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UHPLC-MS Chemical Fingerprinting and Antioxidant, Enzyme Inhibition, Anti-Inflammatory In Silico and Cytoprotective Activities of *Cladonia chlorophaea* and *C. gracilis* (Cladoniaceae) from Antarctica

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Abstract: The lichen species *Cladonia chlorophaea* and *C. gracilis* (Cladoniaceae) are widely distributed in the island archipelago of maritime Antarctica and represent a natural resource of scientific interest. In this work, the metabolomic characterization of the ethanolic extracts of these species and the determination of the antioxidant activity, enzymatic inhibition and anti-inflammatory potential of selected compounds on the 5-lipoxygenase enzyme by molecular docking and cytoprotective activity in the SH-SY5Y cell line were carried out. Nineteen compounds were identified by liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS) in each of the species. The contents of phenolic compounds, antioxidant activity, the inhibition of cholinesterases (acetylcholinesterase and butyrylcholinesterase) and digestive enzymes (α -glucosidase and pancreatic lipase) were variable among species, with better results in *C. chlorophaea*. Molecular docking evidenced significant binding affinities of some compounds for the 5-lipoxygenase enzyme, together with outstanding pharmacokinetic properties. Both extracts were shown to promote cell viability and a reduction in reactive oxygen species production in an H₂O₂-induced oxidative stress model. This study contributes to the chemical knowledge of the *Cladonia* species and demonstrates the biological potential for the prevention and promising treatment of central nervous system pathologies, inflammatory disorders and metabolic alterations.

Keywords: *Cladonia*; secondary metabolites; antioxidant; enzyme inhibition; anti-inflammatory; cytoprotective; Antarctic lichens

1. Introduction

Lichens are defined as a mutualistic relationship between a heterotrophic mycobiont and an autotrophic photobiont (algae and/or cyanobacteria) associated with an abundant and structured microbiome [1,2]. Antarctic lichens have aroused deep interest for their abundance, their sensitivity to environmental variables [3], their natural products and the abundance of particular bioactive compounds derived from the mycobiont that are

organized in different chemical classes, such as depsides, depsidones, dibenzofurans, xanthenes, terpenic derivatives, anthraquinones, etc., and synthesized through the acetyl-polymalonate (APP), shikimic acid (SAP) and mevalonic acid (MAP) pathways in the fungal component with multiple biological activities [4]. Species of the genus *Cladonia* (Cladoniales, Lecanorales) are characterized by an erect thallus consisting of podocia; primary thallus, persistent or absent; apothecia, absent or present and red or brown; and biatorine. They are usually found growing on mosses [5] (Figure 1).

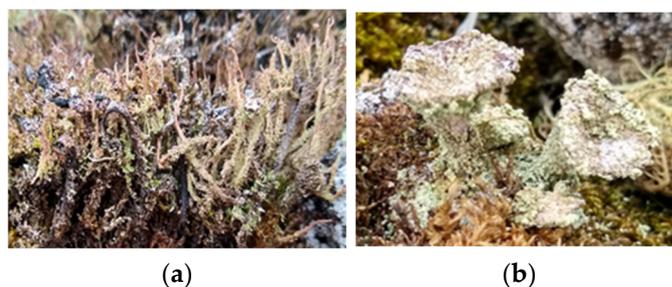


Figure 1. U Lichens thallus in the polar tundra of King George Island, Maritime Antarctic: (a) *C. gracilis*; (b) *C. chlorophaea*.

Lichen secondary metabolites are widely studied by robust techniques such as ultra-high-resolution chromatography (UHPLC) coupled with a mass spectrometer, and more than 1000 bioactive compounds are currently registered [6]. As for the genus *Cladonia*, there is evidence of the potential of extracts with major compounds, such as atranorin and oxalic, propionic, tartaric, butyric, malonic, lactic, citric, maleic, fumaric, stylic, succinic, norstylic, usnic, lecanoric, orsellinic and fumarprotocetraric acids, that show significant antioxidant activity, as in the case of the species *Cladonia foliacea*, *C. fimbriata*, *C. digitata*, *C. verticillaris*, *C. furcata*, *C. pyxidata*, *C. rangiferina*, *C. uncialis*, *C. rappi*, *C. pocillum* and *C. clathrate* [7]; likewise, recent studies show the positive effect of extracts of Antarctic lichens for the enzymatic inhibition of cholinesterases (acetylcholinesterase and butyrylcholinesterase) that influence the interruption of nerve cell communication [8]. As a complement, this work shows the effect of these natural products on the inhibition of other enzymes involved in metabolic alterations associated with the development of chronic non-communicable diseases.

The study of the effect of extracts has also been enhanced on precursor targets of alterations of inflammatory origin; one of them, arachidonate 5-lipoxygenase (5-LOX), is a soluble monomeric enzyme that contains 673 amino acids and has two domains in its structure. The smaller domain is the N-terminal domain, while the larger domain is the area comprising the catalytic C-terminus with a non-heme iron atom. 5-LOX catalyzes the formation of 5(S)-hydroperoxyeicosatetraenoic acid (5-HPETE) from arachidonic acid (AA) by incorporating molecular oxygen, thus generating the dehydration of 5-HPETE to leukotriene A₄ (LTA₄); the conversion of this leukotriene (LTA₄) causes the synthesis of other leukotrienes that, together with LTA₄, are important mediators in the inflammatory response [9]. Therefore, 5-LOX is considered one of the important enzymes in the inflammatory response, it being the main cause of pathologies such as asthma and other inflammatory disorders, such as allergic rhinitis and rheumatoid arthritis [9,10]. On the other hand, recent studies with extracts of lichens such as *Dactylina arctica*, *Nephromopsis stracheyi*, *Tuckermannopsis americana* and *Vulpicida pinastri* and compounds such as evernic acid confirm their potential as neuroprotective agents in cellular models of oxidative stress by H₂O₂ and murine models of Parkinson's disease induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [11,12].

In this work, we report the identification of the compounds present in the ethanolic extract of *C. chlorophaea* and *C. gracilis* species, their content of phenolic compounds, antioxidant activity and enzymatic inhibition on cholinesterases, α -glucosidase and

pancreatic lipase, an in silico analysis of the anti-inflammatory potential on the 5-LOX enzyme and their cytoprotection activity against H₂O₂-induced oxidative stress.

2. Materials and Methods

2.1. Chemicals

Ultrapure water (<5 µg/L TOC) obtained by a water purification system (Mili-Q Merck Millipore, Chile) was used. For mass spectrometry analysis, HPLC-grade methanol and MS-grade formic acid were used, obtained from J.T. Baker (Phillipsburg, NJ, USA). Gallic acid, Folin Ciocalteu commercial reagent, 2,4,6-tris(2-pyridyl)-s-triazine, sodium carbonate, acetic acid, ferric chloride hexahydrate, sodium acetate, Trolox, hydrochloric acid, 2,2'-Azobis(2-amidinopropane) dihydrochloride, absolute ethanol, fluorescein solution, phosphate buffer, acetylcholinesterase enzyme (AChE), butyrylcholinesterase enzyme (BChE), galantamine, Tris-HCl buffer, Ellman's reagent (DTNB), acetylcholine, butyrylcholine, magnesium chloride, sodium chloride, alpha-glucosidase, 4-nitrophenyl α-D-glucopyranoside, acarbose, orlistat, pancreatic lipase, 4-nitrophenyl-dodecanoate, dimethyl sulfoxide (DMSO) and HPLC standard with a purity greater than 95% (usnic acid and atranorin) were obtained from Sigma (Sigma, St. Louis, MO, USA).

2.2. Lichen Material

The specimens of the lichens *Cladonia gracilis* (L.) Willd. (100 g) and *C. chlorophaea* Flörke ex Sommerf. (Cladoniaceae, Lecanorales) (100 g) were collected by A.T.-B. and M.J.S. on Ardley Island, King George Island, South Shetland Archipelago in February 2021. The specimens were determined by botanist Alfredo Torres-Benítez. The specimen numbers HL-01122021 (*C. gracilis*) and HL-01132021 (*C. chlorophaea*) were deposited at the Natural Products Laboratory of the Universidad Austral de Chile, Valdivia, Chile.

2.3. Preparation of Ethanolic Extracts

A total of 5 g of each lichen species was macerated with ethanol (three times with 30 mL each) via ultrasound at room temperature. Each extract was filtered, and the solutions were concentrated under reduced pressure at 38 °C to obtain two final gummy extracts.

2.4. LC Parameters and MS Parameters

The separation and identification of the compounds present in the lichen extracts were performed on a UHPLC-ESI-QTOF-MS system equipped with UHPLC Ultimate 3000 RS with Chromeleon 6.8 software (Dionex GmbH, Idstein, Germany) and Bruker maXis ESI-QTOF-MS with the software Data Analysis 4.0 (all Bruker Daltonik GmbH, Bremen, Germany). A total of 5 mg of each extract was dissolved in 2 mL of methanol for analysis and filtered with a polytetrafluoroethylene (PTFE) filter, and 10 µL was injected into the equipment. The chromatographic equipment consisted of a quaternary pump, an autosampler, a thermostated column compartment and a photodiode array detector. Elution was performed with a binary gradient system with eluent (A) 0.1% formic acid in the water, eluent (B) 0.1% formic acid in the acetonitrile and the gradient: 1% B isocratic (0–2 min), 1–5% B (2–3 min), 5% B isocratic (3–5 min), 5–10% B (5–8 min), 10–30% B (8–30 min), 30–95% B (31–38 min) and 1% B isocratic (39–50 min). Separation was carried out with a Thermo 5 µm C18 80 Å column (150 mm × 4.6 mm) at a flow rate of 1.0 mL/min. ESI-QTOF-MS experiments were recorded in negative ion mode, and the scan range was between 100 and 1200 *m/z*. Electrospray ionization (ESI) conditions included a capillary temperature of 200 °C, a capillary voltage of 2.0 kV, a dry gas flow rate of 8 L/min and a nebulizer pressure of 2 bar. The experiments were performed in automatic MS/MS mode. The structural characterization of secondary metabolites was based on HR full MS, fragmentation patterns and comparisons with the literature data.

2.5. Total Phenolic (TP) Content

The total phenolic content (TPC) of the extracts was measured by the Folin–Ciocalteu (FC) method [13,14]. Briefly, the test mixture was FC reagent (10%), the extract sample, Na_2CO_3 (7%), was kept for 90 min in darkness and absorbance was measured at 750 nm in a microplate reader (BioTek Instrument, Inc., Winooski, VT, USA). The results were expressed as mg of gallic acid per gram of dried lichen.

2.6. Antioxidant Activity

2.6.1. Ferric-Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed following a method previously described in Karadag et al. and Parra et al. [15,16]. Briefly, the assay mixture was FRAP reagent, the extract sample was kept for 30 min in darkness and absorbance was measured at 593 nm in a microplate reader (BioTek Instrument, Inc., Winooski, VT, USA). Trolox was used as the reference compound. The results were expressed as micromoles of Trolox equivalents per gram of dried lichen.

2.6.2. Oxygen Radical Absorbance Capacity (ORAC) Assay

The assay was performed following a method previously described by Huang et al. [17]. Briefly, the assay mixture was fluorescein, Trolox and phosphate buffer, the extract sample was incubated for 30 min at 37 °C and AAPH solution was added as the peroxy generator. Fluorescence intensity was measured at 520 nm on a microplate reader (BioTek Instrument, Inc., Winooski, VT, USA) every 2 min for 1 h and 30 min at an excitation/emission wavelength of 485/520 nm. Trolox was used as the reference compound. The results were expressed as micromoles of Trolox equivalents per gram of dried lichen.

2.6.3. DPPH Scavenging Activity

The assay was determined as previously reported [18]. Briefly, the assay mixture was a solution of DPPH (400 mM), the standard or extract sample, as appropriate, was kept for 30 min in the dark and absorbance was measured at 515 nm on a microplate reader (BioTek Instrument, Inc., Winooski, VT, USA). Gallic acid was used as a reference standard. The results were expressed as IC_{50} values (μg of lichen/mL).

2.7. Enzymatic Inhibitory Activity

2.7.1. Cholinesterase Inhibition

According to Ellman's method [19], the inhibition activity was determined. DTNB solution (3mM), AChE (0.26 U/mL) and BChE (0.26 U/mL) enzyme solution were added, as appropriate, Tris-HCl buffer (50 mM, pH 8.0) and solutions of the extracts at different concentrations were incubated for 20 min at 25 °C, the substrates acetyl thiocholine iodide (15 mM) and butyryl thiocholine chloride (15 mM) were added, as appropriate, and absorbance was measured at 412 nm on a microplate reader (BioTek Instrument, Inc., Winooski, VT, USA). The results were expressed as IC_{50} values (μg of lichen/mL). Galantamine was used as a positive control.

2.7.2. α -Glucosidase Inhibition Assay

α -glucosidase inhibition assay was performed according to the method reported by Costamagna et al. and Burgos-Edwards et al. [20,21]. Briefly, the reaction mixture contained sodium phosphate buffer (200 mM, pH 6.9), solutions of the extracts at different concentrations prepared in the same buffer and α -glucosidase (0.1 U/L). After 15 min of preincubation at 37 °C, the reaction was started by adding p-nitrophenyl- α -D-glucopyranoside (5 mM) into the wells and incubated for 30 min at 37 °C, and absorbance was measured at 415 nm in a microplate reader (BioTek Instrument, Inc., Winooski, VT, USA). The results were expressed as IC_{50} values (μg of lichen/mL). Acarbose was used as a positive control.

2.7.3. Lipase Inhibition Assay

This assay was performed according to the method reported by McDougall et al. and Picot et al. [22,23]. Porcine pancreatic lipase type II was resuspended in ultrapure water at 20 mg/mL. The substrate 4-nitrophenyl-dodecanoate was prepared with DMSO and ethanol. Briefly, the assay mixture was Tris-HCl buffer (0.1 M, pH 8.5), lipase (10 mg/mL) and solutions of the extracts at different concentrations, the substrate solution (5 mM) was incubated for 15 min at 37 °C and absorbance was measured at 410 nm in a microplate reader (BioTek Instrument, Inc., Winooski, VT, USA). The results were expressed as IC₅₀ values (µg of lichen/mL). Orlistat was used as a positive control.

2.8. Calculation of ADME Parameters

Osiris Data Warrior toolkits (v 5.5.0) were used to verify the pharmacokinetic properties of compounds present in extracts of *C. gracilis* and *C. chlorophaea* species (usnic acid, thamnolic acid, fumarprotocetraric acid (FP acid), orsellinic acid, atranorin, cetraric acid, squamatic acid and methylorsellinate), compounds reported for the genus *Cladonia* (13-(beta-D-glucosyloxy)docosanoic acid (13D acid)), squaric acid, perlatolic acid and psoromic acid) and the commercial inhibitor of 5-LOX (Zileuton). The molecular descriptors that were calculated are the logarithm of the partition coefficient (cLogP), the number of hydrogen bond donors, the number of hydrogen bond acceptors, the molecular mass of compounds, the topological polar surface area (TPSA), the number of rotatable bonds and violations of Lipinski's rule of five. The absorption percentage (% ABS) was calculated using the following equation [24–26]:

$$\% \text{ ABS} = 109 - (0.345 \times \text{TPSA}) \quad (1)$$

2.9. Calculation of Risk Toxicity

To calculate the toxicological properties of compounds present in extracts of *C. gracilis* and *C. chlorophaea* species (usnic acid, thamnolic acid, FP acid, orsellinic acid, atranorin, cetraric acid, squamatic acid and methylorsellinate), compounds reported for the genus *Cladonia* (13D acid, squaric acid, perlatolic acid and psoromic acid) and the commercial inhibitor of 5-LOX (Zileuton), the Osiris Data Warrior computational tool was used. The toxicity risks evaluated in each of the molecules were mutagenicity, tumorigenicity, irritation and reproductive effects [25,26].

2.10. In Silico Analysis

The crystallographic structure of the enzyme arachidonate 5-lipoxygenase (5-LOX) was obtained from the RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank) with the PDB ID code 6N2W. This crystallographic structure is crystallized with the nordihydroguaiaretic acid (NDGA) ligand which was used as a reference to carry out a directed coupling in the catalytic site of the NDGA/6N2W complex [25,9].

Enzyme optimization was carried out using UCSF Chimera software (v1.16, San Francisco, California, USA), where water molecules and ligands were removed from the active site of the crystallographic protein. In the same way, all polar hydrogen atoms were added at pH = 7.4. The appropriate ionization states for basic and acidic amino acid residues were considered [25,27].

2.10.1. Ligand Preparation

The two-dimensional structures of the ligands (usnic acid, thamnolic acid, FP acid, orsellinic acid, atranorin, cetraric acid, squamatic acid, methylorsellinate, 13D acid, squaric acid, perlatolic acid and psoromic acid) were prepared with the ChemDraw 8.0 program (PerkinElmer Informatics, Waltham, MA, USA) and imported into the Avogadro program (<https://avogadro.cc>, accessed on 20 April 2022) to optimize the geometry using the field function of MMFF94 [25,26]. All compounds were saved as mol2 files for docking studies.

2.10.2. Docking Simulation

The standard procedure for molecular docking was developed using the rigid crystallographic structure of the enzyme arachidonate 5-lipoxygenase (6N2W), nine flexible ligands that did not present any risk of toxicity (thamnolic acid, 13D acid, perlatolic acid, psoromic acid, orsellinic acid, atranorin, cetraric acid, squamatic acid and methylorsellinate) and a commercial 5-LOX inhibitor ligand (zileuton) whose torsion angles were identified (during 10 independent runs per ligand). Targeted molecular docking was performed using the UCSF Chimera program [25,27,28], where the reference inhibitor catalytic pocket (NDGA) for arachidonate 5-lipoxygenase (5-LOX) was used. Polar hydrogens and Gasteiger partial charges were added, and a bounding box was created, whose size was fixed to a cube with sides that were 20 Å in length, using the Autodock Vina tools at UCSF Chimera. The centroid of the selected residue was chosen based on the enzyme's putative catalytic site (5-LOX), considering its known catalytic amino acids: His367, His372, His550 and Leu673. The coupling and analysis results were visualized using the Discovery Studio Visualizer [25,26]. After docking was complete, the best conformation for hydrogen bonding or π interactions was analyzed, including the free ligand binding energy (ΔG , kcal/mol) [25,26,29].

2.11. Human Neuroblastoma Cell Line (SH-SY5Y Cells)

SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.5% gentamicin at 37 °C and 5% CO₂ and 95% air. The confluence between 80 and 90% was obtained.

2.12. Cell Treatments

A stock solution (1 mg/mL) of each lichen extract was dissolved in DMSO and phosphate-buffered saline (PBS). SH-SY5Y cells were pretreated with different concentrations of the extracts for 24 h, followed by H₂O₂ (1 mM, 1 h). The highest concentration treatment had a final DMSO concentration of less than 0.1%.

2.13. Metabolic Activity Measurement

Cell survival and the cytoprotection rate were determined according to the MTT colorimetric assay [30], with some modifications. After exposure to the extract treatments, MTT stock solution (2 mg/mL) was added to the wells and incubated for 1 h; subsequently, DMSO (100 μ L) was added, and absorbance was measured at 550 nm with a Spectrostar BMG microplate reader.

2.14. Intracellular ROS Production

According to the DCFH-DA assay [31] the intracellular production of ROS was determined. In the 96-well plate, DCFH-DA (24 μ L) was added in the DMEM medium (1%) without phenol red (12 mL) for 30 min, and then the medium was aspirated. The most effective concentrations of the lichen extracts were added; after 1 h, hydrogen peroxide (250 mM) was added. Fluorescence was measured with a microplate reader (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany) at an excitation/emission wavelength of 485/528 nm.

2.15. Statistical Analysis

Data were performed in triplicate and expressed as the mean data with standard deviations (SD) using Microsoft Excel 2019 software (Microsoft Office, Microsoft Corporation, Redmond, WA, USA). Statistical significance between groups was set at $p < 0.05$ and determined by one-way ANOVA with Tukey's post hoc test using the commercial software GraphPad Prism 8.

3. Results and Discussion

3.1. UHPLC Chromatographic Analysis of Lichens Extracts

The fingerprinting of the ethanolic extracts of *C. gracilis* and *C. chlorophaea* were obtained by high-resolution mass spectrometric analysis (UHPLC-MS). The negative mode was used for compound identification. In total, nineteen metabolites were detected in each of the species (Figure 2), and their tentative identification included aromatics, organic acids, lipids, dibenzofurans, depsides and depsidones (Table 1).

Aromatic Derivatives

For *C. gracilis* and *C. chlorophaea*: peak 1 corresponded to Na formiate ($C_4H_2O_4$) as the internal standard. Peak 6 was identified as squamatic acid ($C_{19}H_{17}O_9$), peak 15, with a molecular anion at m/z 355.0454, was identified as a criptostictic acid derivate ($C_{18}H_{11}O_8$) and peak 16, with a molecular anion at m/z 471.0569 and diagnostic peaks at m/z 375.0668, 355.0385, 167.0300 and 943.0975, was identified as fumarprotocetraric acid ($C_{22}H_{15}O_{12}$). In *C. gracilis*, peak 4 was identified as methylorsellinate ($C_9H_9O_4$). In *C. chlorophaea*, peak 3 was identified as orsellinic acid ($C_8H_7O_3$), peak 5, with an $[M-H]^-$ ion at m/z 373.0926, was identified as atranorin ($C_{19}H_{17}O_8$), with diagnostic peaks at m/z 177.0186 and 163.0934, and peak 20 was identified as cetraric acid ($C_{20}H_{17}O_9$), with diagnostic peaks at m/z 281.2464 and 211.0166.

Organic Acids

For *C. gracilis* and *C. chlorophaea*: peak 2 was identified as citric acid ($C_6H_7O_7$).

Lipids

For *C. gracilis* and *C. chlorophaea*: peak 7 was identified as hexahydroxiohexacosanoic acid ($C_{26}H_{49}O_9$), peak 12, with a molecular anion at m/z 489.3432, was identified as pentahydroxyhexacosanoic acid ($C_{26}H_{49}O_8$), with diagnostic peaks at m/z 403.3001 and 979.6848, peak 13, with an $[M-H]^-$ ion at m/z 417.3236, was identified as 9,10,12,13-tetrahydroxytricosanoic acid ($C_{23}H_{45}O_6$), with diagnostic peaks at m/z 235.0538 and 195.0616, and peak 24 was identified as octadeca-9,12,15-trienoic acid ($C_{18}H_{29}O_2$). In *C. gracilis*, peak 8 was identified as 9,10,12,13-tetrahydroxyheneicosanoic acid ($C_{21}H_{41}O_6$), peak 9 was identified as pentahydroxyhexacosanoic acid ($C_{26}H_{51}O_7$), with diagnostic peaks at m/z 448.3405 and 273.0163, peak 10 was identified as 9,10,12,13-tetrahydroxydocosanoic acid ($C_{22}H_{43}O_6$) and peak 11 was identified as stearin ($C_{23}H_{45}O_7$). In *C. chlorophaea*, peak 21, with a molecular anion at m/z 277.2173, was identified as linolenic acid ($C_{18}H_{29}O_2$), and peak 25 was detected but not identified.

Dibenzofurans

For *C. gracilis* and *C. chlorophaea*: peak 14 was identified as pseudoplacodiolic acid ($C_{19}H_{19}O_8$), with diagnostic peaks at m/z 343.0807, 259.0598 and 231.0648, and peak 19 was identified as usnic acid ($C_{18}H_{15}O_7$).

Depsidones

For *C. gracilis* and *C. chlorophaea*: peak 18, with a molecular anion at m/z 401.0593, was identified as constictic acid or siphulellic acid ($C_{19}H_{14}O_{10}$).

Depsides

For *C. gracilis* and *C. chlorophaea*: peak 23 was identified as ramaric acid ($C_{18}H_{17}O_7$). In *C. gracilis*, peak 22 was identified as lecanoric acid ($C_{16}H_{13}O_7$). In *C. chlorophaea*, peak 17, with a molecular anion at m/z 419.0614, was identified as thamnolic acid ($C_{19}H_{15}O_{11}$), with diagnostic peaks at m/z 211.0201 and 317.0592.

Table 1. Identification of metabolites in *C. gracilis* and *C. chlorophaea* by UHPLC-ESI-QTOF-MS.

Peak	Tentative Identification	[M-H] ⁻	Retention Theoretical		Measured Mass (m/z)	Accuracy (ppm)	Metabolite Type	MS Ions (ppm)	Lichens
			Time (min)	Mass (m/z)					
1	Na formiate (internal standard)	C ₄ H ₂ O ₄	1.04	112.9829	112.9856	3.1	A	-	CG, CC
2	Citric acid	C ₆ H ₇ O ₇	1.78	191.0192	191.0184	4.2	OA	111.0074	CG, CC
3	Orsellinic acid	C ₈ H ₇ O ₃	2.2	151.0465	151.0403	5.0	A	-	CC
4	Methylorsellinate	C ₉ H ₉ O ₄	12.8	181.0506	181.0478	-15.4	A	-	CG
5	Atranorin *	C ₁₉ H ₁₇ O ₈	14.3	373.0926	373.0928	0.19	A	177.0186, 163.0934	CC
6	Squamatic acid	C ₁₉ H ₁₇ O ₉	14.8	389.0877	389.0878	0.45	A	181.05000	CG, CC
7	Hexahydroxiohexacosanoic acid	C ₂₆ H ₄₉ O ₉	18.2	505.3338	505.3356	3.5	L	431.3015, 375.2722	CG, CC
8	9,10,12,13-Tetrahydroxyheneicosanoic acid	C ₂₁ H ₄₁ O ₆	18.8	389.2903	389.2841	-15.9	L	-	CG
9	Pentahydroxyhexacosanoic acid	C ₂₆ H ₅₁ O ₇	19.2	475.3315	475.3276	8.1	L	448.3405, 273.0163	CG
10	9,10,12,13-Tetrahydroxydocosanoic acid	C ₂₂ H ₄₃ O ₆	19.8	403.3060	403.3000	-14.8	L	-	CG
11	Stearin	C ₂₃ H ₄₅ O ₇	20.2	433.3117	433.3144	-6.0	L	277.2144	CG
12	Pentahydroxyoxohexacosanoic acid	C ₂₆ H ₄₉ O ₈	20.7	489.3432	489.3403	-5.9	L	403.3001, 979.6848 (2M-H)	CG, CC
13	9,10,12,13-Tetrahydroxytricosanoic acid	C ₂₃ H ₄₅ O ₆	21.1	417.3236	417.3184	-12.4	L	235.0538, 195.0616	CG, CC
14	Pseudoplacodiolic acid or Placodiolic acid	C ₁₉ H ₁₉ O ₈	21.3	375.1080	375.1070	2.7	DBF	343.0807; 259.0598; 231.0648	CG, CC
15	Criptostictic acid derivate	C ₁₈ H ₁₁ O ₈	21.7	355.0454	355.0384	-19.7	A	-	CG, CC
16	Fumarprotocetraric acid	C ₂₂ H ₁₅ O ₁₂	21.9	471.0569	471.0476	-9.6	A	375.0668, 355.0385, 167.0300, 943.0975 (2M-H)	CG, CC
17	Thamnolic acid	C ₁₉ H ₁₅ O ₁₁	22.1	419.0614	419.0586	-6.6	d	211.0201, 317.0592	CC
18	Constictic acid or siphulellic acid	C ₁₉ H ₁₄ O ₁₀	23.1	401.0593	401.0797	50.8	D	-	CG, CC
19	Usnic acid *	C ₁₈ H ₁₅ O ₇	23.5	343.0823	343.0859	-2.91	DBF	-	CG, CC
20	Cetraric acid	C ₂₀ H ₁₇ O ₉	24.1	401.0878	401.0864	-3.4	A	281.2464, 211.0166	CC
21	Linolenic acid	C ₁₈ H ₂₉ O ₂	25.1	277.2173	277.2121	-18.0	L	183.0182	CC
22	Lecanoric acid	C ₁₆ H ₁₃ O ₇	25.3	317.0666	317.0632	-10.7	d	167.034	CG
23	Ramaric acid	C ₁₈ H ₁₇ O ₇	26.1	345.1038	345.1025	-3.7	d	295.1953	CG, CC
24	Octadeca-9,12,15-trienoic acid	C ₁₈ H ₂₉ O ₂	28.0	277.2203	277.2099	-3.2	L	-	CG, CC
25	Unknown	C ₂₇ H ₄₃ O ₄	28.5	431.3228	431.3166	14.3	L	-	CC

* Identified by spiking experiments with an authentic standard compound. A = aromatic; OA = organic acid; L = lipid; DBF = dibenzofuran; D = depsidone; d = depside. CG: *Cladonia gracilis*; CC: *Cladonia chlorophaea*.

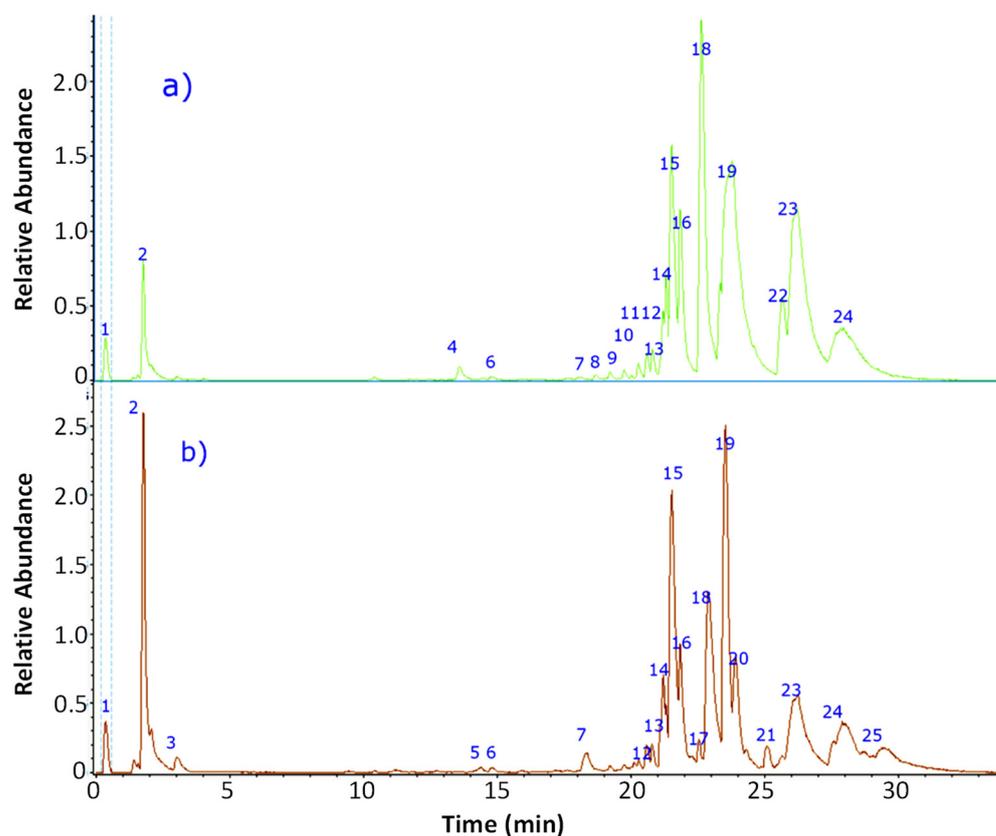


Figure 2. UHPLC-MS Chromatograms: (a) *C. gracilis*; (b) *C. chlorophaea*. The numbers of the peaks correspond to the identification indicated in Table 1.

The group of compounds identified in the ethanolic extracts of the lichens species *C. chlorophaea* and *C. gracilis* form a chemical fingerprint related to the recent metabolomic reports of other Antarctic lichens such as *C. metacorallifera* with 26 compounds [32], *Usnea antarctica* with 21 compounds [33], *Himantormia lugubris* with 28 compounds [8], *Lecania brailmontii* with 18 compounds, *Pseudephebe pubescens* with 18 compounds and *Sphaerophorus globosus* with 14 compounds [26]; some of them, as fumarprotocetraric, lecanoric, squamatic and usnic acids, represent typical compounds in foliose lichens that participate in the biochemical mechanisms for the adaptation of lichens to the habitat [34]. Likewise, the chemical study of extracts is the basis for the detection of new compounds with biological potential, as in the case of the isolation of three p-terphenyls and two fumarprotocetraric acid lactones in the Antarctic lichens *Stereocaulon alpinum* [35] and *C. metacorallifera* [32], respectively.

3.2. Total Phenolic Contents and Antioxidant Activity

The results corresponding to phenolic content and antioxidant activity measured by the different colorimetric assays are detailed in Table 2. The ethanolic extract of *C. chlorophaea* showed six-times-higher phenolic content compared to *C. gracilis*, with 330.276 ± 0.006 and 53.563 ± 0.004 mg gallic acid/100 g of extract, respectively. For the antioxidant activity, in the FRAP and ORAC assays, the *C. chlorophaea* extract presented better results compared to the *C. gracilis* extract; as for the DPPH assay, both extracts presented a high IC_{50} , it being higher for the *C. chlorophaea* extract.

The ethanolic extracts of *C. chlorophaea* and *C. gracilis* showed phenolic contents and antioxidant activities similar to those reported in the methanolic, acetic, chloroformic and hydroalcoholic extracts of other species of *Cladonia*; however, there are variable cases such as the sample of *C. foliaceae*, where the methanolic extract did not show activity in the DPPH assay, and the percentage of phenolic compounds was 1.7% [36]. In *C. furcata*,

an $IC_{50} > 1000$ and 471.3 ± 35.4 $\mu\text{g/mL}$ were reported for DPPH in the methanolic and acetonic extracts, respectively [37]; in other works, an IC_{50} in DPPH of 967.97 ± 2.35 $\mu\text{g/mL}$ and a phenol content of 28.00 ± 1.065 mg GAE/g [38] were reported, as well as a 44.83% effect on DPPH activity and a phenolic content of 12.95 ± 1.065 μg pyrocatechol/g [39].

In other cases, such as *C. pyxidata* and *C. rangiferina*, for the acetone extract, the radical scavenging activity was 461.72 ± 2.93 and 987.64 ± 2.02 $\mu\text{g/mL}$, respectively, and the phenol content was 35.35 ± 1.013 and 22.00 ± 1.065 mg GAE/g, respectively [38]. In *C. am-aurocraea* and *C. mediterranea*, the content of phenolic compounds in acetonic, ethanolic and methanolic extracts was considerably low, with values between 0.005 to 0.174 and 0.006 to 0.009 mg GAE/g, respectively, compared to the amount of proteins and carbohydrates [40]. In *C. clathrata* with the hydroalcoholic extract, a total phenol concentration of 371.8 ± 57.3 mg GAE/g and a DPPH of 69.25 ± 0.65 $\mu\text{g/mL}$ were found [41], similar to *C. verticillata*, with phenol contents of 101.37 ± 3.31 and 48.61 ± 0.87 mg GAE/g in the acetonic and methanolic extract, respectively, and the DPPH assay, with 33.09 ± 0.84 and 17.25 ± 2.98 $\mu\text{g/mL}$ for the acetonic and methanolic extract, respectively [42].

In other species such as *C. rangiformis*, the reported phenolic contents are low, with 0.528 ± 0.007 and 0.103 ± 0.012 mg GAE/g in the chloroform and methanol extracts, respectively [43]. In a work with a majority of compounds from 15 species of the genus *Cladonia* [44], they report, for DPPH radicals, an IC_{50} of 207.8 ± 31.6 , 320.0 ± 48.1 , 145.4 ± 17.3 , 394.1 ± 55.2 , 952.4 ± 85.6 , 153.8 ± 21.5 and 252.4 ± 30.3 $\mu\text{g/mL}$ in atranorin, barbatic, fumarprotocetraric, perlatolic, squamatic, thamnolic and usnic acids, respectively [7]. For specimens of *Cladonia* sp., a DPPH of 38.5 ± 3.8 $\mu\text{g/mL}$ has been reported [45]. In general, Antarctic lichen extracts show significant antioxidant activities that enhance their adaptation to extreme environmental conditions [46].

Table 2. Total phenolic content (TPC) and antioxidant activity (FRAP; ORAC; DPPH) of *C. gracilis* and *C. chlorophaea*.

Assay	TPC (mg GAE/g)	FRAP ($\mu\text{mol Trolox/g}$)	ORAC ($\mu\text{mol Trolox/g}$)	DPPH IC_{50} ($\mu\text{g/mL}$)
<i>C. gracilis</i>	53.563 ± 0.004 *	41.028 ± 0.004 *	223.088 ± 0.761 *	296.737 ± 0.021 *
<i>C. chlorophaea</i>	330.276 ± 0.006 *	142.762 ± 0.002 *	271.483 ± 0.920 *	437.85 ± 0.022 *
Gallic acid #	-	-	-	2.24 ± 0.04

The values represent the means \pm SD of three replicates ($n = 3$). Values marked with * are statistically different using the Tukey test at a 0.05 level of significance ($p < 0.05$). # Positive control.

In comparison with other Antarctic lichens, the ethanolic extracts of the two *Cladonia* species performed better than the ethanolic extracts of *Himantormia lugubris* [8] and *Lecania brialmontii*, *Pseudephebe pubescens* and *Sphaerophorus globosus* [26], confirming the variable antioxidant potential of species from the polar territories.

3.3. Enzymatic Inhibitory Activity

The values obtained with the ethanolic extracts of the two lichen species are shown in Table 3. As for the cholinesterases' inhibition activity, the results of the two extracts were potent and close to the values recorded for the standard compound galantamine; for AChE, the extract of *C. chlorophaea* showed a better IC_{50} value compared to *C. gracilis*, with 4.204 ± 0.061 and 6.211 ± 0.055 , respectively; likewise, for BChE, the extract of *C. chlorophaea* showed better results compared to *C. gracilis*, with 5.938 ± 0.069 and 9.105 ± 0.065 , respectively. As for digestive enzyme activity, the *C. gracilis* and *C. chlorophaea* extracts evidenced higher α -glucosidase inhibition (91.323 ± 0.010 and 108.590 ± 0.006 , respectively) against the reference compound acarbose; for pancreatic lipase, both extracts showed low inhibitory activity (*C. chlorophaea* with 125.310 ± 0.049 and *C. gracilis* with 345.135 ± 0.050) compared to the positive control Orlistat.

Table 3. Enzyme inhibitory activity of *C. gracilis* and *C. chlorophaea* extracts.

Assay	AChE	BChE	α -Glucosidase	Pancreatic Lipase
	IC ₅₀ (μ g/mL)			
<i>C. gracilis</i>	6.211 \pm 0.055 *	9.105 \pm 0.065 *	91.323 \pm 0.010 *	345.135 \pm 0.050 *
<i>C. chlorophaea</i>	4.204 \pm 0.061 *	5.938 \pm 0.069 *	108.590 \pm 0.006 *	125.310 \pm 0.049 *
Galantamine #	0.266 \pm 0.029 *	3.824 \pm 0.024 *	-	-
Orlistat® #	-	-	-	1.9 \pm 0.077
Acarbose #	-	-	192.8 \pm 0.004	

The values represent the means \pm SD of three replicates (n = 3). Values marked with * are statistically different using the Tukey test at a 0.05 level of significance ($p < 0.05$). # Positive control. AChE, acetylcholinesterase; BChE, butyrylcholinesterase.

The anticholinergic activity of ethanolic extracts of *C. chlorophaea* and *C. gracilis* was more effective against the ethanolic extract of Antarctic species such as *H. lugubris*, with an IC₅₀ of 12.38 \pm 0.09 and 31.54 μ g/mL in AChE and BChE, respectively [8]; *L. brialmontii*, *P. pubescens* and *S. globosus* presented similar values in AChE, with 3.949 \pm 0.04, 2.805 \pm 0.07 and 10.422 \pm 0.08 μ g/mL, respectively, and in BChE, with 4.476 \pm 0.06, 8.828 \pm 0.08 and 6.785 \pm 0.04 μ g/mL, respectively [26]. With the compound buruloquinone isolated from *C. macilenta*, an AChE inhibition of 27.1 μ g/mL has been reported [47], demonstrating the inhibitory potential of major components isolated from lichen extracts; in *C. uncialis*, values for AChE between 0.1 \pm 0.0 and 7.9 \pm 0.0 μ g/mL and for BChE between 9.5 \pm 0.4 and 85.9 \pm 0.2 μ g/mL were reported based on reference compounds [48]. Regarding α -glucosidase, the values reported for other lichenic species are lower, with IC₅₀ >3 and 0.6 \pm 0.0, 17.5 and 10.7–17.6 μ g/mL for *Xanthoria elegans* and *X. parietina* [49], *Parmotrema dilatatum* [50] and compounds isolated from *P. tsavoense*, respectively [51]. In the pancreatic lipase, *X. elegans*, *X. parietina* and *X. candelaria* reported lower values, with an IC₅₀ of 79 \pm 5.0, 68 \pm 5.0 and 55 \pm 3.0 μ g/mL, respectively [49].

3.4. In Silico Anti-Inflammatory Effect of 5-LOX

3.4.1. Prediction of Pharmacokinetic Properties—ADME

The analysis of the pharmacokinetic properties of the compounds present in the species of the genus *Cladonia* (usnic acid, thamnolic acid, 13D acid, squaric acid, perlatolic acid, FP acid, psoromic acid, orsellinic acid, atranorin, cetraric acid, squamatic acid and methylorsellinate) was calculated with the Osiris Data Warrior computational tool, where the results of these compounds were compared with the pharmacokinetic properties of a commercial 5-LOX inhibitor (Zileuton) (Table 4). Drugs that are administered orally must meet certain parameters, which are known as the Lipinski rules [25,26]. These rules allow for the evaluation and monitoring of drugs based on biological and pharmacological function. The parameters encompassed by Lipinski's rules are that the drug must have a molecular weight of less than 500 g/mol; cLogP must have a value less than 5; in the molecular structure there must be a maximum of five hydrogen donor sites; the compound must have 10 or fewer hydrogen bond receptor sites; finally, the compound must have less than 10 rotatable bonds in its chemical structure [25,26]. The molecules that had more than one violation of the Lipinski rules were the compound 13D acid (three violations) and perlatolic acid (two violations). These analyses show that these compounds may have a low bioavailability and pharmacokinetic properties, which make it difficult for them to be administered orally. All compounds were compared with the commercial 5-LOX inhibitor (zileuton), showing that usnic acid, squaric acid, psoromic acid, orsellinic acid, atranorin, cetraric acid, squamatic acid and methylorsellinate presented behaviors similar to zileuton, which did not present any violation of the Lipinski rules. Additionally, we observed that the compounds thamnolic acid and FP acid only presented a violation of the Lipinski rules, which are in the limit to be accepted as possible inhibitors of 5-LOX and to be administered orally; however, in silico and in vitro analyses are required to enrich and

further support the *in silico* analysis. The bioavailability of compounds presents in *Cladonia* is also assessed by topological polar surface area analysis (TPSA) [24–26]. This parameter is highly related to the passive molecular transport that a drug can undergo through cell membranes and can predict the transport properties that the compounds can present, as well as their bioavailability. With the TPSA parameter, it was possible to predict the absorption percentage of each of the compounds, comparing them with the commercial inhibitor Zileuton [24]. The compounds that presented a higher percentage of absorption compared to zileuton (76.30%) were squaric acid (83.26%), orsellinic acid (82.17%) and methylorselinate (85.97%).

3.4.2. Toxicity Prediction

To calculate the toxicity risk parameters that the compounds in the species of the genus *Cladonia* could present, the Osiris Data Warrior computational tool was used [25,26]. This computational tool allows for the evaluation of the molecules according to their chemical structure and the possible molecular fragments that can cause some toxicological risk. The toxicity risk parameters that were calculated are mutagenicity, tumorigenicity, irritation and reproductive toxicity [25,26]. The results are displayed using a blue color scale, where the most intense blue color indicates that the compound exhibits a high risk of toxicity, an intermediate blue color indicates that the compound exhibits an intermediate toxicity risk and a weaker blue color indicates that the compound does not present any toxicity risk (Figure 3). The compounds that presented risks of toxicity were usnic acid, squaric acid and FP acid. Usnic acid presented a high risk of toxicity in the reproductive effect because it presented a fragment in its chemical structure that confers this toxicity effect. The squaric acid exhibited a high probability of presenting tumorigenic properties because the entire chemical structure of squaric acid gives it these risks and toxicological properties. The FP acid compound presented various toxicological risks because its chemical fragmentation forms unstable chemical structures that give it these toxicological properties. FP acid exhibited five fragments with a high risk of an irritant effect; likewise, low risks of mutagenicity, tumorigenicity and toxicity in the reproductive effect were observed (Table 5).

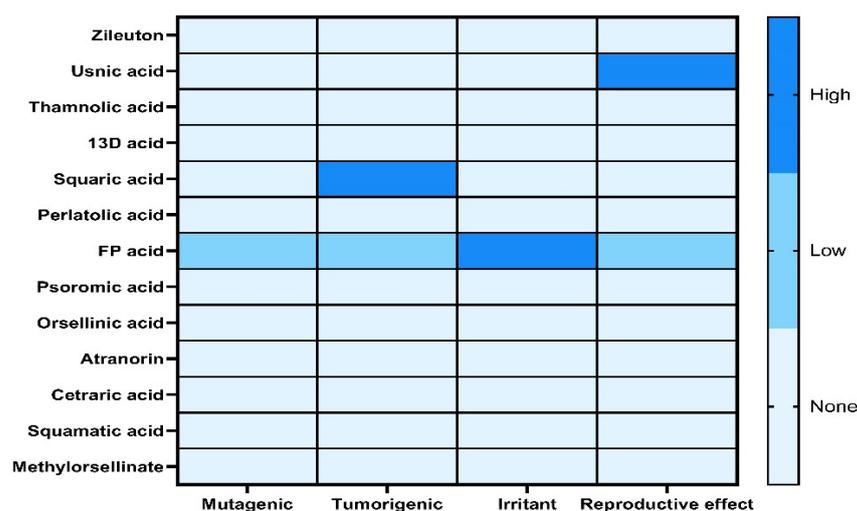


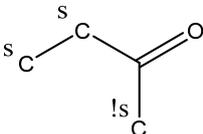
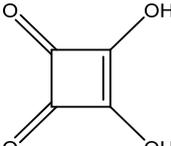
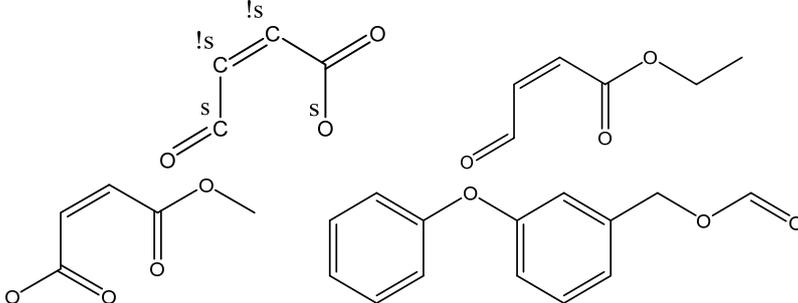
Figure 3. The heat map representation of the toxicity risk of compounds present in the species of the genus *Cladonia* compared to the 5-LOX redox inhibitor (Zileuton) was realized with GraphPad Prism v.7 (GraphPad; La Jolla, CA, USA).

Table 4. Pharmacokinetic properties of compounds present in the species of the genus *Cladonia* in comparison with the commercial 5-LOX redox inhibitor (Zileuton) obtained from Osiris Data Warrior and Molinspiration software (v 5.5.0).

Compound	%ABS ^a	TPSA (Å ²) ^b	MW ^c	cLogP ^d	HBD ^e	HBA ^f	n-ROTB ^g	Violation of Lipinski's Rule
Rule	-	-	<500	≤5	≤5	≤10	≤10	≤1
Usnic acid	68.30	117.97	344.32	0.91	2	7	2	0
Thamnolic acid	44.17	187.89	420.33	1.57	5	11	7	1
13D acid	61.85	136.68	518.73	5.93	5	8	23	3
Squaric acid	83.26	74.60	114.06	-1.33	2	4	0	0
Perlatolic acid	69.91	113.29	444.52	6.06	3	7	13	2
FP acid	42.12	193.86	472.36	1.86	4	12	7	1
Psoromic acid	67.82	119.36	358.30	2.87	2	8	3	0
Orsellinic acid	82.17	77.76	168.15	0.79	3	4	1	0
Atranorin	64.03	130.36	374.34	2.93	3	8	6	0
Cetraric acid	60.84	139.59	402.35	2.83	3	9	5	0
Squamatic acid	57.05	150.59	390.34	2.33	4	9	6	0
Methylorsellinate	85.97	66.76	182.17	1.23	2	4	2	0
Zileuton *	76.30	94.80	236.29	1.23	2	4	2	0

Note: ^aPercentage of absorption (%ABS); ^btopological polar surface area (TPSA); ^cmolecular weight (MW); ^dlogarithm of partition coefficient between n-octanol and water (cLogP); ^enumber of hydrogen bond donors (HBD); ^fnumber of hydrogen bond acceptors (HBA); ^gnumber of rotatable bonds (n-ROTB). * Commercial redox inhibitors of 5-LOX.

Table 5. Structural fragments responsible for the toxicity of the compounds present in the species of the genus *Cladonia*.

Compound	Fraction of the Molecule	Risk of Toxicity
Usnic acid		High-risk fragment indicating a reproductive effect
Squaric acid		High-risk fragment indicating tumorigenicity
FP acid		High-risk fragment indicating an irritant effect

Note: FP acid (fumarprotocetraric acid).

3.4.3. Evaluation of the Docking 5-LOX Inhibition

The directed coupling of 9 of the 12 compounds present in the species of the genus *Cladonia* was performed with the enzyme 5-LOX (PDB ID: 6N2W) to see the possible interactions with the catalytic site. The 5-LOX catalytic site is composed of a tetrad of catalytic residues (His367, His372, His550 and Leu673) that, together with the iron atom, coordinate for its catalysis. Ten conformations were tested in each of the compounds to be

evaluated, selecting the best conformation that presented a better binding affinity (ΔG , kcal/mol) and comparing them with a commercial 5-LOX inhibitor (Zileuton) [25,26].

The results in Table 6 showed that the one with the best binding affinity for the 5-LOX enzyme was psoromic acid (-8.30 kcal/mol); furthermore, the binding energy shown by this compound was very close compared to the commercial inhibitor zileuton (-8.70 kcal/mol). The compounds thamnolic acid, perlatolic acid, atranorin, cetraric acid and squamatic acid exhibited similar behaviors in terms of binding affinity (-7.40 , -7.10 , -7.20 , -7.60 and -7.60 kcal/mol, respectively); however, they were inferior to zileuton and psoromic acid. The compounds that presented a lower binding affinity were 13D acid (-5.60 kcal/mol), orsellinic acid (-5.60 kcal/mol) and methylorsellinate (-5.40 kcal/mol); however, these compounds showed specific interactions with the catalytic site of 5-LOX.

Table 6. Binding energies resulting from molecular docking experiments of the selected compounds in the extracts of *Cladonia*, together with the standard inhibitor zileuton on 5-lipoxygenase (5-LOX).

Compound	Binding Energy (Kcal/mol) 5-Lipoxygenase (5-LOX)
Thamnolic acid	-7.40
13D acid	-5.60
Perlatolic acid	-7.10
Psoromic acid	-8.30
Orsellinic acid	-5.60
Atranorin	-7.20
Cetraric acid	-7.60
Squamatic acid	-7.60
Methylorsellinate	-5.40
Zileuton	-8.70

Figures 4 and 5 show the molecular interactions and distances shown for thamnolic acid, 13D acid, perlatolic acid and psoromic acid with the 5-LOX protein. The thamnolic acid showed a strong interaction and hydrogen bond, with a residue His432 distance of 2.21 Å (Figures 4A and 5A). The carboxylate present in one of the aromatic rings of the thamnolic acid presented strong interactions of the charge attraction type with the following residues: an Arg596 distance of 1.82 Å and a His372 distance of 3.92 Å (Figure 4A and 5A); the latter is highly involved in the catalytic site for the inhibition of the 5-LOX enzyme. Carbon hydrogen bond type interactions were observed, with a His367 distance of 2.83 Å, a Leu368 distance of 2.91 Å and a His600 distance of 2.87 Å, where the His367 residue is directly involved in the inhibition of the 5-LOX enzyme.

The 13D acid presented two strong hydrogen bond type interactions (Figures 4B and 5B) with the residues Arg596 (distance 1.87 Å) and His367 (distance 2.78 Å), which allowed it to stabilize within the catalytic pocket. Pi-sigma interactions (Figures 4B and 5B) with His372 (distance 3.47 Å) were observed between the imidazole chain of His372 and the carbon of the tetrahydropyran group of 13D acid. Alkyl interactions (Figures 4B and 5B) were observed between the methylenes of the alkyl chain of 13D acid and the residues Ala410 (distance 4.37 Å), Leu414 (distance 4.46 Å), Trp599 (distance 5.00 Å) and Ala603 (distance 4.89 Å).

Figures 4C and 5C show the molecular interactions between the catalytic site of 5-LOX and perlatolic acid. In this figure, three hydrogen bonding interactions were presented with the residues Gln363 (distance 1.93 Å), His367 (distance 2.23 Å) and His432 (distance 2.28 Å). Charge attraction interactions were exhibited between the residue Arg596 (distance 3.13 Å), and it was also observed that this residue also presented hydrogen bond type interactions (distance 2.11 and 2.48 Å) with the carboxylate group of perlatolic acid (Figures 4C and 5C).

of the aromatic ring and the residues Arg596 (distance 1.94 Å) and His600 (distance 2.08 Å). Additionally, π - π T-shaped interactions were presented between the π electrons of the aromatic ring of methylorsellinate and the π electrons of the aromatic rings of the residues Phe359 (distance 5.11 Å) and Trp599 (distance 5.38 Å).

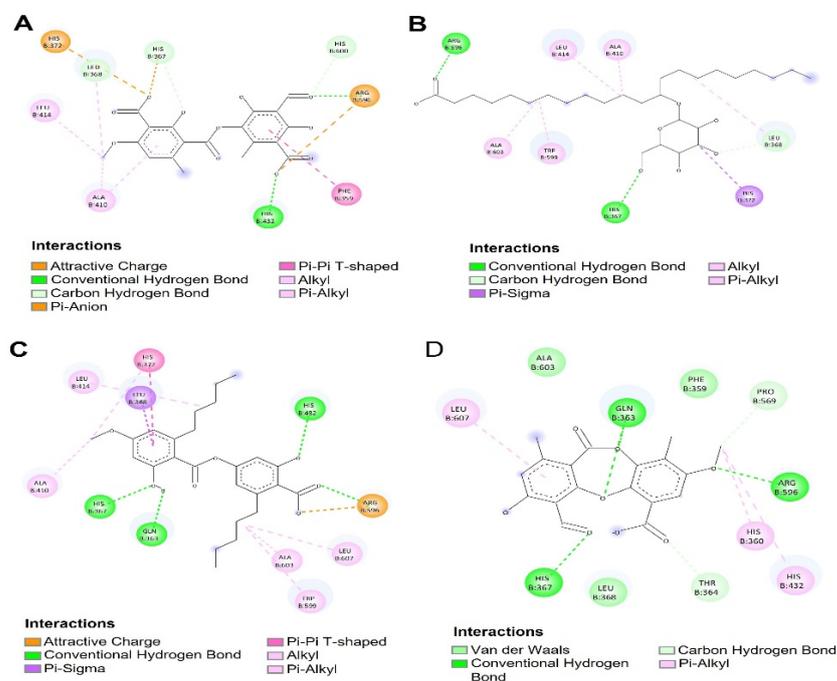


Figure 5. Molecular interactions of the compounds present in the species of *Cladonia*. (A) Molecular interactions of Thamnic acid with the 5-LOX catalytic pocket; (B) Molecular interactions of 13D acid with the 5-LOX catalytic pocket; (C) Molecular interactions of Perlatolic acid with the catalytic pocket of 5-LOX; (D) Molecular interactions of Psoromic acid with the 5-LOX catalytic pocket.

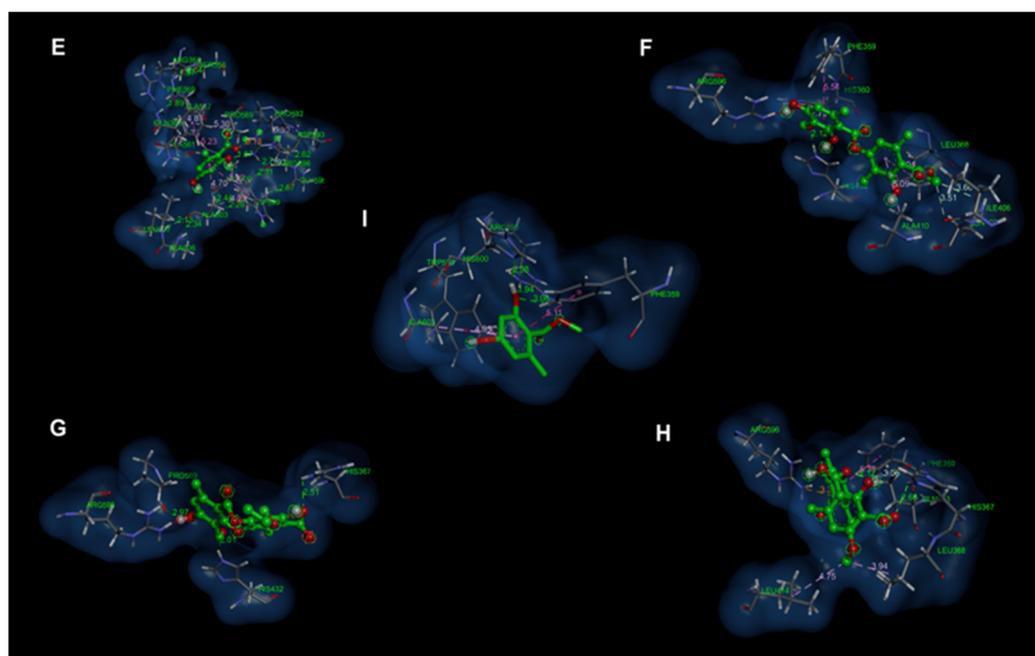


Figure 6. Molecular docking of compounds present in the species of *Cladonia*. (E) Conformation of a higher affinity of orsellinic acid with 5-LOX; (F) Conformation of a higher affinity of atranorin with 5-LOX; (G) Conformation of a higher affinity of cetraric acid with 5-LOX; (H) Higher affinity conformation of squamatic acid with 5-LOX; (I) Higher affinity conformation of methylorsellinate with 5-LOX.

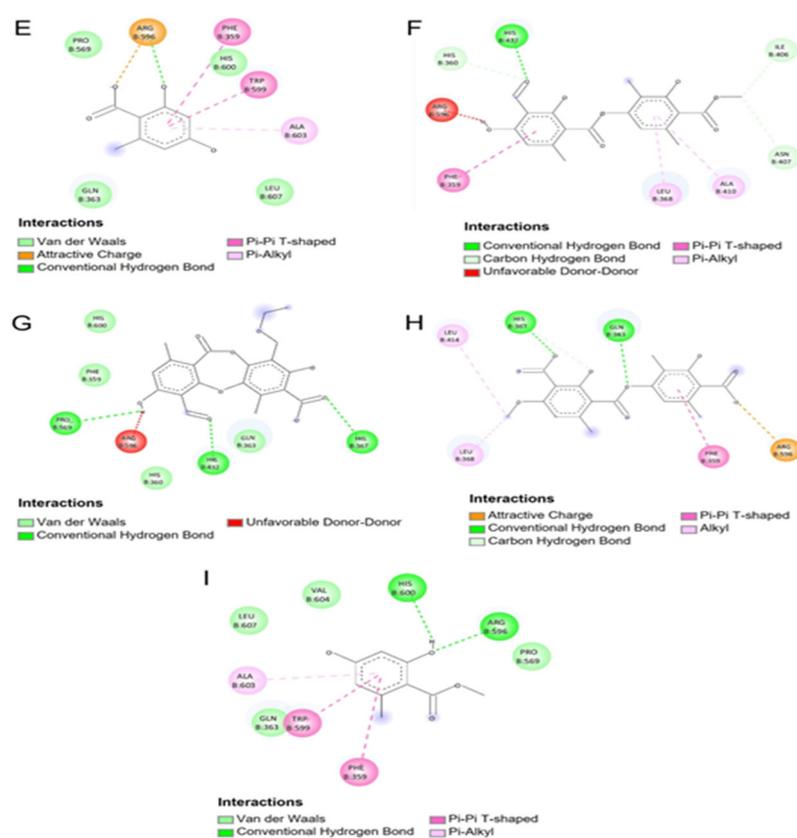


Figure 7. Molecular interactions of compounds present in the species of *Cladonia*. (E) Molecular interactions of orsellinic acid with the catalytic pocket of 5-LOX; (F) Molecular interactions of atranorin with the 5-LOX catalytic pocket; (G) Molecular interactions of cetraric acid with the catalytic pocket of 5-LOX; (H) Molecular interactions of squamatic acid with the 5-LOX catalytic pocket; (I) Molecular interactions of methylorsellinate with the 5-LOX catalytic pocket.

3.5. Cytoprotective Activity

3.5.1. Effect of *Cladonia* Extracts on Cell Viability and Survival under Oxidative Stress Induced by H₂O₂

First, the effect of the ethanolic extracts of *C. chlorophaea* and *C. gracilis* on the viability of the SH-SY5Y cell line was determined by quantification with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. According to previous cytotoxicity assays, five independent concentrations were determined for each extract: *C. gracilis* with 0.5, 1.0, 5.0, 10 and 25 µg/mL and *C. chlorophaea* with 0.5, 0.7, 1.0, 3.0 and 5.0 µg/mL. The *C. gracilis* extract promoted over 90% cell viability at all the concentrations evaluated; on the other hand, the *C. chlorophaea* extract showed optimal cell viability, but at 1.0 and 5.0 µg/mL, a slight reduction in viability was found (85% and 84%, respectively) (Figure 8).

Subsequently, the most optimal concentrations of cell viability in the two extracts were selected to determine the protective effector against H₂O₂-induced oxidative stress: *C. gracilis* at 5.0, 10 and 25 µg/mL and *C. chlorophaea* at 0.5 and 0.7 µg/mL. According to Figure 9, H₂O₂ treatment decreased the viability of SH-SY5Y cells by 75% compared to control cells. On the other hand, the two extracts promoted cell survival compared to H₂O₂ treatment; thus, for *C. gracilis*, the increase in cell viability was 98.7%, 104.7% and 90.2% at 5.0, 10 and 25 µg/mL, respectively, and for *C. chlorophaea*, the increase was 100% and 88% at 0.5 and 0.7 µg/mL, respectively.

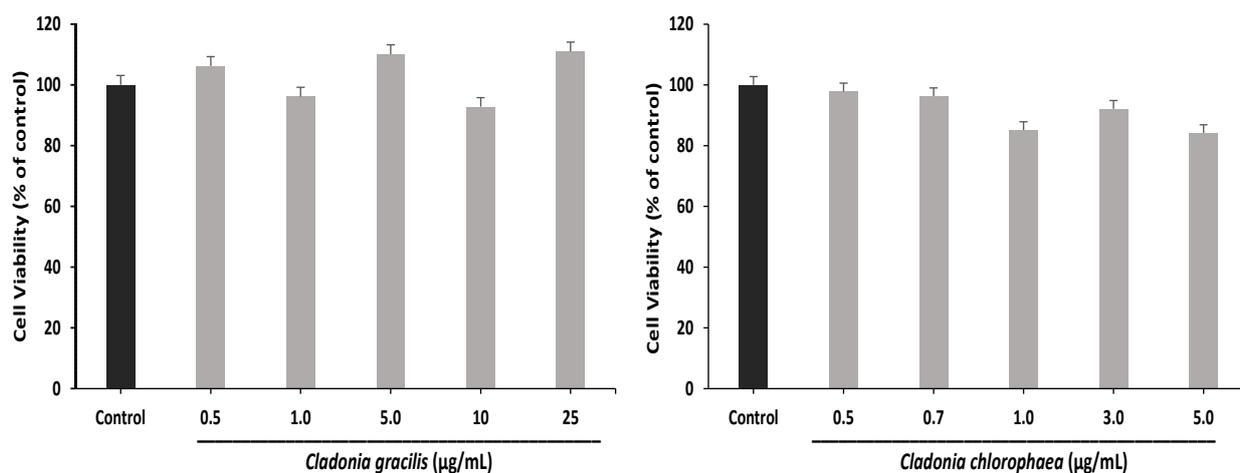


Figure 8. Effect of *C. gracilis* and *C. chlorophaea* extracts on viability in the SH-SY5Y cell line. The values represent the means \pm SD of three replicates ($n = 3$).

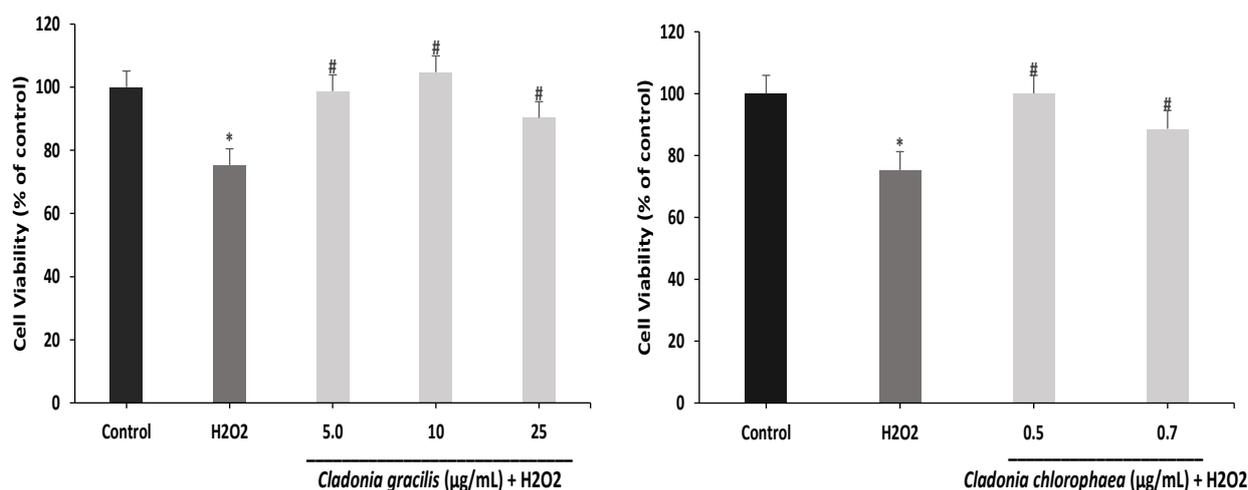


Figure 9. Effect of *C. gracilis* and *C. chlorophaea* extracts on cytoprotection in an H₂O₂-induced oxidative stress model. The values represent the means \pm SD of three replicates ($n = 3$). Values marked with * and # are statistically different using the Tukey test at a 0.05 level of significance ($p < 0.05$) versus the control and versus H₂O₂, respectively.

3.5.2. Effect of *Cladonia* Extracts on the Reduction in ROS Production in an H₂O₂-Induced Oxidative Stress Model

In this experiment, the concentrations of the extracts used in the previous assay were used to deepen their cytoprotective potential. According to Figure 10, a significant increase in intracellular ROS production of 143.9% was evidenced in the treatment of SH-SY5Y cells with H₂O₂ versus the control treatment (100%). In parallel, treatments with ethanolic extracts of *Cladonia* species significantly reduced ROS production; thus, *C. gracilis* showed a reduction of 46.5%, 48.3%, and 38% at 5.0, 10 and 25 µg/mL, respectively, versus treatment with H₂O₂, and *C. chlorophaea* showed a reduction of 26.8% and 43.8% at 0.5 and 0.7 µg/mL, respectively, versus treatment with H₂O₂.

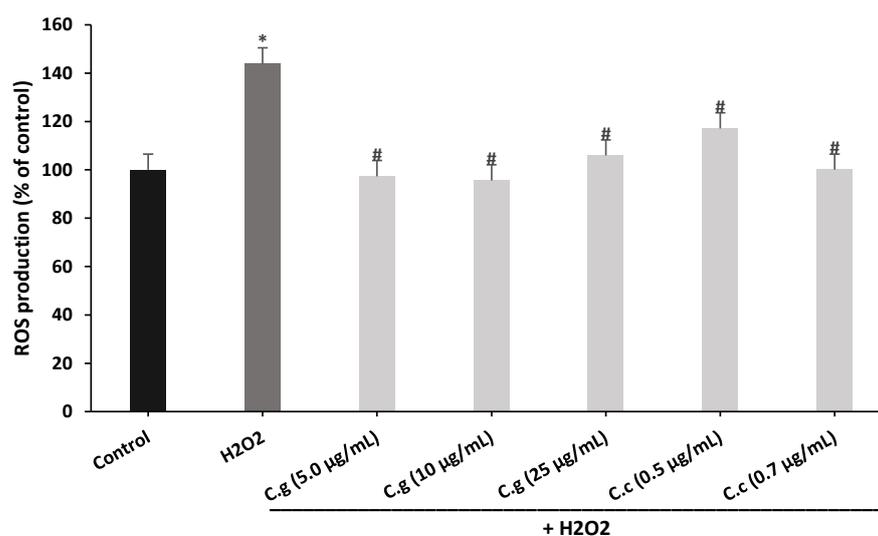


Figure 10. Effect of *C. gracilis* and *C. chlorophaea* extracts on intracellular ROS production. The values represent the means \pm SD of three replicates ($n = 3$). Values marked with * and # are statistically different using the Tukey test at a 0.05 level of significance ($p < 0.05$) versus the control and versus H₂O₂, respectively. C.g: *C. gracilis*; C.c: *C. chlorophaea*.

In other lichen species such as *Dactylina arctica*, *Nephromopsis stracheyi*, *Tuckermannopsis americana* and *Vulpicida pinastri* from cetrarioid clade, ideal viability ranges have been reported with methanolic extracts at concentrations of 5 to 10 µg/mL, along with a reduction of the same between 25 and 50 µg/mL; likewise, cell protection against H₂O₂ was evidenced, especially in *D. arctica* and *V. pinastri*, with a viability percentage of 76.8% at 10 µg/mL and 78.9% at 5 µg/mL, respectively. In addition, for *D. arctica*, a reduction in ROS production of 42.4% was reported at 10 µg/mL compared to H₂O₂ treatment [12], which is similar to that obtained with *C. gracilis* species at concentrations of 5 and 10 µg/mL. This effective cytoprotective activity is triggered by major compounds in the extracts such as usnic acid and fumarprotetratric acid, which report high antioxidant properties that promote the reduction of oxidative damage in different cell lines [52]. In other studies, isolated compounds such as evernic acid, which are abundantly present in lichens of the genera *Ramalina*, *Evernia* and *Hypogymnia*, report a cell viability >50% using primary culture neurons [11]; biruloquinone, isolated from *C. macilenta* [47], showed a protective effect against H₂O₂-induced oxidative stress >80% at a concentration of 25 µg/mL and a reduction in ROS production at 25 and 12.5 µg/mL, which are similar to the values recorded for *C. gracilis* at working concentrations. In parallel, the viability assays can be contrasted with cytotoxicity analyses on cancer cell lines that show the promising use of compounds isolated from lichens for pharmacological treatment, such as the case of (-)-usnic, fumarprotocetraric and 9'(O-methyl)protocetraric acids from *C. convoluta* [53] and diffractaic acid, vicanicin, lobaric acid, variolaric acid, protolichesterinic acid and usnic acid from *Protousnea magellanica*, *Psoroma pallidum*, *Stereocaulon alpinum*, *Ochrolechia deceptionis*, *Cornicularia aculeata* and *C. lepidophora*, respectively [54].

4. Conclusions

This work contributed to the understanding of the metabolomics of the species of the genus *Cladonia*, *C. chlorophaea* and *C. gracilis*, which are present in the Antarctic territory, reporting the identification of 19 compounds for each one by UHCLP-ESI-QTOF-MS analysis, including aromatics, organic acids, lipids, dibenzofurans, depsides and depsidones, which are typical of lichenized fungi. *C. chlorophaea* and *C. gracilis* species presented variable but significant results in terms of the phenolic compound content, FRAP ORAC antioxidant assays, cholinesterases (AChE and BChE) and α -glucosidase inhibition and moderate values in terms of the DPPH antioxidant assay and pancreatic lipase inhibition.

On the other hand, in an anti-inflammatory potential approach, molecular docking evidenced significant binding affinities of some compounds for the 5-LOX enzyme, together with outstanding pharmacokinetic properties that compromise their good bioavailability. Likewise, the two extracts of the *Cladonia* species promoted protective activity in the human neuroblastoma cell line against cytotoxicity effects generated by hydrogen peroxide. These species represent a biological resource of scientific interest for the prevention and treatment of central nervous system pathologies, inflammatory disorders and metabolic alterations. Future research could address the isolation and study of bioactive compounds of both species and their molecular mechanisms of action.

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