SUPPLEMENTARY MATERIAL

The antileishmanial activity of the Antarctic brown alga *Ascoseira mirabilis* Skottsberg

Leandro da Costa Clementino[1,2], Fernando Bombarda Oda[2],Thaiz Rodrigues Teixeira[3], Renata Spagolla Napoleão Tavares[3], Pio Colepicolo[4], André Gonzaga dos Santos[2], Hosana Maria Debonsi[3] and Márcia A. S. Graminha[2*]

[1]*Chemistry Institute, São Paulo State University (UNESP), Araraquara - SP, Brazil.* [2]*School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara - SP, Brazil.* [3]*School of Pharmaceutical Sciences of Ribeirão Preto, São Paulo University (USP), Ribeirão Preto - SP, Brazil.* [4]*Chemistry Institute, São Paulo University (USP), São Paulo - SP, Brazil.*

* Corresponding author: marcia.graminha@unesp.br

Abstract

Leishmaniasis is a group of diseases that have limited and high toxic therapeutic options. Herein, we evaluated the antileishmanial potential and cytotoxicity of hexanic extract obtained from the Antarctic brown alga *Ascoseira mirabilis* using bioguided fractionation against *Leishmania amazonensis* and murine macrophages, which was fractionated by SPE, yielding seven fractions (F1-F7). The fraction F6 showed good anti-amastigote activity (IC_{50} = 73. 4 ± 0.4 μg mL^{-1}) and low cytotoxicity (CC_{50} > 100 μg mL^{-1}). Thus, in order to identify the bioactive constituent(s) of F6, the fraction was separated in a semipreparative HPLC, yielding four fractions (F6.1-F6.4). F6.2 was the most bioactive fraction (IC_{50} = 66.5 ± 4.5 μg mL^{-1}) and GC-MS analyses revealed that the compounds octadecane, propanoic acid, 1-monomyristin and azelaic acid correspond to 61% of its composition. These data show for the first time the antileishmanial potential of the Antarctic alga *A. mirabilis*.

Key-words: Cutaneous leishmaniasis, Antarctic macroalgae, Ascoseiraceae, GC-MS, Brown algae, Natural products

Experimental

The brown alga *A. mirabilis* was collected during OPERANTAR XXXIII, in January 7th 2015 at Admiralty Bay, King George Island, South Shetland Archipelago, Antarctic (61º 50' 62'' S; 57º 30' 59" W). Algae identification was performed by Dr Toyota Fujii Mutue and a voucher specimen was deposited in herbarium "Maria Eneyda Kauffmann Fidalgo" of the Botanical Institute of São Paulo State (São Paulo, Brazil), under reference number SP470094. The collected macroalgae were stored at 4°C.

Algal extract and its fractionation

Algae were cleaned, dried and grounded to yield a powder (440 g). The extraction with hexane (1:5 g mL^{-1}) was performed by maceration with occasional stirring during 24 h. The extractive solution was filtered and dried on a rotary evaporator, under reduced pressure at 40° C, yielding 5.9 g of dry extract. The extract was fractionated by solid phase extraction using silica gel (10 x 3 cm; 63-200 μm, 60Å, Sigma®) and hexane: ethyl acetate 95:05 (fractions 1 and 2, F1 and F2), 1:1 (F3 and F4), ethyl acetate (F5), ethyl acetate: ethanol 90:10 (F6) and ethanol (F7) as mobile phase (eluent volume of 60 mL). Fraction F6 was submitted to semipreparative reversed-phase HPLC-PDA/UV that was performed using a Shimadzu system (Kyoto, Japan) comprising a model Prominence LC-20AT pump, SIL-20A autosampler, DGU-20A5 degasser, CTO-20A column oven, SPD-M10 photodiode array detector and CBM-20A communication bus module, fitted with Supelco® C18 column (250 × 10 mm, 5 μm), with control and data handling managed by Class-VP 5.02 software. The sample (20 $mg \cdot mL^{-1}$; injection volume of 1.0 mL) was eluted with methanol: water 88.5:11.5 (v/v) during 20 min at a flow rate of 2.0 mL min^{-1} and detection was at 250 nm.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of F6.2

The sample was derivatized with common silylation reagents (trimethylsilylethers—TMS) prior to GC–MS analyses. The derivatization was carried out with a mixture of pyridine, *N*, *O*-bis (trimethylsilyl) trifluoroacetamide and chloromethyl-trimethylsilane 98%. The samples were incubated at 60 °C for 45 min and then direct injection was performed in GC-MS (Teixeira et al. 2019). All solvents used were of chromatographic grade and obtained from Sigma-Aldrich®. Subfraction F6.2 (1.0 mg mL^{-1}; hexane) was injected in a gas chromatograph coupled to a mass

spectrometer (GC-MS; Shimadzu), with an DB-5MS (30m x 0.25mm x 0.25µm, SGE Analytical Science) column, equipped with split injector heated to 260 °C. The carrier gas used was helium (99.999%) at a flow rate of 1.40 mL min^{-1}, the injection volume was 1 µL and the injector split ratio was 1:40. The oven temperature program was increased at rate of 8 °C min^{-1} from 70 to 330 °C and was hold isothermally for 20 min. The mass spectrometer was set to observe *m/z* 40-700 in positive EI mode (70 eV) and the ion source temperature at 250 °C. For the compound identification, an EIMS spectrum with library matches (Wiley 7 and Nist 11s library) was considered. Additionally, the relative retention index (RI) values were calculated by evaluating external TMS derivatives of a standard set of *n*-alkanes (C_{10}-C_{40}) under the same conditions and column using Van den Dool and Kratz equation's (Van den Dool and Kratz 1963). The compound confirmation was obtained by comparing the calculated RI values with those previously reported in literature.

Anti-promastigote assays

Parasites of *Leishmania amazonensis* (MPRO/BR/1972/M1841-LV-79) were cultivated in liver-infusion-tryptose (LIT) medium, supplemented with 10% fetal bovine serum (FBS). Promastigote forms were maintained at 28ºC until the exponential growth phase (3-4 days) and seeded at $1x10^7$ parasites mL^{-1} in 96-well flat-bottom plates (TPP®) in a final volume of 100 µL (Velázquez et al. 2016). *A. mirabilis* extracts (3 – 500 µg mL^{-1}) or fractions (from 1.5 - 100 µg mL^{-1}) were dissolved in DMSO and incubated in the parasite suspension for 72 h at 27°C. Amphotericin B treated and untreated parasites were used as positive and negative controls, respectively. After incubation, calculation of the half-maximal inhibitory concentration (IC_{50}) was performed by counting in Neubauer chamber and applying nonlinear regression to the data using Bioestat® software. The experiments were performed in triplicate.

Anti-amastigotes assays

Peritoneal macrophages from Swiss mice were obtained as previously described (Dutra et al. 2014), seeded at 5×10^5 cells per well on coverslips (13 mm diameter). The coverslips containing macrophages were placed in 24-well plates with RPMI 1640 medium, supplemented with 10% FSB and incubated for 6 h at 37 °C in 5% CO_2 for cells adhesion. The adherent macrophages were infected with promastigote forms of *L.*

amazonensis in the stationary growth phase (6-7 days) using a ratio of 5:1 (parasites: macrophage) and further incubated at 37 °C in 5% CO_2 for 8 h in a final volume of 500 µL. The non-internalized parasites were removed by washing the coverslips with PBS (pH 7.4) and the remaining infected macrophages were incubated in RPMI 1640 medium and treated with different concentrations of the samples for 24 h. The cells were then fixed with methanol and Giemsa stained. The 50% amastigote parasites growth inhibition was expressed as the inhibitory concentration ($IC_{50\text{-}AMA}$) in µg. mL^{-1} by counting 100 macrophages and its calculation was performed by nonlinear regression of the data using Bioestat® software. The experiments were performed in independent duplicate and data expressed as mean ± standard deviation.

Cytotoxicity assay

Murine macrophages were obtained as described (Dutra et al. 2014) and seeded in 96-well flat-bottom plates, at 5 x 10^5 cells mL^{-1}, with a final volume 100 µL, and incubated for 6 h at 37°C in a 5% CO_2-air. Subsequently, the medium was renewed containing decreasing concentrations (500-7.81 µg mL^{-1}) of samples. The plates were incubated for 24 hours under the same conditions; next the cell viability was assessed using the MTT method (Velázquez et al. 2016). The cytotoxic concentration of compounds that resulted in 50% of cell growth inhibition (CC_{50}) was determined by non-linear regression of data using Bioestat® software. The assays were performed in triplicate and results expressed as mean ± standard deviation. All experiments involving animals was performed as approved by the ethical committee of the School of Pharmaceutical Sciences, São Paulo State University "Júlio de Mesquita Filho", Araraquara – São Paulo (42/2016).

Table S1. Anti- *L. amazonensis* promastigote activity and cytotoxicity for murine macrophages by an extract and fractions obtained from *Ascoseira mirabilis* Skottsberg.

Table S2. Anti-promastigote, Anti-amastigote activities and cytotoxicity by subfractions of fraction F6 obtained from *Ascoseira mirabilis* Skottsberg against *L. amazonensis* and murine macrophages.

Table S3. Putative identification of compounds in fraction F6.2 from *Ascoseira mirabilis* Skottsberg obtained by GC-MS.

Table S1. Anti- *L. amazonensis* promastigote activity and cytotoxicity for murine macrophages by an extract and fractions obtained from *Ascoseira mirabilis* Skottsberg.

Sample	$IC_{50\text{-}PRO}$ µg. mL^{-1}	CC_{50} µg. mL^{-1}	Selectivity Index
F1	>100	-	-
F2	>100	-	-
F3	61.3 ± 1.9	> 100	>1.6
F4	>100	-	-
F5	>100	-	-
F6	73. 4 ± 0.4	> 100	>1.4
F7	>100	-	-
Hexanic extract	176.0 ± 11.3	46.1 ± 10.4	0.3
Amphotericin B	3.2 ± 0.1	23.1 ± 2.5	7.6

$IC_{50\text{-}PRO}$ – Concentration resulting in 50% growth inhibition of promastigote forms. CC_{50} – Concentration resulting in 50% cytotoxicity to murine macrophages. Selectivity Index - $CC_{50}/CI_{50\text{-}PRO}$.

Table S2. Anti-promastigote, Anti-amastigote activities and cytotoxicity by subfractions of fraction F6 obtained from *Ascoseira mirabilis* Skottsberg against *L. amazonensis* and murine macrophages.

Sample	$IC_{50\text{-}PRO}$ µg. mL^{-1}	Selective Index#	$IC_{50\text{-}AMA}$ µg. mL^{-1}	Selectivity Index*	CC_{50} µg. mL^{-1}
F6.1	>100	-	-	-	-
F6.2	66.5 ± 4.7	1.8	40.0 ± 0.1	3.0	121.3 ± 0.1
F6.3	>100	-	-	-	-
F6.4	>100	-	-	-	-
Amphotericin B	3.2 ± 0.1	7.6	4.9 ± 0.1	4.7	23.1 ± 2.5

$IC_{50\text{-}PRO}$ – Concentration resulting in 50% growth inhibition of promastigote forms. $IC_{50\text{-}AMA}$ – Concentration resulting in 50% growth inhibition of amastigote forms. Selectivity Index = CC_{50}/CI_{50} with #for promastigotes and *for amastigotes.

Table S3. Putative identification of compounds in fraction F6.2 from *Ascoseira mirabilis* Skottsberg obtained by GC-MS.

Compounds	$RI_{calc.}$	$RI_{lit.}$	Identification	Peak area (%)
Octanedioic acid	1702	1702 (Kimura et al., 1999)	RI	1.8
Propanoic acid	-	796 (Tret'yakov 2007)	MS	13.9
Azelaic acid	1805	1800 (Kimura et al., 1999)	RI	10.0
Hexadecanoic acid	2076	2042 (Teixeira et al., 2019)	RI	3.7
Octadecanoic acid	2279	2242 (Teixeira et al., 2019)	RI	2.6
Octadecane	2312	-	MS	25.3
2-Monomyristin	2372	2385 (Isidorov et al., 2007)	RI	2.3
1-Monomyristin	2434	2424 (Isidorov et al., 2007)	RI	11.6
cis-11,14-eicosadienoic acid	2606	2614 (NIST/Standard non-polar)	RI	2.1

RI: Relative retention index corresponding to the TMS derivatives; MS: identification by fragmentation profile.

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