

Effect of hydroalcoholic extract from *Copaifera langsdorffii* leaves on urolithiasis induced in rats

Ana Paula S. Brancalion · Rejane B. Oliveira · João Paulo B. Sousa · Milton Groppo · Andresa A. Berretta · Marcio E. Barros · Mirian A. Boim · Jairo K. Bastos

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Abstract *Copaifera langsdorffii* Desf. commonly known as “copaíba”, produce a commercially valuable oil-resin that is extensively used in folk medicine for anti-inflammatory, antimicrobial and antiseptic purposes. We have found the hydroalcoholic extract of this plant leaf has the potential to treat urolithiasis, a problem affecting ~7% of the population. To isolate the functional compounds *C. langsdorffii* leaves were dried, ground, and macerated in a hydroalcoholic solution 7:3 to produce a 16.8% crude extract after solvent elimination. Urolithiasis was induced by introduction of a calcium oxalate pellet (CaOx) into the bladders of adult male Wistar rats. The treated groups received the crude extract by oral gavage at 20 mg/kg body weight daily for 18 days. Extract treatment started 30 days after CaOx seed implantation. To monitor renal function sodium, potassium and creatinine concentrations were analyzed in urine and plasma, and were found to be in the normal range. Analyses of pH, magnesium, phosphate,

calcium, uric acid, oxalate and citrate levels were evaluated to determine whether the *C. langsdorffii* extract may function as a stone formation prevention agent. The HPLC analysis of the extract identified flavonoids quercitrin and afzelin as the major components. Animals treated with *C. langsdorffii* have increased levels of magnesium and decreased levels of uric acid in urinary excretions. Treated animals have a significant decrease in the mean number of calculi and a reduction in calculi mass. Calculi taken from extract treated animals were more brittle and fragile than calculi from untreated animals. Moreover, breaking calculi from untreated animals required twice the amount of pressure as calculi from treated animals (6.90 ± 3.45 vs. 3.00 ± 1.51). The extract is rich in flavonoid heterosides and other phenolic compounds. Therefore, we hypothesize this class of compounds might contribute significantly to the observed activity.

Keywords *Copaifera langsdorffii* · Urolithiasis · Phenolic compounds · Flavonoid heterosides

A. P. S. Brancalion · R. B. Oliveira · J. P. B. Sousa · J. K. Bastos (✉)
Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/n, CEP 14040-903 Ribeirão Preto, SP, Brazil
e-mail: jkbastos@cfrcp.usp.br

M. Groppo
Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil

A. A. Berretta
Apis Flora Industrial e Comercial Ltda,
Ribeirão Preto, SP, Brazil

M. E. Barros · M. A. Boim
Departamento de Medicina, Divisão Renal,
Universidade Federal de São Paulo, São Paulo, SP, Brazil

Introduction

Plant products continue to be a good alternative source of lead compounds in drug discovery. Only 30% of all new chemical entities launched from 1981 to 2006 were synthetic in origin, suggesting that natural products represent an important part of drug discovery programs [1].

The use of plants in folk medicine in tropical regions has been successful in treating diseases worldwide and particularly in developing countries. For example, a plant used for treating disease is the *Copaifera langsdorffii* Desf. (Fabaceae–Caesalpinoideae), commonly called “copaiba.” This plant is a large tree distributed throughout the northern

and northeastern regions of Brazil, especially in Amazonas, Ceará and Pará states [2]. Species belonging to the genus *Copaifera* secrete a popular and commercially valuable oil-resin extensively used in folk medicine [3]. Biological activities reported for this oil-resin include the following: antinociceptive [4], anti-inflammatory [5], antimicrobial [6], antiseptic [7] and gastroprotective properties [8].

Urolithiasis is a consequence of alterations in the crystallization conditions of urine in the urinary tract [9], and it affects ~7% of the population. The incidence of this pathology in the United States is ~5% in females and 12% in males [10]. In Brazil, ~7% of hospitalizations were associated with urolithiasis in 2004 [11].

Multiple biochemical changes lead to disease development, and renal stones can have different chemical compositions. Treatment consists mainly of using shock wave lithotripsy to disrupt the kidney stone. If this therapy is unsuccessful, surgery is used to remove the stone [12]. The therapeutic alternatives for preventing calculi formation include the following strategies: administration of thiazide diuretics to hypercalciuric patients [13]; allopurinol treatment in patients with a high concentration of uric acid in urine [14]; administration of antibiotics to treat infection and prevent the formation of struvite calculi, which are composed of ammonium magnesium phosphate; and administration of potassium citrate to maintain the pH of the urine >6.5 to prevent cystine stone formation [15].

Several plants are used in folk medicine to treat urolithiasis, but their pharmacological and clinical assays are either inconclusive or have not been investigated [16]. Singh and Sachan [17] reported the evaluation of a formulation, called Trinapanchamool, that was composed of five plants: *Desmostachya bipinnata*, *Saccharum officinarum*, *Saccharum nunja*, *Saccharum spontaneum* and *Imperata cylindrical*. The use of this plant mixture displayed both prophylactic and therapeutic effects in *Albinus* rats, and the activity was related to a diuretic effect produced by the formulation.

The bark of *Crataeva nurvala* functions as both a prophylactic and therapeutic agent in urolithiasis in *Albinus* rats, and lupeol was determined to be the active compound in the bark [18]. Sannidi et al. [19] reported the activity of a combination of *Bergenia ligulata* and *Tribulus terrestris* in 30 patients with urolithiasis. They found that 28 and 75% of the patients eliminated the calculi in the kidney and ureter, respectively. *Costus spiralis* is extensively used in Brazilian folk medicine to treat urolithiasis, and its administration at concentrations of 0.25–0.5 g/kg/day significantly reduced the size of CaOx calculi in rat bladders [20].

Despite intense research efforts devoted to identifying an efficacious natural or synthetic therapeutic drug to treat urolithiasis, no drug is currently on the market to treat this

disease. In addition, neither the role of diet in the development of this disease or the mechanisms involved in the calculi formation in patients has been fully determined [16].

This work is founded on previous reports suggesting that *C. langsdorffii* leaves effectively treated urolithiasis in several patients. The aim of the present study was to induce calculi formation in rats by introducing CaOx pellets into their bladders and subsequently, to determine whether the leaf extract has a therapeutic effect on calculi growth and hardness. Furthermore, we tested the effect of the extract on renal function and other biochemical parameters, such as the concentrations of magnesium, phosphate, calcium, uric acid, oxalate, citrate and urine pH, to validate the therapeutic use of this plant.

Materials and methods

Plant material and extract preparation

Leaves and branches of *Copaifera langsdorffii* Desf. were collected in the University of São Paulo, campus of Ribeirão Preto, SP, Brazil. The plant material was identified by Professor Milton Groppo of the Biology Department of the School of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, SP, Brazil. A voucher specimen (SPFR 10120) was deposited in the herbarium of the same institution.

Plant material was dried at 40°C in an oven with air circulation and ground using a knife mill. The resultant powder (3.1 kg) was submitted to maceration three times sequentially in a hydroalcoholic solution 7:3 for 72 h, followed by filtration using filter paper. The extracts obtained were concentrated under vacuum and lyophilized to furnish the total crude extract (520 g).

HPLC crude extract analysis

The HPLC instrumentation used in our experiments consisted of a Shimadzu SCL-10Avp (Kyoto, Japan) multisolvent delivery system, a Shimadzu SPD-M10Avp photodiode array detector, and an Intel Celeron computer for controlling the analytical system. The analytical chromatography of the crude hydroalcoholic extracts of *C. langsdorffii* was conducted using two monolithic columns that were linked in series (Onyx™ 100 × 4.6 mm – C₁₈ Phenomenex) and were protected by a pre-column from the same company. The mobile phase consisted of water (A) and acetonitrile (B). The elution program was 5–6% of phase B for 1 min, 6–8% of B (1–2 min), 8–10% of B (2–5 min), 10–15% of B (5–12 min), 15% of B (12–22 min), 15–25% of B (22–27 min), 25% of B (27–35 min), 25–40% of B (35–39 min), 40% of B

(39–42 min), 40–100% of B (42–47 min), 100% of B for 1 min, and finally, an 12 additional minutes to return to the initial conditions and re-equilibrate the column. The flow rate was maintained at 1 mL/min, and detection was set at 257 nm. Quercetin-3-O- α -L-rhamnopyranoside (quercitrin) and kaempferol-3-O- α -L-rhamnopyranoside (afzelin) were isolated using column chromatography on a Sephadex LH-20 and semipreparative RP-HPLC [21].

CaOx pellet

CaOx pellets measuring 4 mm in diameter were prepared in a template containing cylinders 4 mm in diameter. To prepare the pellets, a supersaturated solution of CaOx was obtained by reaction of 100 mL of calcium chloride (0.4 mol/L) and 100 mL of potassium oxalate (0.4 mol/L). The reaction was then slowly placed into 300 mL of distilled water and incubated with continuous shaking for 2 h at 75°C. The mixture continued shaking for additional 5 h at 75°C. Crystals of CaOx were washed with distilled water to eliminate excess chloride and potassium. The crystals were incubated in an oven at 37°C for two weeks to allow aggregation and seed formation. The seed crystals were compacted into cylinders to obtain pellets. The pellets were weighed and sterilized before use [11].

Urolithiasis model

Urolithiasis was induced by introduction of CaOx pellets into the bladders of adult male Wistar rats as previously described [11]. Briefly, the bladder was exposed through a suprapubic incision under anesthesia with a ketamine/xylazine (2:1 v/v) solution (0.1 mL/g body weight) by intramuscular injection. One CaOx pellet was introduced into the bladder of each animal. After suturing the bladder wall, muscle and skin, the animals were monitored in individual cages for 24 h. All animals had free access to regular rat chow and tap water. Animals were then housed in cages of five animals per cage in their respective control and experimental groups. The experiments were approved by the Ethics Committee for Animal Care of São Paulo Federal University, protocol no. 1785/08, in accordance with the Federal Government legislation on animal care.

Experimental protocol

The crude *C. langsdorffii* extract was diluted in 1 mL of distilled water to allow administration by oral gavage to rats at a concentration of 20 mg/kg body weight/day.

Animals were divided into four groups: A ($n = 5$), control animals without treatment; B ($n = 5$), control animals treated with *C. langsdorffii*; C ($n = 4$), animals

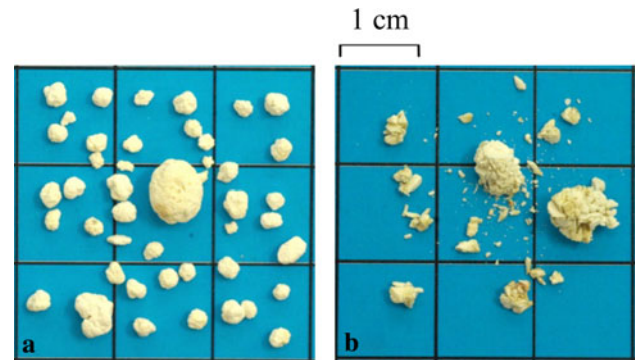


Fig. 1 Calculi obtained from **a** untreated animals and **b** animals treated with *C. langsdorffii*

administered CaOx pellet with no treatment, which were euthanized 48 days after pellet implantation; and D ($n = 5$), animals with CaOx pellet treated with *C. langsdorffii*, which started 30 days after pellet implantation and lasted 18 days. On the last day of the experiment, all animals were transferred to individual metabolic cages (Nalgene, NalgNunc Int., USA) to collect urine from each animal for 24 h. After the urine was collected, animals were anesthetized, and blood was taken from the aorta. The animals were then euthanized. The bladder was opened, and the matrix calculi (CaOx seed) and any satellites (stones formed surrounding the CaOx pellet) were removed (Fig. 1). The stones were then washed with distilled water and dried in an oven at 40°C before weighing. Calculi hardness was measured (Durometer Nova Ética, Brazil). Biochemical assays were performed to determine urinary and plasma concentrations of sodium (Na), potassium (K) (flame photometer B462, Micronal, Brazil), phosphorus (P), magnesium (Mg), calcium (Ca), uric acid and creatinine (Labtest Diagnostics, Brazil). Urinary oxalate concentration was evaluated by direct precipitation followed by titration as previously described [21, 22]. Urinary citrate determination was performed using a colorimetric assay previously described [22, 23]. Urinary pH was measured using pH strips (Chemco, Brazil).

Data are expressed by the mean \pm standard error. Statistical analysis was performed using Student's *t* test (paired and not paired) followed by Mann–Whitney Rank Sum test when appropriate. Results with $P < 0.05$ were considered significant.

Results

The crude hydroalcoholic extract of leaves and branches from *C. langsdorffii* using aqueous ethanol 7:3 yielded a 16.8% of crude extract. Quantitative analysis of the crude hydroalcoholic extract by HPLC showed that it contained

5.4% quercitrin and 7.4% afzelin. The retention times for these components were 31.8 and 33.9 min, respectively. Based on analysis of the UV spectra from the other chromatogram peaks, the majority of other components in the extract also corresponded to phenolic compounds [21].

No significant differences in body weight gain were found between the groups. However, compared to the control group (group A), water intake was significantly decreased in group B, and 24 h diuresis was increased in both groups C and D (Table 1). Urinary excretion of sodium and potassium did not show any significant differences. Animals in group D showed a decrease in creatinine excretion compared to the control group (Table 2).

There were no significant differences between the control group (group A) and the treated group (group B) in urinary pH (~ 7.0), calcium, citrate and oxalate (Table 3). Both phosphate and magnesium urinary levels were

statistically increased in group B compared to controls, while uric acid levels were decreased in group B (Table 3).

Blood concentrations of sodium and potassium were not different between the groups. Groups C and D showed significantly higher blood creatinine concentration than the controls (Table 4). However, the observed alterations in creatinine concentrations could be due to the CaOx pellets and not a result of the *C. langsdorffii* extract treatment because creatinine concentrations were normal in group B.

The mean mass of the CaOx pellets introduced into the animal bladders was similar between the groups (Table 5). Our results demonstrate that animals treated with *C. langsdorffii* extract have a reduction in calculi mass but that this difference is not statistically significant. However, the mean number of satellite stones was significantly reduced in treated animals. We also observed that extract treatment both changed the mean number of satellites formed and

Table 1 General variables

Groups	Body weight (g)		Water intake (mL)		24 h diuresis (mL)	
	Initial	Final	Initial	Final	Initial	Final
A	315.14 \pm 7.12	429.58 \pm 12.51*	38.0 \pm 2.00	30.0 \pm 1.58*	12.40 \pm 0.68	14.40 \pm 1.17
B	348.50 \pm 9.02	424.12 \pm 24.16*	34.0 \pm 4.00	18.0 \pm 2.00**	11.00 \pm 0.63	13.80 \pm 0.49*
C	337.42 \pm 8.34	408.87 \pm 15.16*	30.0 \pm 0.49	37.5 \pm 4.79	12.87 \pm 0.55	23.25 \pm 2.06**
D	347.00 \pm 20.35	392.00 \pm 13.81*	29.0 \pm 2.91	33.0 \pm 5.38	10.70 \pm 0.58	25.20 \pm 4.27**

$P < 0.05$: * versus initial, ** versus group A (control)

Table 2 Biochemical urinary determinations

Groups	Na (mEq/24 h)		K (mEq/24 h)		Creatinine (mg/24 h)	
	Initial	Final	Initial	Final	Initial	Final
A	1.73 \pm 0.05	1.41 \pm 0.16	3.89 \pm 0.18	2.74 \pm 0.21*	18.55 \pm 1.42	27.82 \pm 1.55*
B	1.45 \pm 0.20	1.25 \pm 0.09	3.25 \pm 0.27	2.48 \pm 1.18	19.86 \pm 2.80	26.05 \pm 2.14*
C	1.79 \pm 1.14	1.75 \pm 0.08	3.05 \pm 0.35	2.62 \pm 0.56	18.10 \pm 1.17	23.28 \pm 2.86
D	1.31 \pm 0.06	1.91 \pm 0.21	2.63 \pm 0.26	2.18 \pm 0.09	22.59 \pm 1.50	22.54 \pm 0.55**

$P < 0.05$: * versus initial, ** versus group A (control)

Table 3 Comparison of urinary biochemical parameters between groups A (negative control) and B (*C. langsdorffii*)

Groups	P (mg/dL)	Mg (mg/dL)	Ca (mg/dL)	Uric Acid (mg/dL)	Citrate (mg/mL)	Oxalate (mg/mL)
A	16.49 \pm 0.33	2.91 \pm 0.07	5.40 \pm 0.38	2.20 \pm 0.13	4.80 \pm 0.19	0.02 \pm 0.01
B	23.03 \pm 1.02*	4.14 \pm 0.58*	5.04 \pm 0.33	1.24 \pm 0.21*	5.26 \pm 0.47	0.02 \pm 0.00

$P < 0.05$: * versus group A (control)

Table 4 Biochemical serum determinations

Groups	Na (mEq/L)	K (mEq/L)	Creatinine (mg/dL)
A	136.0 \pm 1.41	5.04 \pm 0.12	0.70 \pm 0.03
B	133.2 \pm 0.86	4.84 \pm 0.24	0.76 \pm 0.08
C	136.0 \pm 1.47	5.17 \pm 0.19	0.85 \pm 0.02*
D	135.4 \pm 1.43	4.86 \pm 0.19	0.89 \pm 0.06*

$P < 0.05$: * versus group A (control)

Table 5 Analyses of calculi

Groups	CaOx seed mass (g)	Calculi mass (g)	Number of satellites	Pressure (kgf/mm ²)
C	0.009 ± 0.001	0.286 ± 0.042	25.25 ± 6.96	6.90 ± 3.45
D	0.010 ± 0.008	0.164 ± 0.066	7.40 ± 3.67*	3.00 ± 1.51

$P < 0.05$: * versus group C (animals with no treatment)

modified calculi morphology. The calculi taken from treated animals were more brittle and fragile than the calculi from untreated animals (Fig. 1). These observations encouraged us to measure and quantitatively compare the hardness of nidus (CaOx seed with crystal deposition) of calculi from treated and untreated animals. The pressure required to break the calculi from the untreated animals was more than twice the pressure required to break calculi from the treated animals (6.90 ± 3.45 vs. 3.00 ± 1.51) (Table 5).

Discussion

It is well known that copaiba oil has several functional properties and has been used as an antiseptic for the urinary tract. This function of copaiba oil is not directly related to calculi disruption [7]; however, there are limited reports in the literature regarding the aerial parts of *Copaifera*. We have determined that the composition of leaf extract is very different than that of the oil-resin.

The leaves and branches of *C. langsdorffii* can be used as a tea. This tea is prepared by boiling 10 g of plant material in 600 mL of water for 10 min. One-third of the obtained extract is consumed three times per day. Our protocol used an aqueous ethanol 7:3 mixture as a solvent to avoid microorganism growth and to facilitate extract concentration. It is important to note that the chromatographic profiles of both extracts are similar and that ~60% of the hydroalcoholic extract are water soluble (data not shown). Sobrinho et al. [24] reported the optimization of the extraction of flavonoids from *Bauhinia cheilantha* and found that the use of aqueous ethanol is one of the most efficient methods of obtaining flavonoid-rich extracts.

We combined phytochemical investigation of the crude hydroalcoholic extract derived from the aerial parts of *C. langsdorffii* with HPLC–DAD analysis. Our analysis revealed that the extract is composed mostly of phenolic compounds, including flavonoid heterosides, such as quercitrin and afzelin [21]. Therefore, we suggest that flavonoids and other phenolic compounds might be responsible for the observed activity. Similarly, Perez et al. [25] reported that the administration of isoflavonoids isolated from *Eysenhardtia polystachya* diminished the size of calculi in rats. The folk use of *Trigonella foenum graecum* for the prophylaxis of urolithiasis has also been corroborated. Laroubi et al. [26] found that a plant extract rich in

flavonoids disperses the particles of CaOx in urine and facilitates elimination.

The use of rats to induce the formation of CaOx urinary calculi is limited because the deposition of calcium oxalate only occurs in hypercalciuric genetically modified rats, as described by Bushinsky et al. [27]. Furthermore, Ogawa et al. reported that modifying the parameters involved in lithogenesis, such as the administration of ethylene glycol or ammonium chloride [28], could reduce urinary pH [29]. Calculi can also be generated surgically by implanting calcium oxalate (CaOx) seeds into rat bladders [11]. In this work, we used the model developed in the 1950s by Vermeulen et al. [30]. As previously described [11], a period of 30 days was used to allow for calculi development. After the calculi formed, treatment with *C. langsdorffii* was started. In this model, the introduction of a CaOx pellet into the animal bladder did not alter the overall metabolism of the animal but induced local modifications [13]. The mechanism of calculi growth is not well described, but the CaOx seed (matrix) serves as surface for the deposition of both organic and inorganic elements, allowing the formation of calculi (satellites) surrounding the matrix [11]. However, Barros et al. [11] used X-ray diffractometry analysis to show that despite the introduction of a calcium oxalate disc, the growth of calculi was composed of struvite. Struvite formation is favored by the alkaline pH of rat urine [13].

We analyzed Na, K and creatinine levels in both serum and urine to determine if the administration of *C. langsdorffii* hydroalcoholic extract interfered with kidney function. Our results show that the administration of this extract did not affect renal function.

Administration of a hydroalcoholic extract from *C. langsdorffii* led to an increase in urinary magnesium levels in treated animals. The increased urine magnesium levels could be a factor responsible for reducing calcium crystals and satellite formation in the bladder. Urinary magnesium is known to have an inhibitory effect on crystallization, nucleation and growth of the calcium oxalate crystals [31]. In addition, the decreased uric acid excretion observed in extract treated animals may indicate that *C. langsdorffii* can be useful in the prevention of uric acid stones. Uric acid stones compose ~3–10% of calculi in the world population [16]. The increased phosphate levels in the treated groups should not affect calculi formation because both the pH and calcium levels remained unaltered and thus prevented the formation of phosphate stones.

It is known that in humans, ~80% of the calculi are formed as calcium salts, mainly as calcium oxalate, while the other 20% are composed of uric acid (5–10%), struvite, cystine and others [13]. We consider that the effect of the assayed extract would be more prominent if the animal calculi were composed of CaOx because extracts from the aerial parts of *C. langsdorffii* were discovered to be useful in treating human urolithiasis. Human calculi are composed mostly of CaOx [32]. Nevertheless, our results are significant and encourage us to further pursue investigations to more fully characterize the potential of the aerial parts of *Copaifera* to treat urolithiasis.

We concluded that the administration of extract from *C. langsdorffii* leaves displays significant activity in our urolithiasis animal protocol because it diminished the number of calculi formed and reduced the pressure required to break the calculi. Biochemical analysis of the levels of P, Mg, Ca, uric acid, citrate and oxalate in urine showed increased levels of Mg and P and decreased levels of uric acid. These data indicate that the increase in Mg concentration may play an important role in calculi formation and hardness. The decrease of uric acid may also indicate a potential for this extract in treating calculi formed by uric acid. The concentrations of Na, K and creatinine in both serum and urine were not modified by the intake of *C. langsdorffii* extract, indicating that the extract does not affect renal function. Nevertheless, further studies should be conducted to fully investigate the mechanism of action of this extract as well as its effectiveness in treating urolithiasis in humans.

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