

Evaluation of antileishmanial activity of harzialactone A isolated from the marine-derived fungus *Paecilomyces* sp.

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Fractionation of extracts from the culture broth of the marine-derived fungus, *Paecilomyces* sp. 7A22, resulted in the isolation of the harzialactone A (**HA**), a known compound previously isolated from fungi of marine environments. The chemical structure of **HA** was determined by spectroscopic analyses. Upon evaluation of **HA** on antileishmanial assays against *Leishmania amazonensis*, **HA** exhibited significant activity against promastigotes forms with IC_{50} of $5.25 \mu\text{g mL}^{-1}$ and moderate activity against intracellular amastigotes with IC_{50} of $18.18 \mu\text{g mL}^{-1}$. This is the first report on the antileishmanial activity of **HA**, and the effects of **HA** presented in this work suggest that this class of compounds are suitable for future biological *in vitro* and *in vivo* studies for the search of natural products with activity against *Leishmania* spp. Furthermore, the present results corroborate marine-derived fungi as a promising source of natural products with antiparasitic activity.

Keywords: *Aplidiopsis* sp.; harzialactone A; leishmanicidal activity; *Paecilomyces* sp.

SUPPLEMENTARY MATERIAL

Experimental section

General

The NMR spectra were run on a Bruker DPX 400 spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C) using TMS as an internal chemical shift reference. Analytical HPLC were performed on a SHIMADZU LC-6AD system equipped with a communication bus module CBM-20A, with a degasser DGU- 20A5, a Photo Diode Array (PDA) detector SPD-M20A series, a Reodyne manual injector, and using a SHIMADZU Shim-pack ODS (particle diameter $5 \mu\text{m}$, $250 \times 4.60 \text{ mm}$) column equipped with a pre-column of the same material. Analytical TLC was performed on silica gel (Merck, 60, F254, 0.25 mm), vacuum liquid chromatography (VLC) was carried out using silica gel 60 H (Merck) in glass column (10 cm i.d.), and column chromatography (CC) was performed on silica gel (Merck, 60, 0.063–0.200 mm). The liquid fungal cultivation procedures were performed on a Cientec CT-713 rotary shaker. The optical rotation of compound was measured in CHCl_3 in a JASCO DIP-370 polarimeter.

Fungal material, fermentation, extraction and isolation

The fungal strain *Paecilomyces* sp. (7A22) has been isolated from the marine ascidian *Aplidiopsis* sp. collected in Ilhabela, on the northern coast of the state of São Paulo, Brazil in September, 2015. The fungus was identified by morphological characters observation and analysis of the ITS regions of its rDNA, whose sequence data have been deposited at GenBank with the accession number MF379654.1. The secondary metabolites fungal production experiments were conducted using submerged shaken liquid culture. An inoculum of 2×10^6 conidia mL^{-1} was aseptically added to 2 L Erlenmeyer flasks (40) containing 800 mL of sterile PDB medium. Cultures were incubated at 30°C using a shaker operating at 130 rpm. After 14 days of incubation, the broth was filtered and extracted with EtOAc (3 x 400 mL). The combined EtOAc extract (2.1 g) obtained was subjected to VLC using hexanes/EtOAc gradient, which afforded eight fractions of 300 mL each (fr.1- fr.8). The fraction fr.4 (305.5 mg; 60% EtOAc) was submitted to CC using an isocratic system of hexanes/EtOAc (4:6) to yield 52 mg of **HA**.

trans-(+)-harzialactone A (**HA**); white solid; $[\alpha]_D^{23.3} = + 30.67$ (c 0.3, CHCl_3); ^1H NMR (400 MHz, CDCl_3): δ_{H} 7.35-7.20 (m, 5H), 4.93-4.87 (m, 1H), 4.11 (t, $J = 7.8$ Hz, 1H), 2.97 (d, $J = 6.1$ Hz, 2H), 2.37-2.24 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3): δ_{C} 177.7 (C, C-2), 135.4 (C, C-1'), 129.5 (CH, C-2' and C-6'), 128.8 (CH, C-3' and C-5'), 127.2 (C, C-4'), 78.4 (CH, C-5), 67.1 (CH, C-3), 41.1 (CH_2 , C-6), 34.6 (CH_2 , C-4).

Anti-promastigote assay

The bioassays were performed using *Leishmania amazonensis* (MHOM/BR/PH8). Promastigote forms of *L. amazonensis* were incubated in RPMI 1640 medium (Sigma Aldrich), supplemented with L-glutamine (2 mM), NaHCO_3 (10 mM), penicillin (100 UI mL^{-1}), streptomycin (100 $\mu\text{g mL}^{-1}$), and 10% bovine fetal serum (Gibco). After 6 days of initial inoculation, promastigote forms (2×10^6 parasites mL^{-1}) were incubated in 96-well microtiter plates containing the tested samples. The plates were incubated at 22 °C for 24 h, and the lysis percentage was determined by an MTT colorimetric method. The bioassays were performed in triplicate, using RPMI 1640 medium with 5% DMSO as negative control and amphotericin B as positive control group.

Anti-amastigote assay

To evaluate activity against intracellular amastigote forms, peritoneal macrophages cells were seeded (2×10^5 cells mL^{-1}) into 24-wells plates containing glass coverslips (13 mm). Non-adherent cells were removed, and the cells were infected with

promastigote forms at a ratio of 1:10 (macrophage/promastigote). Infected macrophages were incubated with the harzialactone A (3.12 - 50 $\mu\text{g mL}^{-1}$) and amphotericin B (0.095 - 1.56 $\mu\text{g mL}^{-1}$) for 48h at 37 °C in a 5% CO₂ atmosphere. Parasites incubated in RPMI 1640 medium (Sigma Aldrich) with 0.1% DMSO served as the negative control. The number of amastigotes was determined by randomly counting 200 cells. The results were calculated using the negative control (0.1% DMSO) as representative of 100% cell survival. Determination of 50% inhibition concentration values (IC₅₀) was carried out by GraphPad Prism version 5.0 Windows software (GraphPad Software, USA) using a nonlinear regression model of variable slope. All tests were conducted in triplicate, and three independent assays were performed.

Macrophages and cytotoxicity assay

Thioglycolate-elicited peritoneal macrophages were obtained from BALB/c mice (weighing 20–25 g) by injection of 500 μL of 3% thioglycolate 3 days prior to peritoneal lavage with 5 mL of ice-cold phosphate-buffered saline (PBS 1 ×), and the cells collected during washing were centrifuged at 1000 rpm and 4 °C for 10 min. The supernatant was removed and the pellet (cells) was added with 10 mL of RPMI 1640 (Gibco) ice cold medium supplemented with 10% inactivated fetal bovine serum and 1% antibiotic (10,000 U/mL penicillin and 10,000 mg/mL streptomycin). The cells were counted in a Neubauer's chamber and adjusted to a concentration of 2×10^5 cells mL^{-1} . The cells were then seeded in a 96- well microtiter plate and incubated at 37 °C in a 5% CO₂ atmosphere for 24 h and when the macrophages had developed a monolayer, the cells were exposed with the compounds at concentrations of 3.12 to 50 $\mu\text{g/mL}$ (harzialactone A) or 0.095 to 1.56 (amphotericin B) for 48 h. The cell viability was determined using the MTT assay. RPMI 1640 medium (Gibco) containing 0.5% DMSO (Synth) (highest concentration) was used as a negative control and 25% DMSO was used as a positive control. Two experiments were performed in triplicate and repeated twice. Determination of 50% cytotoxic concentration values (CC₅₀) was carried out by GraphPad Prism version 5.0 Windows software (GraphPad software, USA) using a nonlinear regression model of variable slope. The experiment was authorized by the University of Franca's Ethics Committee for Animal Care (approval number: 046/15).