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

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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

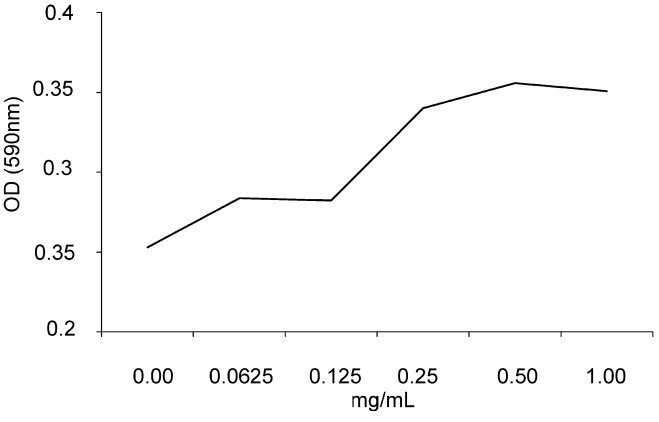

Fig. 1 The induction of CaOx crystallization measured by optical density (OD_{590}), in normal human urine by adding sodium oxalate in the presence of increasing amounts of *P. niruri* extract

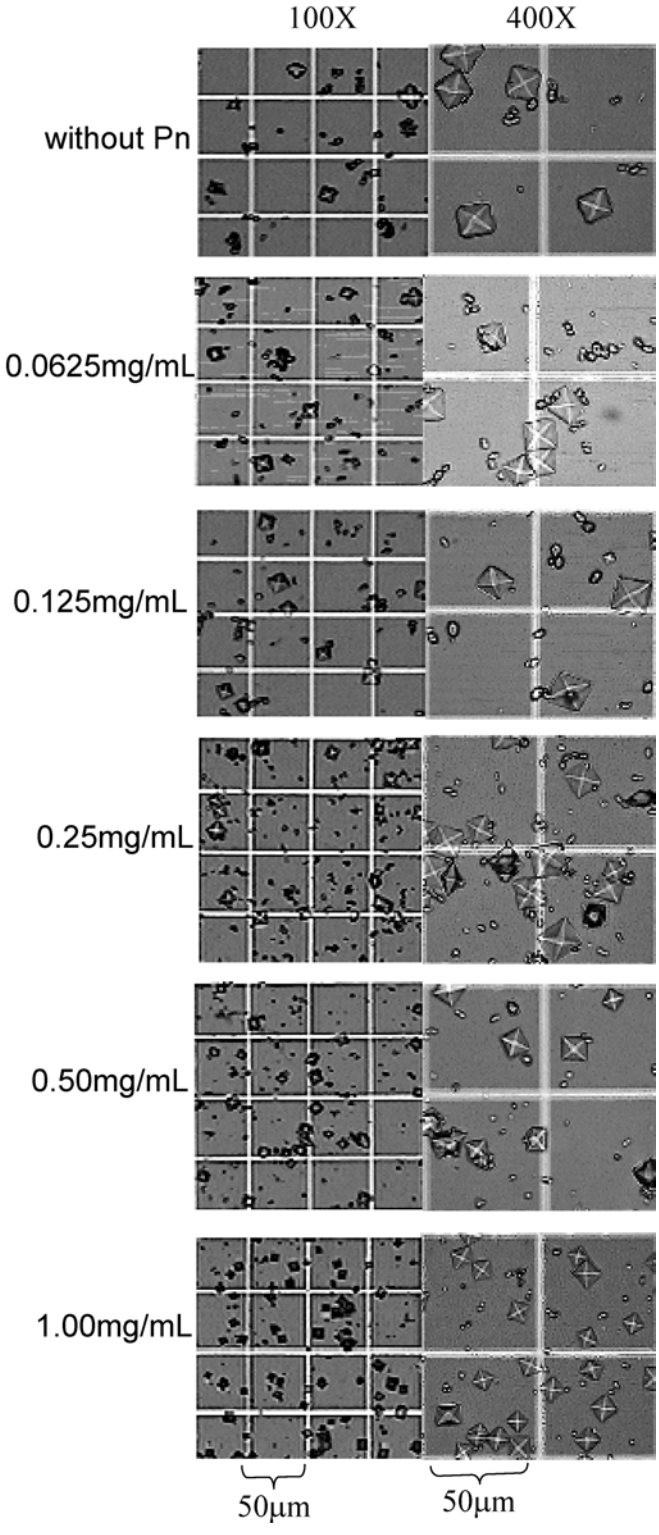

Fig. 2 Light microscopy of CaOx crystals induced in human urine by adding sodium oxalate solution in the absence and presence of *P. niruri* extract at different concentrations. 100× and 400× magnification

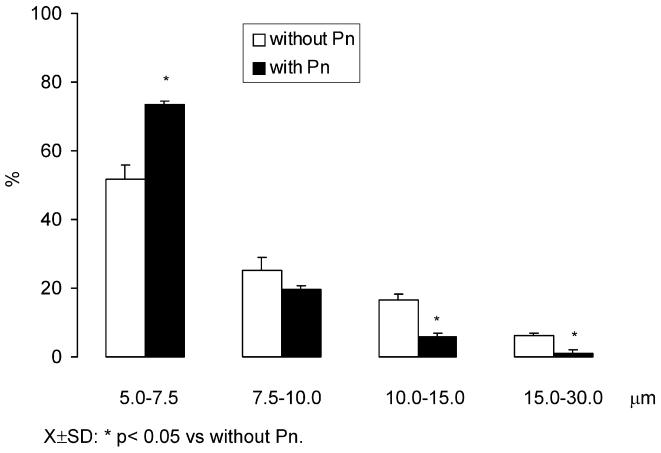

Fig. 3 Number of particles analyzed by automatic counting classified by size in the absence and presence of *P. niruri* extract in human urine

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

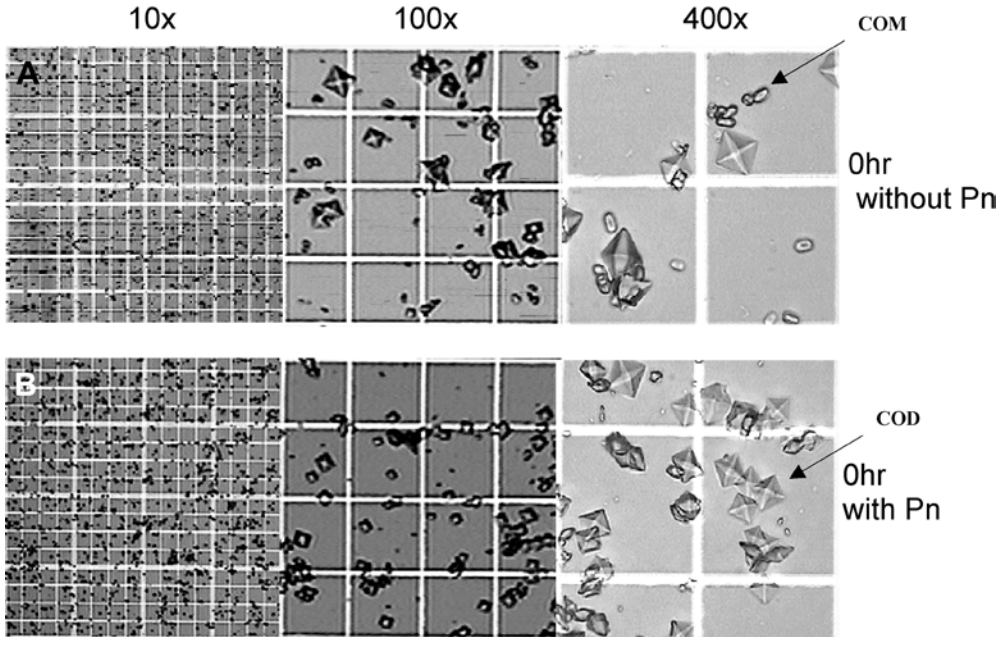

Fig. 4 CaOx crystals observed in human urine by light microscopy in the presence (**A**) and absence (**B**) of *P. niruri* extract. 10×, 100× and 400× magnification

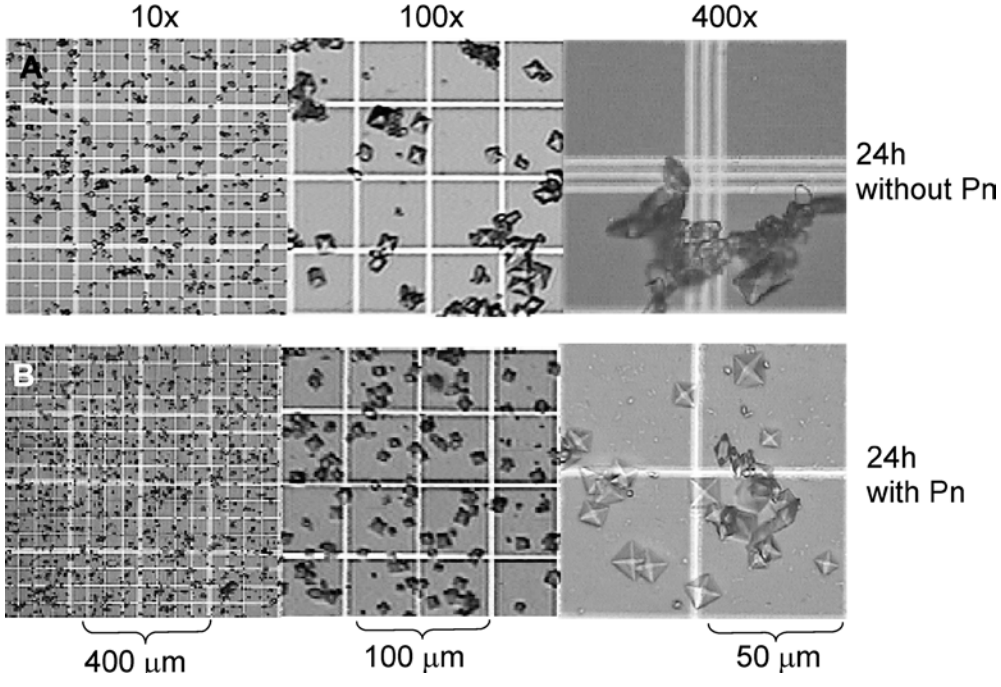

Fig. 5 CaOx crystals observed in human urine 24 h after crystallization by light microscopy in the absence (**A**) and presence (**B**) of *P. niruri* extract. 10×, 100× and 400× magnification

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.

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