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Dehydroabietic acid isolated from *Pinus elliottii* exerts *in vitro* antileishmanial action by pro-oxidant effect, inducing ROS production in promastigote and downregulating Nrf2/ferritin expression in amastigote forms of *Leishmania amazonensis*

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ABSTRACT

Dehydroabietic acid (DHA) is one of the main constituents of the resin that have antiprotozoal activity against *Leishmania* spp., but the leishmanicidal mechanism is unknown. The objective of the study was to investigate *in vitro* the leishmanicidal activity of the natural compound DHA against intracellular and extracellular forms of *L. amazonensis* and the mechanism of action involved. The antileishmanial activity of DHA was evaluated *in vitro* against promastigote forms of *L. amazonensis* by counting in Neubauer chamber. The morphological changes were observed by scanning electron microscopy and cell death mechanism by fluorescence assay using 2′,7′-dichlorofluorescein diacetate probe (H_2DCFDA), tetramethylrhodamine ethyl ester (TMRE), annexin-V and propidium iodide (PI). The antiamastigote effect was observed by counting the number of amastigotes per macrophage and percentage of infected cells. In addition, reactive oxygen species (ROS) production, nitric oxide (NO), cytokines, free iron and total iron-binding capacity (TIBC), expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and ferritin were evaluated. DHA inhibited the proliferation of promastigotes at all times tested. The compound (IC50, 40 ± 0.1458 μg/mL) altered the morphology of the promastigote forms, caused mitochondrial depolarization, induced ROS production, increased phosphatidylserine exposure and caused loss of plasma membrane integrity. DHA also reduced the number of amastigotes and the percentage of infected macrophages by increasing ROS production, free iron and TIBC, and also caused downregulation of Nrf2 and ferritin expression. DHA was effective in the elimination of *L. amazonensis* both in its promastigote forms by apoptosis-like mechanisms and intracellular form by ROS production.

Abbreviations: ACL, American cutaneous leishmaniasis; AMB, amphotericin B; CTLC, comparative thin layer chromatography; DMSO, dimethylsulfoxide; DHA, dehydroabietic acid; FBS, fetal bovine serum; FSC-H, forward scatter-height; H_2DCFDA, diacetate 2′,7′-dichlorofluorescein probe; IC50, Inhibitory concentration of 50%; IL, interleukin; mRNA, messenger RNA; M199, Medium 199; MeOH, methanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; NO, nitric oxide; Nrf2, nuclear factor erythroid 2–related factor 2; OD, optical density; PBS, phosphate buffered saline; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard error of mean; TBIC, total iron binding capacity; TMRE, tetramethylrhodamine ethyl ester probe; TNF, tumor necrosis factor; TPSA, molecular polar surface area; VLC, vacuum liquid chromatography

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1. Introduction

Pinus elliottii is a conifer belonging to the family *Pinaceae*, exploited commercially for its resin. It is a species originally from the Southeast United States, and it is widely found in India, China and subtropical regions of Brazil [1].

Phytochemical studies have shown that the oleoresin of *P. elliottii* is composed of mixtures of monoterpenes, diterpenes (abietane and pimarane) and sesquiterpenes. Diterpene acids are the main constituents of *Pinus* resin and can be found in resins of other plants, such as *Boswellia thurifera* (family Burseraceae) [2], as well as other pine species such as *P. densiflora, P. sylvestris* and *Abies grandis* [3]. Resin is a defense mechanism used by conifers to kill pathogens and herbivores or inhibit or reduce their invasion through its variety of secondary metabolites, being composed of 90% resin acids and 10% neutral substances. Among the resin acids, > 50% are abietic acids that have biological activities such as anti-inflammatory, anti-allergic, phytoalexin and anticonvulsive [4,5].

One of these diterpene acids is naturally occurring dehydroabietic acid (DHA), which has been reported as the main constituent of all coniferous plants such as *Pinus*, *Picea*, *Larix* and *Abies* [6], which has several biological properties, including antimicrobial [6,7], antiulcer [8], anti-inflammatory and antifungal activities [9]. Previous studies have shown a potent antiprotozoal effect of DHA against *Leishmania donovani, L. infantum, L. braziliensis* and *Trypanosoma cruzi* [10,11].

American cutaneous leishmaniasis (ACL) is a polymorphic disease that affects the skin and mucous membranes, and depending on the immune response of the host and protozoan species, it is grouped into different clinical forms (cutaneous, diffuse cutaneous, disseminated cutaneous and mucocutaneous) [12].

The commercial drugs used since 1912 for the treatment of leishmaniasis are the pentavalent antimonial compounds, such as meglumine antimoniate, amphotericin B and pentamidine. Conventional treatments with these drugs, however, show administration difficulties, high cost, high toxicity and several side effects, besides displaying resistance of the parasite in some specific cases [13].

Therefore, given the limitations presented by conventional drugs, it is extremely important to search for new substances that can be used as alternative treatment. Thus, the objective of our study was to isolate the compound DHA from the resin of *P. elliottii* and to evaluate its leishmanicidal activity against *L. amazonensis* and elucidate the mechanisms of action involved.

2. Materials and methods

2.1. Preparation of oleoresin and isolation of major compound

DHA used in this work was obtained from the certified oleoresin of *Pinus elliottii* Engelm, which was kindly donated by ARESB (Associação dos Resinadores do Brasil - Avaré, SP).

The DHA isolation has been previous reported in the literature and was performed as Leandro et al. [14]. Briefly, the resin (14 g) was dissolved in dichloromethane incorporated into a small portion of silica, after complete evaporation of the solvent, the resin was subjected to vacuum liquid chromatography (VLC). The column was prepared with silica gel 60H (Merck Millipore: 1.07736.1000) followed by addition of the resin and eluted with hexane: ethyl acetate (v/v 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 3:7, 0:10) and 100% methanol (MeOH) using an increasing polarity gradient. This procedure yielded 9 fractions which were dried at room temperature until complete evaporation of the solvents, and compared by comparative thin layer chromatography (CTLC). We observed a major compound and the formation of crystals in fraction 3, which were washed with cold methanol at 4 °C for 24 h, yielding the pure crystals with isolation yield of 24,89%.

2.2. Identification of major compound

The melting point was determined with a Microquímica MQAPF 302 hotplate apparatus. The crystals (fraction 3) were subjected to nuclear magnetic resonance (NMR) spectroscopy with a Bruker Avance Model III instrument, using 1D and 2D experiments (400 MHz for ^{1}H and 100 MHz for ^{13}C, $CDCl_3$ and TMS), and the results were compared with data from the literature [15].

2.3. X-ray crystallography

Powder X-ray diffraction was performed using a PANalytical X'pert MPD PRO diffractometer on Bragg-Brentano geometry with the following configurations: radiation CuKα (λ = 1.5406 Å), divergent slit, rotary sample holder (0.5 rev/s), anti-scattering and receiving slits of 1/4° and 1/8° respectively, carbon graphite monochromator and xenon ionization detector. Voltage of 40 kV and current of 20 mA were applied to the X-ray tubes. Stepping angle and time were 0.05° and 1 s, respectively. Crystalline structure refinement was by the Rietveld method using High Score Plus 2.1 software by PANalytical based on the crystallographic identification (cif) number 2223263 from “Crystallography Open Database”.

2.4. In silico study of dehydroabietic acid

For the *in silico* study, the structure of DHA was designed in Molinspiration software (www.molinspiration.com) and calculations of parameters related to oral bioavailability according to Lipinski's rule of five (Ro5) [16], followed by the additional rule proposed by Veber et al. [17].

2.5. Culture of Leishmania (Leishmania) amazonensis

Promastigote forms of *L. (L.) amazonensis* (MHOM/BR/1989/166MJO) were maintained in culture medium 199 (M199) (GIBCO, Invitrogen, New York, USA) supplemented with 10% fetal bovine serum (GIBCO - Invitrogen, New York, USA), 1 M HEPES buffer, 1% human urine, 1% L-glutamine, streptomycin, penicillin (GIBCO, Invitrogen) and 10% sodium bicarbonate. The culture was maintained in an incubator at 25 °C in a 25-cm^2 culture flask for five days (stationary growth phase). All promastigote forms were used in the stationary growth phase.

2.6. Experimental animals and ethics committee

BALB/c mice weighing 25–30 g aged 6–12 weeks were obtained from Instituto Carlos Chagas/Fiocruz-PR, Curitiba, Brazil. The mice were maintained and used according to the protocol approved by the Ethics Committee for the Use of Animals of the State University of Londrina (Protocol No. 6955/2016.59).

2.7. Leishmanicidal activity of dehydroabietic acid against promastigote forms of L. amazonensis

Promastigote forms of *L. amazonensis* (10^6 cells/mL) were treated with different concentrations of DHA (15, 25, 50, 75, 100, 125 μg/mL). The parasites were counted in a Neubauer chamber after 24, 48, 72 and 96 h of treatment. Untreated promastigotes and those treated with 0.1% dimethylsulfoxide (DMSO) were the negative controls, and those treated with 1 μM amphotericin B (AmB) the positive control.

2.8. Morphological analysis of promastigotes by scanning electron microscopy

Analysis of the morphology of promastigote forms was performed according to Tomiotto-Pellissier et al. [18]. The parasites were treated