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Antileishmanial, antitrypanosomal, and cytotoxic screening of ethnopharmacologically selected Peruvian plants

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Abstract Extracts (34) from eight plant species of the Peruvian Amazonia currently used in traditional Peruvian medicine, mostly as antileishmanial remedies and also as painkiller, antiseptic, antipyretic, anti-inflamatory, antiflu, astringent, diuretic, antipoison, anticancerous, antiparasitic, insecticidal, or healing agents, have been tested for their antileishmanial, antitrypanosomal, and cytotoxic activity. Plant species were selected based on interviews conducted with residents of rural areas. The different plant parts were dried, powdered, and extracted by maceration with different solvents (hexane, chloroform, and 70% ethanol–water). These extracts were tested on promastigote forms of *Leishmania infantum* strain PB75, epimastigote forms of *Trypanosoma cruzi* strain Y, and the mammalian CHO cell line. Parasite viability and nonspecific cytotoxicity were analyzed by a modified MTT colorimetric assay method. The isolation and identification of pure compounds from selected extracts were performed by column chromatography, gas chromatography mass spectrometry (GC-MS; mixtures), spectroscopic techniques [MS, infrared (IR), ultraviolet (UV)], and mono and two-dimensional ^{1}H and ^{13}C nuclear magnetic resonance (NMR; COSY, HSQC, NOESY) experiments. *Chondodendron tomentosum* bark and *Cedrela odorata* were the most active extracts against *Leishmania*, while *C. odorata* and *Aristoloquia pilosa* were the most active against *Trypanosoma*, followed by *Tabebuia serratifolia*, *Tradescantia zebrina*, and *Zamia ulei*. Six compounds and two mixtures were isolated from *Z. ulei*

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[cycasin (**1**)], *T. serratifolia* {mixtures 1–2, and naphthoquinones 2-acetyl-4*H*,9*H*-naphtho[2,3-b]furan-4,9-dione (**2**) and 2-(1-hydroxyethyl)-4*H*,9*H*-naphtho[2,3-b]furan-4,9-dione (**3**)}, and *C. tomentosum* [chondrocurine (**4**); (*S*, *S*′)-12-*O*-methyl(+)-curine (**5**); and cycleanine (**6**)]. Four compounds and the two mixtures exhibited significant activity.

Introduction

The Peruvian Amazonia is among the regions of the planet with the greatest plant diversity. Many of these plants are traditionally used in popular medicine (Kvist et al. 2006; Mejia and Rengifo 2000; Perez and Iannacone 2006; Rojas et al. 2003).

Phytotherapy has recently received considerable attention as an alternative to chemotherapy in parasitic disease control. *Trypanosoma cruzi* is the aethiologic agent of Chagas disease, a public health problem in many Latin American countries. Additionally, the population emigration from South American countries to Europe has resulted in the presence of Chagas disease in some European countries (Gascon et al. 2010; Muñoz et al. 2007). The current treatment of Chagas disease is a challenge since the only available drugs, nifurtimox and benznidazole, have severe side effects, and problems are encountered in their production and distribution (Muelas Serrano et al. 2000; Sra et al. 2004).

Leishmaniasis is a protozoan parasitic disease endemic in 88 countries with a high associated morbidity and mortality rate. The drugs most commonly used to treat leishmaniasis are pentavalent antimonials, amphotericin B, paromomycin, and pentamidine, which require high dosage over long periods and are administered parenterally. In the case of amphotericin B, toxicity and high cost are other limitations (Passero et al. 2011). These drugs are far from satisfactory due to their side effects at effective doses. Even miltefosine, the newly introduced drug, has indicated potential resistance against visceral leishmaniasis (Sánchez-Cañete et al. 2009).

In both cases, Chagas disease and leishmaniasis, the search for new compounds to improve current treatments is urgently needed (Correa et al. 2011; Croft et al. 2005; Ghosh et al. 2011; Murray et al. 2005).

In this work, we have screened the in vitro activity of 34 extracts obtained from eight plant species from the Peruvian rain forest against *Leishmania infantum* promastigotes and *T. cruzi* epimastigotes as a first step in the search of new natural active compounds. The cytotoxic effects on mammalian cells (CHO cell line) were tested in all cases. All the plants studied are traditionally used in popular medicine, some of them as leishmanicidal and antiparasitic agents (Estevez et al. 2007; Kvist et al. 2006; Mackinnon et al. 1997; Mejia and Rengifo 2000; Perez and Iannacone 2006; Rojas et al. 2003). Furthermore, the active compounds from *Chondodendron tomentosum*, *Tabebuia serratifolia*, and *Zamia ulei* have been isolated and characterized.

Material and methods

General experimental procedures

Gas chromatography mass spectrometry (GC-MS) analysis was performed using a Hewlett-Packard HP 5890A gas chromatograph coupled to a Hewlett-Packard HP 5971A mass detector in the electron impact mode (70 eV) and equipped with a capillary column. High performance liquid chromatography (HPLC) was carried out in a Gilson apparatus with ultraviolet (UV) detector and SP-4270 integrator. Optical rotations were determined at room temperature using a Perkin-Elmer 241 polarimeter. Nuclear magnetic resonance (NMR) spectra were measured on a Bruker AMX-500 spectrometer with pulsed field gradient, using the solvent as an internal standard ($CDCl_3$, at δ_H 7.26 and δ_C 77.0). Mass spectra and high resolution MS (HRMS) were taken at 70 eV in a Micromass Autospec and electrospray ionization MS (ESI-MS) were recorded in a LCT Premier XE Micromass spectrometer. Silica gel from Merck (15111 and 5554) was used for column chromatography and preparative thin-layer chromatography (TLC). Silica gel G 1500/LS 254 (200×200×0.25 mm plates), Schleicher & Schuell, Ref. 391132 plates were used for semipreparative TLC.

Plant material

The different plant species were selected according to previous reports (Mejia and Rengifo 2000; Rojas et al. 2003) along with the information obtained from interviews conducted with people living in rural areas outside of the City of Iquitos (Table 1).

All plants were collected between July and August 2003 at the biological station Allpahuayo Mishana (Iquitos-Nauta Road, San Juan Bautista District, Province of Maynas, Department of Loreto), except for *C. tomentosum*, which was collected at the Regional Research Centre CRI-IIAP-Pucallpa also in July 2003. Harvested plants were identified and classified in the Herbarium Amazonense of the Universidad Nacional de la Amazonía Peruana, where voucher samples are on deposit.

Preparation of extracts

The different plant parts were dried, powdered, and extracted by maceration with different solvents (hexane,

Table 1 Plants selected for in vitro investigation and parts of plants studied

Botanical name and family	Voucher number	Common name	Plant part	Extract	Yield (%)	Traditional applications
Aristolochia pilosa L.						
Aristolochiaceae	025860	Huancahui sacha	Stem	H	0.18	Snake bite
				C	0.43	
				E-w	0.70	
			Leaf	H	0.33	
				C	0.85	
				E-w	1.00	
Brunfelsia grandiflora L.						
Solanaceae	026365	Chiric sanango	Stem	H	0.19	Leishmaniasis, reconstituent, antirheumatic febrifuge, yellow fever, antisyphilitic, diuretic
				C	0.39	
				E-w	1.70	
			Leaf	H	0.15	
				C	0.46	
				E-w	1.22	
Cedrela odorata L.						
Meliaceae	033574	Cedro amargo	Bark	H	0.37	Astringent, febrifuge, tonic
				C	0.92	
				E-w	2.43	
Chondodendron tomentosum Ruiz & Pavón						
Menispermaceae	033896	Curare	Bark	H	0.53	Antiseptic, anti-inflamatory, rheumatism, jaundice, dropsy, gonorrhoea, anaesthetic
				C	–	
				E-w	1.42	
			Leaf	H	0.90	
				C	–	
				E-w	3.73	
			Bark & leaf	A	0.26	
Paullinia clavigera Schltdl						
Sapindaceae	033368	Sacha yoko	Bark	H	0.16	Insecticide
				C	0.75	
				E-w	–	
Tabebuia serratifolia (Bahl) G. Nicholson						
Bignoniaceae	034665	Tahuarí	Bark	H	0.49	Bronchitis, flu, gallstones, anticancerous, diabetes, fever, leishmaniasis, rheumatism
				C	1.16	
				E-w	1.45	
Tradescantia zebrina (Rose) D.R. Hunt						
Commeliaceae	011382	Oreja de tigre	Aerial part	H	1.36	Insecticidal
				C	2.27	
				E-w	2.57	
Zamia ulei Dammer						
Zamiaceae	027675	Lengua de perro	Underground tuberous stem	H	–	Leishmaniasis, healing
				C	–	
				E-w	2.24	

H Hexane, *C* chloroform, *E-w* Hydroalcoholic extract, *A* alkaloid extract

chloroform, and 70% ethanol–water) for 48–72 h at room temperature. The extracts were filtered and concentrated in vacuo (Table 1). A mixture of CH_2Cl_2 and 0.5 M H_2SO_4 (1:1) was added to the ethanolic extract and stirred for 12 h to prepare the alkaloidal extract from *C. tomentosum*. The aqueous phase was filtered, its pH increased to 9 with NH_4OH and repeatedly extracted with CH_2Cl_2. The evaporation of the solvent gave a crude alkaloidal extract.

Fractionation and isolation of pure compounds

The dry ground rhizome of *Z. ulei* (300 g) was extracted by maceration with ethanol for 48–72 h at room temperature to give 23.1 g of extract (7.7% yield). The extract was chromatographed on a vacuum liquid chromatograph (VLC) column and eluted with an n-Hex: EtOAc gradient of increasing polarity to give seven fractions. Further purification of the active fractions 6 and 7 by CC and/or HPLC (λ 250 nm; flow rate 3 ml/min, Ultrasphere Si column 10×250 mm, 5 μm particle size) eluted with mixtures of n-Hex:EtOAct of increasing polarity resulted in the isolation of cycasin (**1**) (40.6 mg, 13.5×10^{-3}%).

Cycasin (1) ESI-MS: *m/z* 275.0855 $[M+Na]^+$; calculated for $C_8H_{16}N_2O_7Na$; ^{1}H RMN (500 MHz, $CDCl_3$): δ_H 4.06 (3H, br t, *J*=1.5 Hz,=NO–CH_3), 5.05 and 5.15 (1 H each, dq, *J*=14.4 and 1.6 Hz, O–CH_2–N), 4.52 (1H, d, *J*= 12.8 Hz, H-1, anomeric proton), 3.84 and 3.64 (1H, each, dd, *J*=2.2, 12.0 and *J*=5.5, 12.0 Hz, respectively, CH_2OH), 3.20 and 3.40 (4H, m geminal to the hydroxyl proton of the glycoside). ^{13}C RMN (500 MHz, $CDCl_3$): δ_C 55.6 (q, NO–CH_3), 81.2 (t, C-7), 101.1 (d, C-1), 61.2 (t, C-6), 76.7 (d, C-2), 76.5 (d, C-3), 73.5 (d, C-4), 70.1 (d, C-5).

T. serratifolia bark (650 g) was extracted by maceration with ethanol for 48–72 h at room temperature to give 53.6 g of extract (8.25%). This extract was fractionated on a VLC column eluted with an AcOEt:MeOH gradient of increasing polarity to give ten fractions. Further purification of the active fractions 4, 6, and 10 by HLPC (λ 250 nm; flow rate 3 ml/min, Ultrasphere Si column 10×250 mm, 5 μm particle size) eluted with mixtures of n-Hex:EtOAct of increasing polarity to give two compounds [2-acetylnaphtho[2,3-b] furan-4,9-dione (**2**) (2.5 mg, 3.8×10^{-4}%), 2-(1-hydroxyethyl)-naphtho[2,3-b]furan-4,9-dione (**3**) (7.9 mg, 1.22×10^{-3}%)] and two mixtures [mixture 1 (26.2 mg, 4.03×10^{-3}%) and mixture 2 (5.9 mg, 0.9×10^{-3}%)].

2-Acetyl-4H,9H-naphtho[2,3-b]furan-4,9-dione (2) EIMS: *m/z* (rel. int.,%): $[M]^+$ 240 (62), 225 (100), 197 (2). 173 (2). 157 (8), 141 (3), 129 (4), 113 (12), 76(6) y 57 (12). ^{1}H NMR (500 MH_Z, $CDCl_3$): δ_H 2.60 (3H, s, H-11), 7.5 (1H, s, H-3), 7.75 (2H, m, H-6 y H-7), 8.18 (2H, m, H-5 y H-8). ^{13}C NMR (400 MH_Z, $CDCl_3$): δ_C 187.9 (s, C-10), 180.2 (s, C-4), 174.4 (s, C-9), 155.8 (s, C-9a), 153.2 (s, C-3a), 137.4 (s, C-2), 134.8 (d, C-6 and C-7), 134.7 (s, C-8a), 131.1 (s, C-4a), 127.6 (d, C-5 and C-8), 112.8 (d, C-3), 27.0 (q, C-11).

2-(1-Hydroxyethyl)-4H,9H-naphtho[2,3-b]furan-4,9-dione (3) EIMS: *m/z* (rel. int.,%): $[M]^+$ 242 (44), 227 (100), 200 (48), 199 (39), 171 (20), 115 (24), 113 (7), 105 (24), 104 (11). HREIMS: *m/z* 242.0575 $[M]^+$; calculated for C_{14} H_{10} O_4; ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.61 (3H, d, *J*= 6.7 Hz, H-11), 5.03 (1H, q, *J*=6.7 Hz, H-10), 6.84 (1H, s, H-3), 7.74 (2H, m, aromatic protons), 8.19 (2H, m, aromatic protons). ^{13}C NMR (400 MH_Z, $CDCl_3$): δ_C 180.7 (s, C-4), 173.5 (s, C-9), 165.1 (s, C-2), 151.9 (s, C-9a), 151.0 (s, C-3a), 133.9 (d, C-6), 133.8 (d, C-7), 133.0 (s, C-8a), 132.5 (s, C-4a), 127.0 (d, C-5), 126.9 (d, C-8), 103.7 (d, C-3), 63.8 (d, C-10), 21.7 (q, C-11).

Mixtures 1–2 were analyzed by GC-MS. Working conditions were as follows: injector and transfer line temperature was 280°C. A capillary column (HP-1, 30 m×0.25 mm i.d.×0.25 μm d.f.) was temperature-programmed from 70 to 270°C, 4°C min^{-1}. Electron-induced mass spectra (EIMS) and retention data were used to assess the identity of compounds by comparing them with those of standards or those found in the Wiley MS library. Quantitative data were obtained directly from the TIC peak areas.

The dry bark of *C. tomentosum* (700 g) was extracted by maceration with ethanol for 48–72 h at room temperature to give 54.6 g of extract (7.8% yield). The extract was fractionated on a Sephadex column eluted in n-hex: CH_2Cl_2:MeOH 1:1:1, to give three fractions. The alkaloids from fraction 1 were extracted as described (Reina et al. 2001) to give a crude alkaloidal extract (1.8 g, 0.25%). This extract was purified by CC to give the alkaloids chondrocurine (**4**) (3.4 mg, 4.8×10^{-4}%); 12-*O*-methyl-(+)-curine (**5**) (15.4 mg, 2.2×10^{-3}%), and cycleanine (**6**) (5.3 mg, 7.5×10^{-4}%).

Chondrocurine (4) $[\alpha]_D$=+130 (c, 6×10^{-2}, $CHCl_3$). EIMS: *m/z* (rel. int.,%): $[M]^+$ 594 (28), 442 (10), 398 (7), 369 (4), 341 (2), 299 (22), 298 (100), 297 (23), 296 (17), 266 (7), 253 (15), 208 (23), 180 (23), 180 (43), 160 (23), 136 (33), 134 (18), 91 (14), 83 (10), 81 (12), 57 (50). HREIMS: *m/z* 594.2733 $[M]^+$; calculated for $C_{36}H_{38}N_2O_6$. ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 6.57 (1H, s, H-5′), 5.87 (1H, H-8′), 6.74 (1H, s, H-5), 6.27 (1H, d, *J*=2.0 Hz, H-10′), 6.83 (1H, dd, *J*=8.4, 2.2 Hz, H-14), 7.06 (1H, dd, *J*=8.4, 2.2 Hz, H-14′), 6.79 (1H, dd, *J*=8.0, 2.0 Hz, H-13′), 6.70 (1H, dd, *J*=8.0, 2.4 Hz, H-13), 6.54 (1H, dd, *J*=8.0, 2.0 Hz, H-10), 3.92 (1H, d, *J*=8.5 Hz, H-1′), 3.83 (3H, s, OMe), 3.78 (3 H, s, OMe), 2.42 (3H, s, N-Me), 2.18 (3H, s, N-Me), 3.80 (1H, m, H-1), 3.10 and 3.30 (4H, m, H-3 y H-3′), 2.66 and 2.79 (4H, m, H-4, H-4′). ^{13}C NMR (400 MHz, $CDCl_3$): δ_C 155.4

(s, C-12′), 148.0 (s, C-6′), 146.7 (s, C-6), 145.9 (s, C-12), 143.1 (s, C-11), 143.1 (s, C-7′), 137.9 (s, C-8), 137.2 (s, C-7), 132.6 (s, C-9), 132.2 (d, C-10′), 131.7 (s, C-9′), 129.9 (d, C-14′), 128.6 (s, C-4a′), 128.1 (s, C-8a′), 125.7 (d, C-14), 125.4 (s, C-8a), 124.6 (s, C-4a), 121.0 (d, C-10), 117.9 (d, C-8′), 115.4 (d, C-13), 114.9 (d, C-13′), 111.9 (d, C-5′), 107.7 (d, C-5), 64.4 (d, C-1′), 59.3 (d, C-1), 56.0 and 55.8 (s, 2×OMe), 46.5 (t, C-3′), 44.2 (t, C-3), 42.5 and 42.4 (q, 2×N-Me), 25.2 (t, C-4′), 23.2 (t, C-4).

(S,S′)-12-O-methyl(+)-curine (5) $[\alpha]_D$=+ 128.7 (c, 0.282, $CHCl_3$). EIMS: *m/z* (rel. int.,%): $[M]^+$ 608 (23), 607 (17), 593 (9), 313 (7), 312 (31), 31 (3), 298 (19), 297 (8), 296 (18), 190 (13), 86 (81), 84 (100). HREIMS: *m/z* 608.2884 $[M]^+$; calculated for $C_{37}H_{40}O_6N_2$; ^{1}H NMR (500 MHz, $CDCl_3$). δ_H 2.21 (3H, s, N-Me), 2.57 (3H, s, N-Me), 2.43–2.94 (3H, m, H-4 and H-4′), 3.20 (1H, br d, J=9.9 Hz, H-α), 2.8 (1H, m, H-α′), 3.36 and 2.75 (1H each, m, H-3 and H-3′), 3.60 (1H, m, H-1), 3.79 and 2.88 (3H each, s, OMe), 3.92 (1H, m, H-1′), 3.95 (3H, s, OMe), 5.82 (1H, s, H-8′), 6.26 (1H, d, *J*=2.0 Hz, H-10), 6.51 (1H, s, H-5), 6.60 (1H, br d, *J*=8.4 Hz, H-14′), 6.62 (1H, s, H-5′), 6.68 (1H, d, *J*=8.4, 2.4 Hz, H-13′), 6.75 (1H, d, *J*=8.0 Hz, H-13), 6.78 (1H, br dd, *J*=8.5 and 2.4 Hz, H-11), 6.82 (1H, br d, *J*=8.1 Hz, H-14), 7.05 (1H, br d, *J*=8.4 Hz, H-10′). ^{13}C RMN (400 MHz, $CDCl_3$): δ_C 155.5 (s, C-12′), 149.2 (s, C-6′), 148.7 (s, C-7′), 146.7 (s, C-6), 145.4 (s, C-12), 144.1 (s, C-11), 138.2 (s, C-8), 137.5 (s, C-7), 133.7 (s, C-9), 132.6 (s, C-9′), 132.5 (d, C-14′), 131.0 (d, C-10′), 128.8 (s, C-4′a), 128.4 (s, C-8′a), 125.7 (d, C-14), 125.3 (s, C-4a), 125.2 (s, C-8a), 120.1 (d, C-10), 117.9 (d, C-8′), 114.8 (d, C-11′), 114.2 (d, C-13′), 112.7 (d, C-13), 112.7 (d, C-5′), 108.3 (d, C-5), 64.9 (d, C-1), 58.7 (d, C-1′), 56.5 (q, OMe), 56.4 (q, 2×OMe), 48.5 and 44.7 (t each, C-3 and C-3′, respectively), 42.8 and 43.4 (q each, N-Me), 38.2 and 39.7 (t each, C-α and C-α′, respectively), 26.7 (t, C-4′), 23.4 (t, C-4).

Cycleanine (6) EIMS: $[M]^+$ *m/z* (rel. int.,%) 622 (63%), 313 (19), 312 (100), 311 (29), 204 (27), 190 (15), 174 (15), 159 (11), 146 (8), 145 (11). HREIMS: *m/z* 622.3071 $[M]^+$; calculated for $C_{38}H_{42}N_2O_6$. ^{1}H NMR: (500 MHz, $CDCl_3$) δ_H 2.53 (s, 6 H, 2×N-Me), 2.51 (m, H-α), 3.25 (m, H-α′), 3.40 (s, 3H, OMe), 3.81 (s, 3H, OMe), 4.30 (2 H, d, *J*=10.3 Hz, H-1 and H-1′), 2.92 and 3.29 (2 H, m, H-3 and H-3′), 3.01 and 2.92 (2H, m, H-4 and H-4′), 6.57 (s, 2H, H-5 and H-5′), 7.82 (dd, 2H, *J*=8.4, 2.8 Hz, H-13 and H-13′), 6.60 (dd, 2H, *J*=8.4 and 2.8 Hz, H-11 and H-11′), 6.28 (dd, 2H, *J*=8.4 and 2.8 Hz, H-14 and H-14′), 7.06 (dd, 2H, *J*=8.5, 2.1 Hz, H-10 and H-10′); ^{13}C NMR (400 MHz, $CDCl_3$) δ_C 154.2 (s, C-12 and C-12′), 152.1 (s, C-6 and C-6′), 143.8 (s, C-18 and C-18′), 139.1 (s, C-7 and C7′), 130.3 (s, C-9 and C-9′), 129.5 (s, C-4a and C-4′a), 128.7 (d, C-14 and C-14′), 128.3 (d, C-10 and C- 10′), 124.7 (s, C-8a and C-8′a), 117.5 (d, C-11 and C-11′), 114.0 (d, C-13 and C-13′), 109.2 (d, C-5 and C-5′), 60.1 (q, C-7 and C-7′), 59.6 (d, C-1 and C-1′), 56.0 (q, C-6 and C-6′), 44.6 (t, C-3 and C-3′), 42.3 (q, 2×NMe), 37.9 (t, C-α and C-α′), 24.6 (t, C-4 and C-4′). These data coincide with previous results (Scheinmann et al. 1980).

Antileishmanial screening

Leishmanicidal activity was assayed on promastigote forms of *L. infantum* PB75 strain, cultured at 28°C in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum. Parasites in logarithmic growth phase from an initial culture with 1×10^6 promastigotes/ml were distributed in 96-well flat-bottom plates. Each well was filled with 90 μl of culture after 2 days of incubation. Extracts and compounds were tested at several concentrations (extracts at 800, 400, and 100 μg/ml; compounds at 100, 10, and 1 μg/ml) for 48 h. Amphotericin B was used as reference drug and parasite viability was analyzed by modified MTT colorimetric assay (González-Coloma et al. 2002). Briefly, after 48 h, 10 μl MTT/PMS were added to each well. Incubate 75 min to occur the reduction of MTT and 100 μl sodium dodecyl sulfate (SDS) were added to dissolve formazan crystals obtained as a result of the reduction of MTT. Once the crystals have dissolved (15–30 min), the plate was read on a spectrophotometer at 570 nm. The activity was calculated as % growth inhibition (%GI) as follows: $\%\,\mathrm{GI} = 100 - \left[(A_p - A_b)/(A_c - A_b) \times 100\right]$, A_p being the absorbance of problem wells (treated), A_c the absorbance of control wells (not treated), and A_b the absorbance of blank wells (culture medium and vehicle only). When compounds showed activity, intermediate doses were assayed and GI_{50} values (concentration that inhibits 50% the growth of the parasites) were determined from linear regression analysis (STATGRAPHICS Plus, version 5.1). All assays were carried out in triplicate.

Trypanocidal in vitro activity

This activity was assayed on epimastigote forms of *T. cruzi* Y strain, cultured in LIT medium supplemented with 10% fetal calf serum. Parasites in logarithmic growth phase from an initial culture with 2×10^6 epimastigotes/ml were distributed in 96-well flat-bottom plates. Each well was filled with 90 μl of culture after 2 days of incubation. Extracts and compounds were tested at several concentrations (extracts at 800, 400, and 100 μg/ml; compounds at 100, 10, and 1 μg/ml) for 72 h. Nifurtimox was used as the reference drug, and parasite viability was analyzed by a modified MTT colorimetric assay method as described

above (González-Coloma et al. 2002; Muelas Serrano et al. 2000). The activity (%GI and GI_{50}) was calculated as described above for *Leishmania*. All assays were carried out in triplicate.

Cytotoxicity assays

Mammalian Chinese hamster ovary (CHO) cells were used for these assays. Cells were grown in RPMI medium supplemented with 10% L-glutamine, 10% fetal calf serum, and 1% penicillin/streptomycin at 37°C under humidified atmospheric conditions of 5% CO_2/95% air. Cells were seeded in 96-well flat-bottom microplates with 100 μl medium per well (initial densities of 10^4 cells per well) and incubated under the same conditions. After 24 h, the medium was removed and added fresh medium containing the extracts or compounds. Cells were exposed for 48 h to several concentrations of the extracts and compounds (extracts at 800, 400, and 100 μg/ml; compounds at 100, 10, and 1 μg/ml). The toxicity of reference drugs (amphotericin B and nifurtimox) was also evaluated. Cell viability was analyzed by the MTT colorimetric assay method (Mossman 1983). After 48 h, 10 μl of a solution of 5 mg/ml MTT in PBS was added to each well. After 4 h, the supernatant was removed, and 100 μl DMSO were added to each well. The plate is read on a spectrophotometer at 570 and 620 nm. The activity is expressed as the proportion of viable CHO cells (percentage of viability) as follows: $\%\ \text{of viability} = (A_p - A_b)/(A_c - A_b) \times 100$, where A_p is the absorbance of problem wells (treated), A_c is the absorbance of control wells (not treated), and A_b is the absorbance of blank wells (culture medium and vehicle only). When compounds showed activity, intermediate doses were assayed and GI_{50} values (concentration that inhibits 50% the growth of the cells) were determined from linear regression analysis (STATGRAPHICS Plus, version 5.1). All assays were carried out in triplicate.

Results and discussion

Effects of the plant extracts

The antileishmanial and antitrypanosomal activity of 34 extracts (hexane, chloroform, and 70% EtOH) obtained from eight Peruvian plant species is reported in Table 2. We also obtained an alkaloidal extract from the aerial parts of *C. tomentosum* (bark and leaves). Overall, the hexane and chloroform extracts from *Cedrela odorata*, the alkaloid extract from *C. tomentosum* and the chloroform extract from *T. serratifolia* proved to be the most active against both parasites. Hexane extracts from *Tradescantia zebrina* and chloroform extract from *Z. ulei* showed selective activity against *T. cruzi* epimastigotes (at 100 μg/ml). The chloroform and hydroalcoholic extracts from *C. tomentosum*, and the chloroform extract from *Paullinia clavigera* exhibited selective activity against *L. infantum* promastigotes (at 100 μg/ml). We can conclude that *C. tomentosum* bark and *C. odorata* are the most effective extracts against *Leishmania*, followed by *C. tomentosum* leaves, *T. serratifolia*, and *Aristoloquia pilosa*. On the other side, *C. odorata*, *A. pilosa*, and alkaloid extract of *C. tomentosum* proved to be most effective against *Trypanosoma*, followed by *T. serratifolia*, *T. zebrina*, and *Z. ulei*.

Table 3 shows the nonspecific cytotoxicity of the extracts tested at the concentrations showing antiparasitic effects. While most of the extracts studied were cytotoxic, the chloroform extract from *C. odorata* bark, the hydroalcoholic extract from *C. tomentosum* bark, the chloroform extract from *P. clavigera* bark, and the hexane extract from leaves of *Brunfelsia grandiflor*a were not.

T. serratifolia, *Z. ulei*, and *B. grandiflora* are used in traditional medicine to treat ulcers and wounds caused by leishmaniasis. These plants are applied topically, using macerated leaves or resin or latex (Kvist et al. 2006; Mejia and Rengifo 2000). Our results confirm the presence of compounds with leishmanicidal activity in these plants. The extracts from the bark of *T. serratifolia* and the extracts from stem and leaf of *B. grandiflora* exhibited similar activity against both parasites. In the case of *B. grandiflora*, the stem and the root are the most commonly used plant parts for the treatment of parasitic diseases (Mejia and Rengifo 2000). However, our results showed that the leaves also exhibit antiparasitic activity. *C. odorata* and other plants of the Meliaceae family have been used in traditional medicine for the treatments of fevers, a characteristic symptom of malaria. Extracts from this plant and genudin derivatives have been previously assayed against *Plasmodium* with interesting results (Mackinnon et al. 1997). However, this is the first report on its activity against *Leishmania* or *Trypanosoma*. It is interesting to note the strong antiparasitic effects of the alkaloidal extract of *C. tomentosum*. Alkaloid extracts from Menispermaceae exhibited antimalarial activity and in vitro toxicity against the tumoral cell lines KB-3, KB-V1 and P-38878 (Lin et al. 1993). Additionally, we found interesting leishmanicidal activity for *P. clavigera* or *A. pilosa* extracts.

Identification and effects of mixtures and isolated compounds

Two of the four most active plants against both of the parasites (*C. tomentosum* and *T. serratifolia*), and a plant with selective action against one of them (*Z. ulei*) were subjected to bioassay-guided fractionation.

The phytochemical study of the active fractions of the underground tuberous stem of *Z. ulei* resulted in the

Table 2 In vitro activity of hexane (H), chlorophorm (C), hydroalcoholic (E-w), and alkaloid (A) plant extracts against *Leishmania infantum* and *Trypanosoma cruzi*

Plant species and part	Extract	% Growth inhibition					
		L. infantum			*T. cruzi*		
		800 μg/ml	400 μg/ml	100 μg/ml	800 μg/ml	400 μg/ml	100 μg/ml
Aristoloquia pilosa							
Stem	H	99.1±0.6	99.0±0.7	37.8±9.9	100.0±0.0	99.0±0.9	59.1±7.8
	C	95.6±0.1	89.8±0.4	73.3±2.2	98.2±0.5	95.4±0.4	88.4±1.2
	E-w	0.0	0.0	0.0	15.1±3.6	0.0	0.0
Leaf	H	99.9±0.2	98.5±0.2	40.8±5.5	100.0±0.5	99.0±0.4	42.4±1.7
	C	92.2±0.7	84.1±0.7	60.3±1.0	91.0±0.8	86.0±0.5	76.5±1.2
	E-w	22.0±4.0	0.0	0.0	36.7±8.2	20.4±2.9	0.0
Brunfelsia grandiflora							
Stem	H	99.0±0.6	54.6±2.7	0.0	22.0±9.0	0.0	0.0
	C	88.0±3.2	13.0±1.6	12.0±1.2	98.0±1.3	95.0±2.3	27.2±2.0
	E-w	77.2±7.3	8.5±1.8	3.0±4.2	72.6±2.1	46.6±1.6	4.2±3.7
Leaf	H	98.1±0.1	97.4±1.3	47.9±7.1	82.0±1.0	51.5±2.1	0.0
	C	69.2±0.9	38.8±13.8	3.1±3.2	97.7±0.6	93.5±0.2	33.2±2.7
	E-w	42.5±5.8	8.4±6.2	5.0±4.5	46.0±8.7	42.0±4.8	16.1±1.8
Cedrela odorata							
Bark	H	98.1±0.3	97.3±0.6	95.9±0.5	100.0±0.4	100.0±0.2	100.0±0.5
	C	100.0±1.8	100.0±0.2	100.0±0.1	97.9±0.4	95.1±1.1	76.5±1.2
	E-w	0.0	0.0	0.0	72.1±0.7	50.9±2.3	0.0
Chondodendron tomentosum							
Bark	H	15.0±9.9	0.0	0.0	96.5±0.9	21.7±5.6	0.0
	C	93.0±1.0	93.0±0.4	93.0±1.0	100.0±0.5	89.9±2.2	28.0±1.2
	E-w	100.0±1.1	97.0±0.4	91.0±1.2	42.1±2.6	24.0±2.2	0.0
Leaf	H	99.0±0.1	96.0±0.7	19.68±2.9	97.2±0.2	27.3±5.8	0.0
	C	85.6±10.6	80.5±4.8	44.0±6.9	32.8±7.7	14.5±5.9	0.0
	E-w	89.0±3.1	87.0±2.0	84.0±1.0	27.9±0.6	4.0±5.1	0.0
Bark & leaf	A	100.0±4.2	100.0±2.2	100±0.0[a]	82.9±4.0	90.9±0.7	96.5±0.5[a]
Paullinia clavigera							
Bark	H	93.9±1.5	92.1±0.9	20.1±3.4	56.0±2.2	47.2±3.0	5.7±5.2
	C	100.0±0.8	100.0±2.7	74.1±5.6	100.0±0.2	89.9±1.0	38.4±3.6
	E-w	0.0	0.0	0.0	0.0	0.0	0.0
Tabebuia serratifolia							
Bark	H	100.0±0.5	99.1±0.7	37.8±9.9	100.0±0.2	99.5±0.5	24.8±4.0
	C	100.0±0.4	98.9±2.8	90.5±0.7	98.0±1.3	96.0±1.0	94.0±0.7
	E-w	1.5±3.2	0.0	0.0	47.6±1.8	28.5±3.7	14.3±1.6
Tradescantia zebrina							
Aerial part	H	96.4±1.2	93.5±0.4	7.8±1.4	96.0±1.4	95.0±1.0	73.5±3.2
	C	23.8±3.2	6.3±2.0	4.1±2.7	27.0±11.6	0.0	0.0
	E-w	0.0	0.0	0.0	35.7±7.1	17.4±7.4	13.4±4.4
Zamia ulei							
Underground tuberous stem	H	84.7±1.0	72.2±1.3	27.5±3.5	78.2±2.0	77.0±0.6	28.2±6.2
	C	93.3±1.2	84.2±1.3	0.0	92.5±2.3	88.2±0.2	62.9±1.0
	E-w	0.0	0.0	0.0	26.4±5.7	10.1±1.4	7.8±3.3

[a] Concentration assayed: 200 μg/ml

Table 3 In vitro cytotoxicity of hexane (H), chlorophorm (C), hydroalcoholic (E-w), and alkaloid (A) plant extracts on CHO cells

Plant species and part	Extract	% of viability		
		800 μg/ml	400 μg/ml	100 μg/ml
Aristoloquia pilosa				
Stem	H	0.2±0.2	1.2±0.2	9.5±1.6
	C	17.3±3.1	22.3±0.5	33.5±1.6
Leaf	H	0.5±0.4	0.2±0.1	–
	C	1.9±0.4	5.5±2.1	–
Brunfelsia grandiflora				
Stem	H	3.4±0.8	30.9±3.0	35.7±5.7
	C	4.2±1.7	–	–
	E-w	0.4±0.2	–	–
Leaf	H	0.0	58.5±3.4	49.9±6.2
Cedrela odorata				
Bark	H	0.0	0.0	1.72±1.7
	C	67.1±1.6	95.5±7.0	100.0±10.5
Chondodendron tomentosum				
Bark	H	0.7±0.4	1.8±1.2	–
	C	1.9±0.9	1.4±0.7	2.6±1.5
	E-w	48.6±17.0	87.9±13.23	66.0±11.1
Leaf	C	0.0	0.0	–
	E-w	12.7±1.9	4.3±1.8	14.5±1.6
Bark & leaf	A	0.5±0.3	0.2±0.2	0.0[a]
Paullinia clavigera				
Bark	H	0.9±0.6	0.2±0.3	–
	C	1.2±0.5	7.8±0.9	72.7±1.6
Tabebuia serratifolia				
Bark	H	0.0	1.8±0.9	–
	C	0.0	0.0	–
Tradescantia zebrina				
Aerial part	H	0.0	27.9±2.5	–
Zamia ulei				
Underground tuberous stem	H	0.1±0.1	40.0±1.5	–
	C	4.0±3.3	1.0±0.6	–

The activity is expressed as the proportion of viable CHO cells (percentage of viability) after 48 h of contact with the extract. Only the extracts and concentrations active on parasites were assayed on CHO cells

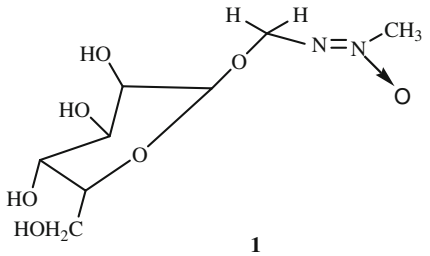
[a]Concentration assayed: 200 μg/ml

isolation of cycasin (**1**) (Fig. 1). Cycasin (**1**) has previously been isolated from other species such as *Cycas revoluta* (Nishida et al. 1955, 1956), *Bowenia spectabilis*, *Stangeria eriopus*, *Ceratozamia mexicana*, *Lepidozamia hopei*, *Encephalartos villosus*, *Macrozamia miquelii*, and *Zamia fischeri* (Yagi 2004).

Mixtures 1 and 2 were isolated from *T. serratifolia*, and they were identified using GC-MS. Their composition is shown in Table 4. Mixture 1 is composed of saturated alkanes and the isoflavonoid rotenalone which is the major component (15.1%), followed by triacontane (6.2%), hentriacontane (6.0%), and an unknown compound of $[M]^+$ 396 (6.0%). Rotenalone has been reported as cytotoxic against the A 2,780 human ovarian cancer cell line (Cao et al. 2004). The major components of mixture 2 were two unidentified compounds of $[M]^+$ 422 (6.7%) and $[M]^+$ 396 (6.9%). Among the compounds present in mixture 2, eicosane, octacosane, and octadecane are present in essential oils and extracts of various plants, and their activity on various bacteria, fungi, and viruses have been reported (Zai-Chang et al. 2005; Kuiate et al. 2006; Karabay et al. 2007; Mazutti et al. 2008).

Naphthoquinones **2** and **3** were also isolated from *T. serratifolia*. They were identified using spectroscopic techniques (MS, IR, UV) and mono and two-dimensional ^1H and ^{13}C NMR (COSY, HSQC, NOESY) experiments (Fig. 1). Both were previously isolated from *Tabebuia cassinoides* and *T. ochracea* (Zani et al. 1991).

The bisbenzylisoquinoline alkaloids (BBIQs) chondrocurine (**4**), (*S*,*S*′)-12-*O*-methyl(+)-curine (**5**), and cycleanine (**6**) were isolated from *C. tomentosum*. They were identified by spectroscopic techniques (MS, IR, UV) and ^1H NMR



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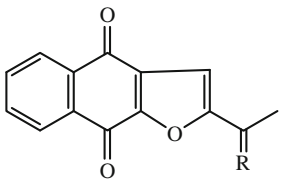

2; R = O

3; R = −OH

Fig. 1 Chemical structures of cycasin (**1**) and naphthoquinones **2** and **3**

and ^{13}C mono and two-dimensional (COSY, HSQC, NOESY) experiments (Fig. 2).

Chondrocurine (**4**) was first isolated from *C. tomentosum* (Everette et al. 1970) and also found in other species of the family. Compound **5** was first identified as (++)-4″-*O*-methylcurine from *Cissampelos pareira* (Haynes et al. 1966). Cycleanine (**6**) is present in a wide range of Menispermaceae and also in other families such as Annonaceae (Leboeuf et al. 1982).

Table 5 shows the antiparasitic and cytotoxic effects of the mixtures and pure compounds. Mixture 1 exhibited moderate antileishmanial effects while mixture 2 showed important activity against both parasites with higher potency than nifurtimox and cytotoxicity values similar to those of the reference drugs. Both naphthoquinones proved to be quite active against both parasites (especially **3**) with GI_{50} values for *T. cruzi* lower than those of nifurtimox and similar to amphotericin B for *L. infantum*. Although these compounds are more toxic to CHO mammalian cells than the reference drugs, it should be noted that compound **3** is 300× more active on epimastigote forms of *T. cruzi* than nifurtimox.

Alkaloid **6** exhibited no significant activity, but **4** and **5** were active against both parasites (with lower potency than the reference drugs). These compounds exhibited strong activity against *Leishmania* but were less toxic to mammalian CHO cells than the reference drugs. This variation in activity may be due to the fact that **6** is composed of two coclaurine-type isoquinoline units linked by two C8-C12′ and C8′–C12 head–tail ether bonds, while in **4** and **5**, the units are linked by C8–C12′ and C11–C7′ head–tail bonds. Previous studies with compounds such as isotetrandrine, pheanthine, cepha-

Table 4 Chemical composition of mixtures 1–2

	Compound	Retention time	Relative abundance (%)
Mixture 1	Tricosane	43.09	1.5
	Tetracosane	45.22	3.1
	Pentacosane	47.25	2.7
	$222[M]^+$	47.43	1.4
	Hexacosane	49.24	3.9
	Heptacosane	51.13	3.7
	Octacosane	52.97	3.0
	Nonacosane	54.72	2.5
	Triacontane	56.49	6.2
	$394[M]^+$	56.88	1.0
	Hentriacontane	57.86	6.0
	$396[M]^+$	57.86	6.0
	$408[M]^+$	62.67	2.7
	Rotenalone	64.06	15.1
% Identified			58.8
Mixture 2	$422[M]^+$	45.28	6.7
	Eicosane	49.27	1.5
	Docosane	51.15	1.7
	Octadecane	52.96	1.4
	$394[M]^+$	57.50	5.4
	$396[M]^+$	57.84	6.9
% Identified			23.6

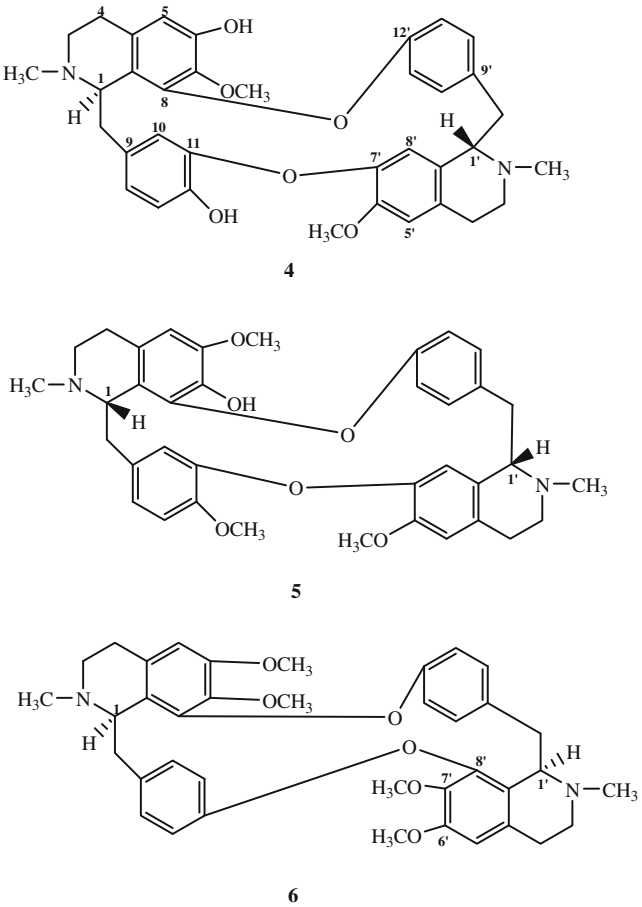

Fig. 2 Chemical structures of chondrocurine (**4**), (*S*,*S*′)-12-*O*-methyl (+)-curine (**5**) and cycleanine (**6**)

ranthine and cycleanine highlight the important role played by stereochemistry in their activity (Thornber 1970).

In our study, cycasin (**1**) was not active. This compound is a toxic azoxyglycoside whose main metabolite, methylazoxymethanol (MAM), is responsible for insect toxicity and genetic alterations in different systems (bacteria, yeasts, plants, mammalian cells, etc.; Schneider et al. 2002; Yagi 2004). Therefore, the lack of activity of **1** may be due to its low bioavailability to the parasites and/or cells due to its high polarity, resulting in low amounts of released MAM (responsible for the toxicity).

The naphthoquinones, lapachol, and lapachone, found in the genus *Tabebuia*, proved active against *Schistosoma mansoni* and *T. cruzi* (Do Campo et al. 1977, 1978). Lapachol showed significant leishmanicidal activity in vitro, high activity against *T. cruzi* and also interferes with oxygen metabolism of tumor cells by blocking celular respiration and the generation of oxygen free radicals (Hussain et al. 2007). Other naphthoquinones act by inducing oxidative stress, reducing the consumption of oxygen in the respiratory chain, and inhibiting electron transport (Colman de Saizarbitoria et al. 1997). It has also been reported that the activity of naphthoquinones against sensitive and resistant strains of *Plasmodium falciparum* to chloroquine may be due to the presence of a methyl group which increases its lipophilicity promoting drug accumulation in the mitochondria of the parasite (Weiss et al. 2000). Naphthoquinones **2** and **3** exhibited significant activity against tumor cells KB (Rao and Kingston 1982; Zani et al. 1991). In addition, naphthoquinone **3** showed significant activity against *Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense*, *P. falciparum*, and *P. berghei* (Weiss et al. 2000; Fournet and Muñoz 2002) and against a wide range of microorganisms (Kuete et al. 2007). Naphthoquinone **3** could have an antiparasitic mode of action similar to that described for other naphtoquinones, while the presence of a hydroxyethyl group in its side chain could be responsible for an increase in activity compared to **2**. This is the first time that the activity of these compounds against *L. infantum* and *T. cruzi* has been described, although their activity has been reported for other species of *Trypanosoma*.

BBIQ alkaloids proved to be active against *T. cruzi* and *P. falciparum*, and some of them enhance the effect of different antimalarial drugs and revert parasite resistance (Lin et al. 1993). *S*,*S*′-12-*O*-methyl-curine (**5**) is present in a root extract of *Cissampelos mucronata* with activity against

Table 5 In vitro activity on *Leishmania infantum*, *Trypanosoma cruzi*, and CHO cells of mixtures 1–2 and compounds **1**–**6**

Compound	*L. infantum*	*T. cruzi*	CHO cells
Mixture 1	35.07 (18.99–64.76)	>100	67.54 (48.11–94.82)
Mixture 2	2.49 (2.22–2.80)	1.57 (0.55–4.49)	11.02 (8.92–13.62)
1	>100	>100	59.54 (37.59–94.31)
2	2.15 (1.97–2.35)	2.36 (2.03–2.75)	5.39 (4.16–6.97)
3	0.19 (0.03–1.09)	0.01 (0.001–0.04)	2.72 (1.12–6.61)
4	0.85 (0.55–1.29)	21.69 (13.50–33.19)	45.48 (37.81–54.69)
5	0.76 (0.46–1.25)	22.83 (15.74–33.10)	41.69 (38.15–45.57)
6	52.96 (29.76–94.21)	>100	51.41 (27.62–57.69)
Nifurtimox		3.39 (1.40–8.19)	13.91 (9.09–21.30)
Amphotericin B	0.04 (0.01–0.12)		10.25 (5.36–19.61)

Results are expressed in GI_{50} (μg/ml). In brackets, confidence limits 95%

chloroquine-sensitive and chloroquine-resistant *P. falciparun* strains (W2 and D6) and showed moderate cytotoxicity against KB cells (Tshibangu et al. 2003). Cycleanine (**6**) showed activity against *P. falciparum* (chloroquine-sensitive clone D6; Angerhofer et al. 1999), in vitro antibacterial and strong fungicidal activity (Lohombo-Ekomba et al. 2004), and in vitro activity against *Leishmania brasiliensis*, *Leishmania amazonensis*, and *Leishmania donovani*, but not to *T. cruzi* (Osorio et al. 2006). Furthermore, **6** showed strong activity in mice infected with CL and Y strains of *T. cruzi* when administered together with other alkaloids such as curine, isotetrandine, limacine, and pheanthine (Fournet et al. 1997). Previous studies have reported that BBIQ alkaloids act on the trypanothione reductase (a pathogen-specific enzyme which is one of the main targets in the search for new drugs against *T. cruzi*) similarly to other inhibitors of this enzyme (nitrofurans and naphthoquinones), which have a positive charge attached to a hydrophobic group. The enzyme is inhibited when binding occurs, interfering with catalysis of trypanothione disulfide reduction (the compound responsible for oxygen exchange), the level of oxygen in the parasite is reduced, and death occurs (Fournet et al. 1998).

Conclusions

The results confirm the presence of leishmanicidal activity for plants used for the treatment of ulcers (*T. serratifolia*, *Z. ulei*, or *B. grandiflora*) and other uses (*C. tomentosum*, *C. odorata*, *P. clavigera*, *T. zebrine*, or *A. pilosa*). Some extracts also exhibited interesting trypanocidal activity. We also isolated the active compounds responsible for the activity of *C. tomentosum* alkaloidal extract and *T. serratifolia* chlorophorm extract. However, the major compounds isolated from the active fractions of *Z. ulei* were not active. One cause may be that the active principle is present in a low proportion in the extract and has not been isolated, or synergistic effects is existent in the extract. To further check this, studies with combinations of different compounds isolated from the extract must be done.

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