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Prospecting and Identifying *Phyllanthus amarus* Lignans with Antileishmanial and Antitrypanosomal Activity

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ABSTRACT

Ten lignans (**1**–**10**) were isolated from the hexane-ethyl acetate extract of *Phyllanthus amarus* leaves. Three of them, cubebin dimethyl ether (**3**), urinatetralin (**4**), and lintetralin (**7**) are described for the first time in this species, while phyllanthin (**1**), niranthin (**2**), 5-demethoxyniranthin (**5**), isolintetralin (**6**), hypophyllanthin (**8**), nirtetralin (**9**), and phyltetralin (**10**) have been already reported from *P. amarus*. Among the lignans tested against *Trypanosoma cruzi* intracellular amastigotes, **2** was the most active with an EC_{50} of 35.28 µM. Lignans **2**, **5**, **7**, and **9** showed inhibitory effects against *Leishmania amazonensis* promastigotes with EC_{50} of 56.34, 51.86, 23.57, and 43.27 µM, respectively. During *in vitro* infection assays, **5** reduced amastigotes by 91% at 103.68 µM concentration, whereas **7** and **9** reduced amastigotes by approximately 84% at 47.5 and 86.04 µM, respectively. Lignans **5**, **7**, and **9** were more potent in intracellular amastigotes with EC_{50} of 2.76, 8.30, and 15.83 µM, respectively, than in promastigotes. CC_{50} for all samples was > 100 µg/mL, thus revealing low cytotoxicity against macrophages, and selectivity against the parasite. *L. amazonensis* promastigotes treated with compounds **2** and **9** showed decreased respiratory control of 38% and 25%, respectively, suggesting a change in mitochondrial membrane potential and lower ATP production.

Introduction

Chagas' disease and Leishmaniasis are parasitic diseases caused by kinetoplastid flagellated protozoans *Trypanosoma cruzi* and *Leishmania spp.*, respectively. These diseases are part of the Neglected Tropical Diseases list and are responsible for high annual mortality in underdeveloped tropical countries, where they mainly affect low-income populations [1–3].

It is estimated that 6 to 7 million people are chronically infected with Chagas' disease, with about 14 000 deaths per y and 70 million people living in risk areas. Chagas' disease is characterized by an acute phase, which sometimes remains unrecognized, and may progress to a chronic phase. In the acute phase, symp-

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ABBREVIATIONS

EE	ethanolic extract
GHIT	Fund Global Health Innovative Technology Fund
HEA	hexane-ethyl acetate extract
MOI	multiplicity of infection
SI	selectivity index
UNICAMP	Universidade Estadual de Campinas

toms such as fever, rash, inflammatory nodules, heart failure, nausea, diarrhea, and vomiting may appear. The chronic phase can lead to lifelong morbidity associated with cardiomyopathy and gastrointestinal problems [2].

Leishmaniasis can be caused by 20 different *Leishmania* species leading to a wide spectrum of clinical manifestations. Cutaneous leishmaniasis and visceral leishmaniasis are the 2 main manifestations of the disease. Also, there are rarer ones, which include mucocutaneous, diffuse, and post-kala-azar dermal forms [3].

About 12 million people are currently infected, and between 20 000 and 30 000 deaths are caused every y by visceral leishmaniasis, the most serious form of the disease. In the last 5 y, 1 million new cases of cutaneous leishmaniasis have been reported, and it is believed that over 1 billion people live in endemic areas at infection risk [4].

As there are no available vaccines, the treatment of infected people is one of the main control strategies of these diseases. However, medications in use have therapeutic complications such as toxicity, relevant contraindications, and complicated administration procedures [5,6]. Therefore, the search for safer alternative drugs for the population continues to be extremely important.

Phyllanthus amarus Schum & Thonn (Euphorbiaceae) is a medicinal plant widely found in South American, Asian, and African countries. It is popularly known as stone breaker and has been traditionally used for the treatment of inflammation, jaundice, diabetes, stomach, liver, and kidney diseases [7–9].

To scientifically support the traditional use of *P. amarus*, several pharmacological studies were conducted with extracts from the leaves and compounds isolated thereof. Antiallodynic [10], anti-inflammatory [11], analgesic [12], cardioprotective [13], hepatoprotective [14], antioxidant [15], cytotoxic [16,17], immunosuppressive [18,19], antiparasitic [20,21], antiviral [22,23], antibacterial [24,25] antidiabetic [26], antihypertensive [27], chondroprotective [28], osteogenic [29], and diuretic properties [30] have been reported for the constituents of *P. amarus*.

The diverse pharmacological profile of *P. amarus* can be attributed to its chemical composition, which mainly comprises lignans belonging to the diarylbutane and aryltetralin classes, as well as alkaloids, flavonoids, hydrolysable tannins, triterpenes, and steroids [8,31,32].

Given the need to search for new, safer, and more effective antichagasic and antileishmanial drugs and in view of the absence of reports on *P. amarus* applied to the treatment of these diseases, as well as considering the already popular therapeutic use of this species for various indications, we have evaluated the antiprotozoal activity of extracts, fractions, and compounds isolated from *P. amarus* leaves.

Results and Discussion

P. amarus leaves were extracted by hexane-ethyl acetate followed by ethanol. The HEA that showed antiprotozoal activity was fractionated by column chromatography on silica gel to provide 7 fractions (F1–F7).

Further chromatography over silica gel afforded 10 lignans (**1**–**10**) that were identified by detailed NMR analysis and by comparison with literature data as phyllanthin (**1**), niranthin (**2**), cubebin dimethyl ether (**3**), urinatetralin (**4**), 5-demethoxyniranthin (**5**), isolintetralin (**6**), lintetralin (**7**), hypophyllanthin (**8**), nirtetralin (**9**), and phyltetralin (**10**) (▶ **Fig. 1**). Cubebin dimethyl ether (**3**), urinatetralin (**4**), and lintetralin (**7**) are have been isolated here for the first time from *P. amarus*, but **3** has been already been described in *P. niruri* while **4** and **7** have been reported from *P. urinaria* [33–35]. The other lignans (**1, 2, 5, 6, 8, 9**, and **10**) have been already described in *P. amarus* [36–38].

The HEA, the F4 fraction enriched with **3** (56.65%) and **4** (37.04%), and the F5 fraction enriched with **5** (57.29%) and **6** (23.44%), as well as the further 6 lignans (**1, 2, 7**–**10**), were submitted to preliminary tests of antileishmanial activity against *L. amazonensis* promastigotes and antitrypanosomal activity against *T. cruzi* epimastigotes and amastigotes. All samples tested had marginal effect on *T. cruzi* epimastigotes. However, compounds **1, 2, 9,** and HEA showed a significant reduction in cell viability in *T. cruzi* amastigotes, especially compound **2,** which showed a 91.58% reduction at 64 µg/mL (148 µM) and an EC_{50} of 15.24 µg/mL (35.28 µM), and compound **9** with an EC_{50} of 49.14 µg/mL (114.28 µM). EC_{50} > 100 µg/mL were found for compound **1** and HAE. Among the compounds tested against intracellular *T. cruzi* amastigotes, compound **2** showed promising antitrypanosomal activity and low cytotoxicity against U2OS host cells with CC_{50} > 100 µg/mL, showing its selectivity toward the parasite.

Compound **2**, fractions F4 and F5, and HEA reduced the cell viability of approximately 60% of *L. amazonensis* promastigotes at 50 µg/mL. Compounds **7** and **9** reduced approximately 75% of *L. amazonensis* promastigotes cell viability, also at concentrations of 50 µg/mL. In order to identify the bioactive constituents of F4 and F5, both fractions were purified to provide lignans **3** and **4** as a mixture and **5**–**7**, respectively. Samples that presented over 50% reduction of *L. amazonensis* promastigotes cell viability were evaluated at different concentrations for the determination of EC_{50}, CC_{50}, and SI values. Compound **6** could not be evaluated due to low yield. HEA showed moderate effects against *L. amazonensis* promastigotes with an EC_{50} of 72.17 µg/mL. The *in vitro* antileishmanial activity increased as the extract was fractionated. F4 and F5 exhibited EC_{50} values of 16.85 and 41.41 µg/mL, respectively. Lignans **2, 3/4, 5, 7**, and **9** showed significantly higher activity than HEA, especially **7** with an EC_{50} of 9.43 µg/mL (23.57 µM), and higher *in vitro* selectivity with IS ≥ 10.60. Lignans **2, 3/4, 5**, and **9** exhibited EC_{50} values of 24.34 µg/mL (56.34 µM), 23.77 µg/mL (61.58 µM), 20.85 µg/mL (51.86 µM), and 18.61 µg/mL (43.27 µM), respectively, and CC_{50} > 100 µg/mL (> 260 µM), showing marginal cytotoxicity against host cells (▶ **Table 1**). This result is important as a major limitation of the drugs used currently to treat leishmaniasis is their high toxicity.

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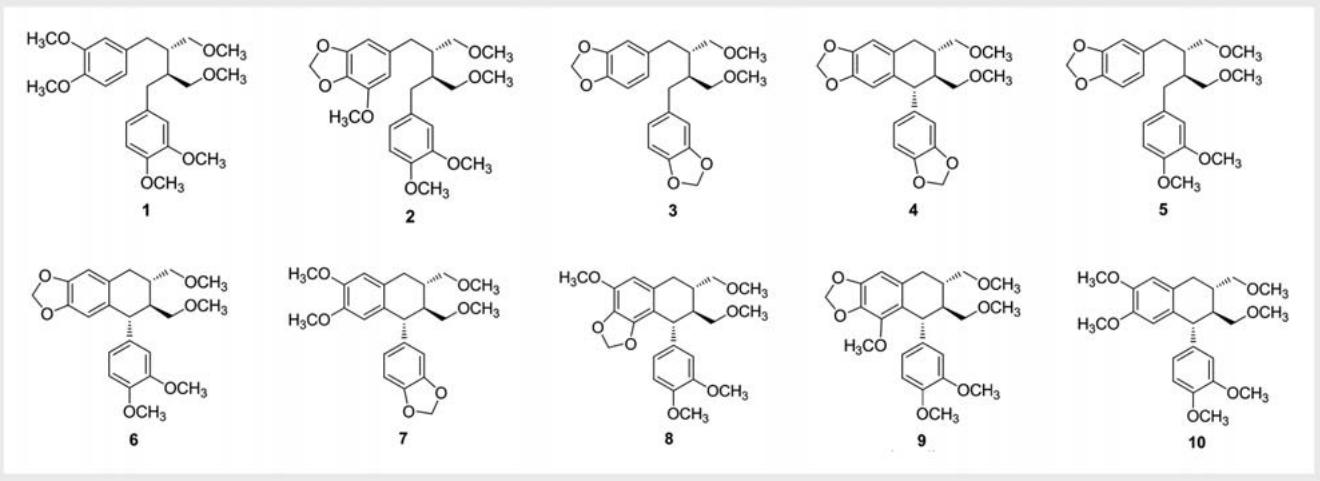

▶ **Fig. 1** Chemical structures of phyllanthin (**1**), niranthin (**2**), cubebin dimethyl ether (**3**), urinatetralin (**4**), 5-demethoxyniranthin (**5**), isolintetralin (**6**), lintetralin (**7**), hypophyllanthin (**8**), nirtetralin (**9**), and phyltetralin (**10**).

Among the lignans evaluated in the present study, only niranthin (**2**) was previously investigated for its antileishmanial activity. Chowdhury et al. (2012) reported that niranthin inhibits the amastigotes proliferation of 2 *L. donovani* strains, AG83 and GE1, which are resistant to antimonials, with EC_{50} of 1.26 and 1.68 µM, respectively, and show low cytotoxicity to host cells [20].

The effects of lignans **5, 7,** and **9** against *L. amazonensis* intracellular amastigotes at nontoxic concentrations to macrophages are shown on ▶ **Fig. 2**. A 91 % reduction was observed in the number of amastigotes treated with 41.68 µg/mL (103.68 µM) of compound **5,** and approximately 84 % reduction was observed at concentrations of 18.86 µg/mL (47.5 µM) and 37 µg/mL (86.04 µM) of compounds **7** and **9**, respectively, when compared to the control groups. Compounds **5, 7**, and **9** showed EC_{50} of 1.1 µg/mL (2.76 µM), 3.32 µg/mL (8.30 µM), and 6.80 µg/mL (15.83 µM), respectively, showing more potent activity against intracellular amastigotes than promastigotes.

The fat-soluble properties of lignans possibly favor their penetration into the host cell membranes, thus promoting their interaction with the parasites. It is suggested that the compounds remain stable after entering the acidic intracellular medium of the parasitophorous vacuole and are able to reach their target.

According to the GHIT Fund, potentially "successful compounds" for leishmaniasis chemotherapy should have EC_{50} values of less than 10 µM against intracellular amastigotes of *Leishmania spp.* in a preliminary *in vitro* infection assay [39]. Therefore, according to the criteria established by the GHIT Fund, compounds **5** and **7** should be considered candidate molecules for further evaluation *in vivo*.

Parasites of the genus *Leishmania*, as well as other trypanosomatid parasites, have only 1 mitochondrion, considered the main source of ATP and therefore a potent chemotherapeutic target. Parasiticidal activity of conventional antileishmanial drugs such as amphotericin B, miltefosine, and pentamidine is attributed to increased membrane permeability and potential collapse of mitochondrial membrane [40].

In order to investigate if the antileishmanial activity presented by lignans **2, 3**/**4, 5, 7**, and **9** can be attributed to some interaction with the parasite's mitochondria, the respiratory control of *L. amazonensis* promastigotes was determined by the measurement of oxygen consumption (▶ **Fig. 3**).

O_2 consumption of promastigotes treated with EC_{25} concentrations showed no significant difference compared with the control group. On the other hand, a decrease in respiratory control of 38 % and 25 % of promastigotes was observed after treatment with compounds **2** and **9** at concentrations of 12.14 µg/mL (28.10 µM) and 9.29 µg/mL (21.60 µM), respectively. Thus, these compounds did not exhibit a direct action on the mitochondrial respiratory chain at the tested concentrations. However, the mitochondrial membrane potential changed and, consequently, respiratory control decreased, suggesting lower ATP production in parasites incubated with these compounds.

HEA and the isolated lignans showed less effect on *T. cruzi* epimastigotes and *L. amazonensis* promastigotes, the parasite forms found only in the transmitter insects. On the other hand, some of the isolated lignans showed promising activity against intracellular amastigotes, which are clinically relevant forms of parasites for the pathogenesis of Chagas' disease and leishmaniasis.

To the best of our knowledge, this is the first report on the antiprotozoal activity of *P. amarus* lignans against *L. amazonensis* and *T. cruzi*. Further studies are warranted to evaluate the effects of these lignans, in particular compounds **5** and **7**, on other species of *Leishmania* and investigate their mechanisms of action, since they may be promising candidates for the development of new antiprotozoal agents.

Materials and Methods

Solvent and chemicals

Solvents for extraction and chromatography, including *n*-hexane, ethyl acetate (EtOAc), dichloromethane, acetonitrile, ethanol, and methanol (analytical grade) were purchased from Labsynth. Deu-

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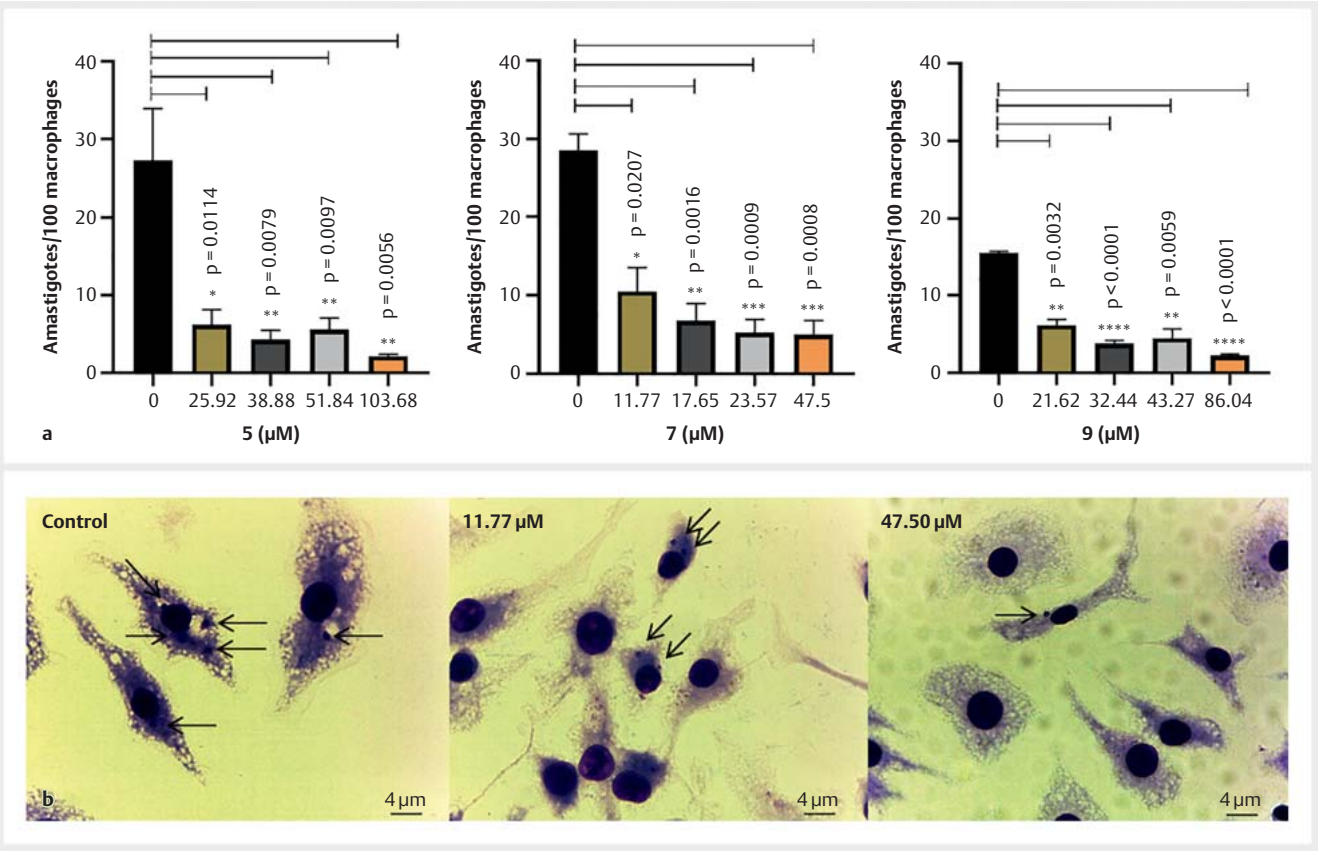
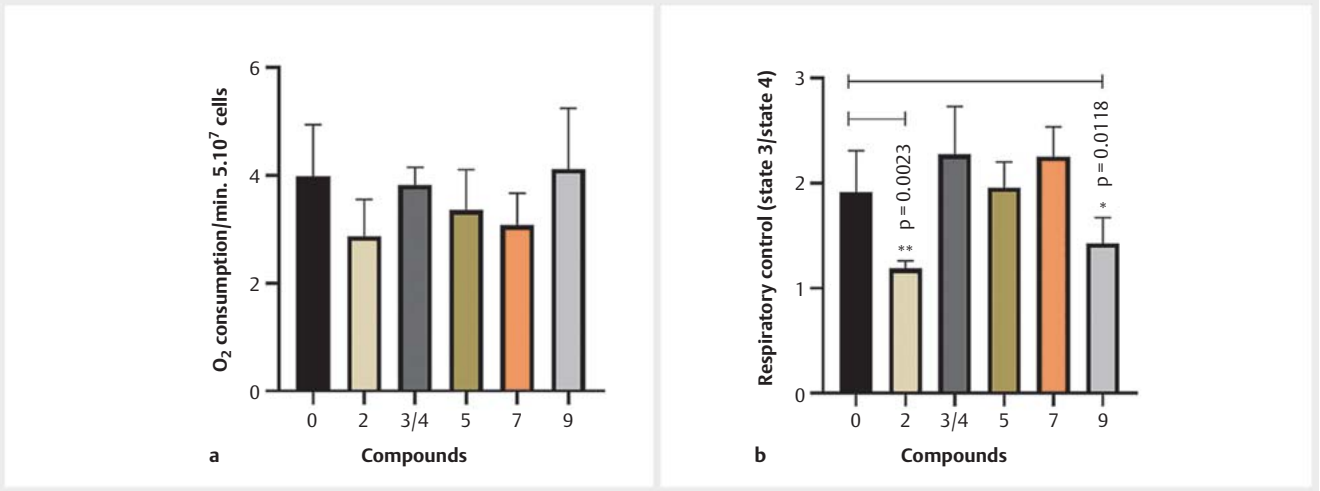
▶ **Table 1** Antiprotozoal and cytotoxic activity of extracts, fractions and isolated compounds from *P. amarus*. EC_{50}: half-maximal effective concentration; CC_{50}: half-maximal cytotoxic concentration; –: not determined; SI: Selectivity Index: CC_{50}/CE_{50} for *L. amazonensis* promastigotes.

Samples	Cell viability (%) *T. cruzi* epimastigotes		Cell viability (%) *T. cruzi* amastigotes	*T. cruzi* amastigotes	U2OS	Cell viability (%) *L. amazonensis* promastigotes		*L. amazonensis* promastigotes	*L. amazonensis* amastigotes	Macrophages (BMDM)	SI (CC_{50}/CE_{50})
	50 µg/mL	100 µg/mL	10 µg/mL	CE_{50} (µg/mL)	CC_{50} (µg/mL)	50 µg/mL	100 µg/mL	CE_{50} (µg/mL)	CE_{50} (µg/mL)	CC_{50} (µg/mL)	
1	> 100	84.6	49.79	≥ 100	≥ 100	69.79	63.85	–	–	–	–
2	70.6	60.6	8.42	15.24	≥ 100	31.25	26.77	24.34	–	≥ 100	≥ 4.10
3/4*	–	–	–	–	–	–	–	23.77	–	≥ 100	≥ 4.20
5	–	–	–	–	–	–	–	20.85	1.12	≥ 100	≥ 4.79
7	–	–	–	–	–	24.65	23.34	9.43	3.32	≥ 100	≥ 10.60
8	≥ 100	≥ 100	≥ 100	–	–	70.87	72.84	–	–	–	–
9	58.45	64.6	33.12	49.14	≥ 100	21.90	19.63	18.61	6.80	≥ 100	≥ 5.37
10	≥ 100	≥ 100	≥ 100	–	–	69.09	69.71	–	–	–	–
F4	58.25	62.6	≥ 100	–	–	31.67	27.46	16.85	–	≥ 100	≥ 5.93
F5	59.15	58.25	≥ 100	–	–	34.60	30.36	41.41	–	≥ 100	≥ 2.41
HEA	65.3	55.6	46.67	≥ 100	≥ 100	39.89	30.13	72.17	–	≥ 100	≥ 1.38
EE	≥ 100	≥ 100	–	–	–	≥ 100	≥ 100	–	–	–	–
Ampho B[1]	–	–	–	–	–	–	–	0.53		–	–
Benz[2]	–	–	–	7.45	≥ 100	–	–	–		–	

[1] Amphotericin B (reference drug for leishmaniasis); [2] Benznidazole (reference drug for Chagas'disease); * the compounds were tested as mixture of 2 : 1 ratio



▶ **Fig. 2** *In vitro* activity of **5**, **7**, and **9** against intracellular *Leishmania* (L.) *amazonensis* amastigotes. Macrophages derived from BALB/c mice bone marrow were infected with *L. amazonensis* stationary promastigotes overnight (MOI = 5). After 24 h, infected cells were incubated with different concentrations of each compound for 24 h. MeOH-fixed cells were stained, and infection was determined by counting 300 cells/coverslip. Experiments were performed in triplicate. The results shown are representative of 2 independent experiments. **a** Bars indicate the number of intracellular amastigotes. **b** Photomicrograph examples showing untreated infected macrophages and infected macrophages incubated with compound **7** at 11.77 and 47.50 μM concentration. Arrows indicate intracellular amastigotes. Bar = 4 μm.



▶ **Fig. 3** (**a**) Oxygen consumption and (**b**) respiratory control of *Leishmania amazonensis* promastigotes treated with compounds **2**, **3/4**, **5**, **7**, and **9** at 28.1, 30.8, 25.9, 11.8, and 21.6 μM (EC_{25}) concentrations, respectively, and untreated promastigotes (0). O_2 consumption was determined at the cell density of 5×10^7 cells/mL. The cells were resuspended in the respiration medium and added to the Oxygraph containing 40 μM digitonin and 5 mM succinate (**a**) and in the presence of ADP (400 μM) and oligomycin (1 μg/mL) (**b**). Data are expressed as the mean ± SD of 2 independent experiments performed in triplicate.

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terated chloroform and tetramethylsilane (TMS) used for NMR were from Merck and Sigma, respectively.

Chemicals for bioassays including DMSO, MTT, DMEM, medium 199, hemin, triethanolamine, adenine, L-glutamine, sucrose, potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), magnesium chloride ($MgCl_2$), HEPES, egtazic acid (EGTA), BSA, digitonin, succinate, adenosine diphosphate (ADP), oligomycin, and carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) were purchased from SIGMA. FBS, penicillin, and streptomycin were obtained from Cultilab. Benznidazole was kindly provided Dr Antônio Eros de Almeida (FCM-UNICAMP). Deionized water was obtained from a Simplicit Millipore water purification system.

General experimental procedures

Extracts, fractions, and isolated compounds were analyzed by TLC using precoated silica gel plates (F_{254}-Merck) and hexane-EtOAc (70 : 30) as mobile phase. Compounds were detected under UV at 254 nm, followed by spraying with an anisaldehyde solution and heating at 100 °C for 5 min. Compounds fractionation was performed on a CombiFlash *Rf+* chromatography system with UV–Vis detector (Teledyne ISCO). GC-MS analysis was performed on an Agilent Gas Chromatograph, model 6890 N coupled to Agilent Mass Spectrometry detector 5975 and a HP5-MS column (J & W Scientific). The chromatographic conditions used for GC-MS were: injector: 280 °C, detector: 300 °C, column: 150 °C for 2 min; 5 °C · min^{-1} until 240 °C; 10 °C · min^{-1} until 300 °C and keeping this temperature during 34 min. The injection volume was 1.0 µL with entrainment gas flow (He) of 1.0 mL/min. All NMR experiments were conducted on a Bruker Avance 500 MHz spectrometer. Data processing was performed using TopSpin software (Bruker) and ACD/NMR Processor Academic Edition.

Plant material

The plant material was collected at the Multidisciplinary Center for Chemical, Biological, and Agricultural Research Experimental Field of UNICAMP. The species was identified by Professor Grady L. Webster (University of California Davis, USA), and a voucher specimen (UEC 127.411) has been deposited in the Biology Institute, UNICAMP.

Extraction and isolation

The dry powders of *P. amarus* leaves (1 kg) were extracted by Soxhlet using *n*-hexane-ethyl acetate (1 : 1 v/v; 3 L) for 16 h followed by ethanol (2 L) for 8 h. The solvents were removed on a rotary evaporator (MOD 802, Fisatom) providing the hexane-EtOAc (HEA, 88 g) and ethanolic (EE, 63 g) extracts.

The HEA was subjected to open column chromatography using silica gel 60 (900 g, Merck, 0.063–0.200 mm). The column was eluted with *n*-hexane and EtOAc with a gradual increase in polarity toward 100% EtOAc. All 7 fractions were concentrated under reduced pressure and analyzed by TLC and GC-MS.

Fraction 4 (11 g) was separated by flash chromatography on a silica gel column (0.040–0.063 mm, 23 cm height × 3.5 cm diameter, 50 g, RediSep Rf) with hexane-EtOAc mixture of increasing polarities, resulting in 28 subfractions. Subfraction 3 (600 mg) enriched in compounds **3** and **4**, was submitted to open column chromatography using Florisil (20 g, Merck, 0.150–0.250 mm, 12.5 cm height × 1 cm diameter) and a gradient of hexane-CH_2Cl_2 until 100% CH_2Cl_2 followed by 100% EtOAc and 100% MeOH. Seven subfractions (A–G) were obtained. Subfraction A (260 mg) which contained compounds **3** and **4** as revealed by GC-MS analysis was further purified by flash chromatography [silica gel, 50 g, 23 cm height × 3.5 cm diameter, RediSep Rf, hexane-EtOAc (90 : 10)] to provide a sample (127 mg) composed of only cubebin dimethyl ether (**3**) and urinatetralin (**4**) in a 2 : 1 ratio as determined by GC-MS and NMR analysis.

Column chromatography of Fraction 5 (11.6 g) on Florisil (380 g, Merck, 0.150–0.250 mm, 12.72 cm height × 3.16 cm diameter), with hexane-CH_2Cl_2 (80 : 20) gave a fraction enriched in compounds **5, 6**, and **7** (F5.1). Fraction 5.1 (4.4 g) was separated by flash chromatography, using silica gel high-resolution stationary phase (RediSep Rf 20–30 µm, 21 cm height × 3.5 cm diameter, 80 g), and hexane-EtOAc (97 : 3) to afford lintetralin (**7**; 154.7 mg), **5** (86.4 mg), and **6** (27.8 mg) after final purification by flash chromatography using C18 (24 g, RediSep Rf 40–63 µm, 4.26 cm height × 0.89 cm diameter) with 0–80% aq, acetonitrile in 65 min. Isolation of lignans **1** (412 mg), **2** (352 mg), **8** (302 mg), **9** (85 mg), and **10** (174 mg) was performed as previously described [10]. These compounds were identified through GC-MS, ^{1}HNMR, and ^{13}CNMR analysis.

In vitro cell culture

Human osteosarcoma cell line U2OS, obtained from the Rio de Janeiro Cell Bank (BCRJ, Brazil), and monkey kidney epithelial cell LLC-MK2, kindly provided by S. Schenkman (Federal University of São Paulo, Brazil), were cultured in DMEM high-glucose media, supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, in a humid atmosphere of 5% CO_2 at 37 °C. LLC-MK2 tissue cultures were used to support *T. cruzi* mammalian cycle *in vitro*. The T. cruzi Y strain was originally provided by S. Schenkman and a clone (Y-H10) was generated through limiting dilution at the Institut Pasteur Korea (South Korea) [41]. Trypomastigote forms were obtained from the supernatant of LLC-MK2 tissue cultures and used to infect U2OS cells. All infected cultures, containing intracellular parasites, were maintained in DMEM low-glucose media (Sigma-Aldrich), supplemented as described for mammalian cells, but with 2% FBS [41]. *T. cruzi* epimastigotes (Y strain) were kindly provided by Dr Maria Júlia Manso Alves (Chemistry Institute-University of São Paulo) and were grown in liver infusion tryptose medium supplemented with 20 mg/L hemin and 10% fetal calf serum at 28 °C [42]. *L. amazonensis* strain MHOM/BR/1973/M2269 was kindly provided by Dr Silvia Uliana (Biomedical Sciences Institute-University of São Paulo). *L. amazonensis* promastigotes were maintained at 26 °C in Medium 199 (Sigma-Aldrich) supplemented with 5% penicillin/streptomycin, 0.1% hemin (25 mg/mL in 50% triethanolamine), 20% heat-inactivated FBS, 10 mM adenine (pH 7.5), and 5 mM L-glutamine [43].

The cytotoxic activity was evaluated using bone marrow macrophages from BALB/c female mice maintained in the animal house of the Dept. of Biology Animal, Biology Institute-UNICAMP. Briefly, femur and tibia were removed from euthanized mice for isolation of bone marrow content by washing with 5 mL of Me-

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dium RPMI supplemented with 20% FBS and 30% of L929 fibroblast supernatant. After 7 days, cells were completely differentiated and used for cytotoxicity and intracellular infection assays, as described below.

Antileishmanial and antitrypanosomal activity assays

Assays with *T. cruzi* amastigotes were performed as previously described [41]. The samples were assayed in concentration-response experiments (10 concentration points and 2-fold dilution), with the highest concentration at 100 µg/mL, except for benznidazole (400 mM). Infected cultures were exposed to compounds for 96 h. All plates included multiple wells of infection treated only with 1% DMSO as negative controls and noninfected wells (blank containing only U2OS cells, also treated with 1% DMSO) as positive controls. High content analysis was performed with High Content Imaging System Operetta (PerkinElmer) at 20 × magnification. Images were analyzed for identification, segmentation, and quantification of host cell nucleus, cytoplasm, and intracellular parasites [41].

The antiparasitic activity of the extracts, fractions, and compounds isolated from *P. amarus* were assessed against *L. amazonensis* promastigotes and *T. cruzi* epimastigotes populations, using a MTT viability assay after 24 h of incubation with the samples previously mentioned [43]. The cytotoxic activity was evaluated by a MTT assay using bone marrow macrophages from BALB/c female mice. Differentiated cells were cultured in 96-well plates at 37 °C for posterior incubation with active samples at increasing concentrations for 24 h. CC_{50} values were calculated.

Intracellular infections with **5, 7**, and **9** compounds were obtained by infecting bone marrow-derived macrophages with stationary phase *L. amazonensis* promastigotes (5 parasites: 1 macrophage; (MOI = 5); 5×10^5 infected macrophages were cultivated on coverslips added to 24-well plates, maintained at 37 °C, 5% CO_2, for 24 h, and exposed to compounds for an additional 24 h in the same conditions. After that, cells were fixed with MeOH for subsequent staining using the Instant Prov kit (Newprov). The number of intracellular amastigotes was obtained by counting 300 cells in triplicate coverslips. Infections photomicrographs were recorded using the Leica LAS Core microscope system.

Experiments using BALB/c mice were approved by the Ethical Committee for Animal Experimentation of the Biology Institute of the State University of Campinas on June 20, 2017 (4535-1).

Oxygen uptake measurements

O_2 consumption was monitored in a computer-interfaced Clark-type oxygen electrode with continuous stirring at 28 °C (Hansatech Systems). *L. amazonensis* promastigotes in logarithmic phase (5×10^7) treated with compounds **2, 3/4, 5, 7**, and **9**, as well as the untreated group (control), were resuspended in a standard intracellular reaction medium (125 mM sucrose, 65 mM KCl, 2 mM KH_2PO_4, 0.5 mM $MgCl_2$, 10 mM HEPES pH 7.2, 1 mM EGTA, and 1 mg/mL BSA) in the presence of 40 µM digitonin and 5 mM succinate. Respiratory control ratio (state 3/state 4) was determined by the addition of 400 µM ADP (state 3) followed by 2 µg/mL oligomycin (state 4). Maximum oxygen consumption rates were determined in the presence of 1 µM carbonyl cyanide m-chlorophenylhydrazone [42].

Statistical analysis

Concentration-response curves were processed with the Analysis Software Origin 8.0, for sigmoidal dose-response nonlinear curve fitting generation, and determination of EC_{50} values by interpolation. EC_{50} was defined as compound concentration corresponding to at least 50% infection reduction (50% normalized activity). CC_{50} is defined as the compound concentration that reduced the cell ratio by half compared with the average number of cells in the negative control wells.

Intracellular infections and oxygen uptake measurement results were analyzed with the Graphpad Prism software and are expressed as the mean ± S. E. M. of 3 independent experiments. Student's t-test was used for statistical analyses. P values > 0.05 were considered significant.

Supporting information

^1H, ^{13}C, COSY, HSQC, and HMBC NMR spectra of compounds **3, 4**, and **7**, as well as ^1H and ^{13}C NMR and GC-MS data of compounds **1–10** and concentration-response curves are available as Supporting Information.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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