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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.

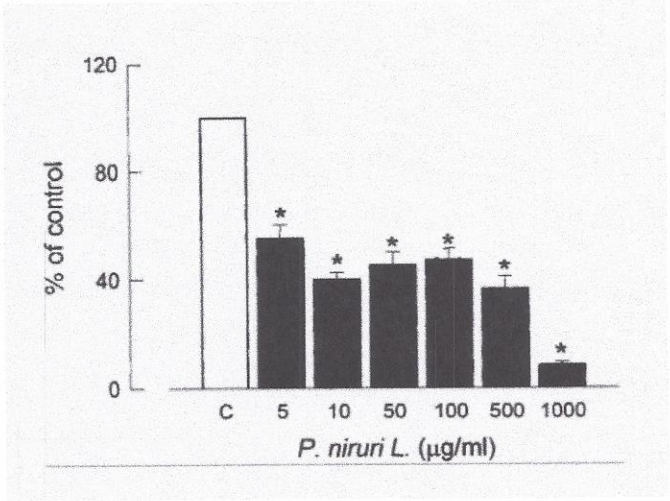
Drugs and Reagents

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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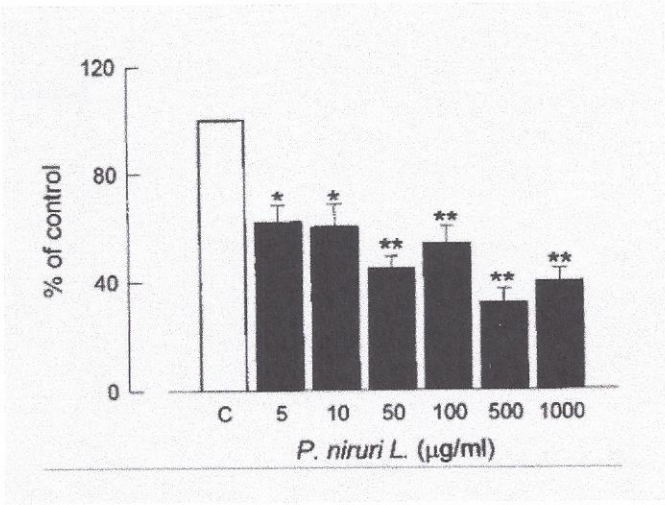

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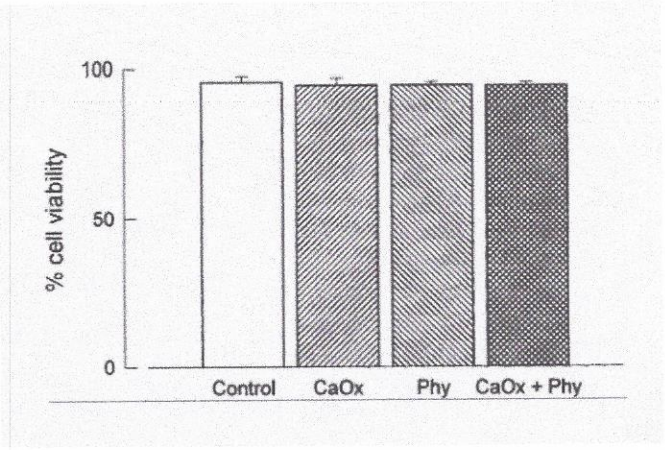
Fig. 1. Calcium oxalate uptake by MDCK cells exposed for 6 h to a crystal suspension (100 µg/ml) in the absence and presence of different concentrations of *P. niruri L.* Columns represent the mean and the bars the SEM of at least 8 experiments. * $p < 0.001$ compared to the control group (C).

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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