

Antileishmanial, Antimalarial and Antimicrobial Activities of the Extract and Isolated Compounds from *Austroplenckia populnea* (Celastraceae)

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Austroplenckia populnea (Celastraceae), known as “marmelinho do campo”, is used in Brazilian folk medicine as antimicrobial, anti-inflammatory, and antitumoural agent. The aim of the present work was to evaluate the antimicrobial, antileishmanial and antimalarial activities of the crude hydroalcoholic extract of *A. populnea* (CHE) and some of its isolated compounds. The phytochemical study of the CHE was carried out affording the isolation of methyl populnoate (**1**), populnoic acid (**2**), and stigmast-5-en-3-*O*- β -D-glucopyranoside (**3**). This is the first time that the presence of compound **3** in *A. populnea* is reported. The results showed that the CHE presents antifungal and antibacterial activities, especially against *Candida glabrata* and *Candida albicans*, for which the CHE showed IC₅₀ values of 0.7 μ g mL⁻¹ and 5.5 μ g mL⁻¹, respectively, while amphotericin B showed an IC₅₀ value of 0.1 μ g mL⁻¹ against both microorganisms. Compounds **1**–**3** were inactive against all tested microorganisms. In the antileishmanial activity test against *Leishmania donovani*, the CHE showed an IC₅₀ value of 52 μ g mL⁻¹, while compounds **2** and **3** displayed an IC₅₀ value of 18 μ g mL⁻¹. In the antimalarial assay against *Plasmodium falciparum* (D6 and W2 clones), it was observed that all evaluated samples were inactive. In order to compare the effect on the parasites with the toxicity to mammalian cells, the cytotoxicity activity of the isolated compounds was evaluated against Vero cells, showing that all evaluated samples exhibited no cytotoxicity at the maximum dose tested.

Key words: *Austroplenckia populnea*, *Leishmania donovani*, Populnoic Acid

Introduction

Leishmaniasis is a group of tropical diseases caused by a number of species of protozoan parasites belonging to the genus *Leishmania* (Kayser *et al.*, 2002). According to WHO, leishmaniasis is among the six most important tropical diseases, affecting around 12 million people in 80 countries (WHO, 1998). Only in Brazil, there are about 26,000 registered cases of leishmaniasis per year. Historically, the chemotherapy of leishmaniasis has been based on the use of pentavalent antimonial drugs. Other medications, such as pentamidine and amphotericin B, have been used as alternative drugs. However, these drugs are not orally active, requiring long-term parenteral administration, and

display serious side effects (Neto *et al.*, 2004; Kayser *et al.*, 2002).

Another important tropical disease is malaria, which has the potential to affect nearly 40% of the world's population, and it is responsible for 1–2 million deaths each year. Human malaria is endemic to 90 countries and is caused by protozoan parasites of the genus *Plasmodium*, mainly *P. falciparum*. Although several research programs are focused on various strategies to control malaria and leishmaniasis, drug discovery is one of the main areas of concentrated effort (Zhang *et al.*, 2001; Kayser *et al.*, 2002).

In the last decade there has been intensification in the search for antiprotozoal and antimicrobial compounds from natural sources (Rocha *et al.*,

2005; Silva *et al.*, 2004; Leitão *et al.*, 2004), mainly from plants, which continue to be a major source of biologically active metabolites that may provide lead structures for the development of new drugs.

Several plant species from the Celastraceae family are used in folk medicine as antiulcerogenic, male anti-fertility, analgesic and anti-inflammatory agents. *Austroplenckia populnea* (Reiss) Lundell (Celastraceae), popularly known as “marmelinho do campo”, is a plant from Brazilian cerrado, which is widely distributed in the northern and central parts of Brazil (Andrade *et al.*, 2006). This plant has been used in Brazilian folk medicine as antidiarrheal, anti-inflammatory, antimicrobial, and antitumoural agent (Andrade *et al.*, 2006; Duarte *et al.*, 2002). Pentacyclic triterpenes from *A. populnea* have been reported to display trypanocidal and anti-inflammatory properties (Andrade *et al.*, 2007; Duarte *et al.*, 2002).

On the basis of the folkloric uses of *A. populnea* and as part of our works on antiprotozoal and antimicrobial activities of medicinal plants (Da Silva Filho *et al.*, 2004a, 2008; Oliveira *et al.*, 2007; Souza *et al.*, 2005), the aim of the present work was to evaluate the antileishmanial, antimalarial, and antimicrobial activities of the crude extract and isolated compounds from *A. populnea*.

Materials and Methods

Plant material

The wood barks of *Austroplenckia populnea* were collected in the “cerrado” area of Botucatu (São Paulo State, Brazil), in December 2003, from a plant approx. 20 years old. The plant material was identified by the staff of State University of São Paulo-UNESP, Institute of Bioscience, Botucatu, SP, Brazil, and a voucher specimen (no. 20415) deposited at the herbarium of the same institute.

Drugs and chemicals

Chloroform was from Acros Co. (New Jersey, USA); ethyl acetate, hexane, and methanol were from Mallinckordt Co. (Xalostoc, Mexico); dimethyl sulfoxide (DMSO) was from Sigma-Aldrich Co. (St. Louis, MO, USA); Sabouraud dextrose broth, Middlebrook 7H9 broth, and Mueller-Hinton broth were from Difco; Alamar Blue was from BioSource International (California, USA); ethanol was bought from a local distillery and purified by distillation. The mammalian Vero cells

(African green monkey kidney fibroblasts) were from American Type Culture Collection (ATCC) (Manassas, VA, USA). All the other chemicals employed in this work were of analytical grade and purchased locally.

General procedures

NMR spectra were recorded on a Bruker ARX 400 spectrometer. Vacuum-liquid chromatography (VLC) was carried out with Silica gel 60H, 100–200 mesh ASTM (Merck Co., Darmstadt, Germany). Column chromatography (CC) was carried out with Silica gel 230–400 mesh ASTM (Merck) in glass columns. The chemical structures of all compounds were established by ^1H (400 MHz) and ^{13}C NMR (100 MHz) data analysis, and by comparison of the data with those of authentic compounds.

Extraction and isolation of compounds

The wood bark was air-dried at 40 °C, powdered, and the dried plant material (3.5 kg) was exhaustively extracted by maceration using aqueous ethanol (96%) at room temperature. The filtered extract was concentrated under vacuum to furnish 406 g of the dried crude hydroalcoholic extract (CHE). The CHE (406 g) was dissolved in methanol/H₂O (7:3), followed by sequential partition with hexane and CHCl₃, furnishing 19.0 g and 17.0 g of these fractions, respectively. The hexane fraction (HF, 7.0 g) was chromatographed over silica gel using a VLC system and hexane/ethyl acetate mixtures in increasing proportions as eluent, giving four fractions. The resulting fractions II (230 mg) and III (2.5 g) were submitted to CC over silica gel, using hexane/ethyl acetate mixtures in increasing proportions as eluent. Fraction II furnished compound **1** (50 mg), while fraction III furnished compound **2** (730 mg). The chloroform fraction (CF, 7.0 g) was submitted to CC over silica gel, using hexane/ethyl acetate mixtures in increasing proportions as eluent, giving six fractions. Fraction II (350 mg) was washed with ethyl acetate to afford compound **3** (150 mg).

Antimicrobial assays

The activity against a panel of microorganisms was evaluated *in vitro*. All organisms were obtained from ATCC and included *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neo-*

formans ATCC 90113, methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA), and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI methods (Muhammad *et al.*, 2003; Da Silva Filho *et al.*, 2008). Susceptibility testing of *M. intracellulare* was done using the modified Alamar Blue procedure of Franzblau *et al.* (1998). Samples (dissolved in DMSO) were serially diluted by using 0.9% saline and transferred in duplicate to 96-well microplates. Microbial inocula were prepared after comparison of the absorbance (at 630 nm) of cell suspensions to the 0.5 McFarland standard and dilution of the suspensions in broth (Sabouraud dextrose and cation-adjusted Mueller-Hinton broth for the fungi and bacteria, respectively, and 5% Alamar Blue in Middlebrook 7H9 broth with oleic acid-albumin-dextrose-catalase enrichment for *M. intracellulare*) to afford the recommended inocula. Microbial inocula were added to the samples to achieve a final volume of 200 μL and final sample concentrations starting with 200 $\mu\text{g mL}^{-1}$ for crude extracts and 50 $\mu\text{g mL}^{-1}$ for pure compounds. Growth, solvent, and media controls were included on each test plate. The plates were read either at 630 nm or at excitation and emission wavelengths of 544 and 590 nm (for *M. intracellulare*) using a Polarstar Galaxy Plate Reader (BMG LabTechnologies, North Caroline, USA) prior to and after incubation. Percent growth was calculated and plotted with the concentration tested to afford the concentration that inhibited 50% of growth (IC_{50}). Minimum inhibitory concentration (MIC) was defined as the lowest test concentration that allows no detectable growth.

Antimalarial assay

The *in vitro* antimalarial assay procedure utilized was an adaptation of the parasite lactate dehydrogenase (pLDH) assay, according to Muhammad *et al.* (2003), using a 96-well microplate assay protocol with two *P. falciparum* clones [Sierra Leone D6 (chloroquine-sensitive) and Indochina W2 (chloroquine-resistant)]. The primary screening involved the determination of pLDH inhibition (percentage) of each sample tested at 15.9 $\mu\text{g mL}^{-1}$ for extracts and 1.59 $\mu\text{g mL}^{-1}$ for pure compounds. The IC_{50} values were determined only for samples that inhibited the parasite growth (one of the clones) by > 50%. The antimalarial agents chloro-

quine and artemisinin were used as positive controls, with DMSO as the negative (vehicle) control.

Antileishmanial assay

A transgenic cell line of *L. donovani* promastigotes, showing stable expression of luciferase, was used as the test organism (Muhammad *et al.*, 2003). Cells in 200 μL of growth medium (L-15 with 10% FCS) were plated at a density of $2 \cdot 10^6$ cells/mL in a clear 96-well microplate. Stock solutions of the standards and the test compounds/extracts were prepared in DMSO. Culture media without cells and the controls were incubated (at 26 °C for 72 h) simultaneously, in duplicate, at six concentrations of the test compounds. An aliquot of 50 μL was transferred from each well to a fresh opaque/black microplate, and 40 μL of Steadyglo reagent were added to each well. The plates were read immediately using a Polar Star galaxy microplate luminometer. IC_{50} and IC_{90} values were calculated from dose-response inhibition graphs. Pentamidine and amphotericin B were tested as standard antileishmanial agents.

Cytotoxicity assay

Cytotoxicity was determined by the neutral red method according to a procedure previously described (Yang *et al.*, 2006). The IC_{50} value for each compound was computed from the growth inhibition curve.

Results

Phytochemical study

The phytochemical study of the CHE led to the isolation of three compounds. The chemical structures of all isolated compounds (Fig. 1) were established by ^1H and ^{13}C NMR data analysis, in comparison with the literature, as follows: methyl populnoate (**1**) (Sousa *et al.*, 1990), populnoic acid (**2**) (Itokawa *et al.*, 1991), and stigmast-5-en-3-*O*- β -(D-glucopyranoside) (**3**) (Kojima *et al.*, 1990).

In vitro antileishmanial, antimalarial and cytotoxicity activities

The *in vitro* antileishmanial, cytotoxicity and antimalarial activities are summarized in Table I. It was observed that the CHE of *A. populnea* showed an IC_{50} value of 52 $\mu\text{g mL}^{-1}$ against *L. donovani*. Among all isolated compounds, popul-

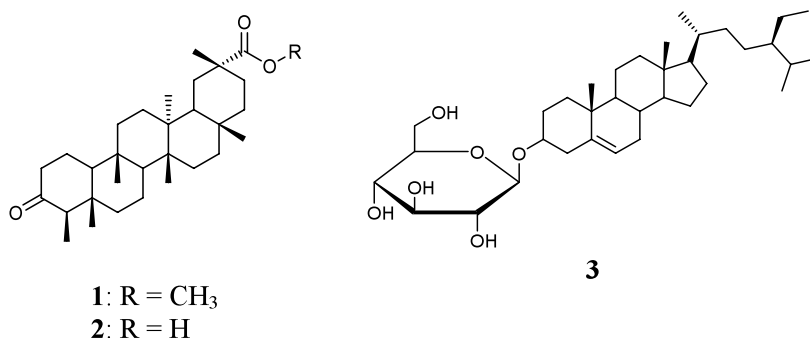


Fig. 1. Chemical structures of compounds isolated from *A. populnea*: methyl populnoate (**1**), populnoic acid (**2**), stigmast-5-en-3-*O*- β -(D-glucopyranoside) (**3**).

Table I. *In vitro* antileishmanial, cytotoxicity, and antimalarial activities of crude extract (CHE) and isolated compounds from *A. populnea*.

	<i>L. donovani</i> [$\mu\text{g mL}^{-1}$]		<i>P. falciparum</i> [ng mL^{-1}]				Cytotoxicity (Vero cells)
	IC ₅₀	IC ₉₀	D6 clone ^a		W2 clone ^b		IC ₅₀ [$\mu\text{g mL}^{-1}$]
			IC ₅₀	SI ^c	IC ₅₀	SI ^c	
CHE	52.0	100.0	— ^d	—	— ^f	—	— ^e
1	— ^d	— ^d	— ^d	—	— ^f	—	— ^e
2	18.0	35.0	— ^d	—	— ^f	—	— ^e
3	18.0	37.0	— ^d	—	— ^f	—	— ^e
Chloroquine	—	—	18.0	> 556	176.7	> 56	— ^e
Artemisinin	—	—	14.3	> 669	8.5	> 1176	— ^e
Pentamidine	1.9	8.5	—	—	—	—	—
Amphotericin B	0.7	1.7	—	—	—	—	—

^a Chloroquine-sensitive clone.

^b Chloroquine-resistant clone.

^c Selectivity index = IC₅₀ (Vero cells) / (IC₅₀ *P. falciparum*).

^d Not active at the highest tested concentration of 47.6 $\mu\text{g mL}^{-1}$ for crude extracts and 10 $\mu\text{g mL}^{-1}$ for pure compounds.

^e Not cytotoxic (up to the maximum doses tested).

^f Not tested.

noic acid (**2**) showed the highest antileishmanial activity, displaying an IC₅₀ value of 18 $\mu\text{g mL}^{-1}$ and IC₉₀ value of 35 $\mu\text{g mL}^{-1}$, while compound **1** was inactive. Compound **3** showed an IC₅₀ value of 18 $\mu\text{g mL}^{-1}$ and IC₉₀ value of 37 $\mu\text{g mL}^{-1}$, while pentamidine and amphotericin B, used as positive controls, showed IC₅₀ values of 1.9 $\mu\text{g mL}^{-1}$ and 0.7 $\mu\text{g mL}^{-1}$, respectively.

In the antimalarial assay, it was observed that both the CHE and its isolated compounds **1–3** were inactive against *P. falciparum* (D6 or W2 clones), while chloroquine and artemisinin (used as positive controls) displayed IC₅₀ values of 18.0 ng mL^{-1} and 14.3 ng mL^{-1} , respectively, against *P. falciparum* (D6 clone).

All evaluated samples showed no cytotoxicity against Vero cells at the maximum dose tested.

Antimicrobial activity

The antibacterial and antifungal activities are summarized in the Table II. The CHE displayed antifungal and antibacterial activities, especially against *Candida glabrata* and *Candida krusei*, with IC₅₀ values of 0.7 $\mu\text{g mL}^{-1}$ and 15 $\mu\text{g mL}^{-1}$, respectively. However, the isolated compounds **1–3** were inactive against all tested microorganisms.

Discussion

Some extracts and several isolated compounds from *A. populnea* have been investigated for their

Table II. *In vitro* antimicrobial activity of crude extract (CHE) and isolated compounds from *A. populnea*.

	IC ₅₀ ^a /MIC ^b [$\mu\text{g mL}^{-1}$]					
	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. krusei</i>	<i>C. neoformans</i>	MRSA ^c	<i>M. intracellulare</i>
CHE	5.5	0.7	15.0	— ^d	65.0	— ^d
1	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d
2	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d
3	— ^d	— ^d	— ^d	— ^d	— ^d	— ^d
Amphotericin B	0.1/0.3	0.1/0.3	1.5/2.5	0.5/1.2	—	—
Ciprofloxacin	—	—	—	—	0.1/0.6	0.3/0.6

^a 50% growth inhibitory concentration.^b Minimum inhibitory concentration.^c Methicillin-resistant *Staphylococcus aureus*.^d Not active at the highest tested concentration of 200 $\mu\text{g mL}^{-1}$ for crude extract and 50 $\mu\text{g mL}^{-1}$ for pure compounds.

biological properties, including anti-inflammatory and antiulcerogenic activities (Andrade *et al.*, 2006, 2007). Studies on the chemical composition of *A. populnea* showed the presence of pentacyclic triterpenes as major compounds of the bark extracts (De Sousa *et al.*, 2006; Duarte *et al.*, 2002; Sousa *et al.*, 1990). Populnoic acid (**2**), which possesses trypanocidal (Duarte *et al.*, 2002) and anti-inflammatory (Andrade *et al.*, 2007) activities, is commonly found in *Austroplenckia* species (Itokawa *et al.*, 1991; Andrade *et al.*, 2006). However, compound **3** is being reported for the first time for *A. populnea*.

Regarding the antileishmanial assay, compounds **2** and **3** as well as the CHE were active against *L. donovani*, while compound **1** was inactive. It may be observed that the chemical structure of **1** is quite similar to that of **2**. Thus, it is suggested that the carboxy group may interfere in the antileishmanial activity of these compounds, once compound **1** was inactive. The trypanocidal and antimicrobial activities of some pentacyclic triterpenes and their semi-synthetic derivatives have been reported in the literature (Cunha *et al.*, 2006, 2007). As previously reported, the presence of polar groups in the pentacyclic triterpene acids may be important for their antimicrobial and trypanocidal activities (Cunha *et al.*, 2006; Da Silva Filho *et al.*, 2004b). Considering the obtained results this class of pentacyclic triterpenes should also be considered for further antileishmanial studies. However, despite their antileishmanial activities, none of the evaluated samples was active against *P. falciparum*.

In order to compare the effect on the parasites with the toxicity to mammalian cells, the cytotoxic-

ity activity was evaluated against Vero cells, showing that all evaluated samples, including the CHE, exhibited no cytotoxicity in the maximum dose tested. These results are important not only for comparing the antiprotozoal activities, but also because this plant is used as antitumoural in Brazilian folk medicine (Vieira Filho *et al.*, 2002).

Moreover, the CHE and its isolated compounds were evaluated against different microorganisms. Since the CHE was more active towards all the evaluated microorganisms than its isolated compounds, it is suggested that its antimicrobial activity may be due to the effect of other compounds present in the crude extract.

In summary, the antimicrobial activity found for the crude hydroalcoholic extract of *A. populnea* gives support for using this plant as antifungal in the Brazilian folk medicine. In addition, our results indicate that pentacyclic triterpenes are promising compounds that could also be evaluated in additional antileishmanial studies. Finally, further studies are in progress to disclose other important biological effects of this medicinal plant.

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