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Biological and chemical studies of *Pera benensis*, a Bolivian plant used in folk medicine as a treatment of cutaneous leishmaniasis

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The stem barks of *Pera benensis* are employed by the Chimane Indians in the Bolivian Amazonia as treatment of cutaneous leishmaniasis caused by the protozoan *Leishmania braziliensis*. The chloroform extracts containing quinones were found active against the promastigote forms of *Leishmania* and the epimastigote forms of *Trypanosoma cruzi* at 10 $\mu g\ ml^{-1}$. The activity guided fractionation of the extract by chromatography afforded active compounds. Their structures were elucidated, by spectral and chemical studies, as known naphthoquinones, plumbagin, 3,3′-biplumbagin, 8-8′-biplumbagin, and triterpene, lupeol. The activity in vitro of each compound was evaluated against 5 strains of *Leishmania* (promastigote), 6 strains of *Trypanosoma cruzi* (epimastigote) and the intracellular form (amastigote) of *Leishmania amazonensis*. The baseline drugs used were Glucantime and pentamidine (*Leishmania* spp.), nifurtimox and benznidazole (*T. cruzi*). Plumbagin was the most active compound in vitro. This study has demonstrated that *Pera benensis*, a medicinal plant used in folk medicine, is an efficient treatment of cutaneous leishmaniasis.

Key words: Chimane Indians; folk medicine; cutaneous leishmaniasis; *Pera benensis;* Euphorbiaceae; *Leishmania* sp.; *Trypanosoma cruzi;* naphthoquinones

Introduction

Cutaneous leishmaniasis is an endemic disease in South America, particularly in the tropical subandean regions of Bolivia. Leishmaniasis is initiated by inoculation of *Leishmania* species into the skin during sandfly bites. Cutaneous disease typically presents as a papule that enlarges over weeks to months to form a shallow ulcer with raised red margins and that is thought to ultimately self-heal with scarring in months to years (Saenz et al., 1990). The cutaneous and mucosal leishmaniasis caused by the parasite *Leishmania braziliensis* are common infections in the subtropical regions of the Yungas, Alto-Beni (Department of La Paz) and the foothills of the Andes (Department of Beni) where the Chimane Indians live. Cutaneous leishmaniasis is popularly known as *espundia* in this region of Bolivia called *Oriente* by the natives. The infection is classically treated with pentavalent antimony in the form of sodium stibogluconate (Pentostam®) or *N*-methylglucamine antimonate (Glucantime®) and with pentamidine or amphotericin B (Berman, 1988). These drugs are parenteral, potentially or frankly toxic and generally administered in a hospitalized setting. In the endemic regions, these treatments are too expensive or unavailable to the population suffering from *espundia*. The impact of this disease was developed by the colonization of sudandean tropical regions of the Departments of La Paz, Beni and Cochabamba by the highlanders. The use of medicinal plants is quite widespread, specially in the Chimanes Indians, a lowland group numbering about 5000 who inhabit the gallery forest and sandbars of the Rio Maniqui and its affluents.

The Instituto Boliviano de Biologia de Altura (IBBA) and ORSTOM (French Institute of Scientific Research for the Development in Cooperation) have initiated investigations in the endemic regions of cutaneous leishmaniasis. We have collected and studied many medicinal plants (Fournet, in preparation) and more specially a tree, called *apaiñiki* by the Chimane Indians, used for the specific treatment of cutaneous leishman-

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iasis (*espundia*) and identified as *Pera benensis* Rusby (Euphorbiaceae). The Chimane Indians use a preparation containing only the yellow fresh stem barks of this 15–20 m tall tree for curing the cutaneous lesion topically. In a preliminary screening, quinonic extracts of stem barks or root barks of *Pera benensis* displayed activity in vitro at a concentration of 10 μg ml^{-1} against three strains of promastigote forms of *Leishmania* species, *L. braziliensis*, *L. amazonensis* and *L. donovani*, and three strains of an other Trypanosomatidae, *Trypanosoma cruzi*, responsible for Chagas disease. Activity-directed fractionation and purification gave three active compounds, identified by their physical and spectral data.

This work is devoted to the isolation, the chemical identification and the antileishmanial and trypanocidal activities of the active compounds.

Materials and Methods

Isolation and chemistry

General experimental procedures. UV spectra were recorded on a Unicam SP 1800 spectrophotometer. IR spectra were measured in KBr with a Perkin Elmer 257. All ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ (δ ppm) on a Bruker AC 200 P spectrometer operating at 200 and 50 MHz, respectively. EI-MS spectra were obtained on a Nermag R 1010 C mass spectrometer operating at 70 eV. GLC analyses were performed on a Hewlett Packard HP 5890 A equipped with a 25 m × 0.2 mm i.d. open HP1 column (0.33 μm film thickness). The temperature for the analyses was programmed from 200° to 330° at 4°/min. Si gel GF_{254} and Si gel 60 N (Merck) were used for TLC and CC, respectively. Naphthoquinones were detected by spraying ethanolic solution containing 5% KOH.

Plant material

Stem barks (4.5 kg) and root barks (1.2 kg) of *Pera benensis* Rusby were collected by A. Fournet (*A.F. 846*) in October 1989, at Fatima de Chimane, in the Department of Beni, altitude 450 m, Bolivia. The plant material has been identified by S. Beck of the National Herbarium of Bolivia, La Paz. Voucher specimens are deposited in the National Herbarium of Bolivia (La Paz).

Extraction and isolation

See Fig. 1 for the isolation procedure of the stem bark of *Pera benensis*. For the root bark, the same procedure provided plumbagin (3.25 g), 3,3′-biplumbagin (180 mg), 8,8′-biplumbagin (240 mg) and lupeol (11.6 g).

Plumbagin (**1**)

Orange needles from chloroform, m.p. 78–79°C; UV, λ_{max} (EtOH) nm (log ϵ) 210 (4.54), 265 (4.10), 419 (3.61); IR (KBr) υ cm^{-1} 1665, 1625; EIMS, *m/z* (%): M$^+$ 188.0469 (100), 173.0245 (30), 160.0523 (19), 131 (35), 120 (22), 92 (27), 63 (33); ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.83 (3H, d, J = 1.5 Hz, Me-11), 6.44 (1H, q, = 1.5 Hz, H-3), 6.89 (1H, dd, J = 7.5 and 2.5 Hz, H-6), 7.23 (1H, t, J = 7.5 Hz, H-7), 7.28 (1H, dd, J = 7.5 and 2.5 Hz, H-8), 9.47 (1H, s, OH-5); ^{13}C-NMR (50 MHz, $CDCl_3$) δ 189.7 (C-4), 184.1 (C-1), 160.7 (C-5), 149.2 (C-2), 135.7 (C-7), 135.0 (C-3), 131.6 (C-9), 123.7 (C-6), 118.8 (C-8), 114.6 (C-10), 16.1 (C-11).

3,3′-Biplumbagin (**2**)

Orange crystals from chloroform, m.p. 213–214°C; UV, λ_{max} (EtOH) nm (log ϵ) 214 (4.96), 250 (4.52), 270 (4.52), 424 (4.06); IR (KBr) υ cm^{-1}: 1665,1625; EIMS, *m/z* (%): M$^+$ 374 (59), 359 (89), 357 (48), 331 (20), 303 (16), 120 (45), 92 (100); ^{1}H-NMR (200 MHz, $CDCl_3$) δ 2.09 (6H, s, Me-11 and Me-11′), 7.29 (2H, dd, J = 7.7 and 1.7 Hz, H-6 and H-6′), 7.67 (2H, t, J = 7.7 Hz, H-7 and H-7′), 7.74 (2H, dd, J = 7.7 and 1.7 Hz, H-8 and H-8′), 11.82 (2H, s, OH-5 and OH-5′); ^{13}C-NMR (50 MHz, $CDCl_3$) δ 187.6 (C-4 and C-4′), 183.5 (C-1 and C-1′), 161.6 (C-5 and C-5′), 147.6 (C-2 and C-2′), 139.7 (C-3 and C-3′), 136.6 (C-7 and C-7′), 131.9 (C-9 and C-9′), 124.5 (C-6 and C-6′), 119.6 (C-8 and C-8′), 114.7 (C-10 and C-10′), 14.5 (C-11 and C-11′).

8,8′-Biplumbagin or maritinone (**3**)

Red needles from chloroform, m.p. 190–191°C; UV, λ_{max} (EtOH) nm (log ϵ) 213 (4.70), 263 (4.56), 435 (4.01); IR (KBr) υ cm^{-1}: 1656, 1647; EIMS, *m/z* (%): M$^+$ 374 (79), 331 (48), 317 (32), 303 (100), 278 (48), 250 (79), 139 (51); ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.99 (6H, d, J = 1Hz, Me-11 and Me-11′), 6.79 (2H, q, J = 1Hz, H-3 and H-3′), 7.18 (2H, d, J = 8.7Hz, H-7 and H-7′), 7.28 (2H, d, J = 8.7Hz, H-6 and H-6′), 12.57 (2H, s, OH-5 and OH-5′); ^{13}C-NMR (50 MHz, $CDCl_3$) δ 190.5 (C-4 and C-4′), 185.2 (C-1 and C-1′), 161.3 (C-5 and C-5′), 150.0 (C-2 and C-2′), 138.0 (C-7 and C-7′), 135.5 (C-8 and C-8′), 134.9 (C-3 and C-3′), 128.2 (C-9 and C-9′), 124.3 (C-6 and C-6′), 115.5 (C-10 and C-10′), 16.6 (C-11 and C-11′).

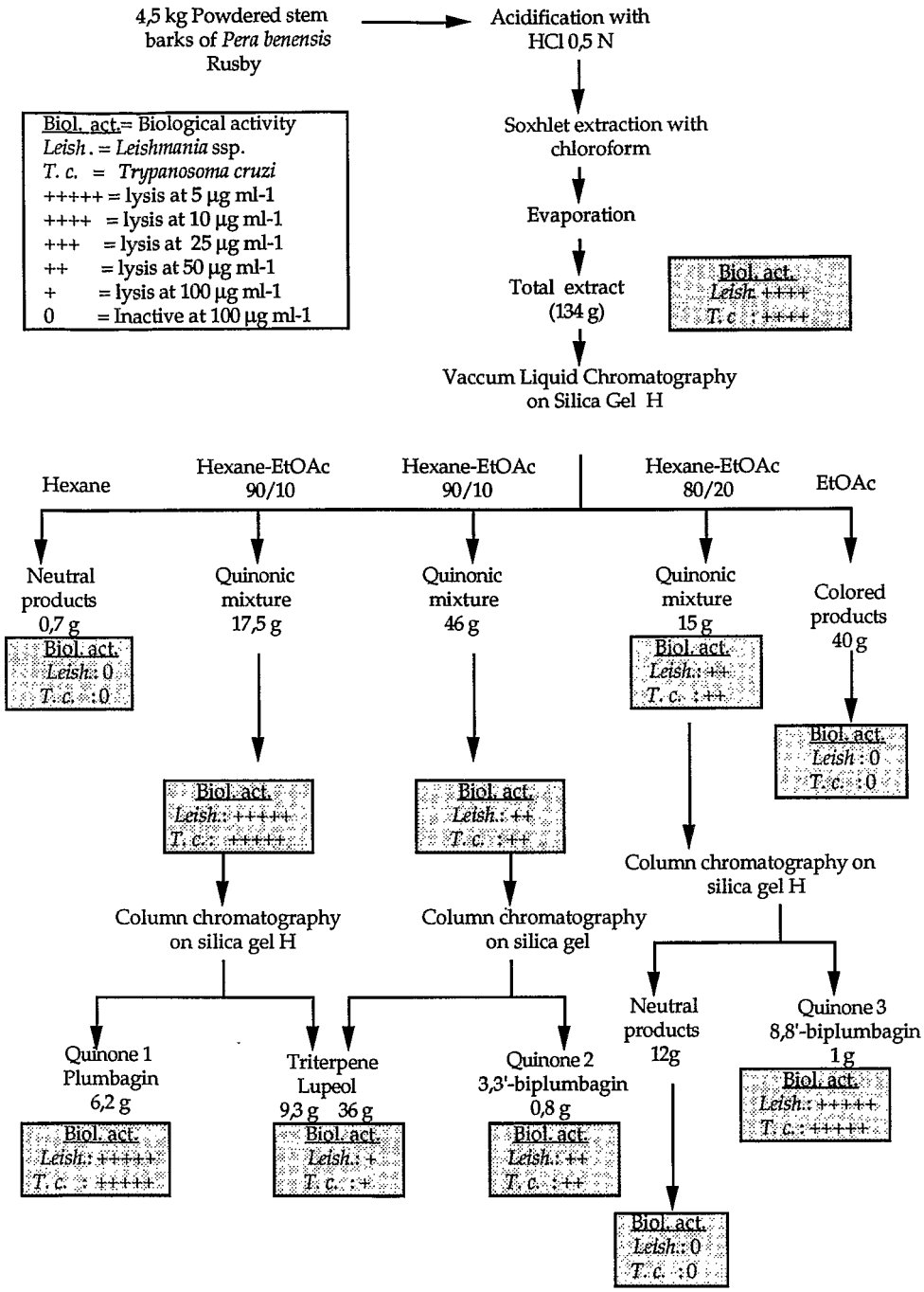

Fig. 1. Bioassay guided fractionation of *Pera benensis*.

Lupeol (**4**)

The identification of this compound was achieved by comparison in GLC analysis with an authentic sample.

Biological assays

Parasites. Cultures of *Leishmania* spp. and *Trypanosoma cruzi* were obtained from IBBA (Instituto Boliviano de Biologia de Altura, La Paz) and identified by isoenzyme analysis.

Five strains of *Leishmania* were used during these investigations: *L. braziliensis* (MHOM/BR/75/M 2903), *L. amazonensis* (IFLA/BR/67/PH8), *L. amazonensis* (MHOM/GF/84/CAY H-142), *L. donovani* (MHOM/IN/83/HS-70) and *L. donovani* (MHOM/BR/00/M 2682).

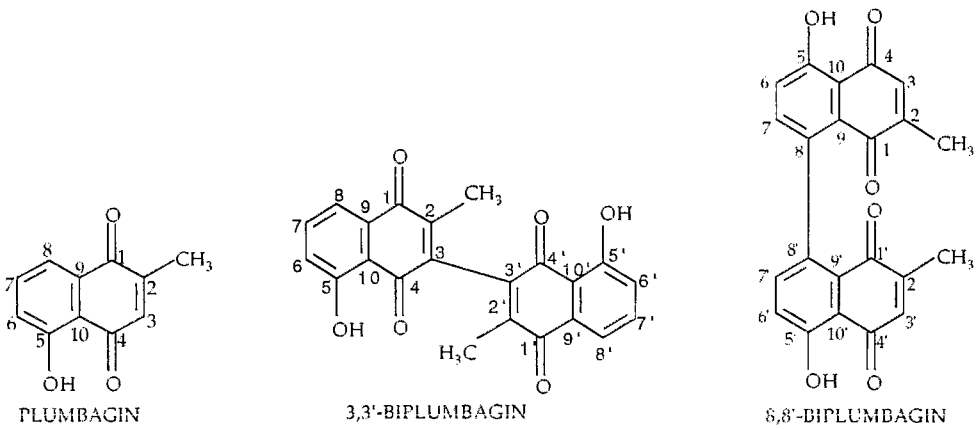

Fig. 2. Structures of plumbagin (**1**), 3,3′-biplumbagin (**2**) and 8,8-biplumbagin (**3**).

Six strains of *Trypanosoma cruzi* were used: S.C. 43 Cl2 (Bolivian strain), C8 Cl1 (Brazilian strain), R 107 Cl8 (isolated from *Rhodnius proxilus*. French Guyana), Tulahuen (isolated from *Triatoma infestans* in Brazil), TeCl2 (Brazilian strain) and 1979 Cl7 (Bolivian strain).

Culture and maintenance of the promastigote forms of Leishmania. Promastigote forms of *Leishmania* were grown at 28°C in USMARU medium (Evans, 1987) containing 10% heat inactivated (56°C for 30 min) foetal bovine serum. Logarithmically growing promastigotes were maintained by transferring 10^6 cells per ml. The extracts and the fractions of *Pera benensis* were dissolved in known volume of DMSO (dimethyl sulfoxide) and then in medium, from which aliquots were drawn. Parasites were counted in a hemacytometer every day and the counts were compared with those of controls grown without drug. The 90% inhibitory concentrations (IC_{90}) were chosen for comparison of susceptibilities of the strains to drugs tested. Glucantime® (Rhône-Poulenc, France) and pentamidine (May and Baker, UK) were used as the baseline drugs against which the efficacy of the naphthoquinones of *Pera benensis* was compared. Each assay was performed in triplicate.

In vitro study of naphthoquinones on the amastigote forms of L. amazonensis.

For the in vitro studies, the mouse active macrophages system described by Neal and Croft (1984) was used. Plumbagin (**1**), 3,3′-biplumbagin (**2**) and 8,8′-biplumbagin (**3**) were tested against *L. amazonensis* (IFLA/BR/67/PH8) and maintained by monthly passage in the feet of hamsters. Cultures were incubated at 37°C in 5% CO_2. The MEM (minimum essential medium) was sup-

TABLE 1

ACTIVITIES OF NAPHTHOQUINONES, LUPEOL, GLUCANTIME AND PENTAMIDINE AGAINST THE PROMASTIGOTE FORMS OF 5 STRAINS OF *LEISHMANIA* IN VITRO

Compounds	IC_{90} (μg ml−1)				
	L.b.[a] (2903)	L.a.[b] (PH8)	L.a.[b] (H-142)	L.d.[c] (2682)	L.d.[c] (HS70)
Plumbagin (**1**)	5	5	5	5	5
3,3′-Biplumbagin (**2**)	50	50	50	50	50
8,8′-Biplumbagin (**3**)	5	5	5	5	5
Lupeol	100	100	100	100	100
Glucantime	>100	>100	>100	>100	>100
Pentamidine	1	1	1	1	1

[a] *Leishmania braziliensis.*
[b] *Leishmania amazonensis.*
[c] *Leishmania donovani.*

TABLE 2

EFFECTS OF NAPHTOQUINONES, LUPEOL AND GLUCANTIME ON THE AMASTIGOTE FORMS OF *LEISHMANIA AMAZONENSIS* (PH8) IN VITRO

Compounds	Concentrations ($\mu g\ ml^{1-}$)	Viability of macrophages (%)	Survival of amastigotes (%)
Plumbagin (1)	50	0	0
	10	100	16.5
	1	100	100
3,3′-Biplumbagin	100	88	76
(2)	50	100	85
8,8′-Biplumbagin	50	6	0
(3)	10	100	66
	5	100	82
Lupeol	100	100	100
Glucantime	100	65	0
	10	85	11

plemented with 10% heat-inactived foetal calf serum, 2% glutamine, 1% antibiotics (10 mg ml^{-1} streptomycin, 1000 I.U. ml^{-1} penicillin). Infected cultures were washed with medium without serum until less than one free parasite per 10 macrophages. The rate of infection was checked by fixing and staining infected macrophages with May-Gründwald-Giemsa. Twenty-four hours after infection, culture medium was replaced by fresh medium either alone (control culture) or containing drugs at different concentrations. Glucantime® (Rhône-Poulenc, France) was used as baseline drug.

Culture and maintenance of the epimastigote forms of Trypanosoma cruzi. Epimastigotes of *T. cruzi* were maintained in continuous exponential growth in liver infusion tryptose medium (LIT, Bacto) supplemented with 10% foetal calf serum at 28°C with an inoculum of 10^6 cells per ml. Aliquots were taken every day, parasites counted in a hemacytometer and the counts compared with those of controls grown without drug. Nifurtimox (Bayer, Germany) and benznidazole (Roche, USA) were used for comparison.

Results

The stem and root bark extracts of *Pera benensis* showed a significant activity on promastigote forms of *Leishmania* spp. and epimastigote forms of *Trypanosoma cruzi.* The fractionation and purification monitored by bioassay led to isolation of three active compounds identified by spectral data to the known naphthoquinones plumbagin (**1**), 3,3′-biplumbagin (**2**) and 8,8′-biplumbagin (**3**) (see Fig. 2). Plumbagin (**1**) and 8,8′-biplumbagin (**3**) are the most potent compounds with a IC_{90} of 5 $\mu g\ ml^{-1}$ on the three strains of *Leishmania* (see Table 1). This antileishmanial activity was confirmed on the intracellular forms (amastigotes) of *Leishmania amazonensis* by plumbagin (**1**), inhibiting 16.5% of the parasites in the host cells at a concentration of 10 $\mu g\ ml^{-1}$ (see Table 2). Plumbagin presented a IC_{90} of 5 $\mu g\ ml^{-1}$ on the six strains of epimastigote forms of *Trypanosoma cruzi.* 3,3′-Biplumbagin (**2**) and 8,8′-biplumbagin (**3**) were weakly active against *T. cruzi,* their IC_{90} varied widely (Table 3). The trypanocidal activity of plumbagin (**1**) was confirmed on biological tests on trypomastigotes, the blood circulating forms of *T. cruzi* (Rojas de Arias et al., 1990). Lupeol, an abundant compound in stems of *Pera benensis,* showed a weak activity against these parasites.

Discussion

In this study, the antileishmanial activity of the stem barks of *Pera benensis* Rusby, a plant used by the Chimane Indians of the Amazonian region of

TABLE 3

ACTIVITY OF NAPHTOQUINONES, LUPEOL, BENZNIDAZOL AND NIFURTIMOX ON 6 STRAINS OF THE EPIMASTIGOTE FORMS OF *TRYPANOSOMA CRUZI* IN VITRO

Compounds	IC_{90} ($\mu g\ ml^{-1}$)					
	Strains of *Trypanosoma cruzi*					
	C8 CL1	R-107	Te CL2	Tula.	1979 CL7	SC43CL1
Plumbagin (1)	5	1	5	5	5	5
3,3′-Biplumbagin (2)	100	100	100	100	100	100
8,8′-Biplumbagin (3)	25	25	25	25	25	25
Lupeol	>100	>100	>100	>100	>100	>100
Benznidazol	100	50	25	50	25	50
Nifurtimox	25	25	25	25	50	50

Bolivia to treat leishmaniasis lesions, was demonstrated. Active compounds against *Leishmania* have been identified to plumbagin and two bis-naphthoquinones, 3,3′-biplumbagin and 8,8′-biplumbagin. In the literature, plumbagin was described active against *Leishmania amazonensis* and *L. donovani* (Docampo and Moreno, 1984; Croft et al., 1985); a bis-naphthoquinone isolated from the Indian plant *Diospyros montana*, diospyrine, a dimer of 7-methyljuglone, is also active in vitro against *L. donovani* (Hazra et al., 1987). Some naphthoquinones have been reported as active against *Trypanosoma cruzi* (Pinto et al., 1987; Carvalho et al., 1988). Our results corroborate the study of Neal and Croft in 1984 showing the effectivity in vitro and in vivo of compounds such as naphthoquinones generating free radicals. The use of topical application of naphthoquinones in skin diseases is described in the literature (Sofowora, 1982; Gujar, 1990).

These ethnopharmacological, biological and chemical studies showed that application of stem barks of *apaiñiki* is really effective against the infection by *Leishmania braziliensis*. The choice of *Pera benensis* as an antileishmanial plant by Chimane Indians is probably due to numerous empirical assays. This tree of 15–20 m tall is rare in the subandean rain forest of the Beni; it was the second identification of *Pera benensis* in Bolivia after the description of the type by Rusby (1920). The Chimane Indians never use the stem barks of *Pera benensis* as a decoction or infusion against *espundia*, because they say that such preparations are too toxic. They use cataplasms of fresh stems directly on the lesion until the complete healing of wound. The applications give an inflammation of the lesion which disappears slowly. The bright black seeds of *Pera benensis* are used as a game by the Chimane children.

In conclusion, the present study has demonstrated that *Pera benensis*, a medicinal plant used in folk medicine, is efficient to treat cutaneous leishmaniasis. If this plant is not toxic in human medicine, it can be a safe and cheap alternative in the endemic rural regions without health centres where drugs are not available. This work on antileishmanial activity of *Pera benensis* should be validated with in vivo studies on BALB/c mice infected by a strain of the cutaneous leishmaniasis of the New World and treated with the naphthoquinones.

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