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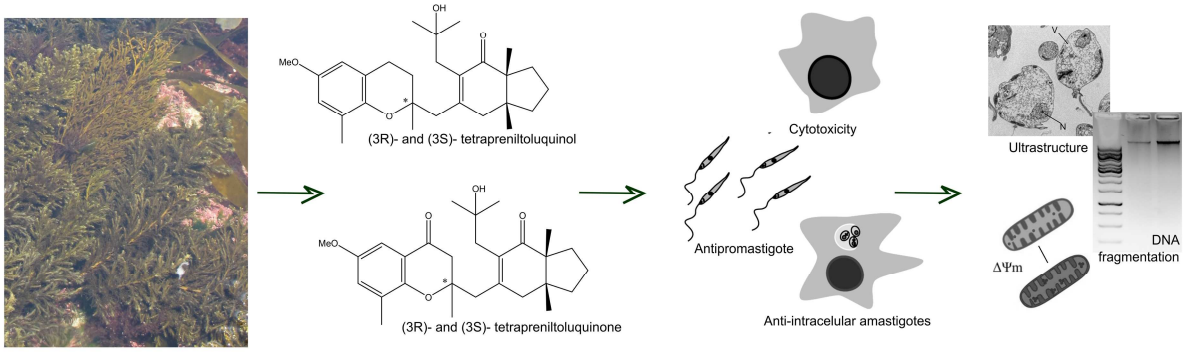

Cystoseira baccata | Epimeric meroterpenoids | Antileishmanial activity | Fffect

Antileishmanial activity of meroditerpenoids from the macroalgae *Cystoseira baccata*

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Abstract

The development of novel drugs for the treatment of leishmaniases continues to be crucial to overcome the severe impacts of these diseases on human and animal health. Several bioactivities have been described in extracts from macroalgae belonging to the *Cystoseira* genus. However, none of the studies has reported the chemical compounds responsible for the antileishmanial activity observed upon incubation of the parasite with the aforementioned extracts. Thus, this work aimed to isolate and characterize the molecules present in a hexane extract of *Cystoseira baccata* that was found to be bioactive against *Leishmania infantum* in a previous screening effort. A bioactivity-guided fractionation of the *C. baccata* extract was carried out and the inhibitory potential of the isolated compounds was evaluated via the MTT assay against promastigotes and murine macrophages as well as direct counting against intracellular amastigotes. Moreover, the promastigote ultrastructure, DNA fragmentation and changes in the mitochondrial potential were assessed to unravel their mechanism of action. In this process, two antileishmanial meroditerpenoids, (3*R*)- and (3*S*)-tetraprenyltoluquinol (**1a**/**1b**) and (3*R*)- and (3*S*)-tetraprenyltoluquinone (**2a**/**2b**), were isolated. Compounds **1** and **2** inhibited the growth of the *L. infantum* promastigotes (IC_{50} = 44.9 ± 4.3 and 94.4 ± 10.1 µM, respectively), inducing cytoplasmic vacuolization and the presence of coiled multilamellar structures in mitochondria as well as an intense disruption of the mitochondrial membrane potential. Compound **1** decreased the intracellular infection index (IC_{50} = 25.0 ± 4.1 µM), while compound **2** eliminated 50% of the intracellular amastigotes at a concentration > 88.0 µM. This work identified compound **2** as a novel metabolite and compound **1** as a biochemical isolated from *Cystoseira* algae displaying antileishmanial activity. Compound **1** can thus be an interesting scaffold for the development of novel chemotherapeutic molecules for canine and human visceral leishmaniases studies. This work reinforces the evidence of the marine environment as source of novel molecules.

Keywords

Leishmania infantum; macroalgae; *Cystoseira baccata*; meroterpenoids; tetraprenyltoluquinol; tetraprenyltoluquinone.

Abbreviations

BALB/c, albino mouse laboratory-bred strain of the house mouse;

CC_{50}, cytotoxic concentration that causes the death of 50% of the viable cells;

COSY, correlation spectroscopy;

DEPT, distortionless enhancement by polarization transfer spectrometry;

FBS, fetal bovine serum;

HMBC, heteronuclear multiple-bond correlation spectroscopy;

HRESIMS; high-resolution electrospray ionisation mass spectrometry;

HSQC, heteronuclear single-quantum correlation spectroscopy;

IC_{50}, half-maximal inhibitory concentration;

IR, infrared;

LRESIMS, low-resolution electrospray ionisation mass spectrometry;

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

NMR, nuclear magnetic resonance spectroscopy;

NOESY, nuclear Overhauser effect spectroscopy;

RCF, relative centrifugal force;

SDS, sodium dodecyl sulfate;

TLC, thin-layer chromatography;

TMS, tetramethylsilane;

UV, ultraviolet;

$\Delta\psi m$, mitochondrial membrane potential.

1. Introduction

Leishmaniases are a group of infectious diseases caused by obligate intracellular protozoa of the *Leishmania* genus. Endemic in 98 tropical and subtropical countries and affecting 12 million people, leishmaniases may entail cutaneous, mucocutaneous and diffuse forms as well as the potentially fatal visceral form (Alvar et al., 2012). Visceral leishmaniasis causes considerable morbidity in 200-400 thousand individuals every year, with extreme suffering and financial loss, especially in the poorest populations of the Indian subcontinent (Mondal et al., 2014). Currently, leishmaniases are among the most neglected tropical diseases, facing problems of resistance of the parasite to the available therapeutic molecules. The need for the discovery and development of alternative drugs allowing more efficient and effective treatments is thus quite urgent (Freitas-Junior et al., 2012).

Nowadays, marine natural products are recognized as powerful reservoirs of novel, chemically diverse molecules with wide applicability to health sciences (Tempone et al., 2011). Occurring worldwide, mainly in the rocky substrates of the Mediterranean Sea and the adjoining Atlantic coasts, *Cystoseira* C. Agardh (1820) genus encompasses 39 species of brown macroalgae (Guiry and Guiry, 2015). Several bioactivities such as anti-inflammatory, antiproliferative, antioxidant (Mhadhebi et al., 2011), enzyme inhibitory (Ghannadi et al., 2013), cytotoxic (Khanavi et al., 2010), antifungal (Lopes et al., 2013), antiviral (Ibraheem et al., 2012), antibacterial (Tajbakhsh et al., 2011) and antiprotozoal (Spavieri et al., 2010) have been detected in this algal genus. Despite the extensive chemical studies available for the *Cystoseira* genus, there have been only a few reports describing the antileishmanial potential effects of its crude extracts, and no information was found on the compounds responsible for the inhibitory effects on the *Leishmania* parasites (Amico, 1995; de Los Reyes et al., 2012). As part of ongoing research on the identification of antileishmanial compounds from the *Cystoseira* genus, this work describes the bioactivity-guided fractionation of the hexane extract from *Cystoseira baccata* and the effect of the extract, fractions and isolated compounds on the promastigote and amastigote forms of *Leishmania infantum*.

2. Material and Methods

2.1 General Experimental Procedures

Optical rotations were measured in a JASCO DIP-370 digital polarimeter (Na filter, λ = 588 nm). UV spectra were recorded using a UV/visible Shimadzu 1650-PC spectrophotometer. IR spectra were obtained with a Shimadzu IR Prestige-21

spectrophotometer. ^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC and NOESY NMR spectra were recorded in a Bruker Avance III 500 spectrometer, operating at 500 and 125 MHz, to ^{1}H and ^{13}C nuclei, respectively. $CDCl_3$ (Aldrich) was used as the solvent with TMS as the internal standard. HRESIMS spectra were measured with a Bruker Daltonics MicroTOF QII spectrometer while LRESIMS spectra were recorded on a VG Platform II spectrometer. Silica gel (Merck, 230–400 mesh) and Sephadex LH-20 (Amersham Biosciences) were used for column chromatographic separation, while silica gel 60 PF_{254} (Merck) was used for analytical (0.25 mm) and preparative TLC (1.0 mm).

2.2 Algal material

Cystoseira baccata biomass was collected in July 2012 in Areosa, Viana do Castelo, Portugal (41º42'27.60''N, 8º51'44.90''W). After collection, biomass was cleaned and cryodesiccated. Voucher specimen (MB-1) was deposited within the Laboratory of the Marine Biotechnology Group - MarBiotech at the Centre of the Marine Sciences of the University of Algarve (Faro, Portugal).

2.3 Extraction and isolation of compounds

Dried and powdered biomass (120 g) was exhaustively extracted with hexane in a Soxhlet apparatus. After evaporation of the solvent under reduced pressure, 1.3 g of crude extract were obtained. Part of this extract (0.6 g) was subject to column chromatography over SiO_2 eluted with hexane containing increasing amounts of EtOAc (up to 100%), followed with $CHCl_3$ containing increasing amounts of MeOH (up to 100%), generating 13 fractions (1 – 13). As fraction 10 (370.0 mg) displayed activity towards promastigote forms of *L infantum*, it was fractionated over SiO_2 column, and eluted with hexane:EtOAc 1:1 yielding 6 sub-fractions (A – F). Bioactive sub-fraction E (195 mg) was purified in a Sephadex LH-20 column being eluted with hexane:CH_2Cl_2 1:4, CH_2Cl_2:Me_2CO 3:2 and 1:1 (Cardellina II, 1983) originating 4 groups (E1 – E4). Bioactive group E4 (65.3 mg) was subjected to preparative TLC (hexane-EtOAc, 7:3, twice) to afford compounds **1a**/**1b** (23.2 mg; 0.30%) and **2a**/**2b** (2.5 mg; 0.04%) (Fig.1).

3R – tetraprenyltoluquinol (**1a**) and *3S – tetraprenyltoluquinol* (**1b**). Yellowish oil; ^{1}H NMR and ^{13}C NMR (500 MHz, $CDCl_3$) data, see Table 1; LRESIMS *m/z* 441 $[M+H]^+$ and 463 $[M + Na]^+$ (calcd for $C_{28}H_{41}O_4$, 441, and $C_{28}H_{40}O_4Na$, 463, respectively).

3R – tetraprenyltoluquinone (**2a**) and *3S – tetraprenyltoluquinone* (**2b**). Colourless oil; $[\alpha]_D^{25}$ = + 0.06 (*c* 0.15, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 352 (2.0), 248 (3.4) nm; IR (KBr) ν_{max} 3400, 1670, 1480, 1180, 1060 cm^{-1}; ^1H and ^{13}C NMR (500 MHz, $CDCl_3$), see Table 1 and Fig. 2; HRESIMS (positive mode) *m/z* 455.2776 $[M+H]^+$ and 477.2604 $[M+Na]^+$ (calcd for $C_{28}H_{39}O_5$ and $C_{28}H_{38}O_5Na$, 455.2797 and 477.2616, respectively).

2.4. Parasites, mammalian cells and animal maintenance

L. infantum strain (MHOM/PT/88/IMT-151) promastigotes were obtained from the cryobank of the Instituto de Higiene e Medicina Tropical (Universidade Nova de Lisboa, Portugal) and cultivated in M199 medium supplemented with 10% foetal bovine serum (FBS), penicillin (10 U/L), streptomycin (0.01 mg/L) and 2% of human male urine at 25 ºC. Peritoneal macrophages from BALB/c mice were cultivated in RPMI-1640 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/L) and streptomycin (0.05 mg/L) at 37 ºC in humidified atmosphere with 5% CO_2. BALB/c mice were obtained in the Animal Facility of the School of Medicine of São Paulo University – Brazil. These animals were maintained in accordance with the institutional guidelines regarding the welfare of experimental animals and with the approval of the Animal Ethics Committee of São Paulo University (322/12).

2.5. Activity against Leishmania *promastigotes*

For the determination of the antileishmanial activity, *L. infantum* promastigotes in stationary phase (2×10^6 parasites/mL) were incubated with the hexane extract at a concentration of 250 μg/mL for 24h on 96-well plates. Using the same methodology, the fractions obtained during the bioactivity-guided fractionation were tested at a concentration of 50 μg/mL. At a later stage, compounds **1** and **2** were added at concentrations ranging from 0.9 to 227.0 and 0.9 to 220.0 μM, respectively. Parasites treated with miltefosine at the half maximal inhibitory concentration (IC_{50} = 23.1 μM) were used as positive control. Promastigotes incubated with M199 medium were used as negative control. Parasite viability was determined by the MTT colorimetric assay (Dutta et al., 2005; Dal Picolo et al., 2014). Briefly, after incubation plates were centrifuged at 10 ºC, using an RCF of 1479 × *g* for 10 min, washed three times with PBS, and supernatants discarded. Afterwards, 50 μL of MTT (5 mg/mL in PBS) were added to each well and plates were re-incubated at 37 ºC for 2 h. Upon incubation, 50 μL of SDS were added to each well and plates were incubated for 18 h in order

to dissolve the formazan crystals. Absorbance was measured at 590 nm using a Thermo Scientifc Multiskan™ FC Microplate Photometer. Results were expressed in terms of parasite viability (%) relative to non-treated parasites and the half maximal inhibitory concentration (IC_{50}; µM).

2.6. Ultrastructural alterations of the promastigotes

L. infantum promastigotes in stationary phase (2 x 10^6 cells/mL) were incubated at 25 ºC for 24 h on 96-well plates with compounds **1** and **2** at their IC_{50} values, i.e. 44.9 µM of 94.4 µM, respectively. Non-treated promastigotes were used as negative control. After incubation, the plate was centrifuged at 1479 × *g* for 10 min at 4 ºC, and washed with PBS three times. Pellets were fixed in 0.1% tannic acid dissolved in 2.0% glutaraldehyde in a 0.15 M phosphate buffer pH 7.2 and incubated for 1h at 4°C. These were afterwards contrasted in 1% osmium tetroxide and a 0.5% uranyl acetate solution for 12 h; then the samples were embedded in araldite resin (Yamamoto et al., 2015). Ultrathin sections (70 nm), obtained with a ultramicrotome Reichert and double contrasted with 2% uranyl acetate and 0.5% lead citrate, were examined using a JEOL 1010 transmission electron microscope.

2.7. Promastigotes DNA integrity

To detect whether the compounds induced fragmentation on *L. infantum* nuclear DNA, promastigote forms in stationary phase of growth (2 x 10^8 cells) were incubated with IC_{50} concentrations of compounds **1** (44.9 µM), **2** (94.4 µM) and hydrogen peroxide (6.2 µM) as an inductor of DNA damage in parasites (Das et al., 2001) for 24 h at 25 ºC. Non-treated cells were used as control. After incubation, plates were centrifuged at 1479 × *g* for 10 min at 4 ºC, and the supernatants discarded. Parasites pellets were extracted with a Macherey-Nagel nucleoSpin® Blood kit according with the manufacturer recommendations and ran on a 2% agarose gel, 100 V for 90 min.

2.8. Promastigote transmembrane mitochondrial potential

In order to evaluate the influence of compound **1** on the promastigote mitochondrial membrane potential (ΔΨm), parasites in the stationary phase (2×10^6 parasites/mL) were incubated with compound **1** and miltefosine at their IC_{50} values (44.9 and 23.1 µM, respectively) for 24h on 96-well plates. Mitochondrial membrane potential was evaluated

using the widefield automated microscope Mitoscreen Kit (BD Biosciences) according to the manufacturer's recommendations (Levy et al., 2014; Yamamoto et al., 2015). Briefly, cells were incubated with working solution, containing the JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide) fluorochrome, for 15 min at 37 °C in an atmosphere of 5% CO_2. ΔΨm induces the uptake of JC-1 monomers into the functional mitochondria. Once inside the organelle, JC-1 monomers aggregate, exhibiting high levels of red fluorescence and ΔΨm is assessed through the determination of the presence of JC-1 fluorochrome inside the mitochondria. ImageXpress® Micro XLS Widefield High-Content Analysis System and transfluor MetaXpress software were used to determine the presence of J-aggregates in nine sites per well and three wells per treatment. ΔΨm was expressed as a percentage of J-aggregates per cell.

2.9. Cytotoxicity against murine macrophages

To determine the compounds toxicity *in vitro*, murine peritoneal macrophages, were seeded in RPMI-1640 at a density of 10^6 cells/mL and incubated overnight at 37 ºC in humidified atmosphere with 5% CO_2, allowing the cells to adhere to the plate background. Compounds **1** and **2** were tested for 24h at concentrations ranging from 0.9 to 227.0 and 0.9 to 220.0 μM, respectively. Miltefosine control cells were incubated with RPMI-1640 medium at concentrations from 3.8 up to 490.7 μM. Cell viability was evaluated by the MTT colorimetric assay (Ferrari et al., 1990; Dal Picolo et al., 2014), as described above, for the determination of the activity against *Leishmania* promastigotes. Absorbance was measured at 590 nm using a Thermo Scientific Multiskan™ FC Microplate Photometer. Results were expressed in terms of the cytotoxic concentration causing a 50% decrease in cell viability (CC_{50}; μM) relative to non-treated cells (100 %).

2.10. Activity against Leishmania *intracellular amastigotes and NO production*

Peritoneal macrophages of BALB/c mice were collected by intraperitoneal lavage, seeded on 24-well plates (10^5 cells/mL) and incubated at 37ºC with 5% CO_2 during 2h for cell attachment. Afterwards, *L. infantum* promastigotes in stationary phase were added to each well at an infection ratio of 10 promastigotes per cell, being further incubated at 37 ºC for 24h. Infected macrophages were treated with compounds **1** and **2** at concentrations ranging from 7 to 90 μM to determine the corresponding IC_{50}. Supernatants were collected for nitric oxide (NO) determination after 24h and intracellular amastigote burden was microscopically

assessed upon Giemsa staining for determination of the infection index [% of infected macrophages × internalized amastigote forms / macrophage)] (Passero et al., 2015) and the inhibitory concentration allowing 50% reduction of the infection index (IC_{50}) was estimated. Miltefosine was used as positive control. Culture supernatants of treated and control macrophages were used for NO determination that was performed using the Measure-iT^{TM} High-Sensitivity Nitrite Assay Kit in accordance with the manufacturer's recommendations (Life Technologies). The NO concentration was determined using a calibration curve prepared with several known concentrations (2.75, 5.5, 11, 22, 33, 44 and 55 μM) of nitrite as standard. Results were expressed as NO production (μM) and compared with untreated infected and non-infected macrophages. The selectivity index (SI) was obtained by calculating the ratio of the CC_{50} of the macrophage by the IC_{50} of the intracellular amastigotes.

2.11. Statistical analysis

Bioassays results were expressed as mean ± standard error of the mean (SEM) of replicates samples from at least two independent assays. The IC_{50} values were calculated fitting the data as a non-linear regression using a dose-response inhibitory model, in the GraphPad Prism V 5.0 program. Student's *t*-test was used to determine whether differences between means were significant at different levels ($p < 0.05$ and $p < 0.01$).

3. Results and Discussion

The hexane extract from the *C. baccata* was incubated with promastigote forms of *L. infantum* for 24h, and cell viability was determined by means of the MTT assay. As this extract decreased the viability of the parasite by 74% at a concentration of 250 μg/mL, it was selected for further study. Bioactivity-guided fractionation afforded compounds **1** and **2** (Fig. 1).

Compound **1** was obtained as an optically active oil $[\alpha]_D$ = + 17.8° ($CHCl_3$, *c* 2.7). Structural evidence was obtained by analysis of NMR (^1H, ^{13}C and DEPT 135°), HREIMS spectra and comparison with those data previously reported in the literature to (3*R*)-(**1a**) and (3*S*)-**(1b**) tetraprenyltoluquinol, previously isolated from *C. baccata* (Valls et al., 1993). In addition, some corrections in the attributions of chemical shifts of C-18 and C-19 in ^{13}C NMR spectrum were carried out, based on the HMBC spectral analysis (Table 1). Compound **2,** also obtained as an optically active colourless oil $[\alpha]_D$ = + 0.06° ($CHCl_3$, *c* 0.15), appeared

to be homogeneous on the TLC chromatograms, revealing that it is a mixture of closely related derivatives. The ^{1}H NMR spectrum of compound **2** revealed some similarities with compound **1** - two peaks assigned to hydrogens of aromatic ring at δ_H 7.15 (d, *J* = 3.0 Hz, H-3') and 7.00 (d, *J* = 3.0 Hz, H-5'), one methoxyl group at δ_H 3.78 (s) as well as five singlets assigned to methyl groups at δ_H 1.20 (H-20), 1.25/1.26 (H-17), 1.13/1.11 (H-16), 1.09/1.04 (H-18), and 0.91/0.83 (H-19). ^{13}C and DEPT 135° NMR spectra confirmed the presence of aromatic ring due the peaks at range δ_C 151.9 – 114.6 (C-1' – C-6'), and one methoxyl group at δ_C 55.7. Additionally, peaks assigned to a carbonyl group at δ_C 192.2/192.1 (C-1), to carbinolic carbons at δ_C 81.3/81.2 (C-3) and 71.0 (C-15) as well as an α,β-unsaturated carbonyl carbon at δ_C 153.3/154.3 (C-5), 133.5/134.0 (C-13) and 208.0/208.1 (C-12) were observed. Finally, HRESIMS showed the $[M+H]^+$ and $[M + Na]^+$ *quasi*-molecular ion peaks at *m/z* 455.2776 and 477.2604, respectively, indicating the molecular formula $C_{28}H_{38}O_5$. The connectivity between hydrogens and carbon atoms was revealed by analysis of the HMBC spectrum as showed in Fig. 2. The correlations between signals at δ_H 7.15 (H-3') and 2.56/2.57 (H-2) with δ_C 192.2/192.1 (C-1) as well as between δ_H 2.70 (H-4) with δ_C 81.3/81.2 (C-3) and 133.5/134.0 (C-13) indicated that compound **2** contained one additional carbonyl group at C-1. Based on these results, it was possible to identify **2** as epimers of (3*R*)-(**2a**) and (3*S*)-(**2b**) tetraprenyltoluquinones.

In vitro antiparasitic activity and cytotoxic studies of the compounds **1** and **2** were evaluated by the colorimetric MTT method against promastigote forms of *L. infantum* and murine macrophages, respectively (Table 2). Compound **1** displayed an IC_{50} value of 44.9 ± 4.3 µM against promastigote forms of *L. infantum*. The cytotoxicity against mouse peritoneal macrophages (CC_{50} = 126.6 ± 21.1 µM) was similar to that of the reference drug, miltefosine (130.3 ± 17.2 µM). Compound **2** showed lower activity against the promastigote forms (IC_{50} = 94.4 ± 10.1 µM), and higher toxicity to the mouse peritoneal macrophages (CC_{50} = 84.5 ± 12.5 µM).

To assess the alterations induced by the compounds on the promastigotes forms of *L. infantum*, transmission electron microscopy images were acquired (Fig. 3). Important changes were observed with both treatments, including loss of the typical fusiform shape (Fig. 3A). Ultrastructural analysis revealed morphologic changes in parasites treated with the IC_{50} concentrations of both compounds **1** (Figs. 3B and 3C) and **2** (Figs. 3D and 3E). Moreover, cellular vacuolization was observed, which might be a consequence of cytoplasmic organelle disruption (Figs. 3B and 3D). When treated with compound **1**, parasites presented coiled

multilamellar structures within the mitochondria (Fig. 3C). These structures have been shown to be a consequence of starvation processes caused by deficient mitochondrial activity or autophagic mechanisms caused by the action of chemical compounds on these organelles (Lockshin and Zakeri, 2004). If left unchecked, both processes may result in the removal of the damaged organelles as well as cell death (Nishikawa et al., 2010). Previous studies have described similar structures in promastigotes of different *Leishmania* species treated with distinct natural products (Monte Neto et al., 2011). Compound **2** induced noticeable changes in the ultrastructure of the cell, in particular the occurrence of pyknotic nuclei, which was accompanied by the disappearance of the chromatin associated with the nuclear inner membrane (Fig. 3D).

Overall, these compounds seem to induce parasite death through different mechanisms. Other reports have shown that *Leishmania* apoptosis occurs in response to different drugs (Holzmuller et al., 2002). In order to evaluate if the alterations observed in the nuclei were associated with DNA fragmentation and consequently with programmed cell death, promastigote DNA was analysed through horizontal electrophoresis. This analysis did not reveal any fragmentation of the genomic DNA when promastigote forms of *L. infantum* were treated with the IC_{50} concentrations of compounds **1** and **2** (Fig. 4A), suggesting that the observed cytotoxic effect might not be associated with programmed cell death. Although chromatin condensation culminating in nucleolytic pyknosis is usually accompanied by macronuclear DNA digestion, generating oligonucleosomal fragments of low molecular weight (Kobayashi and Endoh, 2003), non-nucleolytic pyknotic processes have also been described previously (Burgoyne, 1999).

As *Leishmania* cells have a single mitochondrion, the proper functioning of mitochondria, including the stability of their membrane potential, is vital for the survival of the parasite. This organelle is usually considered as a good indicator of cellular dysfunction and therefore is an interesting target for chemotherapeutic studies (Souza et al., 2009). Because the variation of the mitochondrial membrane potential ($\Delta\Psi m$) in different *Leishmania* species exposed to various drugs has been reported (Britta et al., 2014) and that changes were observed in the morphology of the mitochondria of promastigotes treated with compound **1**, the $\Delta\Psi m$ in cells incubated with the latter chemical was evaluated. This was carried out in order to elucidate possible mechanisms of cell death induced by the compound displaying the most potent activity against *L. infantum* promastigotes. This parameter was determined by assessing the presence of JC-1 fluorochrome inside the mitochondria using a widefield automated microscope. $\Delta\Psi m$ induces the uptake of JC-1 monomers into the functional

mitochondria. Once inside the organelle, JC-1 monomers aggregate, exhibiting high levels of red fluorescence. At the IC_{50}, compound **1** induced a significant ($p \leq 0.01$) decrease in fluorescence-emitting cells (133.3 ± 8.5 J-aggregates/well) as compared to non-treated (762.5 ± 36.7 J-aggregates/well) promastigotes (Fig. 4B), corresponding to a disruption of 83% of the ΔΨm. This effect was higher than that observed with miltefosine (216.0 ± 22.6 J-aggregates/well), which disrupted the ΔΨm by only 72%. Interestingly, similar drops in ΔΨm coupled with changes in the mitochondrial ultrastructure have also been detected when using an iron chelator against *L. (V.) braziliensis* (Mesquita-Rodrigues et al., 2013).

When tested against *L. infantum*-infected macrophages the tetraprenyltoluquinol (**1)** applied at concentrations of 34 and 66 µM decreased the infection index by 64.5% and 66.3%, respectively, showing an IC_{50} of 25.0 ± 4.1 µM and a selectivity index of 5.04 against the peritoneal macrophages (Fig. 5A; Table 2).

Only five compounds isolated from marine algae with antileishmanial activity have been reported previously (da Silva Machado et al., 2011; dos Santos et al., 2010, 2011; Soares et al., 2012). However, none of the studies was about *Cystoseira* macroalgae. Reported sesquiterpenes obtusol (IC_{50} = 9.4 µM; da Silva Machado et al., 2011) and elatol (IC_{50} = 13.5 µM and 0.45 µM) from the red alga *Laurencia dendroidea* (daSilva Machado et al., 2011; dos Santos et al., 2010) showed strong activity against *L. amazonensis* intracellular amastigotes. However, the triquinane sesquiterpene isolated from the same algae was significantly less effective (IC50 = 217.4 µM; da Silva Machado et al., 2011). In addition, 4-acetoxydolastane and dolabelladienetriol, isolated from the brown alga *Canistrocarpus cervicornis* (IC_{50} = 12.3 µM; dos Santos et al., 2011) and *Dictyota pfaffii* (IC_{50} = 44.0 µM; Soares et al., 2012), respectively, were also tested against the same species and form of *Leishmania*. Therefore, the activity of compound **1** was in the range of that reported for the aforementioned diterpenes.

Despite the lower activity of compound **2** against promastigotes (IC_{50} = 94.4 ± 10.1), it was higher than the effect reported for triquinane (IC_{50} = 195.5 µM) on promastigotes. However, similarly to what has been reported for triquinane (da Silva Machado et al., 2011), the treatment with the tetraprenyltoluquinone (**2**) did not decrease the infection index (Fig. 5A).

During the infection by *Leishmania*, NO is released by macrophages to eliminate intracellular amastigotes (reviewed by de Almeida et al., 2003). In addition, NO production can be triggered by natural compounds, including those from algae (Robertson et al., 2015). In the present study, infected peritoneal macrophages treated with compounds **1** and **2**

produced low or undetectable amounts of NO as compared to controls. The NO released when the lowest concentrations (8.4 and 17 µM) were applied to the cells was residual, suggesting that the leishmanicidal effect observed for **1** was not related to NO production by the host macrophages (Fig. 5B) and that these compounds did not display an immunomodulatory effect. These results are in agreement with Silva Machado et al. (2011) who observed that triquinane, elatol and obtusol did not promote enhanced NO levels, indicating that leishmanicidal effect of these compounds might be mediated by a mechanism that does not involve the release of this signalling molecule by the host cell.

In conclusion, this is the first report describing the identification of compounds from *Cystoseira* macroalgae displaying activity against *Leishmania* parasites. In addition, the isolation of tetraprenyltoluquinone (**2**) as a novel metabolite from algae of the *Cystoseira* genus is described. Concerning the particular chemical structure of these compounds, our data suggest that the presence of the carbonyl group in C-1 could play a role in the antileishmanial activity of the compounds **1** and **2**. Although not as active as miltefosine, tetraprenyltoluquinol (**1**) displayed significant antileishmanial activity and could be considered as an interesting scaffold for the development of novel chemotherapeutic molecules for canine and human visceral leishmaniases studies. Furthermore, this work reinforces the evidence of the marine environment as source of novel molecules.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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References

Alvar, J., Velez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., et al. 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 7(5), e35671.

Amico, V. 1995. Marine Brown algae of family Cystoseiraceae: chemistry and chemotaxonomy. Phytochem. 39, 1257-1279.

Britta, E.A., Scariot, D.B., Falzirolli, H., Ueda-Nakamura, T., Silva, C.C., Dias Filho, B.P., Borsali, R., Nakamura, V. 2014. Cell death and ultrastructural alterations in *Leishmania amazonensis* caused by new compound 4-Nitrobenzaldehyde thiosemicarbazone derived from S-limonene. BMC Microbiol. 14, 236.

Burgoyne, L.A. 1999. The Mechanisms of Pyknosis: Hypercondensation and Death. Exp. Cell. Res. 248(1), 214-222.

Cardellina II. 1983. Step gradient elution in gel permeation chromatography. A new approach to natural products separation. J. Nat. Prod. 46(2), 196-199.

da Silva Machado, F.L., Pacienza-Lima, W., Rossi-Bergmann, B., de Souza Gestinari, L.M., Fujii, M.T., Campos de Paula, J., Costa, S.S., Lopes, N.P., Kaiser, C.R., Soares, A.R. 2011. Antileishmanial sesquiterpenes from the Brazilian red alga *Laurencia dendroidea*. Planta Med. 77(7), 733-5.

Dal Picolo, C.R., Bezerra, M.P., Gomes, K.S., Passero, L.F., Laurenti, M.D., Martins, E.G., Sartorelli, P., Lago, J. H. 2014. Antileishmanial activity evaluation of adunchalcone, a new prenylated dihydrochalcone from *Piper aduncum* L. Fitoterapia. 97, 28-33.

Das, M., Mukherjee, S.B., Shaham C. 2001. Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. J Cell Sci. 114(13), 2461–2669.

de Almeida, M.C., Vilhena, V., Barral, A., Barral-Netto, M. 2003. Leishmanial infection: analysis of its first Steps. A review. Mem Inst Oswaldo Cruz. 98(7), 861-870.

de Los Reyes, C., Zbakh, H., Motilva, V., Zubía, E. 2012. Antioxidant and anti-inflammatory meroterpenoids from the brown alga *Cystoseira usneoides*. J. Nat. Prod. 76(4), 621-629.

Dutta, A., Bandyopadhyay, S., Mandal, C., Chatterjee, M. 2005. Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis. Parasitol Int. 54, 119–122.

449 Ferrari, M., Fornasiero, M.C., Isetta, A.M. 1990. MTT colorimetric assay for testing
450 macrophage cytotoxic activity *in vitro*. J Immunol Methods. 131(2), 165-72.
451 Freitas-Junior, L., Chatelain, E., Andrade Kim, H., Siqueira-Neto, J.L. 2012. Visceral
452 leishmaniasis treatment: What do we have, what do we need and how to deliver it? Int J.
453 Parasitol. Drugs Drug Resist. 2, 11-19.
454 Ghannadi, A., Plubrukarn, A., Zandi, K., Sartavi, K., Yegdaneh, A. 2013. Screening for
455 antimalarial and acetylcholinesterase inhibitory activities of some Iranian seaweeds.
456 Res. Pharm. Sci. 8(2), 113-118.
457 Guiry, M.D., Guiry, G.M. AlgaeBase. World-wide electronic publication, National
458 University of Ireland, Galway. http://www.algaebase.org; searched on 12 November
459 2015.
460 Holzmuller, P., Sereno, D., Cavaleyra, M., Mangot, I., Daulovede, S., Vincendeau, P.,
461 Lemesre, J.L. 2002. Nitric oxide-mediated proteasome-dependent oligonucleosomal
462 DNA fragmentation in *Leishmania amazonensis* amastigotes. Infect. Immul. 70, 3727-
463 3735.
464 Ibraheem, I.B.M., Abdel-Raouf, N., Abdel-Hameed, M.S, Kel-yamany, K. 2012.
465 Antimicrobial and antiviral activities against Newcastle disease virus (NDV) from
466 marine algae isolated from Qusier and Marsa-Alam Seashore (Red Sea), Egypt. African
467 J. Biotech. 11(33), 8332-8340.
468 Khanavi, M.; Nabavi, M.; Sadati, N.; Ardekani, M.; Sohrabipour, J.; Nabavi, S.; Ghaeli, P.;
469 Ostad, S. N. 2010. Cytotoxic activity of some marine brown algae against cancer cell
470 lines. Biol. Res. 43, 31-37.
471 Kobayashi, T., Endoh, H. 2003. Caspase-like activity in programmed nuclear death during
472 conjugation of *Tetrahymena thermophila*. Cell Death Differ. 10, 634-640.
473 Levy, D., Ruiz, J.L.M., Celestino, A.T., Silva, S.F., Ferreira, A.K., Isaac, C., Bydlowski, S.P.
474 2014. Short-term effects of 7-ketocholesterol on human adipose tissue mesenchymal
475 stem cells *in vitro*. Biochem. Biophys. Res. Commun. 446 (3), 720–725.
476 Lockshin, R.A., Zakeri, Z. 2004. Apoptosis, autophagy, and more. Int J Biochem. Cell Biol.
477 36, 2405–2419.
478 Lopes, G., Pinto, E., Andrade, P., Valentão, P. 2013. Antifungal activity of phlorotannins
479 against dermatophytes and yeasts: approaches to the mechanism of action and influence
480 on *Candida albicans* virulence factor. PloS One. 8(8), e72203.
481 Mesquita-Rodrigues, C., Menna-Barreton R.F.S., Saboia-Vahia, L., Da-Silva, S.A.G., de
482 Souza, E.M., Waghabi, M.C., Cuervo, P., de Jesus, J.B. 2013. Cellular growth and

mitochondrial ultrastructure of *Leishmania (Viannia) braziliensis* promastigotes are affected by the iron chelator 2,2-Dipyridyl. PLoS Negl. Trop. Dis. 7(10), e2481.

Mhadhebi, L., Laroche-Clary, A., Robert, J., Bouraoui, A. 2011. Anti-inflammatory, antiproliferative and antioxidant activities of organic extracts from the Mediterranean seaweed, *Cystoseira crinita*. African J. Biotechnol. 10(73), 16682-16690.

Mondal, D., Alvar, J., Hasnain, M.G., Hossain, M.S., Ghosh, D., Huda, M.M., Nabi, S.G., Sundar S., Matlashewski, G., Arana, B. 2014. Efficacy and safety of single-dose liposomal amphotericin B for visceral leishmaniasis in a rural public hospital in Bangladesh: a feasibility study. Lancet Glob. Health. 2(1), e51–e57.

Monte Neto, R.L., Sousa, L.M.A., Dias, C.S., Barbosa Filho, J.M., Oliveira, M.R., Figueiredo, R.C.B.Q. 2011. Morphological and physiological changes in *Leishmania* promastigotes induced by yangambin, a lignan obtained from *Ocotea duckei*. Exp. Parasitol. 127, 215-221.

Nishikawa, T., Tsuno, N.H., Okaji, Y., Shuno, Y., Sasaki, K., Hongo, K., Sunami, E., Kitayama, J., Takahashi, K., Nagawa, H. 2010. Inhibition of autophagy potentiates sulforaphane-induced apoptosis in human colon cancer cells. Ann. Surg. Oncol. 17, 592–602.

Passero, L.F., Assis, R.R., da Silva, T.N., Nogueira, P.M., Macedo, D.H., Pessoa, N.L., Campos, M.A., Laurenti, M.D., Soares, R.P. 2015. Differential modulation of macrophage response elicited by glycoinositolphospholipids and lipophosphoglycan from *Leishmania (Viannia) shawi*. Parasitol Int. 64(4), 32–35.

Robertson, R.C., Guihéneuf, F., Bahar, B., Schmid, M., Stengel, D.B., Fitzgerald, G.F., Ross, R.P., Stanton, C. 2015. The Anti-Inflammatory Effect of Algae-Derived Lipid Extracts on Lipopolysaccharide (LPS)-Stimulated Human THP-1 Macrophages. Mar Drugs. 13(8), 5402-24.

Santos, A.O., Britta, E., Bianco, E.M., Ueda-Nakamura, T., Dias-Filho, B.P., Pereira R.C., Nakamura, C.V. 2011. 4-Acetoxydolastane diterpene from the Brazilian brown alga *Canistrocarpus cervicornis* as antileishmanial agent. Mar. Drugs. 9, 2369-2383.

Santos, A.O., Veiga-Santos, P., Ueda-Nakamura, T., Dias-Filho, B.P., Sudatti, D.B., Bianco, E.M., Pereira, R.C., Nakamura, C.V. 2010. Effect of elatol, isolated from red seaweed *Laurencia dendroidea*, on *Leishmania amazonensis*. Mar. Drugs. 8, 2733-2743.

Soares, D.C., Calegari-Silva, T.C, Lopes, U.G., Teixeira, V.L., de Palmer Paixão, I.C., Cirne-Santos, C., Bou-Habib, D.C., Saraiva, E.M. 2012. Dolabelladienetriol, a compound

from *Dictyota pfaffii* algae, inhibits the infection by *Leishmania amazonensis*. PLoS Negl. Trop. Dis. 6(9), e1787.

Souza, W., Attias, M., Rodrigues, J.C.F. 2009. Particularities of mitochondrial structure in parasitic protists (Apicomplexa and Kinetoplastida). Int. J. Biochem. Cell Biol. 41, 2069–2080.

Spavieri, J., Allmendinger, A., Kaiser, M., Casey, R., Hingley-Wilson, S., Lalvani, A., Guiry, M.D., Blunden, G., Tasdemir, D. 2010. Antimycobacterial, antiprotozoal and cytotoxic potential of twenty-one brown algae (Phaeophyceae) from British and Irish waters. Phytother. Res. 24, 1724–1729.

Tajbakhsh, S.; Ilkhani, M.; Rustaiyan, A.; Larijani, K.; Sartavi, K.; Tahmasebi, R.; Asayesh, G. 2011. Antibacterial effect of the brown alga *Cystoseira trinodis*. J. Med. Plants Res. 5(18), 4654-4657.

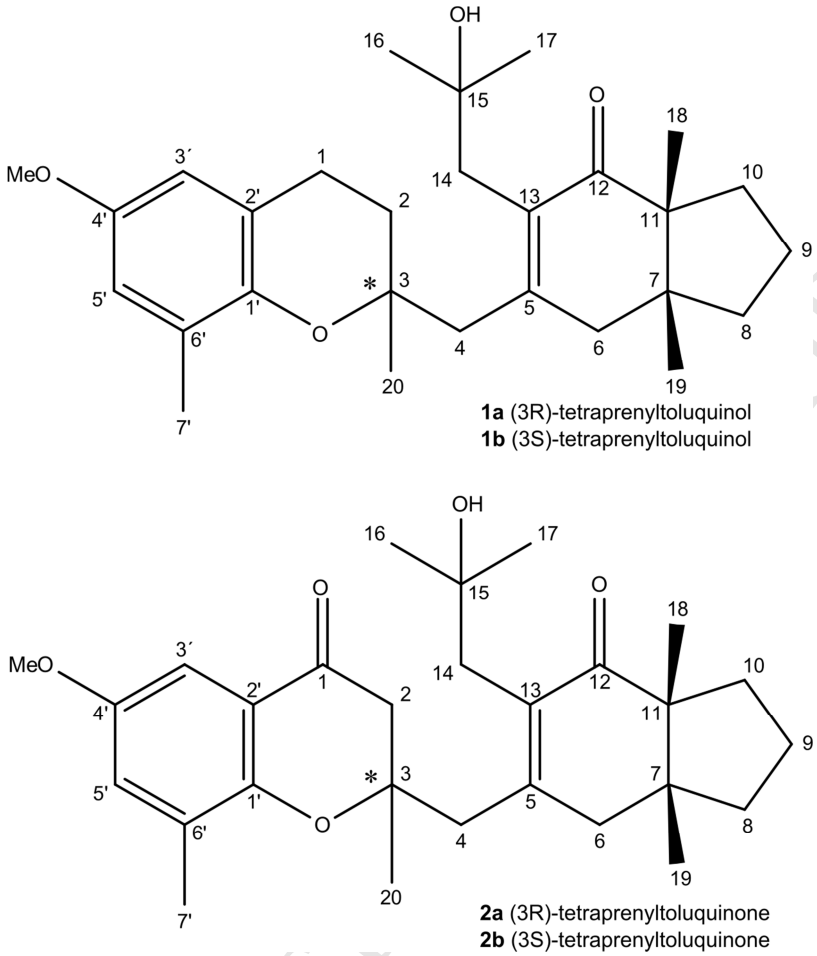
Tempone, A.G., Martins de Oliveira, C., Berlinck, R.G. 2011. Current approaches to discover marine antileishmanial natural products. Planta Med. 77, 572-585.

Valls, R., Piovetti, L., Banaigs, B., Praud, A. 1993. Secondary metabolites from morocco brown algae of the genus *Cystoseira*. Phytochem. 32(4), 961-966.

Yamamoto, E.S., Campos, B.L., Jesus, J.A., Laurenti, M.D., Ribeiro, S.P., Kallás, E.G., Rafael-Fernandes, M., Santos-Gomes, G., Silva, M.S., Sessa, D.P., Lago, J.H., Levy, D., Passero**,** L.F. 2015. The effect of ursolic acid on *Leishmania* (*Leishmania*) *amazonensis* is related to programed cell death and presents therapeutic potential in experimental cutaneous leishmaniasis. PLoS One. 10(12), e0144946.

538 **Figures**

539



540

541 **Fig. 1.** Structures of the tetraprenyltoluquinols (**1a-1b**) and tetraprenyltoluquinones (**2a-2b**)
542 isolated from *C. baccata*

543

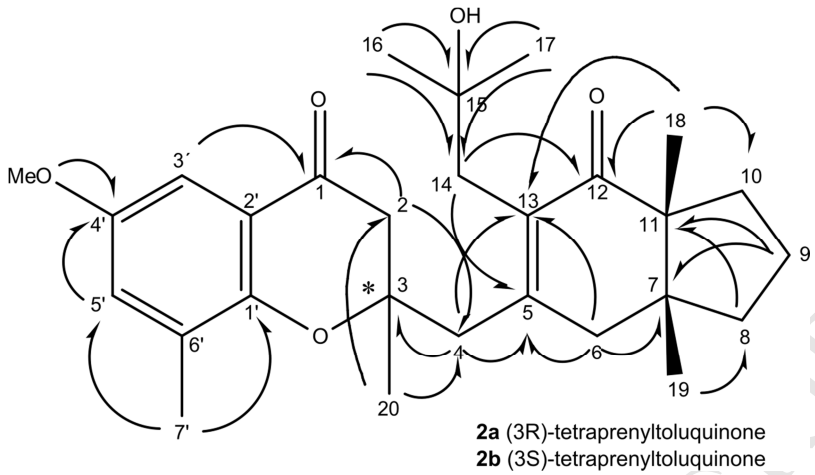
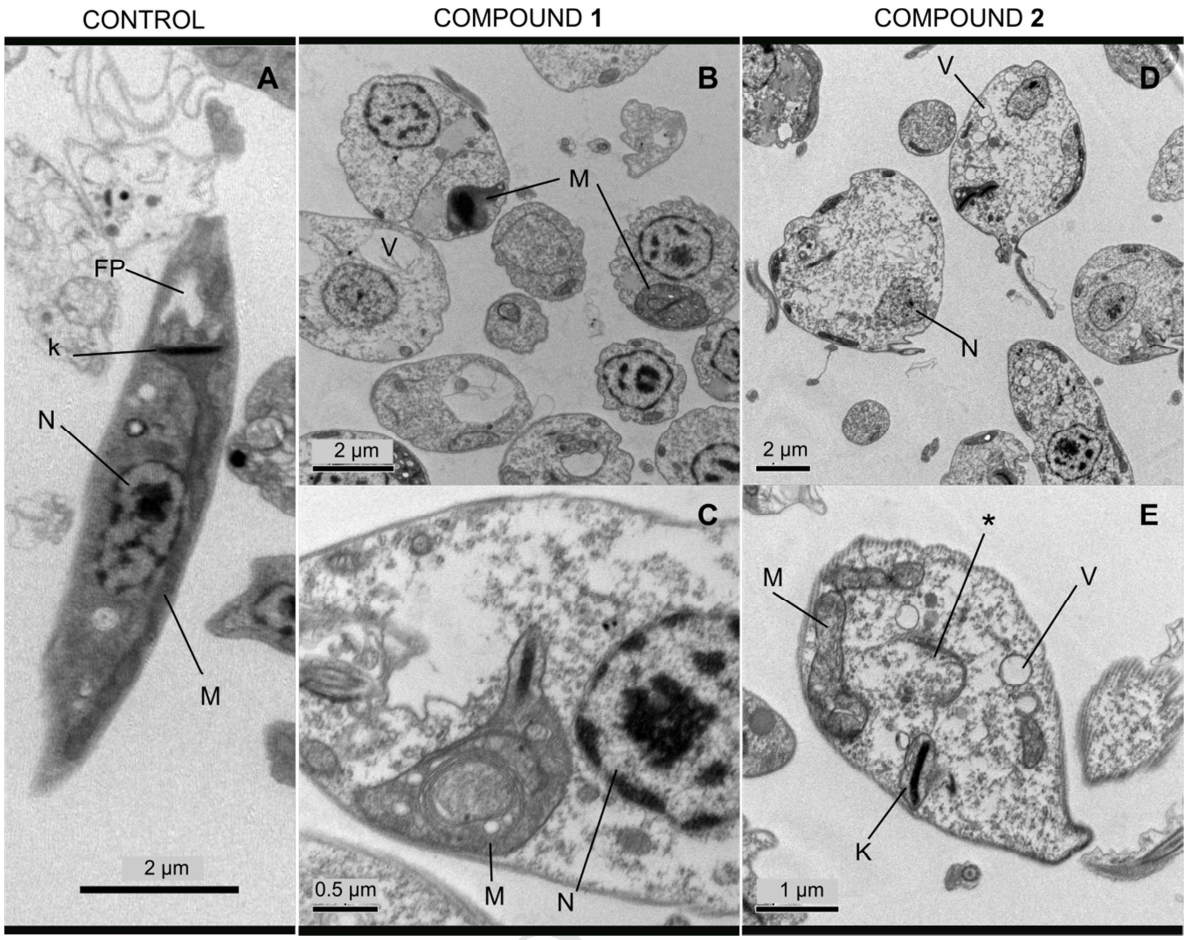


Fig. 2. HMBC of the tetraprenyltoluquinones (**2a-2b**) isolated from *C. baccata*

549



550

551 **Fig. 3.** Effect of compounds **1** and **2** on the ultrastructure of *L. infantum* promastigotes.
552 Parasites were treated with **1a**/**1b** (44.9 µM) and treated with compound **2a**/**2b** (94.4 µM). N
553 – nucleus, FP - flagellar pocket, K – kinetoplast, M – mitochondrion, V – vacuole, * -
554 disappearance of the chromatin associated with the nuclear inner membrane

555

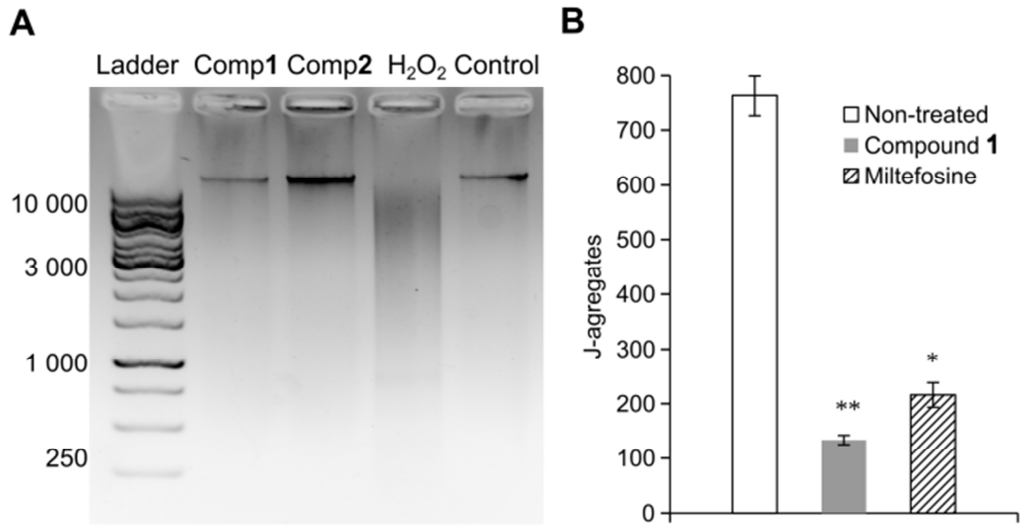
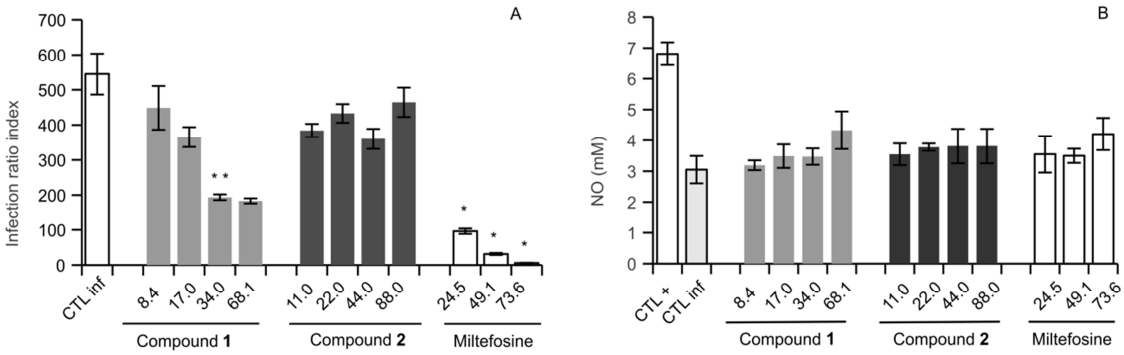


Fig. 4. Effects of compounds **1** (Comp1) and **2** (Comp2) on the nuclear DNA fragmentation (A) and mitochondrial membrane potential (B) of *L. infantum* promastigotes. Parasites were treated with **1a/1b** (44.9 µM) and **2a/2b** (94.4 µM). Hydrogen peroxide (6.2 µM) (A) and miltefosine (23.1 µM) (B) and untreated parasites (A and B) were used as controls. *, $p < 0.05$; **, $p < 0.01$.

564



565

566 **Fig. 5.** Effect of compounds **1** and **2** on the *L. infantum* intracellular amastigotes (A) and on
567 the nitric oxide production (mM) of the infected mouse peritoneal macrophages (B) after a
568 24-h treatment with different concentrations (µM). Untreated non-infected macrophages
569 (CTL+), untreated infected macrophages (CTL inf) and infected macrophages treated with a
570 reference drug, miltefosine, were used as controls.

571

572 **Tables**

573

574 **Table 1.** ^{1}H and ^{13}C NMR data (500 and 125 MHz, $CDCl_3$, δ/ppm) for compounds **1** (**a**/**b**)

575 and **2** (**a**/**b**)

Compound	**1a**		**1b**		**2a**		**2b**	
Position	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	22.7, CH_2	2.79, (m)	22.6, CH_2	2.79, (m)	192.2, C	-	192.1, C	-
2	32.5, CH_2	1.80, (m)	33.6, CH_2	1.80, (m)	47.8, CH_2	2.56, (m)	48.6, CH_2	2.57, (m)
3	76.4, C	-	76.2, C	-	81.3, C	-	81.2, C	-
4	43.6, CH_2	2.66, (s)	45.2, CH_2	2.66, (s)	44.1, CH_2	2.70, (s)	44.8, CH_2	2.70, (s)
5	153.7, C	-	154.5, C	-	153.3, C	-	154.3, C	-
6	44.3, CH_2	2.57, (m)	44.7, CH_2	2.57, (m)	44.1, CH_2	2.60, (d, 4.0)	44.1, CH_2	2.63, (d, 4.0)
		2.60, (m)		2.60, (m)		2.68, (d, 4.0)		2.68, (d, 4.0)
7	44.8, C	-	44.8, C	-	44.9, C	-	44.9, C	-
8	35.0, CH_2	1.54, (m)	35.0, CH_2	1.54, (m)	35.0, CH_2	1.54, (m)	35.0, CH_2	1.54, (m)
		1.73, (m)		1.73, (m)		1.73, (m)		1.73, (m)
9	18.8, CH_2	1.74, (m)	18.8, CH_2	1.74, (m)	18.8, CH_2	1.74, (m)	18.8, CH_2	1.74, (m)
10	29.3, CH_2	1.46, (m)	29.3, CH_2	1.46, (m)	29.4, CH_2	1.40, (d, 13.0)	29.7, CH_2	1.42, (d, 13.0)
11	54.9, C	-	54.9, C	-	54.9, C	-	54.9, C	-
12	208.5, C	-	208.9, C	-	208.0, C	-	208.1, C	-
13	132.9, C	-	133.3, C	-	133.5, C	-	134.0, C	-
14	39.4, CH_2	2.45, (d, 15.0)	39.9, CH_2	2.45, (d, 15.0)	39.4, CH_2	2.54, (d, 15.0)	39.6, CH_2	2.54, (d, 15.0)
		2.73, (d, 15.0)		2.73, (d, 15.0)		2.59, (d, 15.0)		2.59, (d, 15.0)
15	70.8, C	-	71.1, C	-	71.0, C	-	71.0, C	-
16	28.8, CH_3	1.12, (s)	28.8, CH_3	1.14, (s)	29.1, CH_3	1.11, (s)	29.1, CH_3	1.13, (s)
17	30.5, CH_3	1.24, (s)	31.6, CH_3	1.19, (s)	30.8, CH_3	1.25, (s)	31.3, CH_3	1.26, (s)
18	21.1, CH_3	1.09, (s)	21.1, CH_3	1.03, (s)	21.1, CH_3	1.04, (s)	21.1, CH_3	1.09, (s)
19	22.4, CH_3	0.91, (s)	22.5, CH_3	0.83, (s)	22.4, CH_3	0.83, (s)	22.5, CH_3	0.91, (s)
20	24.1, CH_3	1.28, (s)	24.5, CH_3	1.28, (s)	23.9, CH_3	1.20, (s)	24.2, CH_3	1.20, (s)
1'	145.2, C	-	145.3, C	-	167.8, C	-	167.8, C	-
2'	120.4, C	-	120.4, C	-	119.6, C	-	119.6, C	-
3'	111.1, CH	6.45, (d, 3.0)	111.2, CH	6.46, (d, 3.0)	114.6, CH	7.15, (d, 3.0)	114.6, CH	7.16, (d, 3.0)
4'	152.6, C	-	152.6, C	-	151.9, C	-	151.9, C	-
5'	115.2, CH	6.59, (d, 3.0)	115.3, CH	6.60, (d, 3.0)	104.5, CH	7.00, (d, 3.0)	104.5, C	7.01, (d, 3.0)
6'	127.0, C	-	127.2, C	-	126.5, C	-	126.5, C	-
Me-6'	16.6, CH_3	2.16, (s)	16.8, CH	2.17, (s)	16.2, CH_3	2.21, (s)	16.4, CH_3	2.23, (s)
OMe-4'	55.6, CH_3	3.73, (s)	55.6, CH	3.74, (s)	55.7, CH_3	3.78, (s)	55.7, CH_3	3.78, (s)

576

577 **Table 2.** Effect of the compounds **1** and **2** against *L. infantum* promastigotes and intracellular
578 amastigotes and mouse peritoneal macrophages
579

Compounds	Promastigotes[a]	Intracellular amastigotes[a]	Peritoneal macrophages[b]	SI[c]
1	44.9 ± 4.3	25.0 ± 4.1	126.6 ± 21.1	5.04
2	94.4 ± 10.1	> 88.0	84.5 ± 12.5	<0.96
Miltefosine	23.1 ± 0.0	20.3 ± 1.3	130.3 ± 17.2	6.42

[a]IC_{50} - Half maximal inhibitory concentration in µM; [b]CC_{50} - Cytotoxic concentration that causes the death of 50% of the viable cells in µM; [c]SI – Selectivity index concerning the activity against the intracellular amastigotes.

580

Biographies of the authors

Carolina Bruno-de-Sousa - MSc in Animal Production and Post-graduated in Medical Parasitology. As PhD student of the Center of Marine Sciences at the Algarve University, is currently studding marine algae as source of bioactive molecules against *Leishmania* parasites. Main interests include animal parasitological studies and genetic characterization of domestic animals and algae populations. Also collaborated in studies of parasitic diseases with public health significance.

Katkam N. Gangadhar - Post-doctoral Research Fellow at CCMAR, University of Algarve. Works in synthetic/organic lipid medicinal chemistry and pharmaceutical applications: (i) isolation of wound healing and anti-cancer bioactive compounds from natural products; (ii) synthesis of lipid carriers as drug delivery materials for anti-tuberculosis drug and Amphotericin-B; (iii) chemo-enzymatic synthesis of cetyl myristoleates and diacylglycerol and evaluation of their anti-inflammatory, anti-arthritic and nutritional properties; (iv) development of carbon-based solid acid catalyst from crude glycerol for biodiesel production from microalgae and non-edible oils and (v) its application in organic methodologies.

Thiago R. Morais - PhD student of Chemical Biology at UNIFESP, working with Natural Products chemistry, especially with isolation and characterization of micromolecules using NMR and MS data analysis.

Geanne A. A. Conserva - MSc student at the Federal University of São Paulo – UNIFESP – working with Chemistry of Natural Products, mainly in the search and characterization of bioactive derivatives in plant species, particularly those with antitumoral activity.

Catarina Vizetto-Duarte - PhD student at Centre of Marine Sciences (CCMAR) at the University of Algarve. She has an MSc in Molecular Genetics and Biomedicine from the University of Lisbon in 2009. As a PhD student she is evaluating the biomedical applications (especially antioxidant and antitumoral properties) of brown algae, focusing on finding novel bioactive molecules and studying the molecular mechanisms responsible for the said activities in terms of cellular responses to drug exposure, inflammation, cell death (apoptosis/necrosis) versus cell survival.

Hugo Pereira - MSc on Aquaculture and Fisheries, where he worked on the optimization of a novel culture medium for large-scale production of microalgae in photobioreactors at Necton S.A. (Portugal). He is currently a PhD student aiming the development of an algal biorefinery for different biotechnological applications, including the determination of bioactivities to improve the added-value of algal biomass

Márcia D. Laurenti - PhD in Veterinary Pathology; full professor and head chief of Laboratory of Pathology of Infectious Diseases, Department of Pathology, Medical School, University of São Paulo; with experience in the immunopathology of human, canine and experimental cutaneous and visceral leishmaniasis.

Lenea Campino - Full Professor in Medical Parasitology, at the Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa (IHMT/UNL). Main areas of interest are: leishmaniasis and *Leishmania*-HIV co-infections; molecular epidemiology, parasite diversity, vector/host-parasite interactions; immunology of the infection; natural and experimental

leishmaniasis models; vaccine and drug candidates; diagnostics on visceral and cutaneous leishmaniasis; environmental changes and emerging parasitic diseases. She led national and International research projects in those areas, supervised several postgraduate degrees, and acted as a consultant for the Portuguese National Directorate of Health.

Debora Levy - PhD in medical science at Medical School of Sao Paulo University. Currently is scientific researcher at Laboratory of Genetics and Molecular Hematology, and has experience in hematology, genetics and drug development.

Miriam Uemi - Associate Professor at the Federal University of the State of São Paulo - has experience in molecular characterization by nuclear magnetic resonance and mass spectrometry.

Luísa Barreira - Assistant Professor of the Chemistry and Pharmacy Department of the Faculty of Sciences and Technology of the University of Algarve since 2007. She has PhD in Environmental Sciences and Technologies and is currently a senior researcher in MarBiotech for I+D+I of biotechnological applications of marine organisms, from the production of biodiesel and other bioproducts (e.g. phospholipids) from microalgae to the search of natural products with biological activities in marine organisms.

Luísa Custódio - PhD in Biotechnological Sciences and carried out her post-doctoral research at the University of Algarve and CCMAR. Presently she is a research assistant hired by CCMAR under the frame of the FCT investigator programme and her research has focused on the search for bioactive compounds in marine organisms and halophyte species, and the evaluation of the nutritional profile of edible organisms (e.g. algae, halophytes and sea cucumbers).

Felipe Passero - He got PhD in physiopathology at Medical School of Sao Paulo University. Currently is full professor at São Paulo State University, and has experience in Parasitology, mainly with leishmaniasis.

Joao Lago - Full professor at Federal University of Sao Paulo - has experience in Chemistry of Natural Products, mainly in the search and characterization of bioactive derivatives in plant species, including those with antiparasitic anti-inflammatory, antimicrobial and antitumoral activities.

João Varela - Assistant Professor at the University of Algarve and Group Leader of the MarBiotech (Marine Biotechnology) research group at the Centre of Marine Sciences (CCMAR). MarBiotech, which has the following research lines i) search for novel bioactive compounds in marine organisms, with particular emphasis on microalgae, macroalgae and halophytes; ii) design and implementation of biorefineries for the upgrade of algal biomass for biofuel, food and feed production; and iii) marine organisms (e.g. sea cucumbers and halophytes) as innovative gourmet food.

Highlights

- Tetraprenyltoluquinols and tetraprenylquinones from *Cystoseira baccata*.
- Tetraprenyltoluquinols displayed antileishmanial activity
- Tetraprenyltoluquinols induce alterations on promastigotes morphology.
- Tetraprenyltoluquinol disrupt the *Leishmania* mitochondrial membrane potential.