of crystalline material on the CaOx seed. In contrast, *P. niruri* treatment initiated 30 days after CaOx seed implantation (late treatment), and thus in the presence of a preformed calculus, did not prevent the growth of further calculi, but rather induced substantial variations in the spatial arrangement of crystals, resulting in stones with a distinct shape and texture compared to untreated CaOx animals. These differences were analyzed in greater detail by SEM, the results of which revealed that an irregular precipitation of crystals was responsible for the spicule-shaped surface of the calculi taken from untreated CaOX animals. The calculi raised in *P. niruri* treated animals

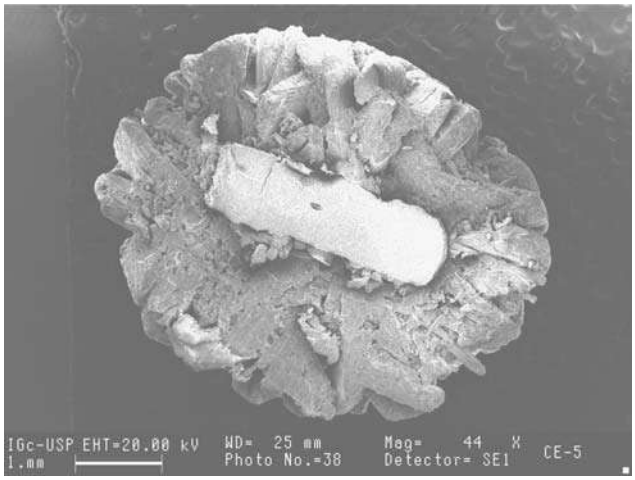

Fig. 5 SEM showing the central nidus consisting of the CaOx seed and the surrounding material formed by struvite

exhibited an anomalous crystal deposition with a filling of the spaces between the spicules, resulting in a more homogeneous and compact surface. We have previously observed that *P. niruri* interfered in the CaOx crystallization process induced in isolated human urine by reducing both crystal size and aggregation, and also by favoring the formation of a less adherent dihydrate CaOx crystalline structure [8]. Taken together, these results suggest that *P. niruri* probably interferes in the biomineralization process by promoting a different interaction between the crystal and the macromolecules of the organic matrix. It is well accepted that this interaction greatly controls crystal nucleation, size, morphology, structure, and growth rate [12–14].

The interaction between matrix proteins and crystals ensures that only crystals of a particular composition are formed, and guarantees that their various faces will grow only in certain directions to produce a structure of defined shape [15, 16]. On the other hand, the mechanisms involved in the pathological mineralization, as observed in urinary stone formation, are not well understood. However, it has become clear in recent years that the interaction between the matrix macromolecules and the nascent crystal may determine the crystal growth rate and morphology [17]. It has been demonstrated, for example, that the different isoforms of nephrocalcin induce different effects on CaOx mineralization [17]. The A and B isoforms of nephrocalcin appear to coat CaOx crystals, leaving their hydrophobic faces exposed, thereby inhibiting further mineralization. In contrast, while the C and D isoforms also coat the crystals, they promote aggregation of multiple crystals by exposing their hydrophilic face [17]. We did not analyze the organic matrix composition in the present study, however, we found in a previous study that used the same model of urolithiasis that calculi taken from CaOx animals treated with *P. niruri* from the first day after the seed implantation showed smaller and softer calculi with a higher incorporation of the matrix macromolecules glycosaminoglycans (GAGs) in the calculus structure [7]. It has been suggested that *P. niruri*-induced retardation of calculus growth was related to an increased incorporation of GAGs into the calculus, which acted to reduce the rate of calculus growth [7]. It is not presently clear if the more regular and smooth surface of the stones in animals with preexisting calculi was also the result of different matrix deposition induced by *P. niruri*.

We found some variability in both the shape and texture of calculi even among animals of the same group receiving the late treatment for 30 or 40 days. Some animals formed stones with a smooth surface (three animals), whereas others formed a less smooth surface, but flattened spicule-shaped stones and a more fragile appearance (two animals). The heterogeneity of the stones raised in this group indicates variability in the biological response to *P. niruri* treatment. Although the alterations induced by *P. niruri* were visible in all animals with preexisting calculi, further analysis of larger populations of calculi will be required to further explore this effect.

It is not clear at this time which substance(s) present in *P. niruri* would be responsible for this effect. More than 50 compounds were identified in *P. niruri*, including alkaloids, flavanoids, lignans, and triterpenes [18]. Among these substances, the triterpenes have been found to inhibit the cytotoxicity induced by calcium oxalate [19], they are also known to reduce excretion of stone forming constituents [20] and the markers of crystal deposition in the kidneys [21]. These findings point to an antilithiasic activity of these compounds, however, their effect on the growth and shape of crystals warrants further investigation.

The present study corroborates previous data suggesting the potential prophylactic effect of *P. niruri* [6–8] on the growth of calculi when the plant extract was administered before calculus development. The novel data shown here is related to the potential therapeutic effect of *P. niruri* on previously formed vesical calculi. Indeed it was clear that *P. niruri* did not prevent the growth of preexisting calculi, however, it certainly interfered with crystal deposition and substantially modified stone shape and texture. This finding raises the possibility for an alternative use of *P. niruri*, namely, to induce changes in calculi that might aid in elimination and/or dissolution of calculi.

Although the effect of *P. niruri* must be further evaluated in lithiasic patients, the results obtained in the

present study suggest that *P. niruri* may have useful therapeutic applications in patients who already have stones, while it might have a prophylactic role in persons who are at high risk but have not yet developed stones.

Acknowledgments We are indebted to Dr. Antonio J. Lapa of the Pharmacology Department, Federal University of São Paulo, for preparing the lyophilized form of *Phyllanthus niruri*. Also to Isaac Jamil Sayeg and Dr. Flávio Machado de Souza Carvalho, of the Geosciences Institute, São Paulo University for XRD and SEM analyses. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Coordenação de Aperfeiçoamento de Nível Superior (CAPES), Conselho Nacional Científico Tecnológico (CNPq), and Fundação Oswaldo Ramos.

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Urol Res (2003) 30:374–379
DOI 10.1007/s00240-002-0285-y

ORIGINAL PAPER

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Effects of an aqueous extract from *Phyllanthus niruri* on calcium oxalate crystallization in vitro

Received: 10 April 2002 / Accepted: 10 October 2002 / Published online: 21 January 2003

Abstract *Phyllanthus niruri* is a plant used in Brazilian folk medicine for the treatment of urolithiasis. It was previously observed that *P. niruri* shows no toxicity, potentially increases calculus voiding by stone forming patients and inhibits the endocytosis of calcium oxalate (CaOx) crystals by MDCK cells. In addition, in a rat model of urolithiasis it reduced calculus growth. In the present study, we evaluated the effect of an aqueous extract of *P. niruri* on CaOx crystallization in vitro. CaOx precipitation was induced by the addition of 0.1 M sodium oxalate to unfiltered urine samples from Wistar rats ($n = 14$) and normal humans ($n = 18$) in the presence or absence of *P. niruri* extract (0.25 mg/ml of urine). The presence of CaOx crystals was evaluated immediately and 24 h later. In vitro crystallization of human urine produced typical mono- and dihydrated CaOx crystals, but only a few typical CaOx crystals were found in rat urine. The presence of *P. niruri* extract did not inhibit CaOx precipitation and even more crystals were obtained, although they were significantly smaller than those in the control urine. Crystal aggregation observed 24 h after crystallization was also inhibited by *P. niruri* extract. The results showed an inhibitory effect of *P. niruri* extract on CaOx crystal growth and aggregation in human urine, suggesting that it may interfere with the early stages of stone formation and may represent an alternative form of treatment and/or prevention of urolithiasis

Keywords Calcium oxalate · In vitro crystallization · *Phyllanthus niruri* · Renal stone · Urolithiasis · Natural products

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Introduction

Urinary stones affect 10–12% of the population in industrialized countries [27]. Their incidence has been increasing over the last years while the age of onset is decreasing [10]. In addition, the recurrence rate is high, being more than 50% after 10 years [28, 29]. In spite of substantial progress in the pathophysiology and treatment of urolithiasis, there is no satisfactory drug to use in clinical therapy. Thus a drug for the prevention of this disease or its recurrence would be of great interest.

Phyllanthus niruri is a plant belonging to the Euphorbiaceae family, which have a worldwide distribution. It is used in Brazilian folk medicine by patients with urolithiasis [18, 21]. Many components of *P. niruri* have been identified, including groups of active substances such as alkaloids, tannin, lignans, phenols, steroids, flavanoids, triterpenes, as well as ricinoleic acid, niruside, and phylitate [4]. However the components involved in lithiasis prevention are not known. We have been evaluating the potential effect of *P. niruri* in the treatment of urolithiasis over the last few years. Experimental and clinical studies have demonstrated that *P. niruri* has no acute or chronic toxicity and preliminary data suggest effects which promote stone elimination in stone forming patients [25]. Moreover, oral administration of *P. niruri* aqueous extract to rats induced an inhibitory effect on vesical calcium oxalate (CaOx) crystal growth, which was associated with a reduction in the urinary excretion of glycosaminoglycans and with an increase in the content of these macromolecules in the calculi compared with untreated animals [11]. Also, *P. niruri* significantly reduced the endocytosis of CaOx crystals in MDCK cells in culture [5]. Despite the beneficial effect of *P. niruri* observed in vivo in rats and humans, its mechanism of action is not fully understood.

Most kidney stones contain calcium oxalate [9], and the formation of urinary calculi involves a CaOx crystallization process that includes nucleation, growth and the aggregation of crystals [15, 16]. Thus, to better

understand the role of *P. niruri* in urinary stone formation in the present study, we evaluated the effect of an aqueous extract of this plant on CaOx crystallization induced in vitro.

Materials and methods

An aqueous extract of *P. niruri* was obtained from the whole plant, as occurs in popular medicine. The plant was grown at the Experimental Center of the Universidade Estadual de Campinas, São Paulo. Plant samples were dried at 50°C for 2 months in a ventilated room. After drying, samples were ground in a mechanical mill and used for tea preparation (5% w/v). The infusion was stirred for 30 min at 72°C and then vacuum filtered, concentrated and lyophilized.

CaOx crystallization was induced in the urine obtained from normal humans and rats. Isolated human urine samples were obtained from six healthy subjects, three males and three females, with no personal or family history of kidney stone disease. Urine was collected on three different occasions from each individual, with an interval of at least 15 days between each sampling time ($n = 18$). Urine samples collected over 24 h were obtained from normal, adult Wistar rats ($n = 14$) in a metabolic cage. Rats were maintained on standard chow and tap water during the collection period. Human and rat urine samples were centrifuged at 5,000 rpm (4,815 *g*) for 8 min, the supernatant was then transferred to a clean tube, and the pH was adjusted to 6.0.

Experimental protocol

CaOx precipitation was induced by adding 40 µl of 0.1 M sodium oxalate per ml of urine (corresponding to 0.536 mg), every 30 min (0, 30, 60 and 90 min) under shaking at 37°C, resulting in a final concentration of 2.14 mg/ml of urine. Each urine sample was divided into two aliquots, one of which was used as a control (crystallization without *P. niruri* extract) while in the other CaOx precipitation was induced in the presence of *P. niruri* extract, which was added to the sample 30 min before the crystallization process. Lyophilized *P. niruri* extract was resuspended in distilled water (25 mg/ml), filtered through a 0.22 µm filter, and used at a final concentration of 0.25 mg/ml urine, based on a dose-response curve.

The present protocol was approved by the Ethics Committee of the Universidade Federal de São Paulo.

Analysis of crystals

The crystals obtained were analyzed immediately and 24 h after the crystallization process. The semiquantitative analysis of crystals was estimated by turbidity [26]. After crystallization, 100 µl aliquots were loaded onto a 96 well microplate and the absorbance was measured with a plate reader (Original Multiskan EX, Labsystens, Finland), at 590 nm (OD_{590}). The absorbance of each sample was measured in quadruplicate and the mean was used to calculate the turbidity index $TI = (DOt \times DOb)/DOb$, where DOt is the mean sample absorbance after CaOX precipitation and DOb is the mean sample absorbance before precipitation. The number and size of the crystals were determined using an automated particle counter [22] (Coulter counter, model Z1, Coulter Electronics, England), using a 50 µm filter calibrated with latex particles measuring 10 µm in diameter. The number of particles was counted according to size, which was classified from 5.0 to 30.0 µm. The number of crystals was expressed on a percentage scale. Crystals were also analyzed by light microscopy. After crystallization, samples were centrifuged at 3,000 rpm (720 *g*) for 5 min and the supernatant was partially discarded, with approximately 10% of the initial volume being left and rehomogenized. One drop was transferred to a Neubauer chamber and the crystals were qualitatively analyzed in terms of size and shape. Images were recorded with a digitalized video-camera (Model SSC-DC54A, Sony Exwave HAD, Japan), transferred to a computer and analyzed using Imagelab 2000 (Brazil) software.

Statistical analysis

All results are reported as means ± SEM. Results for control and experimental samples were compared by the paired Student's *t*-test. Differences between human and rat urine were compared by the unpaired Student's *t*-test. *P* values of less than 0.05 were considered significant.

Results

A dose-response curve was constructed using human urine, based on the effect of different doses of *P. niruri* extract on the turbidity of the solution. Concentrations of *P. niruri* extract varied from 0.00 to 1.00 mg/ml of urine. Figure 1 shows that the absorbance of the solution increased with increasing doses of *P. niruri* extract. Doses above 0.25 mg/ml were not able to induce a further change in absorbance and therefore this dose was used in the subsequent experiments. The increase in absorbance indicates a higher density of crystals, an unexpected result for *P. niruri* extract. However, the observation of crystals by light microscopy showed that increasing doses of *P. niruri* extract actually produced a higher crystal density but of smaller crystals (Fig. 2), explaining why *P. niruri* induced an increase rather than a decrease in absorbance.

The number of crystals related to size was estimated by automatic counting. As shown in Fig. 3, in the presence of 0.25 mg/ml of *P. niruri* extract there was an increase in the number of smaller crystals between 5.0–7.5 µm and a decrease in the number of larger crystals (10–30 µm) compared with urine without *P. niruri* extraxt. Figure 4 illustrates the effect of *P. niruri* on the crystal form of CaOx analyzed by microscopy in human urine. In control urine, crystals were identified as a

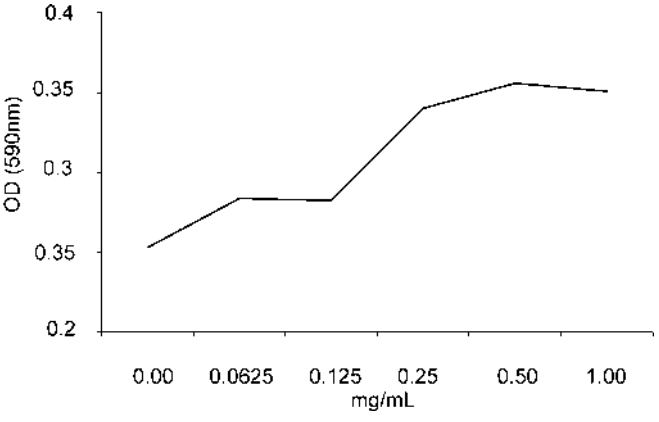

Fig. 1 The induction of CaOx crystallization measured by optical density (OD_{590}), in normal human urine by adding sodium oxalate in the presence of increasing amounts of *P. niruri* extract

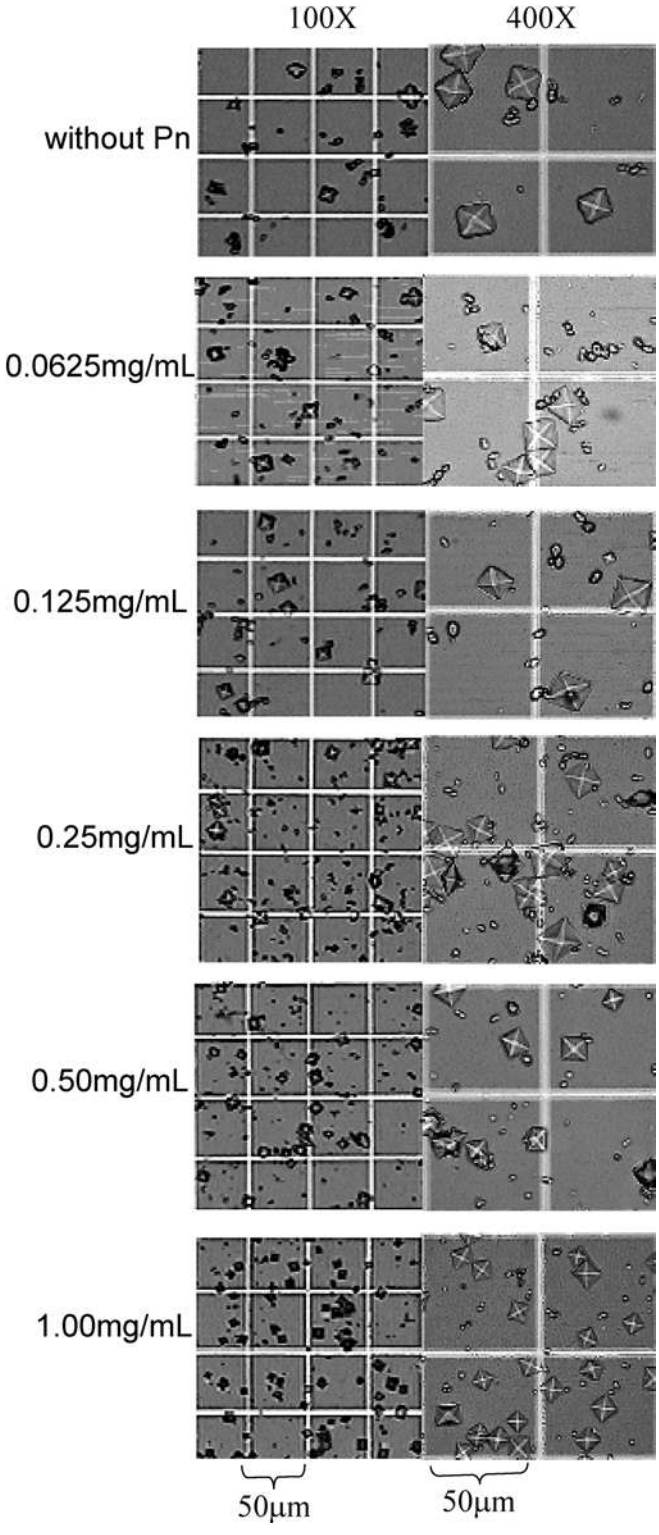

Fig. 2 Light microscopy of CaOx crystals induced in human urine by adding sodium oxalate solution in the absence and presence of *P. niruri* extract at different concentrations. 100× and 400× magnification

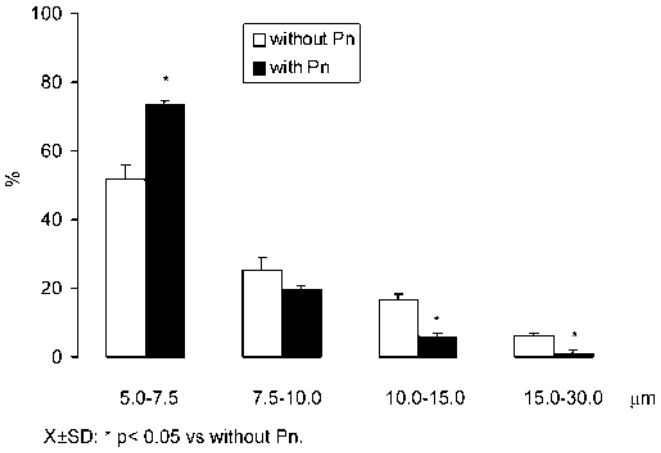

Fig. 3 Number of particles analyzed by automatic counting classified by size in the absence and presence of *P. niruri* extract in human urine

mixture of mono- and dihydrate (COM and COD) CaOx crystals (Fig. 4A). The presence of *P. niruri* extract was associated with a partial shift from the COM form to the COD form (Fig 4B). A semiquantitative analysis of the COM and COD fractions estimated by visual examination under the microscope showed that in the control urine 15% of the crystals were COD and this fraction increased significantly to 30% in the presence of *P. niruri* extract.

An intense aggregation of CaOx crystals was observed 24 h after crystallization in control urine (Fig 5A), a phenomenon that was substantially inhibited in the presence of *P. niruri* extract (Fig. 5B).

In contrast to human urine, typical CaOx crystals were almost absent in rat urine after the induction of crystallization. Small particles, but no typical CaOx crystals were observed in these samples, either immediately or 24 h after the crystallization process. *P. niruri* extract had no effect on rat urine.

Discussion

Increasing evidence has pointed to the beneficial effects of *P. niruri* in the treatment of urolithiasis [18, 21]. In vivo studies have shown that *P. niruri* may be effective in promoting calculus voiding by stone forming patients [25] and significantly reduced calculus growth in a model of vesical calculi in rats [19, 25]. In addition, no toxic effect was observed in individuals ingesting *P. niruri* tea over a period of 3 months [19, 25]. In spite of these exciting results obtained in vivo, little is known about the mechanism of action of *P. niruri*. Many steps and a favorable environment are necessary for the development of a calculus. The supersaturation of urine is a prerequisite for precipitation to occur but not sufficient to produce a stone [15, 16]. Thereafter, nucleation, growth and crystal aggregation take place as a result of a favorable environment, including the adhesion and internalization of the crystal into the tubular epithelial cells [12]. Recently, Campos and Schor [5] showed that an aqueous extract of *P. niruri* significantly reduced the

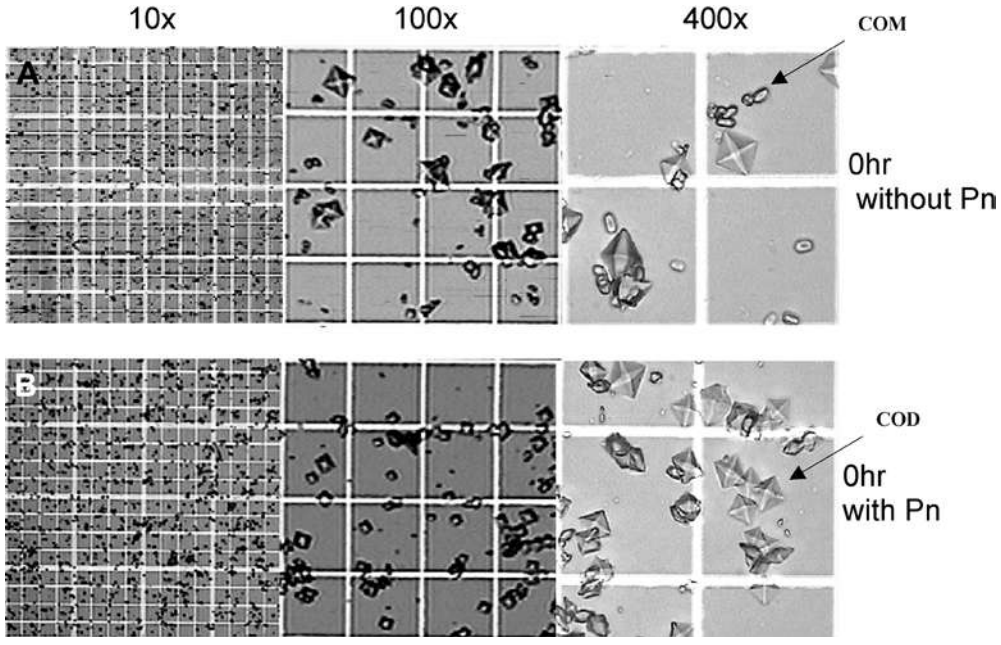

Fig. 4 CaOx crystals observed in human urine by light microscopy in the presence (**A**) and absence (**B**) of *P. niruri* extract. 10×, 100× and 400× magnification

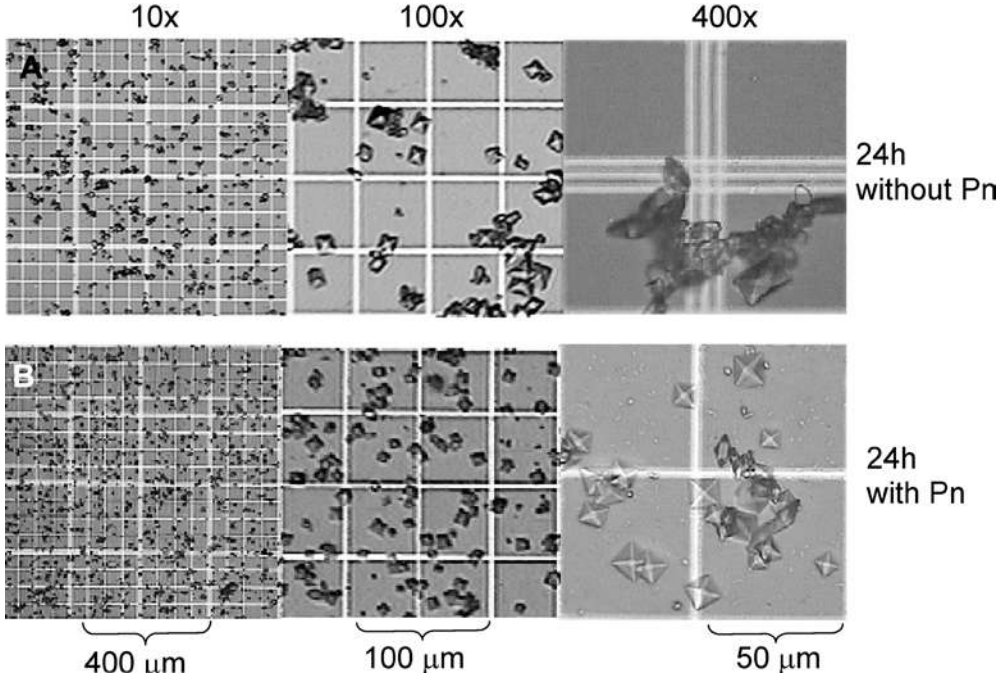

Fig. 5 CaOx crystals observed in human urine 24 h after crystallization by light microscopy in the absence (**A**) and presence (**B**) of *P. niruri* extract. 10×, 100× and 400× magnification

endocytosis of CaOx crystals by MDCK cells. Moreover, the presence of defined substances such as magnesium and citrate or macromolecules such as glycosaminoglycans, osteopotin, nephrocalcin, etc, in urine may act as protectors [7, 8, 23, 31] inhibiting calculus development by interfering with many phases of calculus formation. Studies conducted by Freitas et al. [11] using a model of urolithiasis in rats demonstrated that *P. niruri* did not interfere with the urinary excretion of Mg^{++}, citrate or glycosaminoglycans, but instead promoted adsorption of the latter substances into the calculi, making them softer and smaller. In order to provide further evidence on the potential role of this plant as an inhibitor of stone formation, and to better understand its mechanism of action, we evaluated here the effect of *P. niruri* extract on the CaOx crystallization process induced in vitro in human and rat urine.

In vitro CaOx crystallization in human urine under the conditions employed in the present study produced a mixture of typical mono- and dihydrate CaOx crystals. Crystalluria is a common event observed even in

non-stone forming individuals, and these crystals are predominantly of the COD form [9]. In the present study, the major crystalline form found after CaOx precipitation was COM (85%), prevailing over the COD (15%) form. This discrepancy probably reflects the differences between spontaneous CaOx nucleation in vivo and induced precipitation in vitro. In spite of this difference, *P. niruri* extract induced an increase in the COD fraction from 15% to 30%. It has been suggested that COM has a stronger affinity for cell membranes than COD [17, 30], and thus COM crystals may constitute a form of higher potential risk for stone formation. Moreover, the most common form of CaOx crystals found in kidney stones is COM [10], although many stones contain both crystal forms. Thus, the presence of *P. niruri* extract induced alterations in CaOx crystal morphology, favoring the formation of the CaOx dihydrate (COD) form, which is less likely to bind to renal cells [30].

Our results also showed that *P. niruri* extract did not inhibit CaOx nucleation, but inhibited crystal growth, since the size of the particles was significantly smaller than that of the particles found in control samples. Also, the aggregation of crystals was reduced in the samples containing *P. niruri* extract. These properties may constitute an important advantage in the prevention of lithiasis, inhibiting calculus growth and keeping the crystals dispersed in the urine, with their subsequent easier elimination through the urine. The aqueous extract of *P. niruri* must contain substances that interfere with these processes. Many active compounds have been described in *P. niruri* [3, 13, 20, 24]; however, the isolated effect of these compounds on CaOx crystallization have not been tested. Recently, Atmani and Khan [1] showed similar results of CaOx crystallization in vitro obtained with *Herniaria hirsuta*, a plant from Morocco, but the specific compound(s) involved in this protector mechanism is not known.

Finally it is interesting to note that CaOx crystallization did not occur in rat urine and thus no effect of *P. niruri* extract was observed. In fact, spontaneous precipitation of calcium salts in vivo is a rare event in rats [6, 14] and even in induced urolithiasis in these animals, apatite but not oxalate stones are observed [2, 14]. The relatively alkaline rat urine (pH$\sim$7.0) compared to human urine (pH$\sim$5.5) may have a role in the failure to induce CaOx precipitation [6]. However even at an acidic pH, spontaneous CaOx crystallization in rats may be a sporadic event, even when oxalate is in excess in the urine, because the rat urine has a high level of oxalate relative to that of calcium [14] and thus a further increase in oxalate concentration does not increase the level of supersaturation by very much. In contrast, normal human urine has a calcium concentration about 15 times that of oxalate. Moreover, in the present study the pH was adjusted to 6.0 in all rat and human urine samples, with the subsequent elimination of pH as an interfering factor. Thus, the presence of unknown, specific inhibitor molecules in rat urine should be investigated.

In summary, we showed that *P. niruri* extract interfered with the CaOx crystallization process by reducing CaOx crystal growth and aggregation and that this extract favored the formation of a less adherent dihydrate CaOx crystalline structure. These results contribute to the accumulating evidence obtained over the last 10 years pointing to the beneficial effects of *P. niruri* on many stages of stone formation and/or elimination [25], including crystallization, aggregation, cellular adherence [5] and adsorption of macromolecules into the calculi [11]. Thus, *P. niruri* can potentially interfere with the pathogenesis of urolithiasis and may represent an attractive alternative for the prevention of lithiasis of the urinary tract.

Acknowledgements We are indebted to Dr. A.J. Lapa, Pharmacology Department, Universidade Federal de São Paulo, for preparing the lyophilized form of *Phyllanthus niruri*. This study was supported by Fundação de Amparo à Pesquisa (FAPESP), Coordenação de Aperfeiçoamento de Nível Superior (CAPES), Conselho Nacional Científico Tecnológico (CNPq) and Fundação Oswaldo Ramos.

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Original Paper

NEPHRON

Nephron 1999;81:393–397

Accepted: October 26, 1998

Phyllanthus niruri Inhibits Calcium Oxalate Endocytosis by Renal Tubular Cells: Its Role in Urolithiasis

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Key Words

Phyllanthus niruri · Calcium oxalate · Madin-Darby canine kidney cells · Endocytosis · Renal stone

Abstract

We investigated the in vitro effect of an aqueous extract of *Phyllanthus niruri L.* on a model of CaOx crystal endocytosis by Madin-Darby canine kidney cells. The extract exhibited a potent and effective non-concentration-dependent inhibitory effect on the CaOx crystal internalization. This response was present even at very high (pathologic) CaOx concentrations and no *P. niruri L.*-induced toxic effect could be detected. Biochemical analysis of culture media containing *P. niruri L.* did not provide any clues for the elucidation of the cellular pathways affected by this natural product. Although further studies are necessary for a better understanding of the role of *P. niruri L.* in urolithiasis, our findings show that this natural product could be an attractive alternative for the treatment of urinary stones.

Introduction

Plants of the genus *Phyllanthus* (family *Euphorbiaceae*) have a worldwide distribution and more than 500 different species have been catalogued thus far. Many of these natural products and their derivatives have been reported to be effective in the treatment of several pathological conditions [1, 2].

Plants of the genus *Phyllanthus* are widely employed in Brazilian folk medicine by patients with urinary calculi to control pain attacks and to help eliminate stones. Results from our laboratory [3] have shown a marked inhibitory effect of *Phyllanthus niruri L.* tea on the formation of stones in rat bladders. In the same study, patients drinking the tea for a period of 3 months exhibited a significantly enhanced elimination of calculi compared to controls. Interestingly, even at higher doses of the tea, neither rats nor humans showed any acute or chronic adverse reactions, a fact further supporting the therapeutic potential of *P. niruri L.*

Interactions between CaOx crystals and renal tubular epithelial cells can play a role in the genesis and evolution of urolithiasis. Tubular cells avidly and selectively bind and take up CaOx crystals, a phenomenon followed by a series of intracellular events that culminate in a fibrogenic and proliferative cellular response [4]. Since the model of CaOx internalization by renal tubules represents a valuable tool for the study of urinary calculus formation, the present study was undertaken to investigate the effects of an aqueous extract of *P. niruri L.* on CaOx endocytosis by Madin-Darby canine kidney (MDCK) cell cultures.

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Material and Methods

Cell Culture

MDCK cells obtained from the American Type of Culture Collection from passages 75 to 90 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (FBS; 5%), 2 g/l (23.8 mmol/l) $NaHCO_3$, 2.6 g/l (10.9 mmol/l) HEPES, 10,000 IU/l penicillin, 50 mg/l (3.7 mmol/l) streptomycin and 100 mg/l (16.3 mmol/l) neomycin. Cells were maintained at 37°C in a humidified gas mixture (95% air and 5% CO_2). At subconfluence cells were washed twice with phosphate-buffered saline (PBS) and the medium was replaced with DMEM without FBS for 24 h in order to obtain cells in the G_0 phase of the cell cycle.

Cell Trypsinization

At maximal confluence, cells were washed twice with PBS and exposed to trypsin-EDTA (0.25/0.02% w/v, 0.5 ml/bottle) for 2 min in order to obtain cell suspensions. Trypsin activity was neutralized with equal volumes of DMEM containing FBS.

Cell Viability

Cell viability was evaluated by Trypan blue staining. MDCK cell suspensions were centrifuged, resuspended in PBS and incubated with Trypan blue (final concentration, 0.2% w/v) for 5–15 min. Cells were then observed by light microscopy, and all cells that excluded the dye from the cytoplasm were considered viable. At least 100 cells per culture were counted and a 90% viability rate was judged satisfactory.

Preparation of CaOx Crystals

Equimolar solutions (0.4 mol/l, 100 ml) of calcium chloride and potassium oxalate were combined and the mixture was added to distilled and deionized water (DDW; 300 ml) by constant dripping for 2 h. This suspension was maintained under continuous stirring at 75°C for 5 h and then washed with DDW to remove potassium chloride present in the supernatant. The remaining saturated solution was maintained at 37°C for 15 days until CaOx crystallization. CaOx crystals were then sterilized in ethylene oxide and culture medium without FBS was added, yielding a new suspension. In order to make the particle size uniform, the suspension was sonicated for 12 min. Qualitative analysis of CaOx was performed by X-ray diffractometry and crystal size was evaluated by laser chromatography with a CILAS 330 laser granulometer, with a purity grade higher than 95% and a mean crystal diameter of <5 μm, respectively (data not shown).

P. niruri L. *Aqueous Extract Preparation*

The plant was grown at the experimental center of the Universidade Estadual de Campinas, CPQBA, Paulínia, São Paulo, Brazil, and was classified by Dr. L. Webster. A voucher specimen (ref. 481) is deposited in the herbarium of the same institution. A *P. niruri L.* crude extract was obtained from the whole plant, as done in folk medicine. Plant samples were cut and dried at 50°C for 2 months in a ventilated room. After drying, plants were ground in a mechanical mill and used for tea preparation (5% w/v tea). The tea was stirred for 30 min at 72°C and then vacuum filtered, concentrated and lyophilized. The powder was resuspended in DMEM without FBS (10 mg/ml) and the suspension passed through a 0.22-μm filter. Subsequent dilutions were also made in DMEM. All samples were protected from light, with no storage (single day use only).

MDCK Cell Exposure to CaOx

Cell cultures in the G_0 stage were washed twice with PBS and exposed to the CaOx suspension (100 and 200 μg/ml; 78.1 and 156.3 mmol/l) for 6 h. We previously determined the best incubation time and submaximal CaOx concentrations (data not shown). Cultures were then washed again in PBS and trypsinized. Cell suspensions were analyzed by polarized light microscopy and CaOx crystal endocytosis was scored arbitrarily on a 0–4 scale, according to a previous report [5].

Effect of Phyllanthus niruri L. *on CaOx Crystal Endocytosis*

MDCK cells were exposed to the CaOx suspension (100–200 μg/ml, 6 h) in the absence or presence of the aqueous extract of *P. niruri L.* (5, 10, 50, 100, 500 and 1,000 μg/ml) added to the medium 30 min before CaOx administration. CaOx crystal uptake was then evaluated as described above.

Biochemical Profile

After the addition of different concentrations of *P. niruri L.* aqueous extract to cell cultures, a biochemical analysis of the medium was carried out. Sodium, potassium and calcium ion concentrations were determined with an ion-selective electrode device (AVL 9140) and pH was measured with a Micronal B 371 pH meter. Similar procedures were performed employing pure DMEM and the results were compared.

Statistical Analysis

Data are expressed as mean scores ± standard error of the mean (SEM) or as a percentage of control. The unpaired Student t test was employed to compare each treatment group to the control group. The biochemical data were compared by ANOVA followed by the Newman-Keuls test when necessary. p values <0.05 were considered significant.

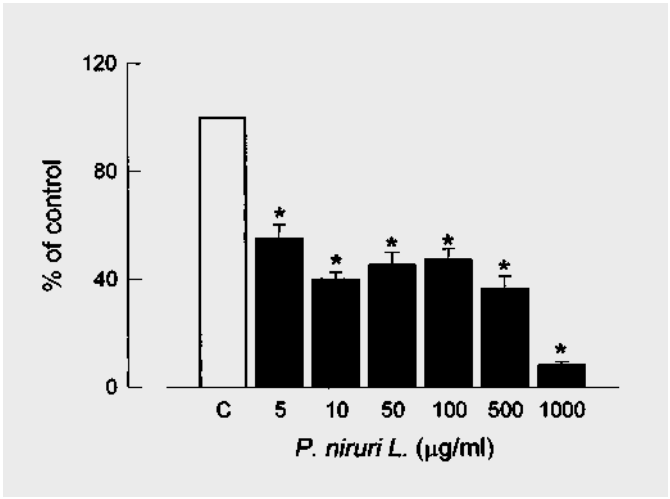
Drugs and Reagents

PBS, DMEM, neomycin/penicillin/streptomycin solution and Trypan blue were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Trypsin-EDTA solution and FBS were obtained from Sigma Chemical Co. or Cultilab (Campinas, Brazil). All salts were purchased from Merck SA (Rio de Janeiro, Brazil).

Results

Effect of P. niruri L. *on CaOx Endocytosis*

As can be seen in figure 1, the addition of the aqueous extract of *P. niruri L.* promoted a marked reduction (45–92%) in the endocytotic response observed in MDCK cells exposed for 6 h to CaOx 100 μg/ml. We did not observe a classical dose-response effect in the presence of the extract, with closely similar inhibitory responses in the intermediate range of *P. niruri L.* concentrations. However, the maximal extract concentration employed (1,000 μg/ml) almost abolished CaOx uptake. In addition, a significant reduction in CaOx endocytosis could also be detected even in the presence of 5 μg/ml of the extract, disclosing a potency not commonly found in non-purified



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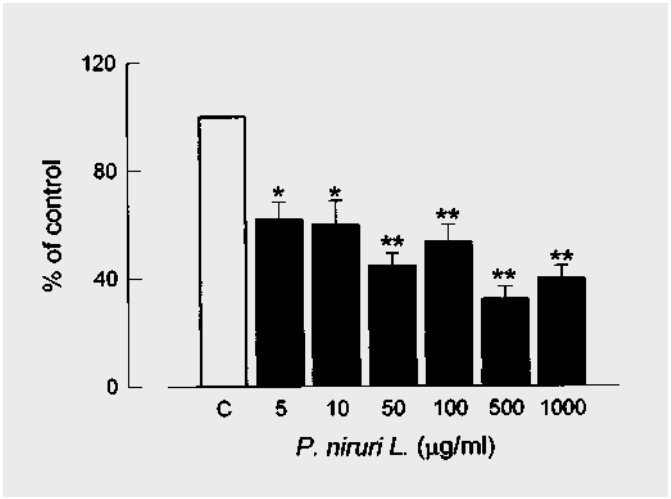

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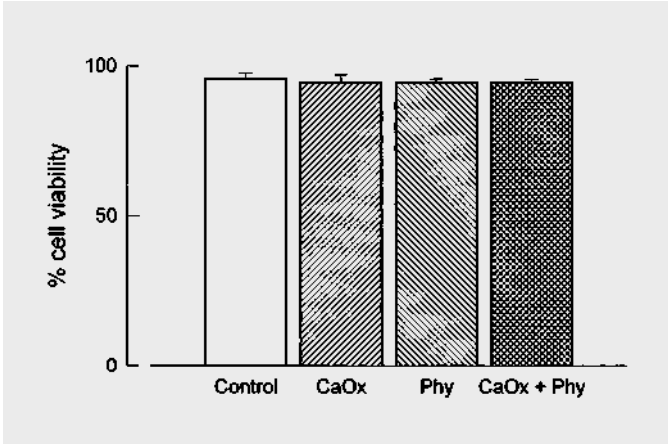
Fig. 1. Calcium oxalate uptake by MDCK cells exposed for 6 h to a crystal suspension (100 µg/ml) in the absence and presence of different concentrations of *P. niruri L.* Columns represent the mean and the bars the SEM of at least 8 experiments. * $p < 0.001$ compared to the control group (C).

Fig. 2. Calcium oxalate uptake by MDCK cells exposed for 6 h to a crystal suspension (200 µg/ml) in the absence and presence of different concentrations of *P. niruri L.* Columns represent the mean and the bars the SEM of at least 11 experiments. * $p < 0.005$; ** $p < 0.001$ compared to the control group (C).

Fig. 3. MDCK cell viability with or without exposure to calcium oxalate (CaOx; 100 µg/ml), *P. niruri L.* (Phy; 1,000 µg/ml), or both, for 6 h, expressed as percentage of cells that excluded Trypan blue dye from the cytoplasm. Columns represent the mean and the bars the SEM of 6 experiments.



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natural products. The absolute scores of control values obtained for groups 5 (n = 9), 10 (n = 8), 50 (n = 8), 100 (n = 12), 500 (n = 14) and 1,000 (n = 8) µg/ml of *P. niruri L.* were 0.78 ± 0.08, 0.75 ± 0.07, 0.86 ± 0.05, 0.76 ± 0.05, 1.07 ± 0.08 and 0.84 ± 0.05, respectively. *P. niruri* concentrations of <5 µg/ml did not induce any inhibitory effect on internalization of CaOx by MDCK cells (data not shown).

The inhibitory response of the *P. niruri L.* aqueous extract was only mildly attenuated when we doubled the concentration of CaOx (38–68%) and, as shown in figure 2, statistically significant decreases in CaOx uptake could still be observed at all concentrations of the extract tested. The absolute control values were 1.05 ± 0.10 for concentrations of 5, 50 and 500 µg/ml (n = 12) and 1.45 ± 0.12 for the concentrations of 10, 100 and 1,000 µg/ml of *P. niruri L.* (n = 12). No significant difference in the inhibitory pattern of *P. niruri L.* was observed if CaOx crystals were added before, simultaneously to or after the administration of the natural product (data not shown).

Cell Viability

No significant difference in cell viability could be detected when we compared control values (n = 6) to MDCK cells exposed to CaOx (100 µg/ml; n = 6) or to the highest concentration of *P. niruri L.* (1,000 µg/ml; n = 6) (fig. 3). Indeed, cell viability was about 95% even in the presence of both substances for 6 h (n = 6), showing that no synergistic cytotoxic effect occurred between the two compounds.

Biochemical Alterations

As depicted in table 1, the addition of the aqueous extract of *P. niruri L.* to the culture media did not promote any significant alteration in sodium or calcium ion concentration compared to control DMEM (n = 4–5). On

Table 1. Sodium, potassium and calcium ion concentrations (mEq/L) in culture medium (DMEM without FBS) in the absence and presence of CaOx and aqueous extract of *P. niruri L.*

	Control	*P. niruri L.*, μg/ml[a]				
		0	50	100	500	1,000
Na^+	140.2 ± 0.4	141.5 ± 0.8	140.6 ± 0.4	140.6 ± 0.3	140.6 ± 0.2	140.7 ± 0.6
K^+	5.66 ± 0.04	5.57 ± 0.02	5.69 ± 0.02	5.69 ± 0.01	6.22 ± 0.03*	6.97 ± 0.10*
Ca^{2+}	1.59 ± 0.01	1.56 ± 0.02	1.58 ± 0.01	1.58 ± 0.01	1.55 ± 0.01	1.54 ± 0.02

Data are reported as the mean ± SEM of at least 4 experiments.
* Values significantly different ($p < 0.05$) compared to the remaining groups.
[a] Plus CaOx 200 μg/ml.

the other hand, higher concentrations of the extract (500 and 1,000 μg/ml) considerably enhanced the potassium ion concentrations of the culture medium, with approximately 10 and 23% increments compared to control (n = 4–5; $p < 0.05$) (table 1). In addition, the pH values obtained for all groups were closely similar, ranging from 7.3 to 7.4 (n = 4; p = n.s.; data not shown).

Discussion

Evidence has accumulated supporting the role of plants of the genus *Phyllanthus* in the treatment of urolithiasis. Some of these data were extracted from popular medicine, with debatable scientific value. On the other hand, experimental and clinical studies performed in our laboratory point in the same direction. Santos [3] did not detect any side effects in individuals ingesting the tea of *P. niruri L.* at high doses (>15 g/d) over a relatively long period of time (3 months). This safety profile makes this natural product an attractive alternative for the treatment of urinary calculi. Although a beneficial action in rats and humans could be demonstrated, the mechanism of action of *P. niruri L.* did not become clear. In the same study [3] no blood or urinary modification occurred to explain the inhibition of calculus formation in rats or the increase in stone elimination in humans. The reduction in CaOx uptake by tubular cells reported in the present study possibly represents one of the mechanisms by which *P. niruri L.* exerts positive effects on urolithiasis. These are, obviously, preliminary results. Nevertheless the potency and the efficacy exhibited by the aqueous extract of *P. niruri L.* deserve special attention. The high CaOx concentrations employed in our experiments (2.5- to 5-fold the upper limit in human urine) reinforce the view of a very strong inhibitory effect of the extract.

CaOx crystals are positively charged and adhere to the plasma membrane syaloglycoproteins exhibiting a negative charge [6]. A series of CaOx internalization inhibitors such as heparin, citrate, nephrocalcin and polylisin [4, 7–10] act by antagonizing this electrical interaction. However, we assume that the mechanism of action of *P. niruri L.* does not involve this effect. First, there was no concentration-dependent inhibition of CaOx endocytosis, a usual characteristic of the above substances; second, when we doubled the concentration of CaOx crystals the inhibitory effect of *P. niruri L.* was maintained at almost all tested concentrations, and, third, previous, simultaneous, or later administration of *P. niruri L.* in relation to CaOx did not reduce the effect of the extract (data not shown), suggesting that there is no competition between the two substances for a putative common site.

As mentioned above, no adverse effects were observed with the in vivo use of the tea of *P. niruri L.* Similarly, the viability of cells exposed to high concentrations of *P. niruri L.* in the absence and presence of CaOx was not impaired. Thus, the reduction in CaOx internalization by MDCK cells could not be attributed to a toxic effect.

Alterations in the physical properties of the plasma membrane change the internalization of CaOx by tubular cells, as described previously [11, 12]. Reductions in potassium concentrations in the culture medium produce increments in CaOx uptake probably by such modifications [13–15]. Thus, the *P. niruri L.*-induced enhancement of the potassium concentration could be responsible for the inhibitory effect demonstrated in MDCK cells. However, this shift in potassium levels was observed only at higher extract concentrations (500 and 1,000 μg/ml) while the reduction in CaOx internalization was evident even at lower *P. niruri L.* concentrations (5–100 μg/ml), when potassium concentrations resembled that of control medium. Furthermore, the reduced CaOx endocytosis

observed with *P. niruri L.* at 500 μg/ml was not significantly different from that obtained with concentrations of 50 and 100 μg/ml. Indeed, when we exposed MDCK cells to CaOx 200 μg/ml, the inhibitory effect of *P. niruri L.* 1,000 μg/ml (with the highest potassium levels observed) was almost identical to that obtained with the other extract concentrations. In addition, blood and urinary potassium concentrations were not modified by acute or chronic administration of high doses of *P. niruri L.* tea in vivo [3]. These findings, taken together, favor a direct action of this plant not dependent on potassium ion concentrations.

By comparing our data with those reported by others, we can make inferences about the possible mechanisms of *P. niruri L.*-induced inhibition of CaOx uptake. Dias et al. [16] reported isolated guinea-pig bladder contractile responses induced by a hydroalcoholic extract of *P. urinaria*, mediated by increased intracellular calcium concentration. We observed a significant reduction in CaOx endocytosis consequent to increases in cytosolic calcium from intra- or extracellular sources (unpublished results). Thus, *P. niruri L.* may prevent the internalization of CaOx crystals by changes in calcium metabolism. In the same study, we demonstrated a markedly diminished endocytotic response of MDCK cells in the presence of staurosporine, a protein kinase C inhibitor. Polya et al. [17] have shown a potent inhibitory effect of tannins purified from *P. amarus* on several kinases, including protein kinase C. This is another explanation for the effects of the aqueous extract of *P. niruri L.* These are, of course, merely assumptions that must be further evaluated. Many compounds from *Phyllanthus* have been isolated, exhibiting a diversity of chemical and biological actions. The study of such compounds in our model certainly will provide useful information about the mechanism(s) of action of this natural product.

In conclusion, our study demonstrated a potent and effective inhibitory effect of the aqueous extract of *P. niruri L.* on the CaOx internalization by MDCK cells. This effect does not seem to be mediated by biochemical alterations and *P. niruri L.* did not promote any cell damage, even at the highest doses tested. Despite the possibility of some cellular alterations produced by plants of the genus *Phyllanthus*, the mechanism of action of this extract remains to be clarified. Nevertheless, *P. niruri L.* may represent a nontoxic, low-cost and bioavailable therapeutic alternative for the management of urolithiasis.

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