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Leishmanicidal activity of a supercritical fluid fraction obtained from *Tabernaemontana catharinensis*

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Abstract

The branches and leaves of *Tabernaemontana catharinensis* were extracted with supercritical fluid using a mixture of CO_2 plus ethanol (SFE), and the indole alkaloid enriched fraction (AF3) was selected for anti-*Leishmania* activity studies. We found that AF3 exhibits a potent effect against intracellular amastigotes of *Leishmania amazonensis*, a causative agent of New World cutaneous leishmaniasis. AF3 inhibits *Leishmania* survival in a dose-dependent manner, and reached 88% inhibition of amastigote growth at 100 μg/mL. The anti-parasite effect was independent of nitric oxide (NO), since AF3 was able to inhibit NO production induced by IFN-γ plus LPS. In addition, AF3 inhibited TGF-β production, which could have facilitated AF3-mediated parasite killing. The AF3 fraction obtained from SFE was nontoxic for host macrophages, as assessed by plasma membrane integrity and mitochondrial activity. We conclude that SFE is an efficient method for obtaining bioactive indole alkaloids from plant extracts. Importantly, this method preserved the alkaloid properties associated with inhibition of *Leishmania* growth in macrophages without toxicity to host cells.

Keywords: Leishmanicidal activity; *Tabernaemontana*; *Leishmania*; Supercritical Fluid Extraction

1. Introduction

Leishmaniasis is a prominent worldwide health problem. It has been estimated that 12 million people are infected in the tropical and subtropical areas of five continents, and that 2 and 0.5 million cases of cutaneous and visceral leishmaniasis are reported each year [1]. Recently, the overlapping geographical distribution of leishmaniasis and human immunodeficiency virus (HIV) infection has contributed to a dramatic increase in the number of individuals infected with *Leishmania* [1].

The first choice treatment for leishmaniasis still relies on pentavalent antimonials, and amphotericin B or pentamidine, can be used as alternatives for resistant parasites [2]. All of these compounds present several problems that limit their use, such as severe side effects, induction of parasite resistance, in-patient administration and high cost [3]. The novel drug miltefosine is an effective treatment for visceral leishmaniasis in India, but has shown limited efficiency in other countries and for other leishmaniasis forms, and is also teratogenic [2,4]. All of these facts have given rise to the need for development of new approaches for leishmaniasis therapy.

Plants have long been used in popular medicine for the treatment of protozoan diseases, and lately have received considerable attention in the search for new compounds with anti-leishmanial activity [5]. *Tabernaemontana catharinensis* A. DC. (syn. *Peschiera catharinensis* A. DC. Miers) is an arboreal species of the *Apocynaceae* family, which resides in Southern Brazil, Argentine, Uruguay and Paraguay. This species has also been denoted *T. affinis*, *T. australis* and *T. hilariana* [6–10], and anti-tumor, anti-microbial, anti-inflammatory and analgesic activities have been reported for its extracts [7,11–14]. We recently demonstrated anti-leishmanial and anti-HIV-1 activities for an extract obtained from *T. australis* as well as the alkaloids purified from this extract [15–17].

Supercritical Fluid Extraction (SFE) is a technology that facilitates the removal of solvents from plant extracts,

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increasing selectivity and permitting the use of moderate temperatures during the extraction. Considering the significant advances resulting from this technology, we employed it to produce *T. catharinensis* fractions [18], which were further tested for anti-leishmanial activity, as well as for toxicity for vertebrate cells. Therefore, we report in this work that the selected alkaloid fraction (AF3) obtained by SFE from *T. catharinensis* extract, presents anti-*Leishmania amazonensis* activity in infected macrophages independent of nitric oxide production. Moreover, AF3 was nontoxic for macrophages, as judged by cell membrane integrity and mitochondrial activity assays. Our results suggest that SFE is a useful methodology for obtaining plant fractions with the potential for anti-leishmania phytotherapy.

2. Materials and methods

2.1. Preparation of the extract

The alkaloid fraction (AF3) of *T. catharinensis* extract was obtained by SFE in a previous work [18]. Briefly, SFE extract was obtained at 250 bar, 45 °C using supercritical CO_2 plus ethanol (4.6%m/m) as co-solvents. A sample of the extract obtained in the third hour of the process was fractionated to obtain AF3, which consists of the major alkaloid compounds coronaridine and voacangine (7% and 53% of AF3, respectively). The remaining content of the fraction (40%), although identified (voacristine, voacristine hydroxylindolenine, voacangine hydroxylindolenine, and 3-hydroxylcoronaridine), was not quantified [18]. Because coronaridine has been described as an anti-leishmanial compound [15,16], this fraction was selected for further studies of leishmanicidal activity and to validate the SFE methodology.

2.2. Parasites

L. (L.) *amazonensis*, (WHOM/BR/75/Josefa) promastigotes were cultured at 26 °C in Schneider Insect Medium (Sigma) supplemented with 10% fetal calf serum (FCS - Gibco BRL, Gaithersburg, MD, USA) and 40 μg/mL of gentamycin (Schering - Plough, Rio de Janeiro, Brazil).

2.3. Anti-amastigote activity

Murine peritoneal macrophages obtained after 3 days of stimulation with thioglycolate were harvested in RPMI 1640 medium (Biochrom KG, Berlin, Germany). Macrophages were plated on 13 mm^2 coverslips inside 24-well plates and allowed to adhere for 2 h at 37 °C in 5% CO_2. Non-adherent cells were removed by washing, and macrophages were incubated overnight in RPMI supplemented with 10% FCS, as described above. Adhered macrophages were infected with *L. amazonensis* promastigotes (stationary growth phase) at a 10:1 parasite/macrophage ratio and incubated for 1 h at 34 °C, 5% CO_2. Free parasites were washed out with 0.01 M Phosphate Buffered Saline (PBS), and cultures were maintained for 24 h at 37 °C in 5% CO_2 in RPMI supplemented with 10% FCS. AF3 from *T. catharinensis* extract was added to the cultures and, after 24 h incubation as above, the cells were washed with PBS at 37 °C, fixed in methanol, and stained with Giemsa. The number of amastigotes and the percentage of infected macrophages were determined by counting at least 200 cells in triplicate cultures. Endocytic indices were obtained by multiplying the percentage of infected macrophages by the mean number of amastigotes per infected macrophage. Glucantime® (Aventis) and 18-metoxy-coronaridine [16] were used as positive controls in these assays. Results are expressed as percentage of survival comparing endocytic indices of treated and untreated macrophages.

2.4. Nitric oxide production

Thioglycolate peritoneal mouse macrophages obtained as above (10^6 cells/well in 24-well plate) were activated or not with 10% IFN-γ (4 days culture supernatant of L1210 cell line transfected with IFN-γ gene [19]) and 100 ng/mL of lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Difco Laboratories Inc., Detroit, MI, USA). After 24 h at 37 °C in 5% CO_2, monolayers were treated with 100 μg/mL of AF3. Alternatively, macrophages were incubated with 100 μg/mL of AF3 in addition to the activators IFN-γ plus LPS, as above. Nitrite concentrations in 48-hour culture supernatants were determined by the Griess method [20]. The reaction was read at 540 nm, and the concentration of NO_2^- was determined with reference to a standard curve using sodium nitrite. Results are expressed as micromolar concentrations of nitrite.

2.5. Cytotoxicity assays

Murine peritoneal macrophages were adhered to 24-well plates and treated with 100 μg/mL of AF3 for 24 h at 37 °C, 5% CO_2. Macrophages were then washed with PBS, incubated with 3% Trypan blue solution and scored for viable cells in an inverted microscope. Additionally, AF3 cytotoxicity to mouse peritoneal macrophages was determined by the reduction of 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxinilide inner salt (XTT, Sigma) assay, according to Roehm et al., 1991 [21].

2.6. Cytokine production

Thioglycolate peritoneal mouse macrophages obtained as above were cultured in 24-well plates and activated as described above. After 24 h at 37 °C, 5% CO_2, cell monolayers were treated with the AF3 fraction. TGF-β1 and TNF-α production were evaluated by sandwich ELISA, using capture and detection antibodies obtained from R&D Systems, Inc. (Minneapolis, MN, USA) and PeproTech (Colonia Portales, Mexico, DF), respectively, according to the manufacturer's instructions. Assays were performed in duplicate.

2.7. Statistical analysis

Data were analyzed by Student's *t*-test when comparing two groups or one-way ANOVA for more than two groups, using the