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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.

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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) *Variable*	*Group, before/after surgery* *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).

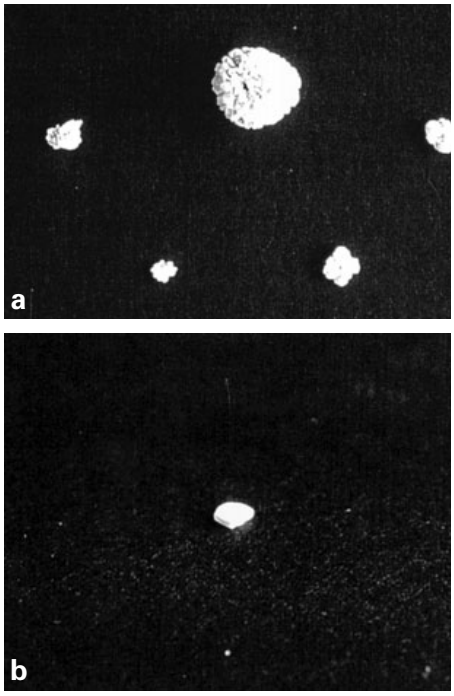

Fig. 1. Calculi (matrix and satellites) taken from an untreated animal (**a**) and a Pn-treated animal (**b**).

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.

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