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ORIGINAL PAPER

Anti-malarial, anti-trypanosomal, and anti-leishmanial activities of jacaranone isolated from *Pentacalia desiderabilis* (Vell.) Cuatrec. (Asteraceae)

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Abstract Leishmaniasis, Chagas disease, and malaria affect the poorest population around the world, with an elevated mortality and morbidity. In addition, the therapeutic alternatives are usually toxic or ineffective drugs especially those against the trypanosomatids. In the course of selection of new anti-protozoal compounds from Brazilian flora, the CH_2C_{l2} phase from MeOH extract obtained from the leaves of *Pentacalia desiderabilis* (Vell.) Cuatrec. (Asteraceae) showed in vitro anti-leishmanial, anti-malarial, and anti-trypanosomal activities. The chromatographic fractionation of the CH_2Cl_2 phase led to the isolation of the bioactive compound, which was characterized as jacaranone [methyl (1-hydroxy-4-oxo-2,5-cyclohexandienyl) acetate], by spectroscopic methods. This compound showed activity against promastigotes of *Leishmania* (L.) *chagasi*, *Leishmania* (V.) *braziliensis*, and *Leishmania* (L.). *amazonensis* showing an IC_{50} of 17.22, 12.93, and 11.86 μg/mL, respectively. Jacaranone was also tested in vitro against the *Trypanosoma cruzi* trypomastigotes and *Plasmodium falciparum* chloroquine-resistant parasites (K1 strain) showing an IC_{50} of 13 and 7.82 μg/mL, respectively, and was 3.5-fold more effective than benznidazole in anti-*Trypanosoma cruzi* assay. However, despite of the potential against promatigotes forms, this compound was not effective against amastigotes of *L.* (L.) *chagasi* and *T. cruzi*. The cytotoxicity study using Kidney Rhesus monkey cells, demonstrated that jacaranone showed selectivity against *P. falciparum* (21.75 μg/mL) and a selectivity index of 3. The obtained results suggested that jacaranone, as other similar secondary metabolites or synthetic analogs, might be useful tolls for drug design for in vivo studies against protozoan diseases.

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Introduction

The genus *Pentacalia* Cass. belongs to Senecioneae tribe, the largest of the Asteraceae, which consists of over 205 species in the tropical Americas (Teles and Stehmann 2008). The chemical composition of *Pentacalia* reports the isolation of terpenoids and phenylpropanoids from *Pentacalia ephiphytica*, *Pentacalia andicola*, *Pentacalia firmipes*, and *Pentacalia archieri* (Bohlmann and Ziesche 1979; Bohlmann et al. 1984) while steroids, triterpenes, coumarins, and flavonoids were obtained from *Pentacalia corymbosa* (Torrenegra et al. 2000). Recently, two quinoids were isolated from *P. ledifolia* and *P. corymbosa*, which showed antifungal activity (Pedrozo et al. 2006). In Brazil, only two species have been described: *P. desiderabilis*, whose occurrence is restricted to the south, southeast, and northeastern regions,

and *P. tropicalis*, found only in the Espìrito Santo and Rio de Janeiro states. *P. desiderabilis* (Fig. 1) is an attractive liane with leaves often fleshy to subfleshy, radiate heads of yellow florets in thyrsoid to corymbose paniculate capitulescences (Teles and Stehmann 2008). However, no information about *P. desiderabilis*' chemistry or pharmacological aspects has been described in the literature.

Neglected protozoan diseases such as malaria, Leishmaniasis, and Chagas disease affect millions of people worldwide, with high morbidity and mortality. Cross-sectional studies in the 1980s indicated that the prevalence of *T. cruzi* in the 18 disease-endemic countries of Latin America was 4.72% (16–18 million) of the population, with an incidence of 700,000–800,000 new cases per year and approximately 45,000 deaths per year due to cardiac disease (Tempone et al. 2007). Presently, the available drugs to treat Chagas disease produce toxic side effects. The nitroheterocycles nifurtimox and benznidazole, the only two drugs used in the early stages of American trypanosomiasis owe their action to the reduction of a nitro group corresponding to an anion radical. This also appears to be the cause of the toxic reactions observed in about 50% of the patients (Tempone et al. 2007).

Fig. 1 Photos of the *P. desiderabilis* (Vell.) Cuatrec. studied specimen

Malaria is an infectious disease caused by the protozoan parasite *Plasmodium* spp. According to the World Health Organization, 243 million cases and 863,000 deaths were registered in all continents in 2008, with most cases related to *P. falciparum*. Malaria occurs in 109 countries and 85% of the registered cases are located in Africa (WHO 2009). The decreased sensitivity of *P. falciparum* to antimalarials is a result of drug pressure in the parasite genome, causing mutations that enable the development of mechanisms for the parasite survival even in the presence of drugs (Samudio et al. 2005). Thus, the emerging resistance of *P. falciparum* to almost all commercialized drugs reveals an alarming need for monitoring the parasite susceptibility, as well as the search for new chemotherapeutic agents (Quashie et al. 2006; Mancini et al. 2008).

Leishmaniasis is among the most neglected diseases, affecting about 12 million people worldwide. Caused by a wide range of species, *Leishmania* could result in a single cutaneous ulceration or a fatal and progressive visceral disease. The chemotherapy is highly toxic, including from metal drugs (antimonials) to the toxic antifungal as amphotericin B (Tempone et al. 2011).

Consequently, a continued search for new and less toxic anti-parasitic compounds is a justification for ongoing screening of natural products as candidate prototypes. In this work, we isolated the quinoid derivative jacaranone from leaves of *P. desiderabilis* (Vell.) Cuatrec. (Asteraceae) and performed in vitro anti-malarial, anti-trypanosomal, and anti-leishmanial assays to test its efficacy as anti-parasitic prototype. This plant was chosen due to their antiradical potential observed in previous DPPH assays with plant extracts from Campos de Altitude (unpublished work).

Material and methods

General procedures

Silica gel (Merck 230–400 mesh) was used for CC separation while silica gel 60 PF_{254} (Merck) was used for analytical (0.25 mm) TLC. LREIMS spectrum was measured at 70 eV on Finnigan-Mat INCOS50 quadrupole spectrometer and ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were measured on a Bruker model DPX-300 spectrometer with sample dissolved in $CDCl_3$ (Tédia Brazil).

Materials

Sodium dodecyl sulfate (SDS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Thiazol blue; MTT), M-199, and RPMI-PR-1640 medium (without phenol red), benznida-

zole, were purchased from Sigma (St Louis, MO USA). Pentavalent antimony (Glucantime-Aventis-Pharma, Brazil) and pentamidine (Sideron, Brazil) were used as standard drugs. Other analytical reagents were purchased from Sigma (St Louis, MO, USA) unless where stated otherwise.

Plant material

Leaves from *P. desiderabilis* (Vell.) Cuatrec. (Fig. 1) were collected in Campos do Jordão, São Paulo, SP, in August 2008. Voucher specimen had been deposited at Herbarium of D. Bento Pickel—Instituto Florestal under number SPSF 37596.

Isolation of jacaranone from leaves of *P. desiderabilis*

Dried and powdered leaves of *P. desiderabilis* (232.5 g) were defatted with hexane and then exhaustively extracted using MeOH. After filtration, the combined extracts were evaporated to yield 13.8 g of MeOH extract. This extract was partitioned using hexane, CH_2Cl_2, and EtOAc to afford the respective phases (hexane—0.41 g, CH_2Cl_2—2.50 g, and EtOAc—1.08 g). Part of CH_2Cl_2 phase (0.4 g) was subjected to silica gel column chromatography eluted with CH_2Cl_2 containing increasing amounts of EtOAc (up to 100%) and EtOAc containing increasing amount of MeOH (up to 100%), to give 12 fractions (1–12). Jacaranone was isolated as colorless needles (132.5 mg) from fraction 4. Purity was assessed by gas chromatography–mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) as >99%.

Jacaranone [methyl (1-hydroxy-4-oxo-2,5-cyclohexandienyl) acetate]. Colorless needles; ^{1}H NMR ($CDCl_3$, 300 MHz): 6.97 (d, J=10.2 Hz, H-2′/H-6′), 6.21 (d, J=10.2 Hz, H-3′/H-5′), 3.75 (s, $OC\underline{H}_3$), 2.72 (s, H-2). ^{13}C NMR ($CDCl_3$, 75 MHz): 171.0 (C-1), 43.4 (C-2), 67.3 (C-1′), 149.0 (C-2′/C-6′), 128.2 (C-3′/C-5′), 185.0 (C-4′), 52.2 (O<u>C</u>H3). LREIMS m/z (rel. int.): 182 [M^+] (3), 166 (2), 150 (19), 122 (16), 109 (84), 94 (8), 81 (44), 74 (100), 69 (7), 59 (19), 53 (36).

Animals

BALB/c mice and golden hamsters (*Mesocricetus auratus*) were supplied by the animal breeding facility at the Adolfo Lutz Institute of São Paulo and maintained in sterilized cages under a controlled environment, receiving water and food ad libitum. Animal procedures were performed with the approval of the Research Ethics Commission.

Parasite maintenance

L. (L.) *chagasi* (MHOM/BR/1972/LD) amastigotes were maintained in golden hamsters, up to approximately 60 to 70 days post-infection. Isolated promastigotes of *L.* (L.) *chagasi* (MHOM/BR/1972/LD), *L. braziliensis* (MHO/BR/75/M2903), and *L.* (L.) *amazonensis* (WHO/BR/00/LT0016) were maintained in M-199 medium supplemented with 10% calf serum and 0.25% hemin at 24°C.

The K1 chloroquine-resistant *P. falciparum* strain was kindly provided by the Institute of Animal Genetics (Edinburgh, Scotland) and maintained in continuous culture in the Center for Research on Malaria-SUCEN. The parasitized blood was diluted to a hematocrit of 5% in culture medium Roswell Park Memorial Institute (RPMI) 1640 (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 30 mM HEPES (Sigma Chemical Co., St. Louis, MO, USA), hypoxanthine 50 mg/mL (Sigma Chemical Co., St. Louis, MO, USA), 10% human serum, gentamicin 20 mg/mL (Schering-Plough, Brazil), and 5% sodium bicarbonate (Rieckmann et al. 1978; Di Santi et al. 1988).

Peritoneal macrophage collection

Peritoneal macrophages were collected from the peritoneal cavity of female BALB/c mice by washing with RPMI-1640 without phenol red, supplemented with 10% calf serum. Cells were dispensed in 96- or 24-well microplate and maintained for 1 h in the same medium at 37°C in a 5% CO_2 humidified incubator for attachment. Non-adherent cells were removed by two step washings with medium.

Determination of the 50% inhibitory concentration (IC_{50}): *Leishmania*

Promastigotes were counted in a Neubauer hemocytometer and seeded at 1×10^6 cells/well in 96-well microplates using pentamidine as standard (Tempone et al. 2004). Jacaranone was dissolved in methanol, diluted in M-199 medium, and incubated with parasites in different concentrations (based on dry weight) for 24 h at 24°C. Parasite viability was determined using the MTT assay at 550 nm (Tada et al. 1986). Each assay was performed in triplicate. The activity against *L. chagasi* intracellular amastigotes was determined with infected macrophages, using pentavalent antimony as standard. Briefly, peritoneal macrophages from Balb/c mice were seeded at 4×10^5 cells/well in 13 mm glass cover slips in 24-well microplates for 24 h at 37°C in a 5% CO_2 humidified incubator. Amastigotes obtained from hamster spleen by differential centrifugation (Stauber et al. 1958) were added to macrophages at 10:1 ratio (amastigotes/macrophage) and incubated for 24 h. Non-internalized parasites were removed by washing and test compounds were then incubated for 120 h at the same conditions. Finally, glass cover slips were fixed with methanol, stained with Giemsa, and observed in a light microscope. The parasite burden was defined as the

mean number infected macrophages out of 600 cells (Tempone et al. 2004).

Anti-trypanosomal activity Cell-culture-derived trypomastigotes from LLC-MK2 cells were counted in a Neubauer hemocytometer and seeded at 1×10^6 cells/well in 96-well microplates. Jacaranone was incubated to the highest concentration of 100 μg/mL for 24 h at 37°C in a 5% CO_2 humidified incubator with benznidazole as the standard drug. The trypomastigotes viability was based on the cellular conversion of the soluble tetrazolium salt MTT into the insoluble formazan by mitochondrial enzymes (Lane et al. 1996). The formazan extraction was carried out with 10% (*v*/*v*) SDS for 18 h (100 m well) at 24°C (Tada et al. 1986) in a spectrophotometer Multiskan MS (Uniscience) microplate reader.

Anti-malarial activity The *P. falciparum* culture was synchronized by centrifugation at 1,200 rpm for 5 min, and the pellet containing the young forms was collected. This procedure was adopted in order to avoid the use of chemicals in synchronization, which could compromise the viability of parasites. The tests were carried out with the stage of young trophozoites and parasitemia of 1% in 96-well sterile flat-bottomed microplates (TPP, Switzerland). Jacaranone was serially diluted in RPMI in order to obtain concentrations from 300 to 0.097 μg/mL. Two wells were used as controls without addition of jacaranone. Finally, 10 μL of human red blood cells (RBC) were added to all wells in order to obtain a hematocrit of 5%. All tests were performed in duplicate. The plate was incubated at 37°C in CO_2 atmosphere for 24 h. After this period, as schizont stage was predominant in the control, thick and thin blood smears were performed with RBC from all different concentrations and controls of the plate. The slides were stained with Giemsa and observed in light microscopy (×1,000 magnification). Evaluation of IC_{50} was performed by counting the number of schizonts with more than three nuclei in 200 parasites. The value found in each well with jacaranone was compared with controls without drug, to calculate the percentage of growth inhibition in the presence of the compound (Rieckmann et al. 1978).

Cytotoxicity studies

Kidney Rhesus monkey cells (LLC-MK2) were cultured using RPMI-1640 medium supplemented with 10% calf serum at 37°C in a 5% CO_2 humidified incubator. Cells were removed by scrapping, seeded at 4×10^4 cells/well in 96-well microplates and further incubated with drugs for 48 h at 37°C, using pentamidine and Glucantime® as control. The viability of the cells was determined using MTT assay at 550 nm (Tada et al. 1986).

Hemolytic activity

Jacaranone was tested in different concentrations on red blood cells in order to evaluate the possibility of causing hemolysis. A stock solution (30 mg/mL in MeOH) was serially diluted to obtain concentrations ranging from 300 to 1.17 μg/mL. Three wells were included as controls: one containing 100 μL of distilled water equivalent to 100% hemolysis, one with 100 μL of phosphate-buffered saline (PBS) representing absence of lysis and the last one with methanol (MeOH), in order to confirm the lack of the solvent toxicity. Human blood was collected aseptically in EDTA tube (BD Vacutainer, Franklin Lakes, NJ, USA) and centrifuged at 1,000 rpm for 5 min. The RBC was washed with PBS, the supernatant was discarded and 200 μL of the pellet were diluted 10× in PBS. The solution was distributed in a volume of 100 μL/well and added to a 96-well U-shaped microplate (Costar, Cambridge, MA, USA). Finally, it was added 100 μL of RBC solution in all wells. The plate was gently homogenized and incubated for 2 h at room temperature. After this time, 100 μL of supernatant from each well were transferred to a new plate, which was read at Organon Teknika Microwell plate reader 530 (Organon Teknika, West Chester, PA, USA), 540 nm.

Statistical analysis

The data obtained represent the mean and standard deviation of triplicate samples from two independent assays. The IC_{50} values were calculated using sigmoid dose–response curves performed using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego, CA, USA.

Results

Chemical characterization of jacaranone

The ^{1}H NMR spectrum showed two doublets at 6.97 (J=10.2 Hz, 2H) and 6.21 (J=10.2 Hz, 2H), which could be assigned, respectively, to H-2′/H-6′ and H-3′/H-5′ of a quinoid derivative (Hase et al. 1995). Additionally, this spectrum

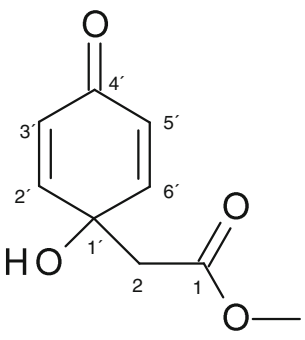

Fig. 2 Structure of jaracarone, isolated from leaves of *P. desiderabilis*

Table 1 Anti-leishmanial and anti-trypanosomal activity of jacaranone

IC_{50} (μg/mL; 95% CI)

	L. (L.) *chagasi* promastigotes	*L.* (L.) *chagasi* amastigotes	*L.* (V.) *braziliensis* promastigotes	*L.* (L.) *amazonensis* promastigotes	*T. cruzi* trypomastigotes	*T. cruzi* amastigotes	MK2 toxicity
Jacaranone	17.22 (15.64 to 18.97)	ne	12.93 (11.79 to 14.19)	11.86 (10.97 to 12.82)	13.02 (10.47 to 16.19)	ne	21.75 (15.89 to 29.79)
Pentamidine	0.23 (0.18 to 0.28)	nd	0.37 (0.31 to 0.43)	0.33 (0.27 to 0.39)	nd	nd	7.96 (7.34 to 9.73)
Glucantime	nd	26.75 (23.87 to 29.65)	nd	nd	nd	nd	>100
Benznidazole	nd	nd	nd	nd	45.74 (42.89 to 49.65)	>50	>100

The viability of parasites was determined by the colorimetric method of MTT at 550 nm

IC_{50} effective concentration 50%, *95% CI* 95% confidence interval, *nd* not determined, *ne* not effective

showed two singlets attributed to one α-carbonyl methylene group at 2.72 (2H, H-2) as well as to one methoxyl group linked to C-1 at 3.75 (3H). The LREIMS spectrum showed the molecular ion peak at *m/z* 182, which allowed to assign the molecular formula $C_9H_{10}O_4$ to the isolated compound. Finally, ^{13}C NMR spectrum indicated the presence of seven signals, including two carbonyl carbons at 185.0 and 171.0, assigned to C-4′ and C-1, respectively, two sp^2 carbons at 149.0 (C-2′/C-6′) and 128.2 (C-3′/C-5′), as well as three sp^3 carbons at 67.3 (C-1′), 43.4 (C-2), and 52.2 (OCH_3). Comparison of spectroscopic data with those reported in the literature (Lajide et al. 1996) allowed the identification of bioactive compound as methyl (1-hydroxy-4-oxo-2,5-cyclohexandienyl) acetate, known as jacaranone (Fig. 2) and previously isolated from *P. corymbosa* and *P. ledifolia* (Pedrozo et al. 2006).

Anti-protozoal activities of jacaranone

Leishmania parasites were incubated for 24 h with jacaranone and the MTT was used for viability. *Leishmania* spp. were in vitro susceptible to jacaranone, resulting in an IC_{50} range between 11 to 17 μg/mL (Table 1). It was also observed that 100% of all tested species were killed at the highest tested concentrations of 100 μg/mL (data not shown). When jacaranone was tested against the clinical relevant form of *L.* (L.) *chagasi*, the intracellular amastigotes, it was observed lack of parasite selectivity, as no efficacy could be observed at those non-toxic concentrations.

Jacaranone was also tested against the etiologic agent of American Trypanosomiasis, the *T. cruzi*. The trypomastigotes also demonstrated susceptibility to jacaranone, with an IC_{50} value of 13 μg/mL (Table 1). Using the MTT assay, it was possible to demonstrate that jacaranone was trypanocidal, eliminating 100% of parasites at the highest tested concentration of 100 μg/mL (data not shown).

P. falciparum chloroquine-resistant parasites (K1 strain) were also susceptible to jacaranone, showing an IC_{50} of 7.82 μg/mL (95% CI of 7.47–8.20; Fig. 3). After 24 h incubation, a strong reduction in the number of schizonts was observed by light microscopy at the highest concentrations, confirming the anti-malarial potential of jacaranone. The cytotoxicity study using Kidney Rhesus monkey cells demonstrated that jacaranone showed to some extent selectivity against *P. falciparum*, resulting in an IC_{50} value of 21 μg/mL (95% CI of 15.89 to 29.79) against mammalian cells, and resulting in a selectivity index of approximately 3 (Fig. 2). Furthermore, no damages could be detected to human erythrocytes (RBC; hemolytic activity) in concentration about 40-fold higher than the IC_{50} value in *P. falciparum* (data not shown).

Discussion

Considering the *Leishmania* parasites, *L.* (L.) *amazonensis*, one of the most important species responsible for the cutaneous and diffuse Leishmaniasis in Brazil, was the

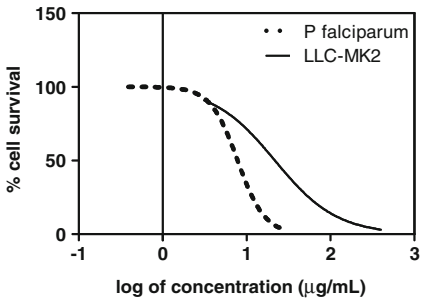

Fig. 3 Selectivity of jacaranone against *P. falciparum* chloroquine-resistant parasites (K1 strain)—anti-malarial and mammalian cytotoxicity of jacaranone. The growth inhibition of schizonts of *P. falciparum* K1 strain was determined by light microscopy. The viability of LLC-MK2 cells was determined by the colorimetric method of MTT at 550 nm

most susceptible protozoan parasite to the obtained quinoid. In contrast, the etiologic agent of visceral leishmaniasis in Brazil, the *L.* (L.) *chagasi*, was the most resistant specie. Differences in drug susceptibility have been shown among *Leishmania* parasites, including isolated compounds from plants (Velozo et al. 2006; Santin et al. 2009; Sartorelli et al. 2009; Sharma et al. 2009; Grecco et al. 2010; Guimarães et al. 2010; Kahla-Nakbi et al. 2010; Corrêa et al. 2011; Duran et al. 2011; Ghosh et al. 2011). In the test with the clinical relevant form of *L.* (L.) *chagasi*, the lack of selectivity toward amastigotes could be described to the higher metabolic resistance, a natural consequence of adaptative response to the hazardous intracellular milieu of macrophages.

The IC_{50} of 7.82 μg/mL showed by the jacaranone for the in vitro activity against the *P. falciparum* K1 strain was also displayed for other quinoids as phenylethanoid glucosides containing jacaranone-type moieties, with IC_{50} ranging from 0.55 to 4 μg/mL (Gachet et al. 2010). Other biological activities include the anticancer potential of this compound, with IC_{50} values ranging from 4 to 7 μg/mL (Loizzo et al. 2007). To our knowledge, this is the first antiprotozoan activity attributed to jacaranone.

Considering the potential anti-protozoal activity of jacaranone, further exploitation of similar secondary metabolites or synthetic analogs could contribute to the design of more active and less toxic candidate compounds for neglected diseases as malaria, Leishmaniasis, and Chagas disease.

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