

Ethnopharmacological communication

Spirolactone iridoids might be responsible for the antileishmanial activity of a Peruvian traditional remedy made with *Himatanthus sucuuba* (Apocynaceae)

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Abstract

Extracts of seven medicinal plants used specifically against cutaneous leishmaniasis in the Madre de Dios region of Peru were evaluated *in vitro* against promastigote and axenic amastigote forms of *Leishmania amazonensis*. One of them showed interesting leishmanicidal activities (IC₅₀ = 5 µg/ml in amastigotes). Bio-guided isolation of the stem bark's ethanol extract of *Himatanthus sucuuba* (Spruce ex Müll. Arg.) Woodson (Apocynaceae) afforded the spirolactone iridoids isoplumericin and plumericin. The latter showed a reduction of macrophage infection similar to that of the reference drug Amphotericin B (IC₅₀ = 0.9 and 1 µM, respectively). These findings validate the traditional use of *Himatanthus sucuuba* in the treatment of cutaneous leishmaniasis (Uta) in Peru.

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American tegumentary leishmaniasis (ATL) is an endemic parasitic protozoal disease in most countries of Latin America that can present itself either as cutaneous (CL) or as mucosal leishmaniasis (ML) (Walton, 1987). It is a major public health problem in Peru where the endemic range covers 74% (951.820 km²) of the country. The endemic distribution pattern of cutaneous and mucosal leishmaniasis is found throughout the Andean and inter-Andean valleys in its cutaneous form, and in the tropical rain forest (low jungle and high jungle) in its mucosal form (Lucas et al., 1998). Antimonials and amphotericin B are currently used as standard drugs. However, they are associated with toxic side effects and have a restricted therapeutic spectrum

for all clinical forms of leishmaniasis (Davis et al., 2004). In this context, the discovery of new active compounds with antileishmanial potential remains essential for the control and prevention of leishmaniasis. Contrary to other diseases, leishmaniasis is properly identified as a disease by the natives of the Amazonian forest (Dedet et al., 1989). This justifies the search for new leads based on ethnomedicine. Few Latin American plants have been reported as useful for the treatment of leishmaniasis. Studies focusing on herbal remedies for this disease were carried out in French Guiana (Sauvain et al., 1993), in Bolivia (Fournet et al., 1994), in Colombia (Weniger et al., 2001), in Panama and Mexico (Camacho et al., 2003), in Brazil (Muzitano et al., 2006), and in Peru (Kvist et al., 2006). Literature dealing with leishmanicidal activity in natural products from the mid-1980s to late 2001 is concentrated in three published reviews (Chan-Bacab and Peña-Rodríguez, 2001; de Carvalho and Ferreira,

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2001; Rocha et al., 2005). The present work investigates plants used against leishmaniasis by people of the Peruvian Amazon region to purify very active compounds against *Leishmania* parasites.

1. Materials and methods

1.1. Collection of plants and ethnomedical information

The field work was carried out in the Madre de Dios department on the road between the city of Puerto Maldonado and the village of Yanapari. Health promoters were contacted and interviewed. The questions concerned the occurrence of leishmaniasis in the local area with a focus on most recent cases. Patients who were confirmed to have recently suffered from cutaneous leishmaniasis were contacted and asked about the treatment they had received. If the patient had used medicinal plants either to cure or alleviate symptoms of leishmaniasis, an ethnobotanical field work was carried on. Plants were systematically collected and their respective uses recorded with the aid of the informant who had given his formal agreement to the study.

1.2. Vouchers in herbarium

Voucher specimen was deposited at the Herbarium of Universidad Nacional de la Amazonia Peruana in Iquitos. A first assessment of the identity of the plants was conducted by the ethnobotanist of the team with the help of specialists of the plant family of interest.

1.3. Preparation of extracts

Fifty grams of air-dried powdered plant material was extracted during 10 days at room temperature with 500 ml of 95% ethanol. After filtration, the solvent was evaporated to dryness under reduced pressure at a temperature lower than 40 °C. For the bioassay, the extracts were resuspended in DMSO at a concentration of 10 mg/ml.

1.4. Bioassay-guided isolation and structure elucidation of active principles

Dried powdered bark (700 g) of *Himatanthus sucuuba* was extracted by maceration with 95% ethanol. The solvent was then evaporated to dryness under reduced pressure at temperature lower than 40 °C to obtain the crude extract (20.8 g). Part (19 g) of this ethanol extract was partitioned with solvents of different polarities. The yields of the obtained fractions were 3.3, 0.93, 4.7 and 8.7 g for the solvents hexane, ethylacetate, 90% methanol and water, respectively. The antileishmania activity was concentrated in the 90% methanol fraction. Two grams of this fraction was subjected to silica gel-column chromatography using mixtures of hexane–ethylacetate–methanol of increasing polarities. Ten fractions (F1–F10) were obtained and evaluated for biological activity. The most active fractions, F4 (50 mg) and F6 (231 mg), were finally purified by recrystal-

lization in ethylacetate to afford isoplumericin (18 mg, 0.007% yield) and in methanol for plumericin (70 mg, 0.026% yield), respectively. Structures of isolated compounds were identified by nuclear magnetic resonance (Bruker AC300) using ¹H NMR (300.13 MHz), ¹³C NMR (75.47 MHz), 2D NMR analysis, and by comparison of spectral data with those available in the literature (Abdel-Kader et al., 1997). CDCl₃ was used as solvent for both compounds. For the X-ray crystallographic analysis, all data were collected at low temperatures (173 K) on a Bruker-AXS CCD-1000 diffractometer with Mo K α radiation ($\lambda = 0.71073 \text{ \AA}$).

1.5. Parasites and cultures

A cloned line of *Leishmania amazonensis* (strain MHOM/BR/76/LTB-012) was used in all experiments. Axenically grown amastigote forms of *Leishmania amazonensis* were maintained by weekly subpassages in MAA/20 medium (Serenó and Lemesre, 1997a) at $32 \pm 1 \text{ }^\circ\text{C}$ with 5% CO₂ in 25 cm² tissue culture flasks. Promastigote forms of *Leishmania amazonensis* were maintained at $25 \pm 1 \text{ }^\circ\text{C}$ by weekly subpassages in RPMI 1640 medium with 25 mM HEPES and 2 mM NaHCO₃ (pH 7.2), supplemented with 20% heat-inactivated foetal bovine serum.

1.6. In vitro activity on amastigotes and promastigotes

All experiments were performed in triplicate. The *in vitro* leishmanicidal activity of plant extracts were determined in the axenic cultures of amastigotes and promastigotes forms of *Leishmania amazonensis*. To estimate the 50% inhibitory concentration (IC₅₀) of the extracts, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) micromethod was used as previously described (Serenó and Lemesre, 1997b). Results were expressed as percentage reduction of parasite burden compared to the level in untreated control wells, and the 50% inhibitory concentration (IC₅₀) was determined from the concentration–response curves.

1.7. Macrophages

Peritoneal macrophages were prepared according to Sauvain et al. (1993). Non-inflammatory macrophages (10⁵) were collected from 6-week-old male BALB/c mice. The adherent cells were incubated at 37 °C under 5% CO₂.

1.8. Determination of toxicity to macrophages

Appropriate dilutions of compounds were added to a part of the macrophages prepared above (Sauvain et al., 1993). The contact between drugs and macrophages alone lasted 48 h at 37 °C under 5% CO₂. The percentage of live macrophages was determined microscopically by means of the trypan blue dye exclusion test and the IC₅₀ was determined by the same method as for amastigotes.

1.9. Infection of macrophages by amastigotes

To assess intracellular antileishmanial activity, medium in the wells containing the macrophages was replaced by the suspension of amastigotes using an infection ratio of 7/1 amastigotes/macrophages according to Sauvain et al. (1993). Twelve hours after infection, a solution of the compounds to be tested was added to the cultures at various concentrations and maintained at 37 °C under 5% CO₂ for another 48 h. Plates were fixed with methanol and stained with 10% Giemsa stain. The percentage of infected macrophages was determined microscopically at 100 times magnification. The results were statistically analyzed by the ANOVA test, using the statistics for Windows program ($P < 0.05$).

1.10. Cell cytotoxicity bioassays

Cytotoxicity of the compounds was determined using BALB/3T3 (non-tumorigenic, BALB/c mouse embryo cells) and Vero (African green monkey kidney cells) cells. Briefly, growth inhibition was evaluated by preparing serial dilutions

of each compound and incubating the cells in 96-well plates in the presence or absence of these compounds for 48 h at 37 °C. Appropriate solvent controls were tested for comparison. The percent inhibition of cell growth relative to control was evaluated calorimetrically using a sulforhodamine B dye according to a published procedure (Skehan et al., 1990) in comparison to control. The IC₅₀ value was defined as the concentration of test sample resulting in a 50% reduction of absorbance as compared with untreated controls that received a serial dilution of the solvent in which the test samples were dissolved, and was determined by linear regression analysis.

2. Results and discussion

Table 1 summarizes the ethnobotanical data of the seven plant species selected for this study (Lacaze and Alexiades, 1995; Mejia and Rengifo, 2000; Pinedo and Rengifo, 1997; Vasquez, 2005) and shows the results of the screening against axenic amastigote and promastigote stages *in vitro*. The most active plant extract (IC₅₀ = 5 µg/ml) on the amastigote model was the ethanol extract obtained from the *Himatanthus sucuuba* barks.

Table 1
Ethnobotanical information of the plants collected in the Department of Madre de Dios and results of the antileishmanial screening using promastigotes and axenic amastigotes of *Leishmania amazonensis*

Species (family, vernacular name, voucher number)	Traditional use reported in the literature	Place and date of collection	Preparation of the plants used by the informants against leishmaniasis ulcers	IC ₅₀ (µg/ml) amastigotes	IC ₅₀ (µg/ml) promastigotes
<i>Brunfelsia grandiflora</i> D. Don ssp. Shultessi Plow. (Solanaceae Chiric sanango PROCOMD-004-001/2005)	Leishmaniasis (Mejia and Rengifo, 2000)	Community of Planchón, road between Puerto Maldonado and Iñapari, 38 km. February 2005	The powder of the bark is applied directly on the affected area	>100	>100
<i>Capirona decorticans</i> Spruce, Rubiaceae, Capirona de altura, PROCOMD-005-001/2005	Warts, infected wounds, mycosis, scabies (Pinedo and Rengifo, 1997)	Community of Planchón, road between Puerto Maldonado and Iñapari, 38 km. February 2005	The bark is rasped and applied as a poultice on the affected area. The bark juice is also poured into the ulcer	>100	>100
<i>Cyathea</i> sp., Cyatheaceae, Sano sano, PROCOMD-006-001/2005	Wounds (Lacaze and Alexiades, 1995)	Community of Planchón, road between Puerto Maldonado and Iñapari, 38 km. February 2005	The powder of the bark is applied directly on the affected area	>100	>100
<i>Dracontium lorentense</i> K. Krause, Araceae, Jergón sacha, PROCOMD-002-001/2005	Leishmaniasis (Vasquez, 2005)	Community of Sudadero, road between Puerto Maldonado and Iñapari, 21 km. February 2005	The powder of sun-dried tubers is sieved and applied on the ulcer four times a day until healing	60	>100
<i>Himatanthus sucuuba</i> (Spruce ex Muell. Arg.) Woodson, Apocynaceae, Bellaco caspi, PROCOMD-008-002/2005	Herpes and wounds (Mejia and Rengifo, 2000; Villegas et al., 1997)	Community of Pacahuara, Near from Iberia, February 2005	The fresh bark and latex are applied directly on the affected area	5	20
<i>Paullinia bracteosa</i> Radlk., Sapindaceae, Abuta colorada, PROCOMD-001-003/2005	Leishmaniasis, wounds (Lacaze and Alexiades, 1995; Vasquez, 2005)	Community of Bello Horizonte, road between Puerto Maldonado and Iñapari, 12 km. February 2005	The powder of the bark is applied on the ulcer	60	>100
<i>Zamia</i> sp., Cycadaceae, Zanahoria del monte, PROCOMD-009-001/2005	Leishmaniasis, cicatrization. (Mejia and Rengifo, 2000; Vasquez, 2005)	Community of Pacahuara, near Iberia, February 2005	The rhizome is crushed and the obtained juice is poured into the affected area	>100	>100

Table 2

In vitro activities of plumericin (1), isoplumericin (2) and Amphotericin B (3) against *Leishmania* models and cytotoxicity assays of 1 and 2 against 3T3 and vero cells

Activities on axenic amastigotes of <i>Leishmania amazonensis</i> (IC ₅₀)	Activity on macrophages infected with <i>Leishmania amazonensis</i> (IC ₅₀)	Toxicity on mice peritoneal macrophages (IC ₅₀)	Cytotoxicity assays (IC ₅₀)	
			3T3	VERO
(1) 0.21 μM	(1) 0.9 μM	(1) 1.86 μM	(1) 2.00 μM	(1) 1.72 μM
(2) 0.28 μM	(2) NA (*)	(2) 1.86 μM	(2) 1.10 μM	(2) 1.03 μM
(3) 0.52 μM	(3) 1 μM	Amphotericin B > 10 μM		

NA*: not available. Isoplumericin tested concentrations affected macrophages.

The barks also showed a moderate activity on a promastigote stage. *Himatanthus sucuuba*, a well-known medicinal plant from the Peruvian Amazon, had been previously reported as inactive against another protozoan parasite, *Plasmodium falciparum* (Kvist et al., 2006). On the other hand, in our plant extracts screening against axenic amastigotes of *Leishmania amazonensis* (one of the etiologic species of cutaneous leishmaniasis in the Amazon region), the bark of *Himatanthus sucuuba* showed the highest direct activity against the intracellular form of the parasite. According to this result, no significant wound-healing activity had been detected in *Himatanthus sucuuba* in a previous work about plant preparations used by traditional medicine practitioners in Peru as wound-healing agents (Villegas et al., 1997). Bio-guided fractionation of the stem bark from *Himatanthus sucuuba* using axenic amastigotes resulted in the isolation of two known spiro lactone iridoids: plumericin and isoplumericin. The structures of the compounds have been confirmed by means of X-ray crystallography (Fig. 1). Both compounds show a strong activity against *Leishmania amazonensis* axenic amastigotes as shown in Table 2. The two compounds evaluated for their cytotoxicity on mice peritoneal macrophages and on tumoral cells have shown less toxicity than in their activity on *Leishmania* amastigotes (Table 2). In the case of the tumoral assays isoplumericin is more toxic than plumericin. Plumericin and its isomer were evaluated on *Leishmania amazonensis* *in vitro*

infected macrophages. Isoplumericin showed a toxicity against infected macrophages which did not allow an evaluation of its activity against intracellular amastigotes. Plumericin caused a reduction of the macrophage infection similar to Amphotericin B, the drug used more frequently when patients do not respond to antimonials, the first line chemotherapy (IC₅₀ of 0.9 μM for plumericin and of 1 μM for Amphotericin B). This difference between the effects of the two isomers on infected macrophages should be of great interest, since the radical group orientation influences the antiparasitic activity of some compounds (Montero-Torres et al., 2005). As far as we know this is the first time that antileishmanial activity is demonstrated for these types of iridoids. This kind of spiro lactone iridoid is not so commonly found in nature. Only around 10 structurally related compounds have been isolated from the genera *Himatanthus*, *Plumeria* and *Allamanda* (Abdel-Kader et al., 1997). The results of our work indicate that it would be very interesting to isolate or synthesize structurally related compounds (Troost et al., 1986) in order to establish structure–antileishmania activity relationships.

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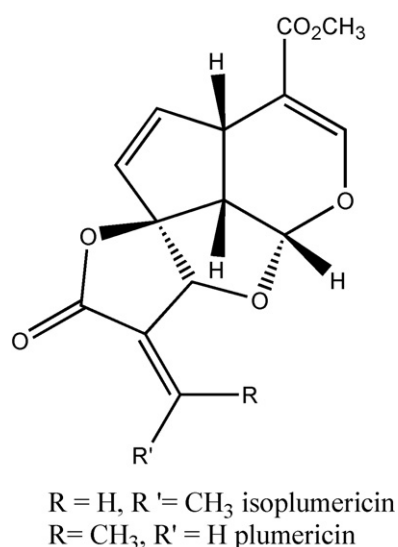


Fig. 1. Structure of plumericin and isoplumericin.

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