

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

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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

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 cytotoxicity or biochemical alterations of the

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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-K α 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 \pm 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 smoothing 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)

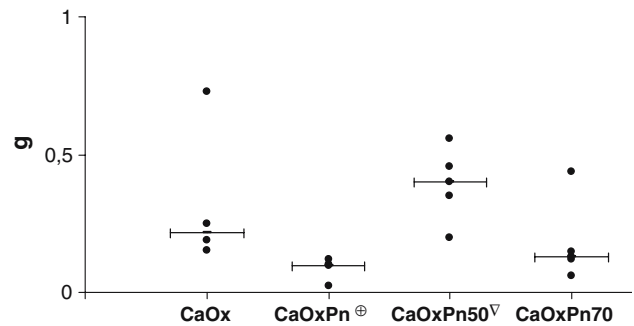


Fig. 2 Weight of calculi. Values represent the sum of the main calculus and the satellites. Horizontal bars indicate the median. $P < 0.05$: ⊕versus CaOx; ∇versus CaOxPn

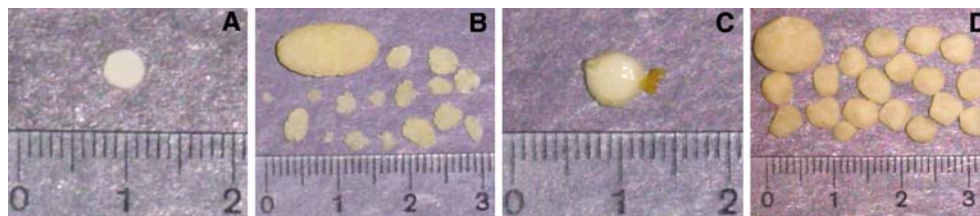


Fig. 1 Optical microscopy of the calculi. **a** CaOx seed. **b** Main calculus and satellites obtained from untreated CaOx animals. **c** Main calculus coated with an organic material from a *P. niruri* treated CaOx animal. **d** Main calculus and satellites from a CaOx

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	$0.3283 \pm 0.1350^*$	9.5 ± 1.6
CaOxPn	0.0131 ± 0.0002	$0.0850 \pm 0.0206^{***}$	$2.5 \pm 0.3^{**}$
CaOxPn20	0.0136 ± 0.0006	$0.3924 \pm 0.0595^*$	$15.3 \pm 1.7^{*****}$
CaOxPn40	0.0131 ± 0.0004	$0.1793 \pm 0.0660^*$	$13.0 \pm 2.6^{***}$

Values represent the mean \pm 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

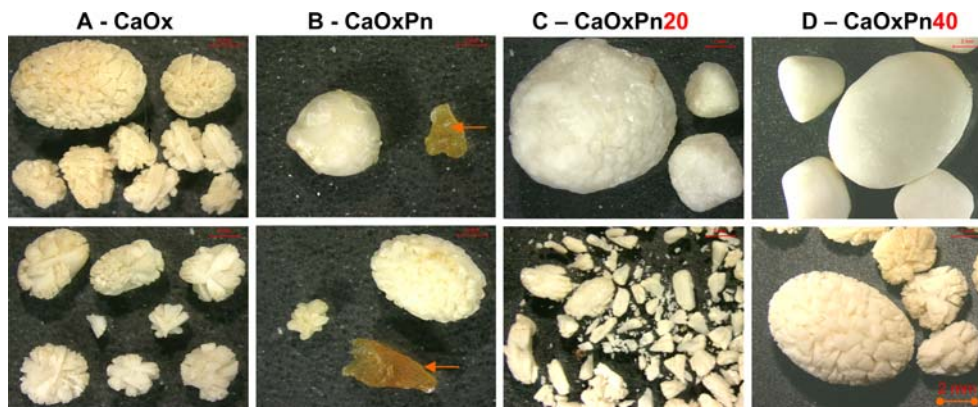


Fig. 3 Stereomicroscopy of the calculi taken from two representative animals (upper and lower panels) of each group. **a** Calculi with a homogeneous spicular shape from untreated CaOx animals. **b** Calculi from CaOx animals treated with *P. niruri* from the first day after CaOx seed implantation showing smaller calculi and very few satellites, mostly composed of an amorphous mate-

rial (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

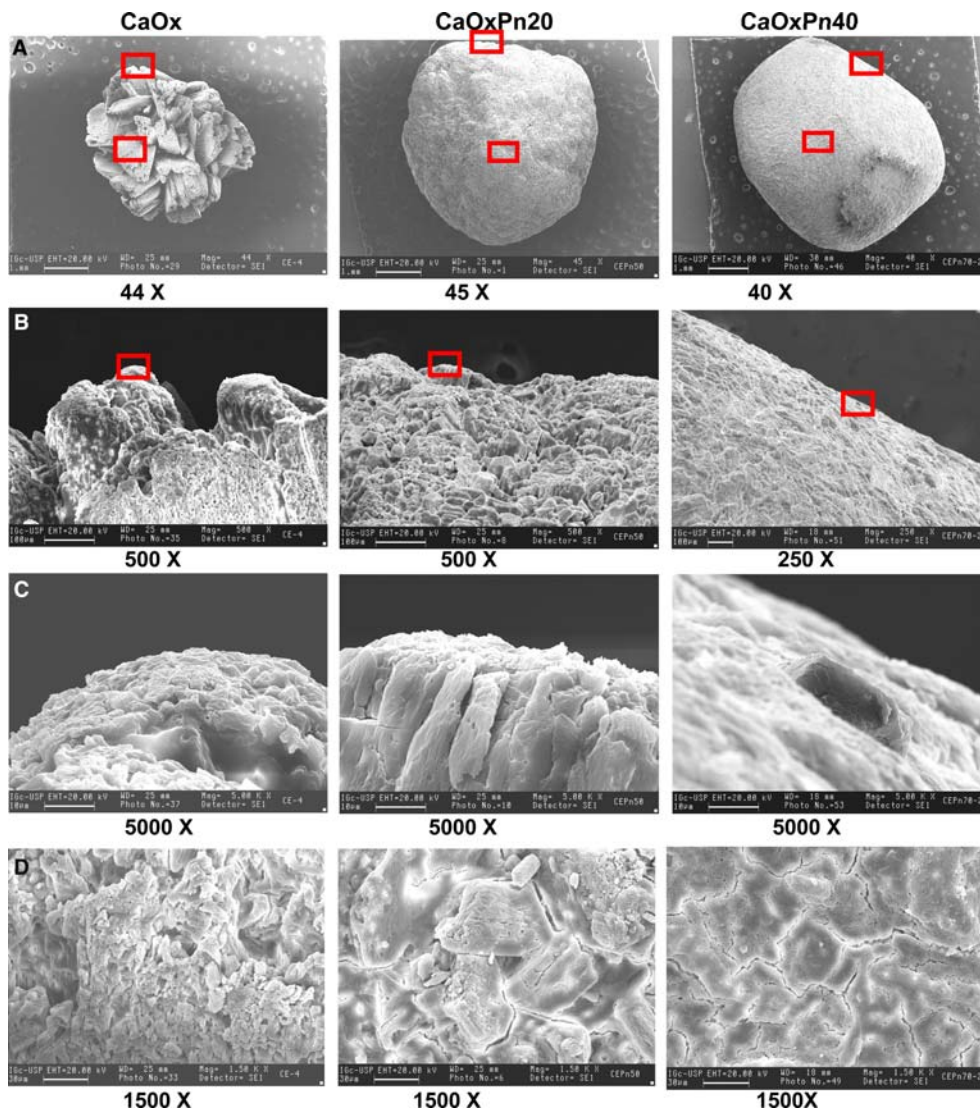


Fig. 4 Scanning electronic microscopy (SEM) of the calculi taken from CaOx untreated and late treated animals during 20 days (CaOxPn20) or 40 days (CaOxPn40). **a** Pictures show a general visualization of the calculi. *P. niruri* treated animals exhibited calculi with more regular shapes. **b** Images 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

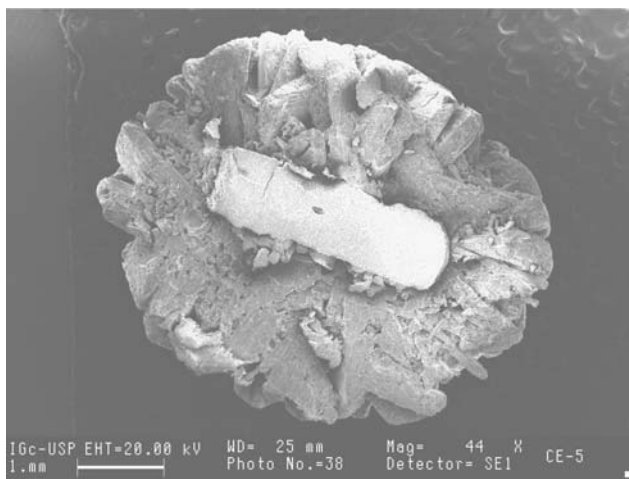


Fig. 5 SEM showing the central nidus consisting of the CaOx seed and the surrounding material formed by struvite

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.

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