Antileishmanial and trypanocidal activity of Brazilian Cerrado plants

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The side effects and the emerging resistance to the available drugs against leishmaniasis and trypanosomiasis led to the urgent need for new therapeutic agents against these diseases. Thirty one extracts of thirteen medicinal plants from the Brazilian Cerrado were therefore evaluated in vitro for their antiprotozoal activity against promastigotes of Leishmania donovani, *and amastigotes of* Trypanosoma cruzi. *Among the selected plants,* Casearia sylvestris *var.* lingua *was the most active against both* L. donovani *and* T. cruzi. *Fifteen extracts were active against promastigotes of* L. donovani *with concentrations inhibiting 50% of parasite growth (IC_{50}) between 0.1-10 µg/ml, particularly those of* Annona crassiflora *(Annonaceae),* Himatanthus obovatus *(Apocynaceae),* Guarea kunthiana *(Meliaceae),* Cupania vernalis *(Sapindaceae), and* Serjania lethalis *(Sapindaceae). With regard to amastigotes of* T. cruzi, *extracts of* A. crassiflora, Duguetia furfuracea *(Annonaceae), and* C. sylvestris *var.* lingua *were active with IC_{50} values between 0.3-10 µg/ml. Bioassay fractionations of the more active extracts are under progress to identify the active antiparasite compounds.*

Key words: Brazilian Cerrado plants - leishmaniasis - Chagas disease - *Leishmania donovani* - *Trypanosoma cruzi* - Brazil

Leishmaniasis and trypanosomiasis are a group of globally widespread parasitic diseases responsible for considerable mortality and morbidity, affecting millions of people every year (WHO 2002). Chagas disease (American trypanosomiasis) is caused by the flagellated protozoan *Trypanosoma cruzi* and is transmitted to humans by triatomine (hematophagous) insects known popularly as "kissing bug", "vinchuna", and "barbeiro". The two drugs used in clinical practice, nifurtimox and benznidazole, do not eliminate the parasites, and resistance was reported (Urbina 1999, Urbina & Docampo 2003). Leishmaniasis is transmitted by the bite of an infected female sand fly of the genera *Phlebotomus* and *Lutzomya*. *Leishmania donovani* is the causative agent of visceral leishmaniasis, which is fatal in the absence of treatment (Paris et al. 2004). In the treatment of leishmaniasis, drugs that can be used are pentavalent antimonials (meglumine antimoniate or sodium stibogluconate), amphotericin B, and pentamidine salts, which may cause serious side-effects and unresponsive therapies (Desjeux 1998, Funasa/MS 2000).

The various side-effects and the resistance to available drugs, in addition to the increase in new cases, have led to the urgent need for new therapeutic agents to treat these diseases. We have evaluated the trypanocidal and leishmanicidal activity in vitro of crude Brazilian Cerrado plant extracts, the country's second most important biome. Plants were selected among traditional healers for their use against cutaneous and infectious diseases, such as ulcers, diarrhea, fever, and malaria. In Brazil, populations in rural areas rely on traditional medicine for the treatment of many infectious diseases.

MATERIALS AND METHODS

Plant material and extract preparation - The plants were collected in Brasília, the Federal District of Brazil, in 2002/2003. Botanical identification was performed by Professor José Elias de Paula of the Vegetal Anatomy Laboratory, Institute of Biology, University of Brasília (UnB). The voucher botanic specimens are deposited at the Herbarium (UB) of that institution (Table I).

The air-dried and powdered parts of the different plants (400 g) were submitted first to exhaustive extractions with hexane (4×2 l), and successively with ethanol (4×2 l) through a maceration process. The crude extracts were obtained after the evaporation of the solvents under reduced pressure at 40°C, which is a technique routinely used at the Pharmacognosy Laboratory of the University of Brasília Health Science School.

Biological assays - Assays were performed by the Department of Parasitology of the Faculty of Pharmacy of Châtenay-Malabry (Paris XI, France), and the Muséum d'Histoire Naturelle de Paris, France.

Trypanocidal assay - In vitro assays against the intracellular amastigote form were performed as described

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(Grellier et al. 2001) using a β-galactosidase-expressing parasite (Tulahuen *LacZ* clone 4) kindly provided by W Van Voorhis (University of Washington, Seattle, Washington, US). Briefly, murine muscle L6 cells were seeded in a 96-well plate at 5×10^3 cells/well. After 24 h, 10^5 trypomastigote forms obtained from infected cultures of L6 cells were added to each well for 6 h. Cells were washed twice to remove extracellular trypomastigotes, and incubated with 2-fold dilutions of the drug, at 37ºC, under a 5% CO_2 atmosphere for 5 days in RPMI 1640 medium supplemented by 25 mM HEPES, pH 7.35 and 10% fetal calf serum (FCS). In all these experiments, untreated parasite cultures were used as negative controls. Growth inhibition was quantified using the chromogenic substrate red β-D-galactopyranoside according to Buckner et al. 1999. IC_{50} values were calculated from dose-response curves obtained from several independent experiments. Benznidazole was used as reference drug.

Cytotoxicity test on mammalian cells - L-6 cells was seeded into 96-well microplates at 5000 cells/well in 100 µl. After 24 h, the cells were washed and maintained with different concentrations of extracts for 5 days, at 37°C under a 5% CO_2 atmosphere. Cytotoxicity was determined using the colorimetric tetrazolium-dye (MTT) assay following the manufacturer's recommendations (Cell proliferation kit I, Boehringer Mannheim S.A., Meylan, France). The IC_{50} values were obtained from the drug concentration-response curve. The results were expressed as the mean ± the standard deviations determined from three independent experiments.

Antileishmanicidal assay - L. donovani (MHOM/ET/L82/LV9) promastigotes were kindly provided by Pr SL Croft, from the WHO collection at the London School of Hygiene and Tropical Medicine. Assays were performed as previously described by M'Bongo et al. (1997) and Okpekon et al. (2004). Briefly, promastigotes were grown at 27ºC in HEPES (25 mM) buffered RPMI 1640 medium containing 10% FCS and 50 µg/ml gentamycin. Assays were performed in 96-well microtitre plates. Drugs were serially diluted in culture medium (100 µl/well); 100 µl of parasites from a logarithmic phase culture (1.75×10^6 promastigotes/ml) were then added to each well and plates were maintained at 27ºC under a 5% CO_2 atmosphere. Biological tests were performed four times, and each tested concentration in duplicate. The viability of parasites was evaluated by the MTT colorimetric method. The antileishmanial activity was expressed as the IC_{50} after a 72 h incubation period. The initial concentration for screening was 15 µg/ml. Pentamidine, amphotericin B and miltefosine were used as reference drugs.

RESULTS AND DISCUSSION

In our search for natural products with leishmanidal and trypanocidal activity, we assayed hexanic and ethanolic crude extracts of native plants from the Brazilian Cerrado. Table I lists the thirteen plants selected, their botanical families and the voucher number. Thirty one extracts were prepared from different parts of the plants.

Nineteen crude extracts showed an inhibition greater than 50% at a concentration of 15 µg/ml against promastigote forms of *L. donovani* and were further investigated to determine their IC_{50} values (Table II). The strongest activity, with IC_{50} values ranging from 0.1 to 4.9 µg/ml, was found for *Casearia sylvestris* var. *lingua*: the hexanic extracts of leaves, stem wood and bark, and root bark, and the ethanolic extract of root bark and fruits, as well as the ethanolic extracts of *Annona crassiflora* root bark. The results also indicated IC_{50} values ranging from 5 to 10 µg/ml for the hexanic extracts of *Cupania vernalis* leaves, *Guarea kunthiana* root and for the ethanolic extracts of *A. crassiflora* stem wood and root wood, *Himatanthus obovatus* root wood, and *Serjania lethalis* root bark.

For *T. cruzi*, among the plants tested, the most effective extracts were the hexanic extract of *C. sylvestris* var. *lingua*, which showed IC_{50} values ranging from 0.3 to 3.4 µg/ml: leaves, stem wood and bark, and root wood and bark. The results also indicated IC_{50} values ranging from 5 to 10 µg/ml for the hexanic extract of *Duguetia furfuracea* bark root, as well as the ethanolic extracts of *C. sylvestris* var. *lingua* root bark, and *A. crassiflora* root wood and bark.

A. crassiflora is traditionally used against Chagas disease (Queiroz et al. 1996), as well as snake bites (seeds) (Correia 1984). The total alkaloids of *A. crassiflora* were shown to be active against *L. chagasi* (IC_{50} value = 24.9 µg/ml), and trypomastigote forms of *T. cruzi*, killing 100% of the parasites at 100 µg/ml (Tempone et al. 2005). It must be noted that other studies have reported the leishmanidal and trypanocidal activities of extracts and isolated compounds from other species of this genus (Sahpaz et al. 1994, Jaramillo et al. 2000, Tempone et al. 2005). Various acetogenins were isolated from the genera *Annona* (Gleye et al. 2001, Wang et al. 2002, Bermejo et al. 2005); acetogenins are known for their antiprotozoal activity, including against *L. donovani* (Raynaud-Le Grandic et al. 2004), which could explain the antiparasite activity observed for *A. crassiflora*.

C. sylvestris var. *lingua* demonstrated to be more potent than the other species tested. In both assays, almost all the plant organs showed antiprotozoal activity, indicating that there might be similar types of secondary metabolites. This variety was also active against epimastigote forms of *T. cruzi* (Espindola et al. 2004). *Casearia* ssp. showed activity in other biological systems, as the down-modulation of nitric production in murine macrophages (Napolitano et al. 2005) or in neutralizing proteases from venoms (Borges et al. 2001), which are probably linked to the presence of diterpenes (de Carvalho et al. 1998, Oberlies et al. 2002).

Two different species of *Guarea* (*G. guidonia*, *G. polymera*) were evaluated against *Plasmodium falciparum*, *T. cruzi*, and *Leishmania* sp. showing good antiprotozoal activity in vitro (Weniger et al. 2001).

Preliminary cytotoxicity assays upon the mammalian L6 cells showed that the hexanic extract of *A. crassiflora* root bark is weakly cytotoxic (IC_{50} value = 45.2 ± 2.2 µg/ml), as well as the hexanic extract of *D. furfuracea* root bark (IC_{50} value = 62.6 ± 2.2 µg/ml). In contrast *C. sylvestris* var. *lingua* showed marked cytotoxicity effect (IC_{50} value = 1.7 ± 0.8 µg/ml).

TABLE I
Plants studied and their traditional use

Plant species (family)	Voucher No.	Vernacular name	Traditional use in medicine
Annona crassiflora Mart. (Annonaceae)	(UB) 3700	marôlo, araticum-do-Cerrado	fever, Chagas disease
Cardiopetalum calophyllum Schl. (Annonaceae)	(UB) 3703	imbirinha, imbiribeira, imbira-amarela	fever
Duguetia furfuracea (A. St. Hil.) Benth & Hook(Annonaceae)	(UB) 3679	araticum-seco, araticunzinho, pinha-de-guará	dysenteric syndrome fever
Xylopia aromatica (Lam.) Mart.(Annonaceae)	(UB) 3699	pimenta-de macaco, pimenta-do-campo	condiment, carminative
Xylopia emarginata Mart. (Annonaceae)	(UB) 3690	pindaíba-reta, pindaíba d'água, pindaíba-do-brejo	antibacterial
Aspidosperma macrocarpon Woodson(Apocynaceae)	(UB) 3692	guatambu-do-cerrado, pau-pereira, peroba-do-campo	fever, malaria
Himatanthus obovatus (Müll. Arg.) Woodson (Apocynaceae)	(UB) 3678	tiborna, pau-de-leite, janaguba	cancer, herpes, verminosis
Casearia sylvestris Sw.var. *lingua* (Camb.) Eichl.(Flacourtiaceae)	(UB) 3693	erva-de-lagarto, cafezeiro-do-mato	analgesic (pain-reliever), anti-inflammatory, antibacterial, anticancerous
Guarea kunthiana A. Juss. (Meliaceae)	(UB) 3710	jataúba da Guiana	antiinflammatory
Cupania vernalis Camb. (Sapindaceae)	(UB) 3695	camboatã-vermelho, olho-de-cotia	fever, tonic, inflammation
Matayba guianensis Aubl. (Sapindaceae)	(UB) 3697	camboatá, assa-leitão	comestible fruit
Serjania lethalis A. St. Hil. (Sapindaceae)	(UB) 3716	timbó	ichtyotoxic
Pouteria gardneri (Mart. & Miq.)(Sapindaceae)	(UB) 3672	leiteiro-da-folha-miúda, sapotinha, aguaí-guaçu	construction, comestible fruit

C. sylvestris var. *lingua* and *A. crassiflora* extracts are as efficient as the reference drugs used against both parasites. As these plants are widely used in traditional medicine in Brazilian Cerrado against cutaneous and infectious diseases, these results prompted us to perform bioassay guided fractionations, and further experiments with *T. cruzi* and *Leishmania* animal models to evaluate their efficacy against both parasites.

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TABLE II

In vitro activities of extracts of Brazilian Cerrado plants against amastigote forms of *Trypanosoma cruzi* and promastigote forms of *Leishmania donovani*

Species	Parts and solvents	*T. cruzi* amastigotes IC_{50} (µg/ml)	*L. donovani* promastigotes IC_{50} (µg/ml)
Annona crassiflora	SB (e)	14.9 ± 2.3	12.4 ± 0.3
	SW (e)	20.5 ± 1.1	8.3 ± 0.8
	SW (h)	45.9 ± 3.1	
	RB (e)	5.9 ± 1.3	3.7 ± 0.3
	RB (h)	18.6 ± 6.8	
	RW (e)	9.9 ± 0.5	8.7 ± 0.6
Cardiopetalum calophyllum	SB (h)	60.4 ± 2.6	
Duguetia furfuracea	S (h)	50.0 ± 1.6	
	RB (e)	30.4 ± 1.3	
	RB (h)	6.6 ± 0.6	
	RW (e)	25.6 ± 1.5	
Xylopia aromatica	RW (h)	21.6 ± 6.0	
	RB (h)	23.5 ± 4.7	
Xylopia emarginata	L (h)	57.6 ± 2.4	
Aspidosperma macrocarpon	L (h)	59.2 ± 1.2	
Himatanthus obovatus	RW (e)	15.7 ± 0.5	7.5 ± 0.9
Casearia sylvestris var. *lingua*	L (h)	3.40 ± 0.35	3.7 ± 0.3
	SB (e)		11.4 ± 0.2
	SB (h)	0.44 ± 0.02	0.2 ± 0.0
	SW (h)	0.44 ± 0.05	0.3 ± 0.0
	RB (e)	5.6 ± 0.4	0.1 ± 0.0
	RB (h)	0.3 ± 0.04	1.0 ± 0.1
	RW (e)		5.0 ± 0.1
	RW (h)	0.86 ± 0.05	11.4 ± 0.2
	F (h)		9.5 ± 0.6
	F (e)		4.9 ± 0.2
Guarea kunthiana	R (h)		7.9 ± 1.3
Cupania vernalis	L (h)		7.1 ± 0.6
Matayba guianensis	SB (h)	14.8 ± 0.5	10.7 ± 0.7
Serjania lethalis	RB (e)		5.2 ± 0.3
Pouteria gardneri	RW (h)	45.5 ± 5.9	
Reference drugs			
Benznidazole		1.0 ± 0.1	
Amphotericin B			0.8 ± 0.1
Pentamidine			3.1 ± 0.6
Miltefosine			3.1 ± 0.1

L: leaves; R: roots; F: fruits; S: stem; SB: stem bark; SW: stem wood; RB: root bark; RW: root wood; e: ethanol; h: hexane

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