Report of *in vitro* antileishmanial properties of Iberian macroalgae

Carolina Bruno de Sousa[1], João Henrique G. Lago[2], Jorge Macridachis[1], Marta Oliveira[1,3], Luis Brito[1], Catarina Vizetto-Duarte[1], Cláudia Florindo[4,5], Sarah Hendrickx[6], Louis Maes[6], Thiago Morais[2], Miriam Uemi[2], Luís Neto[7], Lídia Dionísio[7], Sofia Cortes[3], Luísa Barreira[1], Luísa Custódio[1], Fernando Alberício[8], Lenea Campino[3] and João Varela[1*]

[1] *Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.*

[2] *Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, São Paulo 09972-270, Brazil.*

[3] G*lobal Health and Tropical Medicine Centre, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, 1349-008 Lisboa, Portugal.*

[4] *Programa de Medicina Regenerativa, Departamento de Ciências Biomédicas e Medicina, UAlg, 8005-139 Faro, Portugal.*

[5] *Centro de Investigação Biomédica, University of Algarve, 8005-139 Faro, Portugal.*

[6] *Department of Biomedical Sciences, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Antwerp University, Antwerp, Belgium*.

[7] *Faculdade de Ciências e Tecnologia, University of Algarve, Faro, Portugal.*

[8] *Institute for Research in Biomedicine, Barcelona Science Park, Barcelona, Spain.*

* Corresponding author. Email: jvarela@ualg.pt

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Abstract

Here is reported the anti *Leishmania infantum* activity of 45 hexane, dichloromethane and ethanol extracts from 16 macroalgae collected on the Iberian Coast. Seven hexane and dichloromethane *Cystoseira baccata, C.barbata, C. tamariscifolia, C. usneoides, Dictyota spiralis* and *Plocamium cartilagineum* extracts were active towards promastigotes (IC_{50} 29.8-101.8 µg/mL) inducing strong morphological alterations in the parasites. Hexane extracts of *C. baccata* and *C. barbata* were also active against intracellular amastigotes (IC_{50} 5.1 and 6.8 µg/mL, respectively). Fatty acids, triacylglycerols, carotenoids, steroids, and meroterpenoids were detected by nuclear magnetic resonance (NMR), and gas chromatography (GC) in the *Cystoseira* extracts. These results suggest that *Cystoseira* macroalgae contain compounds with antileishmanial activity, which could be explored as scaffolds to the development of novel sources of antiparasitic derivatives.

Keywords: Antiparasitic, *Leishmania infantum*; Leishmaniasis; Macroalgae; *Cystoseira*; Secondary metabolites.

1. Introduction

Leishmaniases are vector-borne diseases caused by *Leishmania* parasites, and transmitted by phlebotomine sand flies, which affect more than 12 million people in 98 countries (Alvar et al. 2012). Drugs available for controlling leishmaniases have serious side effects and limited effectiveness due to growing parasite resistance. In this context, the development of novel drugs is of paramount importance (Sundar & Chakravarty 2015). Marine resources are recognized as rich sources of compounds for the biodiscovery of novel drug leads which could also be used for antileishmanial therapy and control (Tempone et al.2011). Several macroalgae species show to be toxic to *Leishmania* parasites however few publications described the identification of promising molecules for future studies regarding leishmaniases treatment (Yamthe et al., 2017). This study evaluated the *in vitro* activity of hexane (HEX), dichloromethane (DCM) and methanolic (MeOH) extracts of 16 macroalgae species from the Iberian coast against *L. infantum*, the species responsible for the canine leishmaniasis and the most severe form of human disease, thevisceral leishmaniasis. Results suggested that *Cystoseira* contain compounds with antileishmanial activity.

2. Results and discussion

Screening of 16 macroalgae extracts using the MTT colorimetric assay enabled the detection of activity against *L. infantum* promastigotes in six species (Tables S1 and S2). Most of the identified bioactive extracts that decreased promastigote viability by 50% at concentrations lower than 94 µg/mL belonged to *Cystoseira* species, effects similar to those obtained for crude extracts of other algae of the same phylum (Yamthe et al., 2017). Altough axenic promastigotes are often used for the *in vitro* preliminary drug screening, promising products should be evaluated on the clinical relevant stage in the vertebrate host, the intracellular amastigotes (Tempone et al. 2011). *C. tamariscifolia* and C. *usneoides* HEX and DCM extracts were the most active (IC_{50} 29.8 - 33.6 µg/mL) against promastigote forms. However, its high toxicity against THP-1 mammalian cells prevented its evaluation upon the intracellular model (Figure 1). Conversely, *C. baccata* and *C. barbata* HEX extracts that decreased by half the promastigote viability at 94.1 and 78.7 µg/mL presented good selectivity when assayed against the intracellular model. Incubation with the aforementioned extracts decreased infection by 50% at 5.1 and 6.8 µg/mL, respectively, with cytotoxicity similar to that of the reference drug miltefosine (Figure 1; Table S2). These results showed that the *C. baccata* and *C. barbata* HEX extracts displayed higher leishmanicidal activity than those described for other *Cystoseira* against axenic (IC_{50} > 23.5 µg/mL; Süzgeç-Selçuk et al. 2010) and intracellular (IC_{50} > 15.7 µg/mL; Spavieri et al. 2010) *L. donovani* amastigotes.

The effect of *Cystoseira* extracts on promastigotes was also analysed by optical microscopy, revealing the occurrence of morphological changes (Figure S1) similar to those observed after treatment of *Leishmania* promastigotes with extracts from marine sponges (Kahla-Nakbi et al., 2010). Extracts induced motility loss, cell shrinkage, abnormal round cell shapes, vacuolated and slightly denser cytoplasm as well as reduction of flagellar length in parasite cells (Figure S1D). Loss of motility and cellular vacuolization could be consequence of starvation processes caused by a deficient mitochondrial activity, autophagic mechanisms or cytoplasmic organelle disruption (Lockshin & Zakeri 2004; Monte Neto et al., 2011). These results together with the absence of phosphatidylserine externalization, observed in promastigotes treated with the extracts for 48h (data not shown), suggest that crucial organelles, such as mitochondria, are compromised and that the observed leishmanicidal effect might not be associated with programmed cell death.

Chemical profiles of the active *Cystoseira* extracts investigated using different analytical methods (NMR, GC/FID and GC/LREIMS) reveal the occurrence of different classes of

metabolites (Table S3; Figures S2 and S3)in agreement with previous studies focusing on *Cystoseira* (Bruno de Sousa, et al. 2017a). Unsaturated fatty acids such as arachidonic and eicosapentaenoic (EPA) acids were found in the HEX extracts. EPA was found to be effective against *L. donovani* promastigotes (Vassallo et al. 2011) and triacylglycerols against *L. aethiopica* (Bekele et al. 2013) and *L. amazonensis* (Rodrigues et al. 2014). Steroids such as cholesterol, sitosterol, stigmasterol and fucosterol, also identified in all the analysed HEX extracts. The antileishmanial effect of fucosterol and sitosterol isolated from other biological sources has already been reported (Bruno de Sousa et al., 2017b). Concerning meroterpenoids, E- and Z-usnoidones were identified in the *Cystoseira* studied extracts. Other meroterpenoids were also detected in bioactive CH_2Cl_2 extracts, except from *C. tamariscifolia*. Meroterpenoids have already been described as having several bioactive properties (Reyes et al. 2013) including antileishmanial (Bruno de Sousa et al. 2017a). Although their effect on *Leishmania* has been associated with the inhibition of adenosine phosphoribosyltransferase (Gray et al. 2006), their antileishmanial activity remains poorly studied.

3. Conclusions

Our results suggest that *Cystoseira* are a source of metabolites with activity against *L. infantum*. In that context, further bioguided fractionation of the active *Cystoseira* extracts should be carried out in order to identify the active components.

Disclosure statement

No potential conflict of interest was reported by the authors.

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FIGURE CAPTIONS

Figure 1. Effect of the *L. infantum* and mammalian cells treatment with *Cystoseira* spp. active hexane extracts and the reference drug Miltefosine. Significant differences between extracts and reference drug are indicated as *,$p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's t-test).

SUPPLEMENTARY MATERIAL

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[1] *Centre of Marine Sciences, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.*

[2] *Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, São Paulo 09972-270, Brazil.*

[3] G*lobal Health and Tropical Medicine Centre, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, 1349-008 Lisboa, Portugal.*

[4] *Programa de Medicina Regenerativa, Departamento de Ciências Biomédicas e Medicina, UAlg, 8005-139 Faro, Portugal.*

[5] *Centro de Investigação Biomédica, University of Algarve, 8005-139 Faro, Portugal.*

[6] *Department of Biomedical Sciences, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Antwerp University, Antwerp, Belgium*.

[7] *Faculdade de Ciências e Tecnologia, University of Algarve, Faro, Portugal.*

[8] *Institute for Research in Biomedicine, Barcelona Science Park, Barcelona, Spain.*

* Corresponding author. Email: jvarela@ualg.pt

Abstract

Here is reported the anti *Leishmania infantum* activity of 45 hexane, dichloromethane and ethanol extracts from 16 macroalgae collected on the Iberian Coast. Seven hexane and dichloromethane *Cystoseira baccata, C.barbata, C. tamariscifolia, C. usneoides, Dictyota spiralis* and *Plocamium cartilagineum* extracts were active towards promastigotes (IC_{50} 29.8-101.8 µg/mL) inducing strong morphological alterations in the parasites. Hexane extracts of *C. baccata* and *C. barbata* were also active against intracellular amastigotes (IC_{50} 5.1 and 6.8 µg/mL, respectively). Fatty acids, triacylglycerols, carotenoids, steroids, and meroterpenoids were detected by nuclear magnetic resonance (NMR), and gas chromatography (GC) in the *Cystoseira* extracts. These results suggest that *Cystoseira* macroalgae contain compounds with antileishmanial activity, which could be explored as scaffolds to the development of novel sources of antiparasitic derivatives.

Keywords: Antiparasitic, *Leishmania infantum*; Leishmaniasis; Macroalgae; *Cystoseira*; Secondary metabolites.

Experimental Section

1. Algal Material

Samples from 15 different species belonging to the Rhodophyta, Chlorophyta and Heterokontophyta phyla were collected between July, 2010 and July, 2013 at different locations of the Portuguese and Spanish coasts (Table S1). Morphological identification was made by Dr Aschwin Engelen, Dr Tânia Pereira and MSc Mafalda Tavares (CCMAR-UAlg), Dr Javier Cremades (Facultad de Ciencias, University of A Coruña, Spain) and Dr Ricardo Bermejo (University of Cadiz, Spain). Biomass was washed with water to remove epiphytes, before freeze-drying, then grounded and stored at -20 °C until use. Vouchers specimens are kept at MarBiotech herbarium at the Centre of Marine Sciences, Faro, Portugal.

2. Extracts preparation

Dried biomass was blended with hexane (1:10 w/v) by means of an IKA Ultra-Turrax disperser for 1 min for cell wall disruption, and after centrifugation (5000 × *g*, 10 min) the supernatants were recovered. The extraction was repeated three times. The residue was then sequentially extracted with CH_2Cl_2 and MeOH in a similar manner. All extracts were evaporated under reduced pressure at 40 ºC and stored at 4 °C. For the bioactivity assays, extracts were dissolved in DMSO (dimethyl sulfoxide) at the concentration of 50 mg/mL.

3. Chemical characterization of Cystoseira *extracts*

3.1. NMR analysis

Hydrogen nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker Avance III 500 spectrometer, using 5 mm TXI probe, operated at 500.13 MHz for ^{1}H nucleus. Approximately 10 mg of each sample was dissolved in 0.6 mL of $CDCl_3$ with tetramethylsilane (TMS) as a standard reference with a chemical shift $\delta_H = 0.00$ ppm. Spectra were obtained at 25 °C, with 64 scans, 2 dummy scans, using a 90-degree high power pulse of 8.3 µs, a recycle delay of 1s, and 64 K data points covering a spectral width of 7684 Hz. All spectra were processed using Topspin 3.2 software.

3.2. GC/FID and GC/LREIMS analysis

Gas chromatography with flame ionization detector (GC/FID) chromatograms were recorded on a Shimadzu GC-2010 gas chromatograph equipped with an FID-detector and an automatic injector (Shimadzu AOC-20i) using a RtX-5 capillary column (5% phenyl, 95% polydimethylsiloxane, 30 m × 0.32 mm × 0.25 μm film thickness; Restek, USA). These analyses were performed by injecting 1.0 μL of a 1.0 mg/mL solution of crude hexane extract in hexane in a split mode (1:30) employing helium as the carrier gas (1 mL/min) under the following conditions: injector and detector temperatures of +270 ºC and 300 ºC, respectively; oven programmed temperature from 120–290 °C at 8 °C/min, holding 20 min at 290 ºC. Gas chromatography–low resolution electron ionization mass spectrometry (GC/LREIMS) analysis was conducted in a Shimadzu GC-17A chromatograph interfaced with a MS-QP-5050A mass spectrometer operating using ionization voltage of 70 eV and an ion source temperature of +350 °C with the same conditions described above. Helium was used as the carrier gas.

4. Antileishmanial and cytotoxicity assays

4.1. Cytotoxicity against human acute monocytic leukaemia cell line THP-1

Cytotoxicity of all extracts was performed on human acute monocytic leukaemia cell line THP-1 (ATCC TIB-202). Cells were cultivated in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (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. For the assay, 10^5 THP-1 cells per well were seeded onto the 96-well plates. Extracts were added at concentrations ranging from 4 to 125 μg/mL for 48h, and cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, as described elsewhere (Dutta et al., 2005). Cells used as negative control were treated with DMSO at the highest concentration used in test wells (0.5% v/v). Results were expressed in terms of cell viability (%) and half maximal inhibitory concentration values (IC_{50} – in μg/mL).

4.2. Antipromastigote assay

Promastigote forms of a *L. infantum* strain (MHOM/PT/88/IMT-151) were obtained from the cryobank of the 'Instituto de Higiene e Medicina Tropical' (Universidade Nova de Lisboa, Portugal) and maintained in RPMI-1640 medium supplemented with 10% FBS, L-glutamine, penicillin and streptomycin, at 24 ºC. About 2 x 10^6 parasites per well were incubated in 96-well

plates with the extracts at concentrations ranging from 4 to 125 μg/mL for 48h. Negative control cells were treated with DMSO at the highest concentration used in test wells (≤1% v/v). Amphotericin B (0.2 μg/mL), miltefosine (12.7 μg/mL) and pentamidine (0.5 μg/mL) were used as positive controls. The effect of the extracts on parasite viability was assessed by the MTT colorimetric assay. Results were expressed in terms of cell viability (%) and IC_{50} values (μg/mL).

4.3. Activity against intracellular amastigotes

L. infantum intracellular amastigotes (MHOM/MA(BE)/67) were collected from the spleen of heavily infected donor hamsters and used to infect primary peritoneal mouse macrophages (PMM). PMM (3×10^4 cells per well) were seeded on 96-well plates, and incubated for two days for cell attachment. Then, 5×10^5 amastigotes were added to each well (infection ratio about 16 amastigotes per cell) and infected macrophages were further incubated at 37 ºC for 2 h. The extracts at concentrations ranging from 0.25 to 64.0 μg/mL were added and the plates were further incubated at 37 ºC and 5% CO_2. After 5 days, intracellular amastigote burdens were microscopically assessed upon Giemsa staining and the inhibitory concentration conferring a 50% reduction of the intracellular amastigote burden compared to the non-treated infected positive controls (CC_{50}) was determined. In addition, cytotoxicity of the extracts was evaluated on PMM cells and carried out as previously described (Cos et al., 2016). The reference drug (miltefosine) was used as positive control.

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4.4. Microscopic analysis

Leishmania promastigotes were incubated with the extracts (125 μg/mL) and with amphotericin (0.2 μg/mL) for 48h. After incubation and centrifugation, parasites were smeared on microscope slides, fixed with methanol and stained with Giemsa solution and observed using a Zeiss AXIOMAGER Z2 microscope, equipped with a cool SNApHQ2 camera and AxioVision software version 4.8 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

4.5. Apoptosis detection through annexin V-FITC staining

Promastigotes (4 x 10^6/mL) cultured in RPMI medium with DMSO (0.1%) were treated with IC_{50} concentrations of with Cystoseira extracts at IC_{50} concentrations for 48h. Negative and positive controls cells were treated with DMSO at the highest concentration used in test wells (≤

1% v/v) and amphotericin B (0.6 µg/mL), respectively. Apoptotic effect of the tested extracts on *L. infantum* promastigotes was evaluated by flow cytometry using the Annexin V-FITC (fluorescein isothiocyanate) Apoptosis Kit - KA0714 (Abnova) in accordance with the manufacturer's recommendations. Briefly, treated and control promastigotes were washed with culture medium and resuspended in 500 µL binding buffer and stained with 5 µL of annexin V-FITC (10 mg/mL) and 5 µl of propidium iodide (PI; 50µg/ml) and incubate at room temperature for 5 min in the dark. Results were obtained by flow cytometry using a FACS Calibur Flow Cytometer (Becton-Dickinson, East Rutherford, NJ, USA) using the Cell Quest software (BD Biosciences, San Jose, CA, USA) for acquisition and result analysis. Ten thousand events were analysed and apoptosis evaluated based on the geometric mean of the fluorescence intensity detected in channels 1 (for annexin) and 2 (for PI) (Farias et al., 2013).

6. Statistical analysis

The antileishmanial and cytotoxic assays were conducted in triplicate and the results were expressed as mean and standard error of the mean (SEM). The IC_{50} values were calculated using sigmoid regression on the logarithm of the concentration-response data in the GraphPad Prism V 5.0 software. Student's t-test was used to determine whether differences between means were significant at different levels ($p < 0.05$ and $p < 0.01$).

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Tables

Table S1. Species, date of collection and collection site of the macroalgae used in this study

Phylum /Species	Date	Local	Country
Chlorophyta			
Cladophora albida (Nees) Kutzing	July 2010	Olhos de Água[1]	Portugal
Codium sp. Stackhouse	July 2010	Olhos de Água[1]	Portugal
Heterokontophyta			
Cladostephus spongiosus (Hudson) C. Agardh	July 2010	Olhos de Água[1]	Portugal
Cystoseira baccata (S. G. Gmelin) P. C. Silva	July 2012	Areosa[2]	Portugal
Cystoseira barbata (Stackhouse) C. Agardh	March 2013	Cadiz Bay[1]	Spain
Cystoseira humilis Schousboe ex Kützing	May 2012	Almograve[3]	Portugal
Cystoseira nodicaulis (Withering) M. Roberts	April 2013	Santa Mariña[2]	Spain
Cystoseira tamariscifolia (Hudson) Papenfuss	July 2012	Areosa[2]	Portugal
Cystoseira usneoides (L.) M. Roberts	September 2012	Olhos de Água[1]	Portugal
Halopteris scoparia (L.) Sauvageau	July 2010	Olhos de Água[1]	Portugal
Sargassum muticum (Yendo) Fensholt	July 2010	Olhos de Água[1]	Portugal
Taonia atomaria (Woodward) J. Agardh	July 2010	Olhos de Água[1]	Portugal
Rhodophyta			
Peyssonnelia squamaria (S. G. Gmelin) Decaisne	July 2013	Arrifes[1]	Portugal
Plocamium cartilagineum (L.) P. S. Dixon	July 2012	Olhos de Água[1]	Portugal
Scinaia furcellata (Turner) J. Agardh	July 2013	Olhos de Água[1]	Portugal

[1]Southern coast, [2]Northwestern coast and [3] Southwestern coast of the Iberian Peninsula.

Table S2. Inhibitory concentrations of algal extracts against *L. infantum* and mammalian cells

Species	Extract/ Compound	IC_{50} (µg/mL)[a] Promastigotes	CC_{50} (µg/mL)[b] Amastigotes	IC_{50} (µg/mL)[a] THP-1
Cladophora albida	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cladostephus spongiosus	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cystoseira baccata	Hexane	94.1 ± 1.5	5.1 ± 0.0	>125
	CH_2Cl_2	>125	-	76.2 ± 3.9
	MeOH	>125	-	>125
Cystoseira barbata	Hexane	78.7 ± 3.2	6.8 ± 0.0	79.5 ± 2.3
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cystoseira humilis	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cystoseira tamariscifolia	Hexane	31.2 ± 0.9	-	30.9 ± 0.4
	CH_2Cl_2	29.8 ± 0.5	-	19.9 ± 0.5
	MeOH	>125	-	>125
Cystoseira nodicaulis	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Cystoseira usneoides	Hexane	59.9 ± 0.2	-	16.6 ± 0.3
	CH_2Cl_2	33.6 ± 0.6	-	12.6 ± 0.4
	MeOH	>125	-	45.0 ± 0.4
Codium sp.	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Dictyota spiralis	Hexane	48.3 ± 0.7	-	-
	CH_2Cl_2	>125	-	-
	MeOH	>125	-	-
Halopteris scoparia	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Peysonnelia squamaria	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Plocamium cartilagineo	Hexane	101.8±2.8	-	51.6±0.1
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Sargassum muticum	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Scinaria funcellata	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125

Species	Extract/ Compound	IC_{50} (μg/mL)[a] Promastigotes	CC_{50} (μg/mL)[b] Amastigotes	IC_{50} (μg/mL)[a] THP-1
Taonia atomaria	Hexane	>125	-	>125
	CH_2Cl_2	>125	-	>125
	MeOH	>125	-	>125
Positive controls	Miltefosine	12.7 ± 1.8	8.8 ± 0.0	8.8 ± 0.0
	Amphotericin	0.2 ± 0.0	-	-
	Pentamidine	0.5 ± 0.1	-	-

[a]IC_{50} - Inhibitory concentration of extract / compound causing 50% reduction of the promastigote and human acute monocytic leukaemia cell line THP-1 cells growth; [b]CC_{50} - Inhibitory concentration of extract / compound causing 50% reduction of the intracellular amastigote burden compared to the non-treated infected controls. IC_{50} and CC_{50} values represent the mean ± standard error of the mean of three experiments performed in triplicate.

Table S3. Class of metabolites found in *Cystoseira* extracts

Species	Extracts	Class of metabolites[1]
C. baccata	Hexane	FA, TAG, CAR, ST
	CH_2Cl_2	MT, FA
	MeOH	MT
C. barbata	Hexane	FA, TAG, CAR, ST
	CH_2Cl_2	MT, FA
	MeOH	MT
C. humilis	Hexane	FA, TAG, CAR
	CH_2Cl_2	FA
	MeOH	FA, MT
C. nodicaulis	Hexane	FA, TAG, CAR, ST
	CH_2Cl_2	MT, FA
	MeOH	FA
C. tamariscifolia	Hexane	FA, TAG, CAR
	CH_2Cl_2	MT, FA
	MeOH	MT
C. usneoides	Hexane	MT, FA
	CH_2Cl_2	MT, FA
	MeOH	MT

[1]FA - fatty acids, TAG - triacylglycerols, CAR - carotenoids, ST - steroids, MT - meroterpenoids

Figures

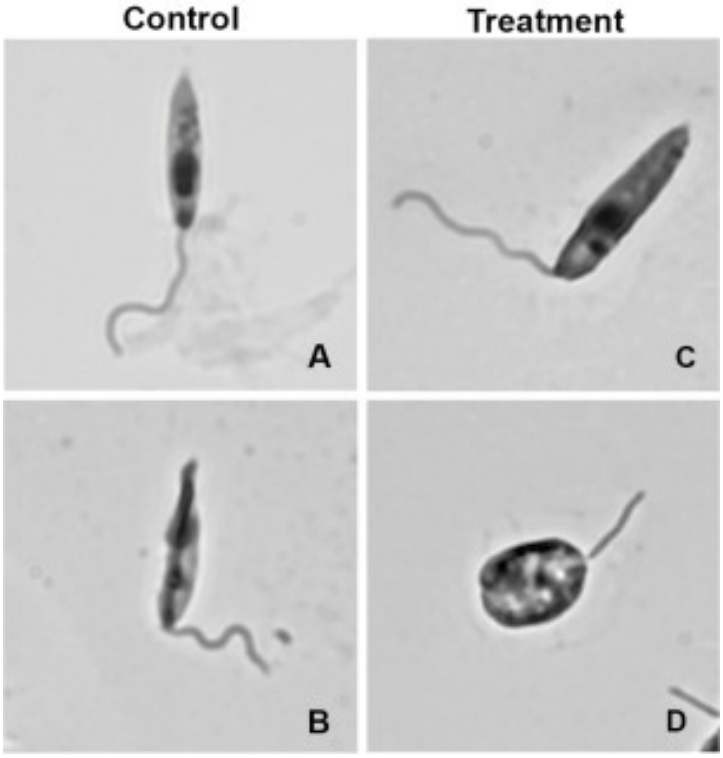


Figure S1. Effect of *C. nodicaulis* and *C. barbata* hexane extracts (125 µg/mL, 48h) on the morphology of *L. infantum* promastigotes. Control cells cultured in RPMI medium alone (**A**) treated with the control drug amphotericin B (0.2 µg/mL; **B**), and treated with *C. nodicaulis* (**C**) or *C. barbata* (**D**) extracts. Bright field images; the scale bar corresponds to 5 µm.

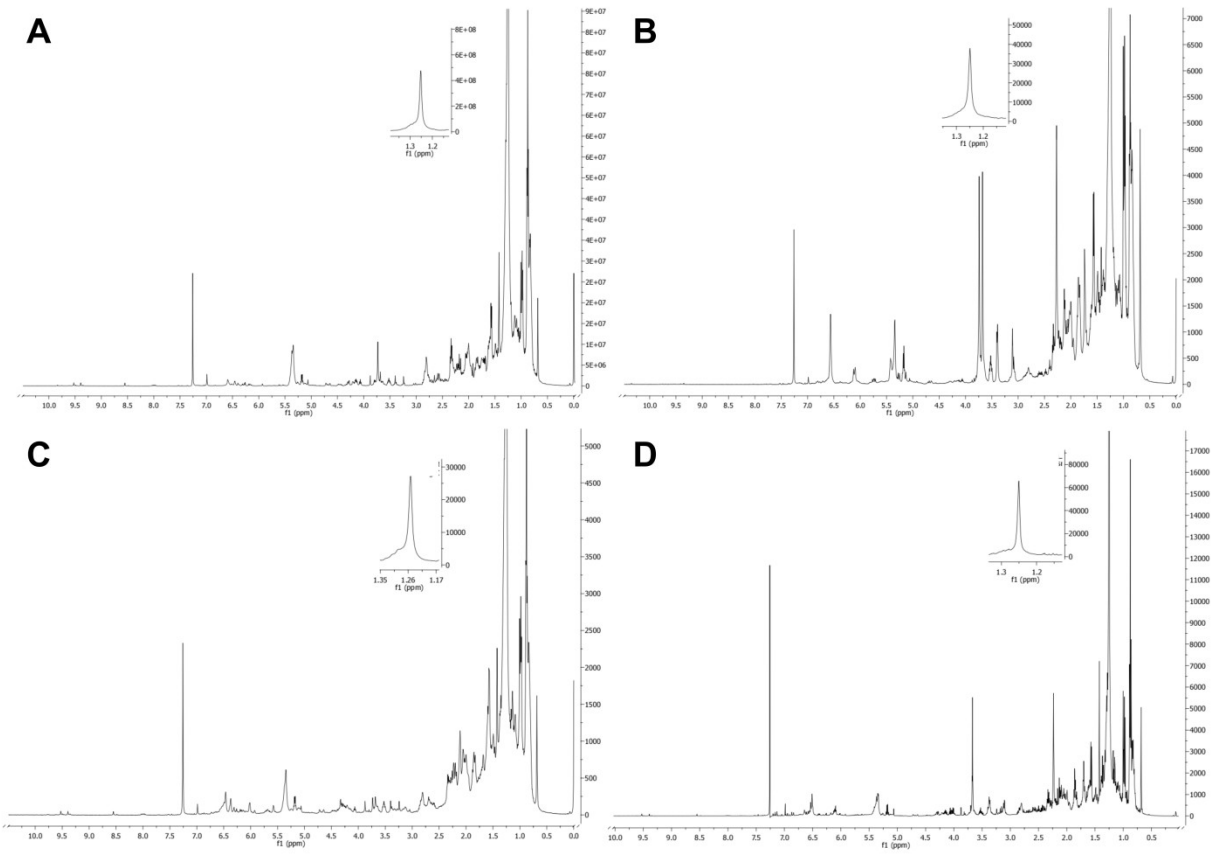
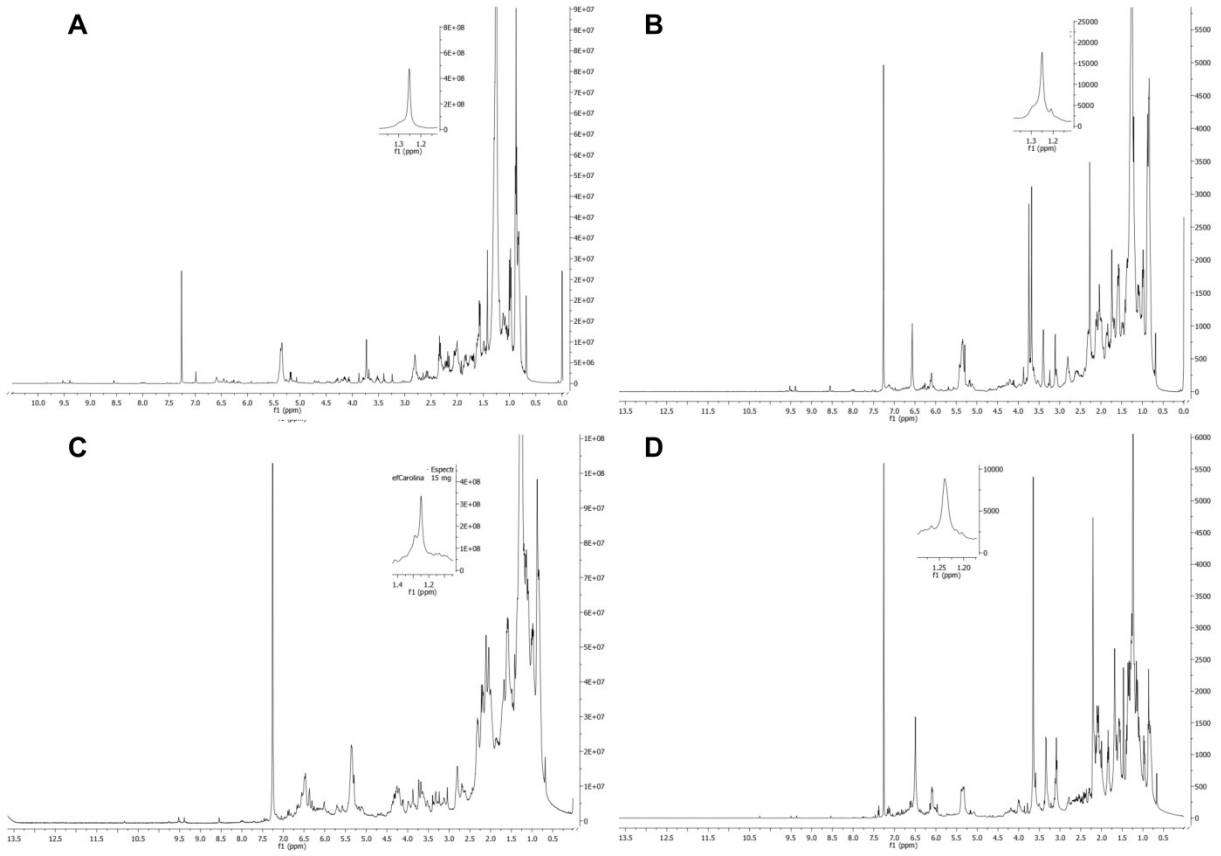


Figure S2. ^{1}H NMR spectra (500 MHz, $CDCl_3$) of the *Cystoseira baccata* (**A**), *C. barbata* (**B**) *C. tamariscifolia* (**C**) and *C. usneoides* (**D**) hexane extracts.



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179**Figure S3.** ^{1}H NMR spectra (500 MHz, $CDCl_3$) of the *Cystoseira. baccata* (**A**), *C. barbata* (**B**), 180*C. tamariscifolia* (**C**) and *C. usneoides* (**D**) CH_2Cl_2 extracts.

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