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Annotation of GC-MS Data of Antimicrobial Constituents in the Antarctic Seaweed *Phaeurus antarcticus* by Molecular Networking

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Phaeurus antarcticus is a member of the Desmarestiaceae family endemic to the Antarctic Peninsula. Reports addressing its chemical composition and biological activities are scarce. Herein, bioactive non-polar compounds of *P. antarcticus* against pathogenic bacteria, *Leishmania amazonensis* and *Neospora caninum* parasites were targeted through GC-MS Molecular Networking and multivariate analysis (OPLS-DA). The effects on horseradish peroxidase (HRP) were also evaluated. *P. antarcticus* exhibited selective bacteriostatic and bactericidal activities against *Staphylococcus aureus* with MIC and MBC values from 6.25–100 $\mu g\,mL^{-1}$. Fractions HX-FC and HX-FD were the most active against *L. amazonensis* with EC_{50} ranging from 18.5–62.3 $\mu g\,mL^{-1}$. Additionally, fractions HX-FC and HX-FD showed potent inhibition of *N. caninum* at EC_{50} values of 2.8 and 6.3 $\mu g\,mL^{-1}$, respectively. All fractions inhibited HRP activity, indicating possible interactions with Heme proteins. It was possible to annotate compounds from tree mains clusters, containing terpenoids, steroids, fatty acids, and alcohols by correlating the spectral data of the GC-MS analysis with Molecular Networking and the OPLS-DA results.

Introduction

In the Antarctic coastal ecosystem, seaweed communities thrive, covering over 80% of the seabed while sustaining biomass levels similar to those found in temperate kelp forests. Furthermore, they play a crucial role in organic matter cycling and mediating ecological interactions by exerting dominance on shallow benthic communities inhabiting hard substrates.[1]

In addition to their role in supporting primary production within coastal ecosystems, Antarctic seaweeds biosynthesize a range of secondary metabolites featuring a diverse array of biological properties. While numerous natural products sourced from these organisms have been identified and characterized for their bioactivities, limited research was conducted into their potential as sources of antimicrobial agents.[2–6] Seaweed secondary metabolites, including peptides, polysaccharides, polyphenols, polyunsaturated fatty acids, steroids, and pigments, have shown promise as potential scaffolds in the development of new drug leads, but current research remains concentrated on tropical and subtropical organisms.[7]

Overall, the discovery of compounds with therapeutic activity from natural products is slow, intensely laborious, and sometimes inefficient since low yielding but highly potent new bioactive compounds cannot be isolated. Advances in bioinformatics and analytical methods have provided new strategies for the discovery of these compounds, such as the Global Natural Products Social Molecular Networking (GNPS).[8–10] Recently, researchers have developed algorithms that allow the auto-deconvolution of gas chromatography-mass spectrometry (GC-MS) data and execution of molecular networks from this data within the GNPS platform. This tool uses MS/MS spectra to group and form clusters of chemically similar compounds.[11] In this context, the implementation of GNPS has played a crucial

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role in preventing the loss of chemical information and enhancing the process of bioassay-guided fractionation and isolation of novel bioactive molecules.[9]

In recent years, two main trends have stimulated the interest in new natural antimicrobial compounds. First, the rising of microbial infections resistant to currently available antibiotics highlights the urgent need for new drugs against pathogens like *Staphylococcus aureus* and parasites such as *Leishmania amazonensis* and *Neospora caninum*. The drug-resistant bacteria and parasites represent a dangerous challenge to healthcare systems. Additionally, the toxicity of current *Leishmania* treatments and the absence of an approved drug for *Neospora caninum* highlight the critical importance of prospecting new antimicrobial compounds in the battle against infectious diseases[12] Secondly, there is a growing demand for natural ingredients with preservative properties to substitute synthetic adjuvants with potentially toxic effects in human and environmental health.[13–15] Seaweeds have shown antimicrobial potential and, in some cases, a synergistic effect with conventional antibiotics against drug-resistant pathogens showing their potential as a source of new antimicrobials.[13] Furthermore, the search for new horseradish peroxidase (HRP) inhibitors is needed, since this enzyme is extensively used in various biotechnological applications, including the development of diagnostic tests (ELISA assays), bioremediation, and cancer therapy. However, the current arsenal of HRP inhibitors is limited, and lack the specificity required for more precise applications.[15]

The macroalga *Phaeurus antarcticus* Skottsberg (Phaeophyceae) is endemic to Antarctic and sub-Antarctic regions.[16] This species remains underexplored in terms of its chemical and biological activity profiles, with a limited number of reports related to its polyphenol and lipid contents.[17–19] Therefore, this study aimed to evaluate the biological activities of *P. antarcticus* towards pathogenic bacteria, and parasites such as *Leishmania amazonensis*, *Neospora caninum*, and, the enzyme HRP, targeting its bioactive compounds by coupling GC-MS molecular networking to multivariate analysis.

Results and Discussion

Herein we evaluated the bactericidal/bacteriostatic, leishmanicidal, and anti-*Neospora* activities, as well as the inhibitory potential against the enzyme HRP, of the Antarctic alga *P. antarcticus*. For this, the crude extract was obtained and analyzed regarding their potential against the bacteria *Proteus mirabilis* ATCC 29906, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Staphylococcus saprophyticus* ATCC 15305, the parasites *L. amazonensis, N. caninum* as well as cytotoxic activities and inhibition of HRP.

Antibacterial activity

Unlike previous data previous showing the lack of antibacterial effect of *Phaeurus antarcticus* ethereal and butanoic extracts against pathogenic and marine derived Antarctic bacteria,[20] herein the *n*-hexane crude extract exhibited selective bacteriostatic and bactericidal activities against *S. aureus* displaying MIC and MBC values ranging from 12.5 to 100 $\mu g\,mL^{-1}$. After chromatographic fractionation, fractions HX-FB, HX-FD, HX-FA, and HX-FC showed MIC values of 6.2, 12.5, 25 and 100 $\mu g\,mL^{-1}$ respectively. Additionally, fractions HX-FA, HX-FB, and HX-FD exhibited bactericidal effects with MBC concentration of 100 $\mu g\,mL^{-1}$. Fraction HX-FC exhibited a weak bacteriostatic activity against *P. mirabilis*, *S. saprophyticus* and *P. aeruginosa* with MIC values ranging from 200 to 400 $\mu g\,mL^{-1}$, the lower antibacterial effects were observed towards *P. aeruginosa* (Table 1). Fraction HX-FB afforded the lowest MIC value (6.2 $\mu g\,mL^{-1}$) but also demonstrated a cytotoxic profile against human fibroblasts (CC_{50} 3.4 $\mu g\,mL^{-1}$), pointing fraction HX-FD as the most promising against *S. aureus* with a MIC value of 12.5 $\mu g\,mL^{-1}$.

S. aureus is classified as both commensal and pathogenic bacteria while approximately 30% of the world population is infected by this microorganism.[21] The main clinical manifestations caused by *S. aureus* include bacteremia, endocarditis, osteoarticular infections and skin and soft tissue damage.[22]

Table 1. Antibacterial activity of *P. antarcticus* crude n-hexane extract and fractions against pathogenic bacteria expressed in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in $\mu g\,mL^{-1}$.

Sample	*P. mirabilis* $\mu g\,mL^{-1}$		*S. aureus* $\mu g\,mL^{-1}$		*S. saprophyticus* $\mu g\,mL^{-1}$		*P. aeruginosa* $\mu g\,mL^{-1}$		*E. coli* $\mu g\,mL^{-1}$	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
n-Hexane extract	400	>400	12.5	100	400	>400	400	>400	>400	>400
HX-FA	400	>400	25	100	400	>400	>400	>400	400	>400
HX-FB	200	400	6.2	100	400	>400	400	>400	400	>400
HX-FC	200	400	100	400	200	>400	400	>400	200	>400
HX-FD	200	>400	12.5	100	400	>400	200	>400	400	>400
Positive Control	2.95 [*]	5.9 [*]	0.18 [**]	0.36 [**]	1.47 [**]	5.9 [**]	5.9 [*]	>5.9 [*]	0.184 [*]	2.95 [*]

[*] Streptomycin and [**] Penicilin G.

Furthermore, this bacterium also has the notorious ability to become resistant to antibiotics, emphasizing the need for a continuous search of new antibacterial molecules.[12]

Antileishmanial activity

The antileishmanial potential of the aforementioned fractions were also evaluated (Table 2), indicating the presence of antileishmanial compounds in fraction HX-FC ($EC_{50\text{-}PRO}$ 62.3 $\mu g\,mL^{-1}$) and HX-FD ($EC_{50\text{-}PRO}$ 52.8 $\mu g\,mL^{-1}$) which were further investigated regarding their anti-*L. amazonensis* amastigote properties. To continue the evaluation regarding their potency against amastigotes in infected murine macrophages, the cytotoxicity properties of both fractions were evaluated and, unlike HX-FD that present high cytotoxicity, causing macrophages lysis, HX-FC exhibited anti-amastigote effect with $EC_{50\text{-}AMA}$ of 18.5 ± 2.7 $\mu g\,mL^{-1}$). Leishmaniasis affects humans and animals, causing public health problems especially in underdeveloped and developing countries. Currently treatments for leishmaniasis are represented by few chemotherapeutic agents, such as pentavalent antimonials, amphotericin B, paromomycin, and miltefosine, which have several limitations regarding toxicity and lack of efficacy in endemic areas,[23] evidencing the need for the search of new antileishmanial compounds. There are only few works available indicating the Antarctic macroalgae antileishmanial potential, including the red alga *Iridaea cordata* and the brown algae *Himantothallus grandifolius*, *Ascoseira mirabilis* and *Desmarestia antarctica*.[4,5,24,25]

Anti-Neospora activity

Neosporosis is caused by the etiological agent *Neospora caninum* infects mammalian species, like cattle, sheep, goats, horses, and dogs, leading to aborts in cattle and neuromuscular disorders in dogs.[26] The economic loss related to neosporosis is estimated at billions of dollars per annum. Additionally, neosporosis still does not have a specific treatment.[27] The n-hexane extract and its derived fractions inhibited *N. caninum* proliferation in concentrations below 23 $\mu g\,mL^{-1}$ (Table 3). The EC_{50} concentrations for *N. caninum* were 20.1, 22.9, 3.9, 2.9 and 6.3 when treated with the n-hexane extract, HX-FA, HX-FB, HX-FC, and HX-FD, respectively. Fraction HX-FC was the most active with the lowest EC_{50} of 3.9 $\mu g\,mL^{-1}$ and low cytotoxicity against human fibroblasts (CC_{50} 32.4 $\mu g\,mL^{-1}$). Fraction HX-FB inhibited the fibroblasts in concentrations above 3.48 $\mu g\,mL^{-1}$. This pattern probably indicates a cytotoxic component in HX-FB. Therefore, the fractions HX-FC and HX-FD demonstrated an interesting activity against *N. caninum*, amplifying the candidates for neosporosis control from natural resources. The use of vegetal or algae-derived compounds in *N. caninum* is poorly described. Extracts of Thai *Piperaceae*, *Thalassomya japonica*, and *Sophora flavescens* have been tested in *N. caninum* proliferation assays.[28,29] However, these extracts demonstrated lower activity on *N. caninum* ($EC50 > 22.1$ $\mu g\,mL^{-1}$) compared to *P. antarcticus* fractions. Similarly, our results were comparable to previous data from the alga *D. antarctica*, which inhibited the *N. caninum* proliferation in concentrations between 1.6–20.6 $\mu g\,mL^{-1}$.[5]

Inhibition of Horseradish peroxidase

The *n*-hexane extract and its derived fractions partially inhibited the horseradish peroxidase activity compared to the controls (including DMSO 1%) (Figure 1A). This inhibitory pattern was similar to the one observed in *N. caninum* proliferation assay (Table 3). The curve slope of the control and DMSO were 0.154 and 0.152, respectively. After incubation with the extract and fractions, the curve slopes decreased to 0.104 (*n*-hexane extract), 0.091 (HX-FA), 0.105 (HX-FB), 0.107 (HX-FC), 0.096 (HX-FD) (Figure 1B). HRP is a member of the Heme peroxidase family, enzymes related to the detoxification of H_2O_2 in several models.[30] HRP is widely applied as a reporter enzyme in diagnostics and histochemistry, usually ligated to secondary antibodies.[31] Consequently, the components of the extract and fractions of *P. antarcticus* may interfere in procedures based on

Table 2. Leishmanicidal activity of *P. antarcticus* *n*-hexane extract and derived fractions against *Leishmania amazonensis*.

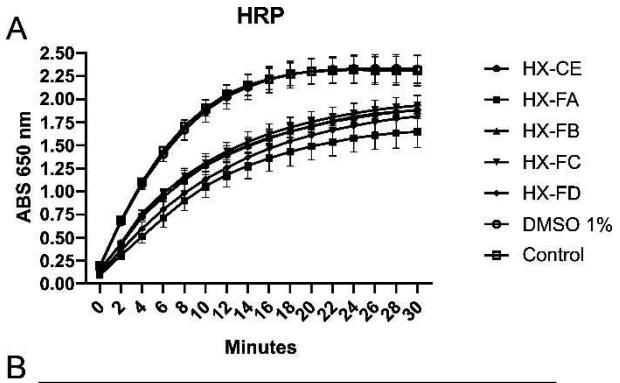
Sample	$EC_{50[PRO]}$ ($\mu g\,mL^{-1}$)	$EC_{50[AMA]}$ ($\mu g\,mL^{-1}$)	$CC_{50[HF]}$ ($\mu g\,mL^{-1}$)	SI[*]
n-Hexane extract	18.45 ± 2.3	–	38.9 ± 13.5	–
HX-FA	205.8 ± 14.8	–	63.6 ± 57.6	–
HX-FB	241.0 ± 0.9	–	3.4 ± 5.5	–
HX-FC	62.3 ± 0.0	18.5 ± 2.7	32.4 ± 20.6	1.7
HX-FD	52.8 ± 0.0	NC [**]	53.7 ± 41.8	–
Amphotericin B	3.2 ± 1.0	3.7 ± 0.1	23.1 ± 2.5	6.2

[PRO] Antiparasitic activities against promastigotes, and [AMA] amastigotes of *L. amazonensis* are expressed as half-maximal effective concentrations (EC_{50}); [HF] Cytotoxicity to non-tumoral human fibroblasts is expressed as half-maximal cytotoxic concentrations (CC_{50}); [*] Selectivity Index; NC [**] Not counted.

Table 3. Anti-Neospora activity of *P. antarcticus* n-hexane extract and derived fractions.

Sample	EC_{50} [a] ($\mu g\,mL^{-1}$)	$CC_{50[HF]}$ ($\mu g\,mL^{-1}$)	SI[*]
n-Hexane extract	20.1 ± 2.1	38.9 ± 13.5	1.9
HX-FA	22.9 ± 2.1	63.6 ± 57.6	2.7
HX-FB	3.9 ± 3.7	3.4 ± 5.5	< 1
HX-FC	2.8 ± 1.7	32.4 ± 20.6	11.2
HX-FD	6.3 ± 3.4	53.7 ± 41.8	8.5
Pyrimethamine	0.56 ± 0.02	> 10	> 17.8

[a] Antiparasitic activity against tachyzoites of *N. caninum* expressed as half-maximal effective concentrations (EC_{50}); Cytotoxicity to [HF] non-tumoral human fibroblasts expressed as half-maximal cytotoxic concentrations (CC_{50}); [*] Selectivity Index.



B

HX-CE	HX-FA	HX-FB	HX-FC	HX-FD	DMSO 1%	Control
0.104	0.091	0.105	0.107	0.096	0.152	0.154

Figure 1. Inhibitory activity of *n*-hexane extract and fractions of *P. antarcticus* on the HRP enzyme. The HRP enzyme was incubated with the n-hexane extract (HX-CE) and derived fractions (HX-FA, HX-FB, HX-FC, HX-FD) of P. antarcticus for 30 min and the absorbance (650 nm) was acquired in 2-min intervals. The enzyme incubated alone (control) or with DMSO 1% were applied as negative controls. The data were collected and plotted (A), and the curve slope (B) was calculated by linear regression with the support of the GraphPad Prism 8.0 software.

the HRP activity. Moreover, our model was the first to describe the inhibition of HRP by a crude extract and fractions of macroalga. Likewise, the HRP activity has been inhibited by β-caryophyllene oxide derivatives[32] indicating an interesting potential of compounds from natural sources in Heme enzymes. The interaction of the extract and fractions in Heme proteins also suggests mechanisms related to the inhibition of *N. caninum* and Apicomplexa. For example, chloroquine binds to haematin, avoiding the molecule incorporation into the hemozoin crystal[33] and blocking the mechanism of the hemoglobin degradation in *Plasmodium*.[34] Although *N. caninum* lacks an erythrocytic cycle, the parasite has a de novo system of Heme synthesis in the apicoplast.[35] The Heme production is fundamental for the activity of *N. caninum* (and other members of the Apicomplexa phylum) cytochromes and catalase, indicating potential novel targets for compounds from *P. antarcticus*.

Annotation of GC-MS data, Molecular Networking and OPLS-DA

Through the combination of GC-MS molecular networking and multivariate analysis (OPLS-DA), it was possible to predict and annotate the components responsible for the bioactivity of the tested samples. Additionally, through this combination, the annotation of compounds was more accurate since it was possible to verify the identity of neighboring nodes in the network as implied by the similar fragmentation patterns (Figures S1–S15) presented by specific classes of compounds. Since the OPLS-DA analyzes pre-defined groups, samples were classified according to their bioactivity results, active versus inactive. In the OPLS-DA scores scatter plot (Figure 2A) the distribution of samples (fractions) is shown. In the OPLS-DA loadings scatter plot (Figure 2B) the observations are the features in each sample, which are labeled as identity numbers (ID) generated during the deconvolution of GC-MS spectral data by the GNPS platform.

In the OPLS-DA loadings plot (Figure 2B) phytol (ID 610), stearic acid (ID 564) and 9-octadecenal (ID 538) were pinpointed as the discriminant features related to the cytotoxic effects of fraction HX-FB. In the GC-MS molecular network cluster containing these compounds (Figure 3), 2-hexadecanoic acid methyl ester (ID 567) and neophytadiene (ID 506) were also annotated, confirming the cluster contained fatty acids and fatty alcohols derivatives. The cytotoxic and antiparasitic effects of phytol have been previously reported in MCF-7 tumor cells and against the parasite Schistosoma mansoni.[36,37] The cytotoxic potential of stearic acid towards DLD-1 cell line has also been reported.[38]

The constituents responsible for the antibacterial and antiparasitic potential of fraction HX-FD were targeted through the OPLS-DA analysis (Figures 4A–B). Discriminant features in this fraction pointed to a single cluster in the GC-MS molecular network (Figure 5). This particular cluster was found to comprise terpenes derived from the natural breakdown of carotenoids such as fucoxanthin and β-carotene.[39] The discriminant features ID 519 and 269 were pointed as the discriminant features. However, only ID 269 was putatively annotated as 1,3,3-

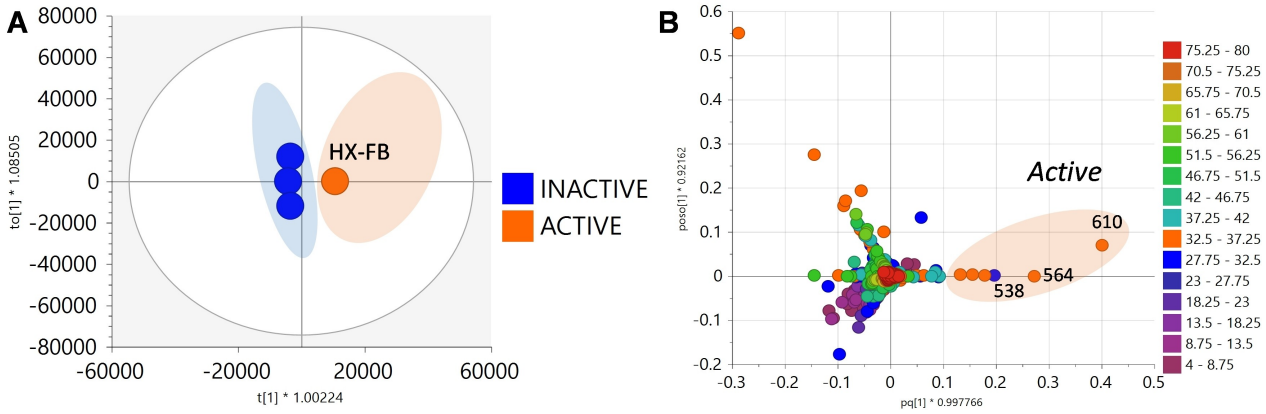


Figure 2. OPLS-DA scores **(A)** and loadings **(B)** of GC-MS data of *P. antarcticus* fractions grouped by the bioactivity profile. In the loadings **(B)** the detected compounds are colored according to their retention time in minutes.

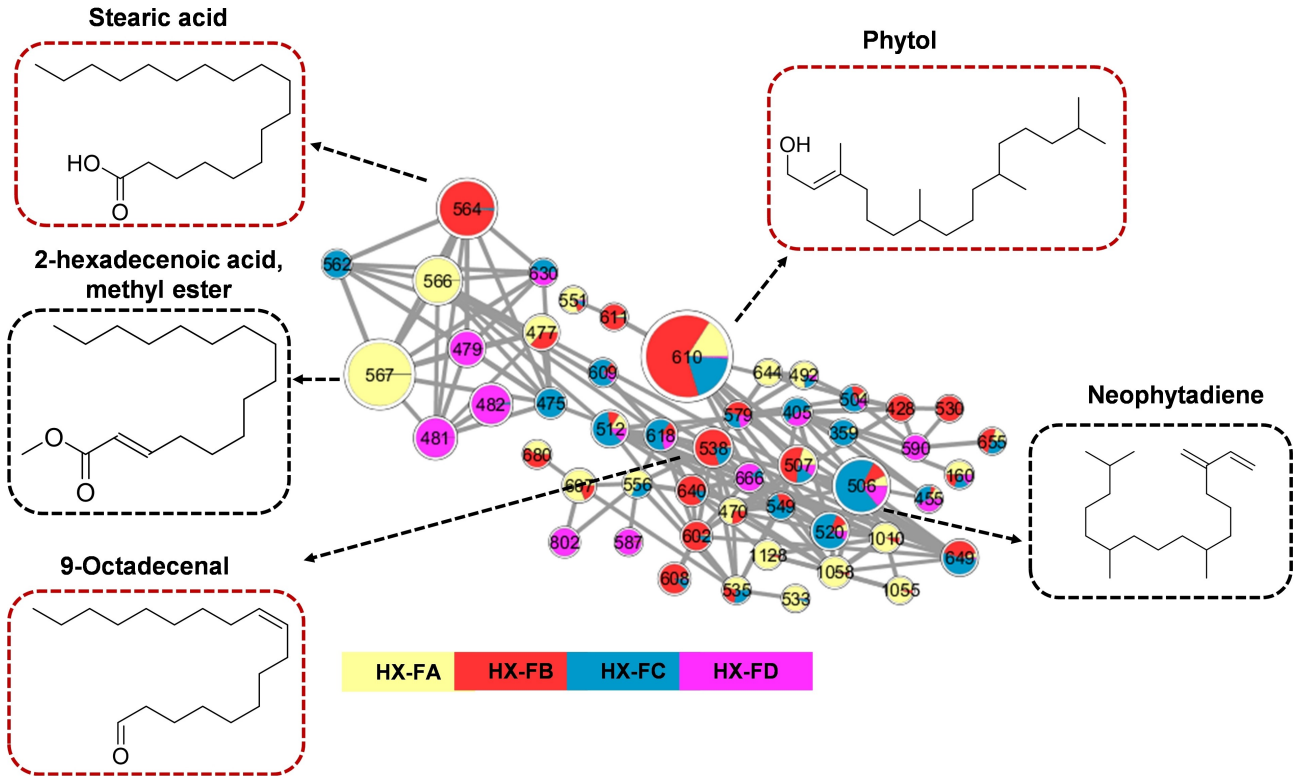


Figure 3. GC-MS molecular network showing the cluster containing the discriminant IDs pinpointed by the OPLS-DA (circled in red) as the bioactive constituents in fraction HX-FB. Nodes size represent the relative abundance of the compound, and are colored according to the fractions of origin.

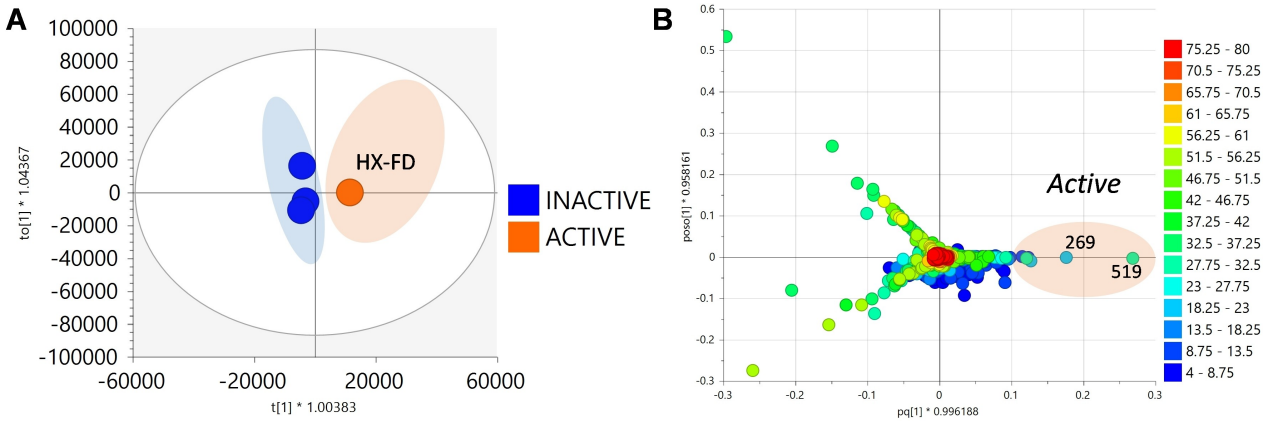


Figure 4. OPLS-DA scores **(A)** and loadings **(B)** of GC-MS data of *P. antarcticus* fractions grouped by the bioactivity profile. In the loadings **(B)** the detected compounds are colored according to their retention time in minutes.

Trimethyl-2-oxabicyclo octan-5-yl acetate. The ID 519 did not present any plausible hit in the dereplication step, but through the MN cluster where it was annotated it possible to hypothesize the compound belongs to the terpenoid class. Loliolide (ID 466), annuionone D (ID 438), 4-hydroxy-beta-ionol (ID 484) and β-ionol (ID 437) were also annotated.

Carotenoid metabolites such as loliolide, annuionone D, β-ionol and 4-hydroxy-beta-ionol, have been found in a variety of algal and plant species.[39–46] Loliolide is known to possess antioxidant, immunosuppressant, and repellent activities, additionally, this compound is also reported as an herbivory inducer in plants.[39,40,42,47] Compounds such as loliolide, neophytadiene and phytol, have been annotated in seaweed's extracts and fractions with antiparasitic activity. The red seaweed *Centroceras clavulatum* was active towards *Trypanosoma cruzi* and the Antarctic alga *Desmarestia antarctica*, active against *L. amazonensis* and *N. caninum*.[5] Our findings align with prior data and expand insights into the biotechnological potential of Antarctic organisms.[48]

Fraction HX-FC showed the highest antiparasitic activity against *L. amazonensis* and *N. caninum*. Thus, an OPLS-DA model was used to target the discriminant features in this

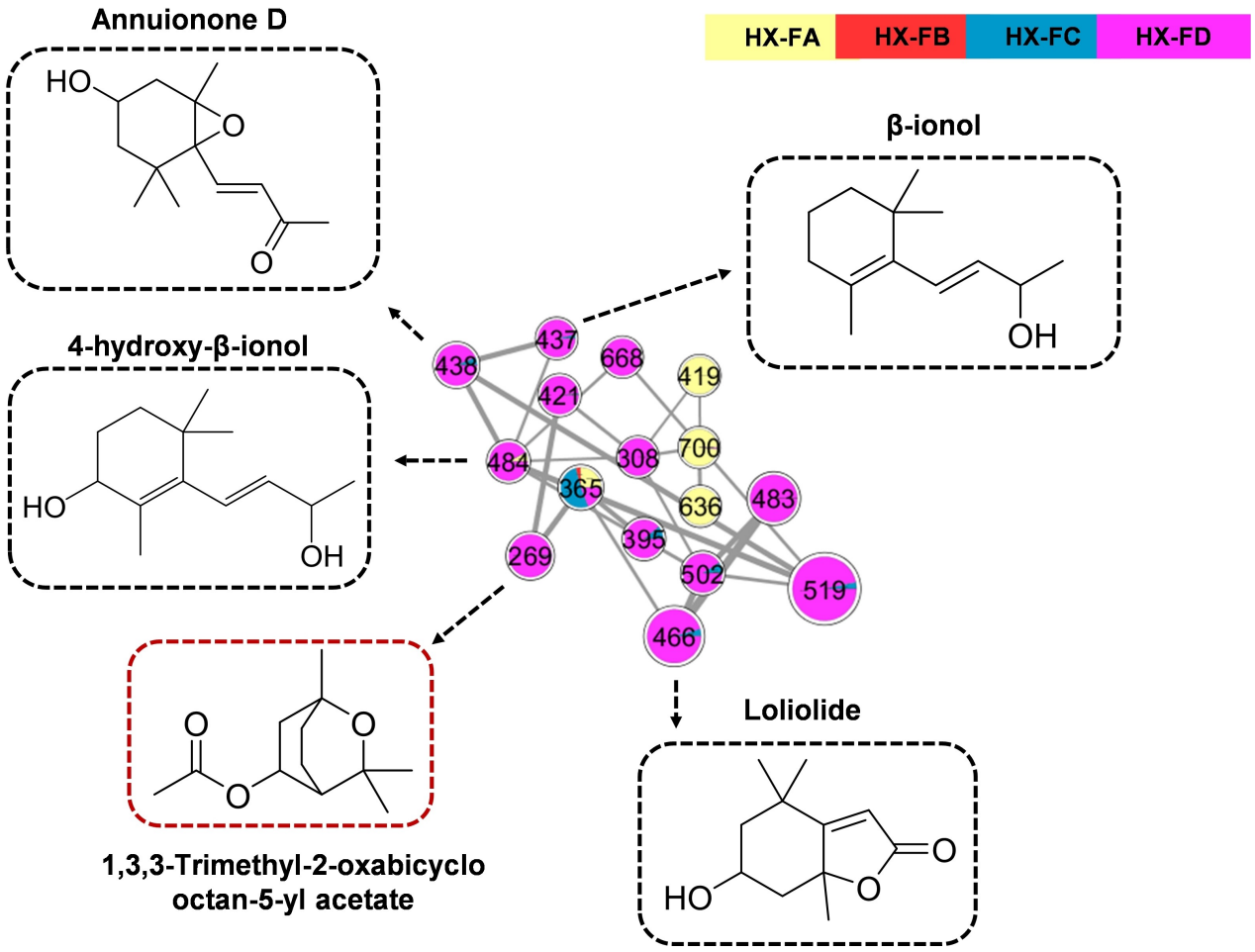


Figure 5. GC-MS molecular network showing the cluster containing the discriminant IDs pinpointed by the OPLS-DA (circled in red) as the bioactive constituents in fraction HX-FD. Nodes size represent the relative abundance of the compound, and are colored according to the fractions of origin.

fraction. Results revealed that fucosterol (ID 998) and 24-methylenecholesterol (ID 974) were pointed out as the main antiparasitic constituents towards *N. caninum* and *L. amazonensis* as shown in Figure 6A–B.

The GC-MS molecular network cluster containing fucosterol (Figure 7), also contained usual macroalgal steroids such as stigmasterol (ID 1015), desmosterol (ID 993), and pregn-11-ene-3,20-dione (ID 1040). The steroidal composition of *P. antarcticus* has been previously reported using a derivatization methodology prior to GC-MS analysis.[18] Despite that we didn't use the derivatization step prior to the GC-MS analysis performed in this work, the steroidal composition of P. antarcticus is in

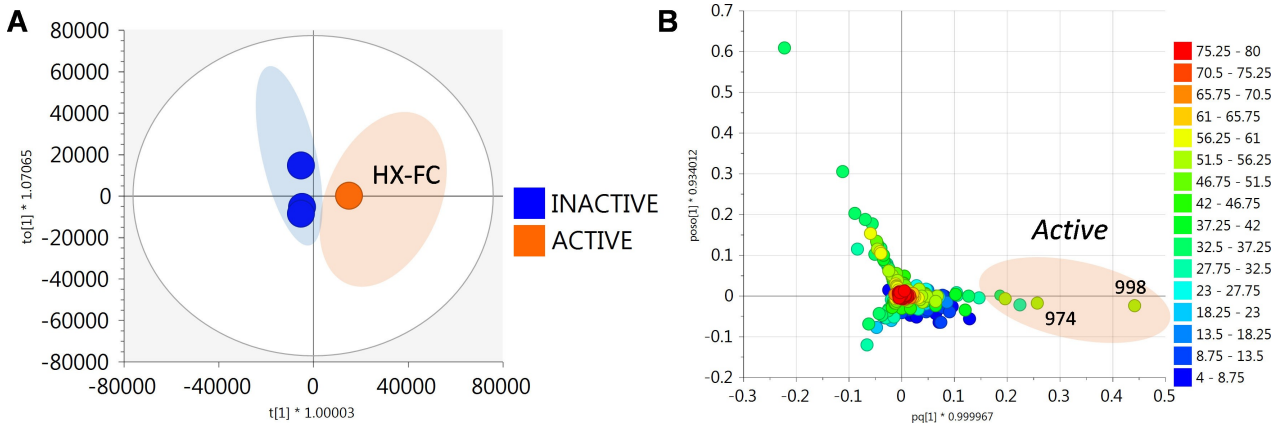


Figure 6. OPLS-DA scores **(A)** and loadings **(B)** of GC-MS data of *P. antarcticus* fractions grouped by the bioactivity profile. In the loadings **(B)** the detected compounds are colored according to their retention time in minutes.

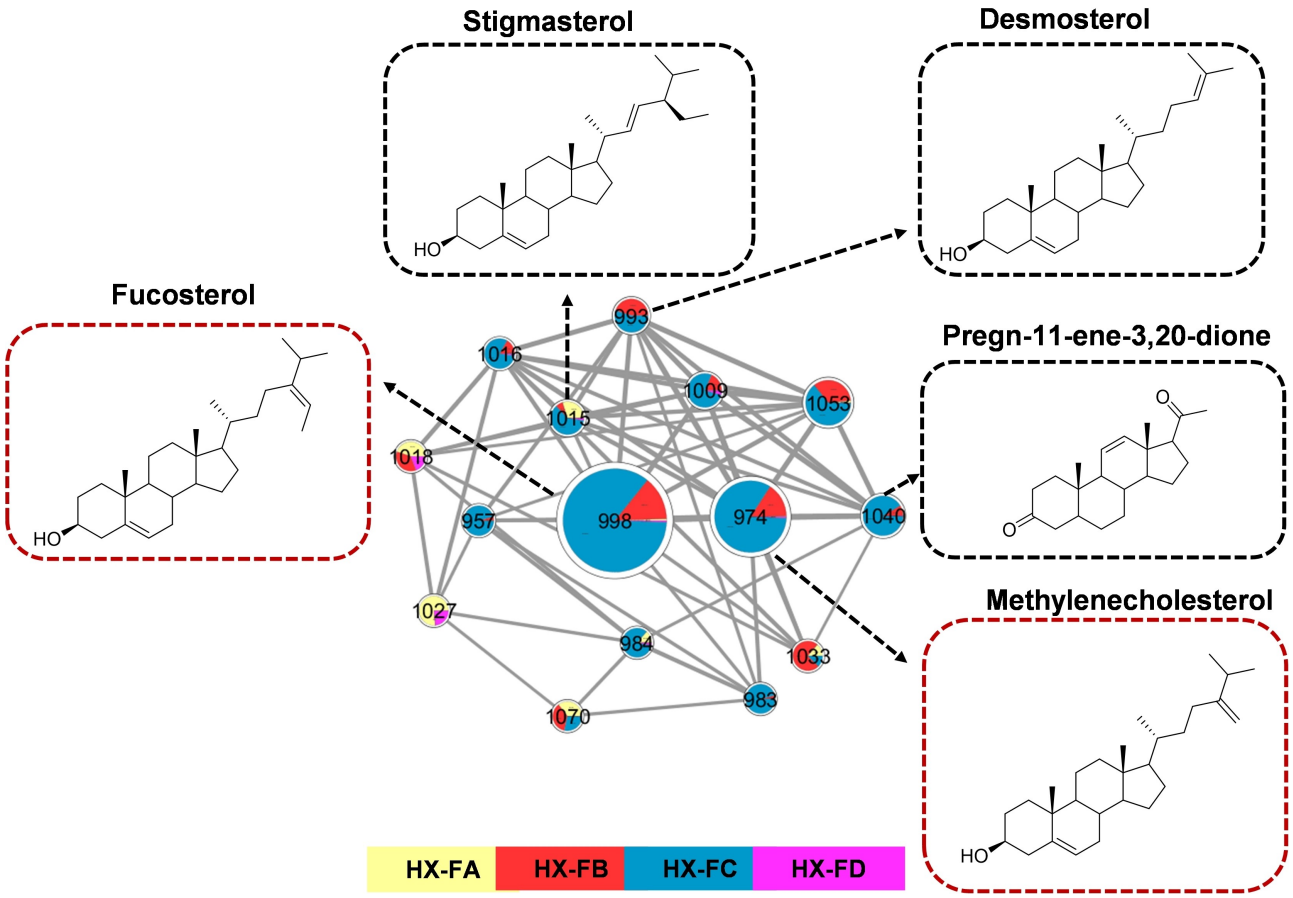


Figure 7. GC-MS molecular networking showing the cluster containing the discriminant IDs pinpointed by the OPLS-DA (circled in red) as the bioactive constituents in fraction HX-FC. Nodes size represent the relative abundance of the compound and are colored according to the fractions of origin.

accordance with previous literature data.[18] Fucosterol isolated from the alga *Lessonia vadosa* showed antileishmanial activity against amastigote forms of *L. infantum* and *L. amazonensis*.[49] Additionally, fucosterol was found to be the major compound in *D. antarctica* fractions which exhibited anti-*Neospora* activity.[5]

Conclusions

The n-hexane extract and derived fractions from the macroalga *P. antarcticus* afforded promising antibacterial (HX-FD) and antiparasitic activities (HX-FC), and inhibited HRP. Thus, the combination of GC-MS molecular networking coupled to multivariate analysis provided a straighter forward approach to the prediction of bioactive metabolites and their annotation. This work is a part of a continuous effort to investigate the diversity and chemical fingerprints of underexplored Antarctic organisms.

Experimental Section

Algal material

Samples of *P. antarcticus* were collected during the PROANTAR expedition (OPERANTAR XXXIV) in November/December 2015 in the Halfmoon Island, South Shetland Islands Archipelago, Antarctic Peninsula (62°35′×59°53′). A voucher specimen was authenticated by Dr. Cesar Bertagia Pasqualetti and Dr. Maria Beatriz Barbosa de Barros Barreto and is deposited at the Herbarium Maria Eneyda Kauffmann Fidalgo – Botanical Institute of São Paulo (São Paulo, Brazil) under the voucher number SP 470404. The collected samples were stored in plastic bags at – 20 °C until further use.

Extraction and fractionation

The algal material was air dried at room temperature (24 °C) for 72 hours. The dried biomass (66 g) was broken down into smaller pieces with a mortar and pestle under liquid nitrogen. The material was submitted to sequential extraction with n-hexane, ethyl-acetate, and methanol (600 mL each) for 30 minutes under stirring in a thermal blanket with a controlled temperature (35 °C) followed by 5 minutes in ultrasound bath (75 W). The procedure was repeated three times for each solvent. The combined resulting solutions (1,5 L) for each solvent was evaporated under reduced pressure at 30 °C. Preliminary antibacterial and antileishmanial assays revealed that the n-hexane extract (0.9 g) was the most active, in comparison to the ethyl acetate and methanolic extracts.

For this reason, the n-hexane extract (HX) was fractionated by vacuum liquid chromatography (VLC) using silica gel 60 (Mesh 70–230) in a 500 mL glass Buchner funnel. The elution was carried using 300 mL of each mobile phases consisting in an increasing proportion of n-hexane (HX) in ethyl acetate (EtOAc). Mobile phases consisted of a stepwise polarity gradient and yielded four fractions: HX-FA (HX/EtOAc, 9:1), HX-FB (HX/EtOAc, 8:2), HX-FC (HX/EtOAc, 6:4) and HX-FD (HX/EtOAc, 4:6).

GC-MS molecular networking and multivariate analysis

GC-MS analysis was performed on a Gas Chromatograph Mass Spectrometer Mod GCMS-QP2010-Ultra (Shimadzu). Analyses were performed using a non-polar DB-5 MS capillary column (30 m×0.25 mm×0.25 μm, SGE AnalyticalScience) and coupled with a quadrupole mass detector equipped with split injector heated to 260 °C. The carrier gas used was helium (99.999%) at a flow rate of 1.1 mL min^{-1}, the injection volume was 1 μL and injector split ratio was 1:4. The GC temperature was initially set at 60 °C and held for 4 minutes, then the temperature was increased at the rate of 5 °C per minute from 60 to 320 °C and isothermally held for 15 minutes, then increased to 330 °C and held for 5 minutes. The mass spectrometer ion source was maintained at 250 °C. Metabolites detection was carried out in full scan (speed 2000) mode ranging between 35 and 600 *m/z*. The ionization mode employed was electron impact (EI) with a collision energy of 70 eV. The spectral deconvolution of GC-MS data and molecular library search was performed following the workflow described[11] and available at https://gnps.ucsd.edu. A molecular network was then created with the Library Search/Molecular Networking GC workflow at GNPS.[50] The precursor ion mass tolerance was set to 0.5 Da and the MS/MS fragment ion tolerance to 0.7 Da. A molecular network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The molecular networks were visualized using Cytoscape software. The Multivariate analysis (OPLS-DA) was performed using the data output from the deconvolution workflow generated by the GNPS platform to highlight metabolites variation in relation to bioactivity. The variables obtained were classified as chemical variables (primary ID) and the bioactivity results for each fraction (secondary variable ID). The dataset was scaled using a Pareto algorithm in the SIMCA software (Umetrics, Sweden). The annotation of compounds of interest was achieved by firstly comparing the experimental spectra with the in house NIST11 library (to be considered a hit, the percentage of fragmentation similarity should be above 80%). Following, the putative annotation results were compared with the hits provided by the Molecular Library Search of the GNPS. To be considered a hit for annotation, the results provided for both searches should be the same, and the value of the cosine score provided by the GNPS, should be above 0.80. Additionally, Kovats retention indices were determined with reference to an external mix of standards of n-alkanes (C_9–C_{40}) under the same conditions with the same column, and calculated values are shown in Table S1. A manual curation of the data was carried out to eliminate hits that were not plausible from the biosynthetically point of view or that have not been previously isolated/reported in related organisms such as plants or algae. Additionally hits that were not structurally related to the closest node with higher confidence of annotation were not considered. As recommended, all the annotations provided in this work should be considered as level 2, following the guidelines.[11]

Antibacterial activity

The minimal inhibitory concentration (MIC - lowest concentration which extract was capable of inhibit microorganism growth) and the minimal bactericidal concentration (MBC – lowest concentration which extract killed 99.99% or more of initial inoculum) was determined against three gram-negative bacteria *Proteus mirabilis* ATCC 29906, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and two gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Staphylococcus saprophyticus* ATCC 15305. The microorganisms were acquired from American Type Culture Collection (ATCC). The experiments were carried out by micro-dilution broth method in 96-well microplates in triplicates as recommended by Clinical Laboratory Standard Institute (CLSI) protocol M07-A10.[51] The extract, fractions and positive controls concentrations ranged from 400 to 0.781 μg mL^{-1} and 5.9 to 0.0011 μg mL^{-1}, respectively. The inoculum was adjusted to 2.5×10^5 colony forming units per mL (UFC/mL) and the microplates were incubated at 37 °C for 24 h. After time incubation an aqueous solution of resazurin (0.02%) was added to the microplates to indicate microorganism viability and determine the MIC value.[52] To determine the MBC value, before the addition of resazurin an aliquot of the inoculum was aseptically transferred from each well and plated on solid medium adequate and the plates were incubated at 37 °C for 24 h.

Antileishmanial activity

Promastigotes – Promastigote forms of *L. amazonensis* (MPRO/BR/1972/M1841-LV-79) were cultivated at 27 °C in liver infusion tryptose medium supplemented with 10% FBS (Sigma-Aldrich), penicillin, and streptomycin (Sigma-Aldrich). Cultured promastigotes at the mid-log exponential growth phase were seeded at 1×10^7 parasites mL^{-1} in 96-well flat-bottom plates (TPP; Sigma-Aldrich). Samples were solubilized in DMSO (Sigma-Aldrich) (highest concentration 1.0%) then added to the parasite suspension at final concentrations ranging from 7.8–500 μg mL^{-1} to the crude extract and from 3.9–250 μg mL^{-1} to the fractions and incubated at 27 °C for 72 h. Amphotericin B (purity > 95%; Sigma-Aldrich) was used as reference drug (1.6–100 μg mL^{-1}). The assays were carried out in triplicate. The cell viability was assessed by the MTT method.[53] Briefly, the plates were kept at 28 °C for 72 h. Then, an aliquot of 10 μL of 6 mM MTT and 0.7 mM PMS (phenazine methosulfate) was added to each well, and the plates were incubated at 28 °C for 75 min. Subsequently, 100 μL of 10% sodium dodecyl sulfate (SDS) were added and maintained at room temperature for 30 minutes, and, finally, the samples were read at 570 nm. All the incubations were performed in the dark. The 50% of promastigote parasite growth inhibition is expressed as the half maximum inhibitory concentration ($EC_{50\text{-}PRO}$).

Amastigotes – Peritoneal macrophages of swiss mice were obtained as previously described,[54] 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 coverslips wash with PBS (pH 7.4) and the remaining infected macrophages were incubated in RPMI 1640 medium and treated with different concentrations 3.9–250 μg mL^{-1} the samples for 24 h. The cells were then fixed in a methanol and Giemsa stained. The 50% amastigote parasites growth inhibition was expressed as the inhibitory concentration ($EC_{50\text{-}AMA}$) in μg mL^{-1} by counting of 100

macrophages and its determination was performed by nonlinear regression using Bioestat® software. The experiments were performed in duplicate in two independent experiments and data expressed in mean ± standard deviation. The experiments involving animals were conducted according to the ethical committee of the School of Pharmaceutical Sciences, São Paulo State University, Araraquara, São Paulo, Brazil (CEUA 11/2018).

Anti-Neospora activity

In *N. caninum* proliferation assays, the β-galactosidase-expressing tachyzoites (NcLacZ) were applied as described.[55] Purified LacZ *N. caninum* tachyzoites were cultured (1×10^3/well) on human fibroblast cultures in a 96-well plate for 2 h at 37 °C (5% CO_2). After tachyzoite invasion, seven serial dilutions (starting from 100 μg mL^{-1}) of the crude extract and fractions of *P. antarcticus* were added to the cultures and incubated for 72 h at 37 °C (5% CO_2). The wells were washed with PBS and lysed with the lysis buffer [100 mM 4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 8.0; 1 mM $CaCl_2$; 1% Triton X-100, 0.5% SDS; 5 mM dithiothreitol] for 1 h at 45 °C. The lysed cultures were incubated with chlorophenol red-β-D-galactopyranoside (CPRG) buffer (5 mM CPRG, 5 mM 2-mercaptoethanol in PBS) for 2 h at 37 °C and the plates were read with an ELISA reader (Synergy H1, Biotek) at 570 nm. Pyrimethamine (Pyr; Sigma-Aldrich) was used as a control drug. The mean absorbance of samples in relation to the non-treated controls was applied for the calculation of the percentage of parasite inhibition and cell toxicity. Three independent assays were performed.

Cytotoxicity to human fibroblasts

The cell toxicity was evaluated by the MTT assay, as described.[56] Human fibroblasts were cultivated in Roswell Park Memorial Institute (RPMI) supplemented with 5% Fetal Bovine Serum (FBS) in 75 cm^2 flasks. For the MTT assay, the cultures were treated with trypsin (15 minutes, 37 °C) and distributed in 96-well plates (5×10^3/well in RPMI-FBS). The cells were incubated at 37 °C and 5% CO_2, until confluence. The plates were incubated with serial dilutions of *P. antarcticus* crude extract and fractions (starting from 100 μg mL^{-1} in phenol-free RPMI) for 72 h, 37 °C, and 5% CO_2. As performed for *N. caninum* proliferation assays, Pyr (25 μg mL^{-1}was applied under the same conditions. After treatment, the media was removed, and the treated cultures were incubated with 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) solution (500 μg mL^{-1}) for 4 h, 37 °C, and 5% CO_2. The MTT solution was discarded, and the formazan crystals were diluted with DMSO. The plates were read at 570 nm in an ELISA reader (Synergy H1; Biotek) and the percentage of cytotoxicity was calculated in relation to non-treated controls. DMSO 5% (purity > 98%; Sigma-Aldrich) was used as a positive control, which led to > 95% cytotoxicity compared to the non-treated group. Moreover, the DMSO concentration was < 1% for all groups treated with *P. antarcticus* fractions or Pyr. From the percentages of tachyzoite/Vero cell inhibition, the EC_{50} and CC_{50} values were calculated using Compusyn software (http://www.combosyn.com/).[57] Three independent assays were performed.

Inhibition of Horseradish Peroxidase

The horseradish peroxidase activity assay was performed as described.[32] Briefly, *P. antarcticus n*-hexane extract and fractions were diluted in water (100 μg mL^{-1}) with horseradish peroxidase (Goat anti-Rabbit IgG Highly – HRP, ThermoFisher, A16110, 1/50000) and incubated in ice for 15 minutes. For HRP reaction development, the samples were incubated with TMB reagent (TMB Substrate Reagent Set, BD) for 30 minutes, at room temperature. During the reaction, the absorbance at 650 nm was measured in intervals of 2 minutes in an ELISA reader (Sinergy H1 BioTek). After the reaction, the absorbance values were plotted against time and the curve slope was calculated using linear regression (GraphPad 8.0, San Diego, California USA).

Author Contributions

Debonsi, H.M, Santos, G.S, Teixeira, T.R and Colepicolo, Pio were responsible for the study design and phytochemical experiments and together with Edrada-Ebel, R analyzed the GC-MS/OPLSDA data and were the principals in the writing process. Teixeira, M.V.S and Furtado, N.A.J.C performed the antibacterial experiments. Gama-Filho, P.A, Pereira, L.M and Yatsuda, A.P were responbile for the design and execution of anti-Neospora, cytotoxic and Horsearadish peroxidase assays. Clementino, L.C and Graminha, M.A.S were responsable for the antileishmanial experiments.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: antiparasitic · antibacterial · antarctic seaweed · neglected diseases

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