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Bioactivity-guided isolation of laevicarpin, an antitrypanosomal and anticryptococcal lactam from *Piper laevicarpu* (*Piperaceae*)

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A B S T R A C T

Crude CH_2Cl_2 extract from leaves of *Piper laevicarpu* (*Piperaceae*) displayed antitrypanosomal activity against trypomastigote forms of *Trypanosoma cruzi* (Y strain) and antimicrobial potential against *Cryptococcus gattii* (strain-type WM 178). Bioactivity-guided fractionation of crude extract afforded one new natural bioactive lactam derivative, named laevicarpin. The structure of isolated compound, which displayed a very rare ring system, was elucidated based on NMR, IR and MS spectral analysis. Using MTT assay, the trypomastigotes of *T. cruzi* demonstrated susceptibility to laevicarpin displaying IC_{50} value of 14.7 μg/mL (49.6 μM), about 10-fold more potent than the standard drug benznidazole. The mammalian cytotoxicity of laevicarpin was verified against murine fibroblasts (NCTC cells) and demonstrated a CC_{50} value of 100.3 μg/mL (337.7 μM–SI = 7). When tested against *Cryptococcus gattii*, laevicarpin showed an IC_{50} value of 2.3 μg/mL (7.9 μM) and a MIC value of 7.4 μg/mL (25 μM). Based in the obtained results, laevicarpin could be used as a scaffold for future drug design studies against the Chagas disease and anti-cryptococosis agents.

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1. Introduction

Tropical neglected diseases are characterized by a number of infectious diseases with high morbidity and mortality, affecting the life quality of poor population, mainly in developing countries [1–3]. As part of these illnesses, the Chagas disease, caused by the parasite *Trypanosoma cruzi*, affects millions of people worldwide mostly in Latin American countries [4,5]. The clinical treatment still uses toxic nitro compound derivatives, such as benznidazole [6]. Another disease that affects poor regions, the cryptococosis, is caused by encapsulated yeasts such as *Cryptococcus neoformans* and *C. gattii* [7,8]. A major problem of this disease is the fact that is one of the leading causing of mortality and morbidity in AIDS patients, about 600.000 people per year [9–11]. The treatment consists on administration of amphotericin B, 5-fluorocytosine and fluconazole – alone or in combination therapy with adverse effects as toxicity, persistence and recurrence of the disease [12]. Thus, the discovery of new bioactive compounds to the treatment of these diseases is mandatory and could be performed from different sources, including natural products [13–15].

In this aspect, species from *Piper* genus could be considered an important source of antiprotozoal/antimicrobial compounds - including flavonoids, phenolic derivatives, amides, benzoic acids, and chromenes [16–25]. In the course of our continuous selection of new bioactive compounds from Brazilian flora [26–28], in the present work the CH_2Cl_2 extract from leaves of *Piper laevicarpu*, which is known as "falsa-pimenteira", displayed *in vitro* potential against trypomastigote forms of *T. cruzi* and antimicrobial activity against *C. gattii* and was subjected to a bioactivity-guided fractionation. This procedure afforded one new bioactive natural lactam, characterized as laevicarpin after extensive analysis of NMR, IR, and MS spectral data.

2. Material and methods

2.1. General experimental procedures

^1H, ^{13}C, DEPT 135°, COSY, HSQC, HMBC, and NOESY spectra were recorded in Bruker Avance III spectrometers, operating at 300/75 and 500/125 MHz, to $^1H/^{13}C$ nuclei, respectively. $CDCl_3$ and CD_3OD (Tedia Brazil) were used as the solvent and the residual peak of the non-deuterated solvent as internal standard. Chemical shifts (δ) are reported in ppm and coupling constant (J) in Hz. HRESIMS spectrum was measured on a Bruker Daltonics MicroTOF QII spectrometer. IR spectra were obtained

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on a Nicolet – Nexus in a KBr pellets. Silica gel (Merck, 230–400 mesh) and Sephadex LH-20 (Sigma-Aldrich) were used for column chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical TLC (0.25 mm).

2.2. Plant material

Piper laevicarpu (*Piperaceae*) leaves were collected in January 2005 at city of Ubatuba, an Atlantic Rain forest region of the São Paulo State, Brazil. The specimen was identified by Prof. Dr. Elsie F. Guimarães from Botanic Garden of Rio de Janeiro/RJ, where a voucher specimen has been deposited.

2.3. Extraction and purification

Dried and powdered leaves of *P. laevicarpu* (210 g) were exhaustively extracted with CH_2Cl_2 (10 × 250 mL) using an automatized accelerated solvent extraction system (Dionex ASE350) at room temperature. The concentration of solvent under reduced pressure yielded 1.2 g of crude extract. As antitrypanosomal/antimicrobial activities were detected in this extract, part of the crude material (1.0 g) was subjected to CC over SiO_2 eluted with increasing amounts of MeOH in CH_2Cl_2, resulting in fifty-four fractions of 50 mL each, which were pooled in seven groups (A–G). As group D (553 mg), eluted with CH_2Cl_2:MeOH 9:1, has shown activity it was chromatographed over Sephadex LH-20 (4 × 50 cm) eluted with MeOH resulting in forty seven fractions (7 mL each) which, after TLC analysis, were pooled to eight groups (D1–D8). Group D3 (107 mg) displayed anti-trypanosomal/antimicrobial activities and was subjected to CC over SiO_2 eluted with a gradient of n-hexane in EtOAc resulting in eight seven fractions (4 mL each) which were pooled in ten groups (D3/1–D3/10). Bioactive fraction D3/6 (45 mg), eluted with n-hexane:EtOAc 8:2, yielded 30 mg of pure laevicarpin.

2.4. Laevicapin

Amorphous colorless solid. HRESIMS *m/z* calcd. $C_{18}H_{19}NO_3Na$ $[M + Na]^+$: 320.1258; found 320.1263. IR (KBr) ν_{max}/cm^{-1}: 3590, 2926, 1644, 1510, 1262, 1125, and 827; ^1H NMR (300 and 500 MHz, $CDCl_3 + CD_3OD$) and ^{13}C NMR (75 and 125 MHz, $CDCl_3 + CD_3OD$), see Table 1.

2.5. Mammalian cells and parasite maintenance

T. cruzi trypomastigotes (Y strain) were maintained in LLC-MK2 (ATCC CCL 7) cells using RPMI-1640 medium supplemented with 2% calf serum at 37 °C. LLC-MK2 and NCTC cells (NCTC clone 929) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum at 37 °C in a 5% CO_2-humidified incubator [29].

2.6. Determination of the antitrypanosomal activity

Trypomastigotes were counted in a Neubauer hemocytometer and seeded at 1×10^6 cells per well in 96-well microplates. Crude CH_2Cl_2 extract from leaves of *P. laevicarpu* as well as isolated laevicarpin were added to the highest concentration of 300 μg/mL for 24 h at 37 °C in a 5% CO_2-humidified incubator, with benznidazole as standard drug [30]. The trypomastigote viability was based on the cellular conversion of the soluble tetrazolium salt MTT into the insoluble formazan by mitochondrial enzymes. The formazan extraction was carried out with 10% (v/v) sodium dodecyl sulfate for 18 h (100 μL per well) at 24 °C [27,31].

2.7. Cytotoxicity against mammalian cells

Mice conjunctive cells, NCTC clone 929 (ATCC) were seeded at 4×10^4 cells per well in 96-well microplates and incubated with laevicarpin at different concentrations for 48 h at 37 °C in a 5% CO_2 incubator. The cell viability was determined using the MTT assay as previously described [31].

2.8. Antimicrobial assay - minimum inhibitory concentration (MIC) and inhibitory concentration (IC_{50}) determination

The *in vitro* activity of laevicarpin against *C. gattii* molecular VGII strain-type (FIOCRUZ WM 178) was investigated according to the *Clinical Laboratory Standards Institutes* (CLSI) modified method M27-A3 [32]. Using a 96-wells microplates, *C. gattii* was inoculated (0.5×10^3 to 2.5×10^3 cells/mL) in RPMI 1640 medium with 0.2% glucose with laevicarpin in a 8-range concentration of 200 to 1.56 μM and incubated for 48 h at 30 °C.·The 100% inhibition was assessed by visual and spectrophotometer (570 nm) reading. All experiments were performed in triplicate. *Candida krusei* (ATCC 6258) and *Candida parapsilosis* (ATCC 22019) were included as quality control in all tests. Amphotericin B was used as standard drug [32]. To determine the 50% inhibitory concentration (IC_{50}) the compound was tested as the same way that was showed in MIC test and the viability was verified by the colorimetric Alamar Blue® Assay [33].

2.9. Minimal fungicidal concentration (MFC)

The activity of laevicarpin against *C. gattii* strain-type was determined according to described in the literature [34]. MFC was obtained

Table 1
NMR spectroscopic data (300/500 and 75/125 MHz, $CDCl_3 + CD_3OD$) to laevicarpin.

Position	δ_H (mult., *J* in Hz)	δ_C	HMBC (H → C)	COSY	NOESY
1	–	172.0 (C)	–	–	–
2	3.51 (t, 6.2)	39.7 (CH_2)	C-1, C-4	H-3	H-3, H-5/H-9
3	2.83 (t, 6.2)	34.6 (CH_2)	C-2, C-4, C-5/C-9	H-2	H-2, H-5/H-9
4	–	136.7 (C)	–	–	–
5	7.20 (d, 8.5)	131.1 (CH)	C-3, C-7, C-9	H-6	H-2, H-3, H-6
6	6.95 (d, 8.5)	124.3 (CH)	C-4, C-6, C-7	H-5	H-5, H-8′
7	–	156.3 (C)	–	–	–
8	6.95 (d, 8.5)	124.3 (CH)	C-4, C-7, C-8	H-9	H-9, H-8′
9	7.20 (d, 8.5)	131.1 (CH)	C-3, C-5, C-7	H-8	H-2, H-3, H-8
1′	2.09 (t, 5.2)	33.8 (CH_2)	C-1, C-2′, C-3′	H-2′	H-2′, H-8′
2′	2.72 (t, 5.2)	24.4 (CH_2)	C-1′, C-3′, C-4′, C-8′	H-1′	H-1′, H-4′, H-8′
3′	–	133.8 (C)	–	–	–
4′	6.53 (dd, 8.2 and 2.1)	120.7 (CH)	C-2′, C-6′, C-8′	H-5′, H-8′	H-2′, H-5′, H-8′
5′	6.69 (d, 8.2)	111.4 (CH)	C-3′, C-4′, C-6′, C-7′	H-4′	OCH_3, H-4′
6′	–	145.7 (C)	–	–	–
7′	–	152.3 (C)	–	–	–
8′	4.58 (d, 2.1)	114.4 (CH)	C-2′, C-4′, C-6′, C-7′	H-4′	H-6/H-8, H-1′, H-2′, H-4′
OCH_3	3.86 (s)	56.0 (CH_3)	C-6′	–	H-5′

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