Exploring marine resources against neurological disorders– the neuroprotective and anti-inflammatory potential of the brown seaweed *Bifurcaria bifurcata*

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Research

Keywords: Antioxidants, Mitochondrial dysfunction, Neuroinflammation, Parkinson's Disease, SH-SY5Y cells, RAW 264.7 cells, Seaweeds

Posted Date: August 6th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-764213/v1>

Abstract

Oxidative stress is strongly involved in the pathogenesis of neurodegenerative diseases, like Parkinson's disease (PD). Particularly, an excess of reactive oxygen species (ROS) released by the cells promotes an oxidative stress condition, which is a main cause of tissue injury leading to nervous system dysfunction. In this work, the antioxidant, neuroprotective and anti-inflammatory activities of different fractions from *Bifurcaria bifurcata* are presented and related with their chemical profile. The antioxidant capacity was evaluated by the Folin-Ciocalteu method, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Neuroprotective capacity was evaluated to prevent neurological cell death mediated by the neurotoxin 6-hydroxydopamine (6-OHDA) on SH-SY5Y cells, and their anti-inflammatory effects on RAW 264.7 macrophages. Regarding results, ethyl acetate fractions (100 µg/mL) exhibited significant antioxidant and neuroprotective activities in the *in vitro* models assayed. Furthermore, two of the most polar fractions obtained with methanol and water, also evidenced a significant neuroprotective potential. *B. bifurcata* fractions treatment decreased ROS production, mitochondrial dysfunction, and Caspase-3 activity. Regarding the anti-inflammatory potential, five fractions (100 µg/mL) inhibited nitric oxide (NO) production and reduced the interleukin – 6 (IL-6) and tumor necrosis factor (TNF-α) levels. Mannitol, identified as the major component of the most bioactive fraction, protected SH-SY5Y cells against the 6-OHDA neurotoxicity mediating ROS generation mitigation, mitochondrial dysfunction, and DNA damage, together with the Caspase-3 activity inhibition. Results suggest that *B. bifurcata* is a relevant source of neuroprotective agents, with particular interest for preventive therapeutics.

1. Introduction

The degenerative brain disorders such as Parkinson's disease (PD) are the consequence of pathological brain aging, which are characterized by the loss of neurons in *substantia nigra* region (Hannan et al. 2020). The current therapeutic options only alleviate symptoms but do not halting the progression of PD. Therefore, several research groups continue to make efforts to discover the potential therapeutic agents that can target disease pathogenesis, but without causing adverse effects on patient's health (Hannan et al. 2020). The mechanisms responsible for neuronal degeneration in PD are complex and remain to be fully elucidated. However, several studies indicate that oxidative stress (ROS generation), nitric oxide (NO) production, mitochondrial dysfunction, inflammation and accumulation of misfolded proteins contribute to the cascade of events leading to degeneration of dopaminergic neurons (Hwang 2013; Blesa et al. 2015; Hemmati-Dinarvand et al. 2019).

Oxidative stress triggers several molecular pathways leading to the progressive loss of neuronal structures and functions, a process termed neurodegeneration, and is considered a major factor in PD development (Chen et al. 2017). The endogenous triggers of ROS generation include biological processes that release ROS as by-products, such as the mitochondrial electron transport chain. Example of this is the membrane-bound NADPH oxidase (NOX) family and nitric oxidase synthase, as responses to bacterial invasions and cytokines' release (Zorov et al. 2014; Di Meo et al. 2016). The most of free

radicals in cells are generated by the mitochondria oxidative phosphorylation, through the electron leak from the electron transport chain (Zhao et al. 2019). These radicals, as superoxide radical, are formed at complexes of the electron transport chain that lead to ROS formation, through O₂ consumption by mitochondria (Zhao et al. 2019). Thus, several studies have been demonstrated that ROS production is strongly dependent from the membrane potential of the mitochondria, showing high values resulting in increased rates of ROS generation (Kausar et al. 2018; Warraich et al. 2020). Another radical that is produced in the mitochondria through the oxidation of L-arginine to L-citrulline is NO, that can mediate neurotoxicity causing neuronal cell death, being suspected to be directly related with PD pathogenesis (Abou-sleiman et al. 2006). Nitric oxide radical is synthesized by cytosolic NO synthases (NOSs) using NADPH and molecular oxygen. It has been described three isoforms of NOS: nNOS (neuronal NOS), eNOS (endothelial NOS) and inducible NOS (iNOS) (Kleinert et al. 2010). nNos and eNos are constitutively expressed with their activity depending on intracellular calcium levels both present in the central nervous system, while the iNOS is expressed in astrocytes and microglia and their activity is induced during cellular inflammatory response (Aquilano et al. 2008). Upregulation of iNOS generates high levels of nitric oxide that breaks down into hydroxyl radicals promoting further intensification of the inflammatory response, leading to the expression of cytokines. These cytokines play an important role in the regulation of the inflammatory response in neuronal cells, such as tumor necrosis factor (TNF- α) and interleukin 1 β (IL-1 β), which are positively regulated in the brain tissue during the inflammatory response in PD (Wink et al. 2011). In addition, other interleukins, such as IL-6 and IL-10, are also secreted by microglia and astrocytes during inflammatory response (Burmeister and Marriott 2018). An excessive inflammatory response in PD leads to an increased release of pro-inflammatory cytokines and production of free radicals, causing neuronal damage (Guo et al. 2018). All of these consequences result in the activation of protein kinases cascades, which can lead to the activation of the transcription nuclear factor kappa B (NF- κ B), which migrates to the nucleus intervening in the transcription of many proteins involved in the inflammatory process, such as COX-2, TNF- α , IL-1 β , IL-6, iNOS and metalloproteinases (MMP) (Liu et al. 2017).

In recent years, several researchers have studied the neuroprotective effects of exogenous antioxidant molecules, since they have been shown to be fundamental in preventing the damage induced by oxidative stress reducing the production of ROS and reactive nitrogen species (RNS) (Tan et al. 2018). As a consequence, the interest in antioxidant for therapeutic applications molecules has been increasing. For example, Anh et al. (2019) (Man Anh et al. 2019) studied the neuroprotective effect of vitamin C in a *Drosophila* model with PD-like phenotype and demonstrated its potential to reduce PD symptoms. However, the authors observed out that its administration in high doses as well as long-term use, lead to significant side-effects. On the other hand, Ma and collaborators (2018) (Man Anh et al. 2019) verified that pretreatment with proanthocyanidins, a class of natural flavonoids, reduced the rotenone-induced oxidation in human neuroblastoma cells SH-SY5Y. In addition, the authors also observed that flavonoids were able to considerably block rotenone-induced apoptosis through Caspase-9 and Caspase-3 activity inhibition, as well as the inhibition of the mitogen-activated protein kinases, p38, JNK and ERK (Man Anh et al. 2019).

Therefore, there is still a medical need to search for new PD therapeutic agents that not only treat symptoms, but also have the capacity to reduce disease the progression. In this way, new naturally occurring antioxidant molecules could be of utmost interest. For example, marine organisms such as seaweeds are a rich source of new bioactive compounds that are not found in other organisms, and exhibit diverse biological activities, including strong antioxidant capacity (Salehi et al. 2019; Cotas et al. 2020b). Among the various species of seaweeds, brown algae have shown the ability to produce a wide variety of secondary metabolites with unique structural features such as phlorotannins (Cotas et al. 2020a). They belong to a group of phenolic compounds identified in several families of brown algae like *Alariaceae*, *Fucaceae* and *Sargassaceae* and previous studies have shown that phlorotannins are the main group of phenolics detected in this group (Sathya et al. 2017).

Bifurcaria bifurcata is a brown seaweed that belongs to the Sargassaceae family. Most of the studies regarding *B. bifurcata* in recent years have been related to its content of specific diterpenoids such as eleganolone, eleganonal and others, which have shown antiprotozoal, antifouling and antioxidant activities (Culioli et al. 1999; Gallé et al. 2013; J. et al. 2013; Silva et al. 2019). However, the place and season of collection, the maturation stage, and extraction procedures are factors that can influence the composition and bioactivities of the extracts.

In this study, twenty-three fractions from *B. bifurcata* harvested on the Portuguese coastline were investigated regarding their antioxidant, neuroprotective and anti-inflammatory potential in different *in vitro* models. Additionally, fractions showing the greatest potential were analyzed by the means of chromatographic and spectroscopic techniques, aiming the identification of compounds responsible for the above described bioactivities.

2. Materials And Methods

2.1. Extraction and fractionation of *Bifurcata bifurcata*

The algal biomass was prepared as previously described [26]. Freeze-dried samples of *B. bifurcata* were extracted with methanol (MeOH) and ethyl acetate (in a biomass/solvent ratio of 1:40), overnight, under constant stirring. Crude extracts were dried at 30°C, under vacuum in a rotary evaporator (IKA HB10, Staufen, Germany) and in a speed vacuum equipment (Concentrator Plus, Eppendorf, Spain). The MeOH dried extract (8.2 g) was subjected to a normal phase vacuum liquid chromatography (VLC) on silica gel 60 (0.06–0.2 mm; VWR, Leuven, Belgium), with different mixtures of cyclohexane/ethyl acetate (v/v) of increasing polarity, 1:0 (F1, 0.02 g), 2:1 (F2, 0.25 g), 2:2 (F3, 0.47 g) 1:2 (F4, 0.29 g) 0:1 (F5, 0.09 g) 0:1 MeOH (F6, 0.04 g), 0:1 acetone (F7, 0.40 g) (400 mL of each eluent). In parallel, an aliquot of the MeOH extract (2.6 g) was also subjected to a reversed phase chromatography using a SPE C18 cartridge (CHMLAB group, Terrassa, Barcelona, Spain) and elution performed with different mixtures of H₂O/MeOH/DCM (v/v) of increased polarity, 1:0:0 (F1, 0.83 g), 1:1:0 (F2, 0.14 g), 0:1:0 (F3, 0.26 g), 0:1:1 (F4, 0.05g), 0:0:1 (F5, 0.038 g) (2 mL of each eluent). The ethyl acetate extract (7.0 g) was subjected to a column chromatography (CC) on silica gel 60 (0.06–0.2 mm), with different mixtures of MeOH /DCM

(v/v) of increasing polarity, 100:00 (F1, 0.24 g), 99.5:0.5 (F2, 0.04 g), 98.5:1.5 (F3, 0.02 g), 98:2 (F4, 0.02 g), 95:5 (F5, 0.03 g), 80:20 (F6, 0.81 g), 70:30 (F7, 1.08 g), 60:40 (F8, 0.06 g), 50:50 (F9, 0.13 g), 100% MeOH (F10, 0.06g), 100 % ethyl acetate (F11, 0.04 g) (100 mL of each eluent). The correspondent fractions were subjected to a series of *in vitro* studies, in order to evaluate their antioxidant, neuroprotective and anti-inflammatory potential. An overview of the fractionation process is depicted in Fig. 1.

2.2. Evaluation of *Bifurcaria bifurcata* bioactivities

2.2.1. Quantification of Total Phenolic Content (TPC)

TPC of *B. bifurcata* polar fractions was determined using the Folin–Ciocalteu reagent as described by Singleton et al. (1965) (Singleton and Rossi 1965) with slight modifications. After 1 h of reaction in the dark, the absorbance was measured at 755 nm (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA). Gallic acid was used as standard, and TPC was expressed in milligrams of phloroglucinol equivalents per gram of dry extract (mg PE/g of extract).

2.2.2. Evaluation of the antioxidant capacity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity

DPPH free radical scavenging effect was determined according to the protocol described by Pinteus et al. (2017) (Pinteus et al. 2017). Posteriorly, the reaction mixtures were incubated in the dark, for 30 min, at room temperature. The absorbance was then measured at 517 nm. The samples were tested at a maximum concentration of 100 µg/mL. Dose-response analysis (10–100 µg/mL) was performed for the samples with high activity (DPPH reduction > 50%) for EC₅₀ determination.

Oxygen Radical Absorbance Capacity (ORAC-fluorescein)

ORAC assay was performed according to the protocol described by Dávalos et al. (2004)(Dávalos et al. 2004). The samples (20 µL) and fluorescein (120 µL; 70 nM, final concentration) were placed in the wells of a 96-wells microplate. The mixture was pre-incubated for 15 min, at 37°C. Then, AAPH (2,2'-azobis(2-methylpropionamidine) dihydrochloride solution) (60 µL; 12 mM, final concentration) was added rapidly. The microplate was immediately placed in the reader and the fluorescence (λ excitation: 458 nm; λ emission: 520 nm), recorded every minute for 240 min, and automatically shaken prior to each reading. Trolox was used as antioxidant standard. ORAC values were expressed as trolox equivalents by using the standard curve calculated for each assay. The results were expressed in micromol of trolox equivalents per gram of dry extract (µmol TE/g of extract).

Ferric Reducing Antioxidant Power (FRAP)

The capacity of seaweed fractions to reduce Fe (III) to Fe (II) through electron donation was performed according to Benzie and Strain (1996) with slight modifications (Silva et al. 2019). The seaweed fractions

(100 µg/mL) were mixed with FRAP reagent (0.3 M acetate buffer (pH = 3.6), 10 mM of 2,4,6-tri(2-pyridyl)-s-triazine, (TPTZ) in 40 mM HCl and 20 mM ferric solution using FeCl₃ (10:1:1)) pre-heated at 37 °C, in the dark, for 30 min. The absorbance was then read at 593 nm. The difference between the absorbance of test fractions and the blank reading was calculated and results expressed as micromolar of FeSO₄ per gram of extract (µM FeSO₄ /g of extract).

Evaluation of Biological Activities of Bifurcaria bifurcata on an In vitro Cellular Model of Parkinson's Disease

2.2.3. Cell Culture Maintenance

Biological activities of fractions were conducted on an *in vitro* cellular model of human

neuroblastoma (SH-SY5Y cells, strain number ACC 209) previously acquired from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) biobank. SH-SY5Y cells grew in Dulbecco's Modified Eagle's Medium: F12 (DMEM:F12) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/ streptomycin, and kept at 37°C in a humidified incubator with 5% CO₂.

2.2.4. Evaluation of the Neuroprotective Potential and Study of Associated Mechanisms

Cell viability

The neuroprotective activity was evaluated on SH-SY5Y cells according to Silva [26]. Cells were treated with seaweed fractions (100 µg/mL) for 24 h, and cell viability estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Briefly, MTT (0.5 mg/mL) was added to SH-SY5Y cells and the cells incubated for 1 h at 37°C. After this time, DMSO was added and absorbance was read at 570 nm (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA).

Determination of intracellular reactive oxygen species (ROS) levels: Intracellular levels of ROS were determined with carboxy-H2DCFDA (carboxy-2',7'-dichlorodihydrofluorescein diacetate) according to Silva et al. (2019) (Silva et al. 2019). SH-SY5Y cells were treated with 6-OHDA (100 µM) in presence/absence of seaweed fractions (100 µg/mL) for 6 h. After this time, cells were washed and carboxy-H2DCFDA (20 µM) probe was added and incubated by 1 h at 37 °C. The fluorescence was then read (λ excitation: 527 nm; λ emission: 495 nm) and the results were expressed in percentage of control.

Mitochondrial membrane potential (MMP) assay

The MMP (ΔΨ_m) was assessed using JC -1 probe according to Silva et al. (2019) (Silva et al. 2019). The cells were exposed to 6-OHDA (100µM) in the presence/absence of seaweed fractions (100 µg/mL) for 6 h. After this time, SH-SY5Y cells were washed twice with phosphate-buffered saline (PBS) and incubated with JC-1 solution (3 µM) in the dark, for 15 min, at 37 °C. The fluorescence was then read at 530 nm (monomers) and at 590 nm (aggregates) and 490 nm emission/ excitation wavelengths, respectively. The

results were calculated from the ratio between JC-1 monomers and aggregates and expressed in percentage of control.

Measurement of Caspase – 3 activity

The induction of apoptosis via 6-OHDA has been shown to increase the activity of Caspase-3 involved in the executioner pathway. Thus, cells were exposed to 6-OHDA (100 μ M) in the presence/absence of seaweed fractions (100 μ g/mL) for 6 h. Ending this time, the Caspase–3 activity was evaluated using the Caspase-3 fluorometric assay kit (Sigma, CASP3F-KIT, St. Louis, MO, USA) according to the manufacturers' instructions. Cells were then washed with PBS, harvested, and enzyme activity determined by reading the fluorescence at 360 nm and 460 nm emission and excitation wavelengths, respectively. The results were expressed as percentage of control.

2.3. Evaluation of Biological Activities of *Bifurcaria bifurcata* on an *In vitro* Cellular Model of Inflammation

2.3.1. Cell Culture Maintenance

RAW 264.7 macrophages were obtained from American Type Culture Collection (ATCC) (TIB – 71). Cells grown in Dulbecco's Modified Eagle's Medium without phenol red and supplemented with 10% (v/v) FBS, 1% penicillin/streptomycin, 1% of sodium pyruvate and kept at 37°C in a humidified incubator with 5% CO₂.

2.3.2. Evaluation of the Anti-inflammatory Potential

Cytotoxicity

The cytotoxicity of seaweed fractions was evaluated on RAW 264.7 macrophages (5 x 10⁴ cells/mL) after seeding in 96-well plates and incubated overnight. Cells were treated with fractions (100 μ g/mL) for 24 h. Cellular viability was then estimated through the MTT method and absorbance was measured at 570 nm using a microplate reader (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Vermont, VT, USA). The results were expressed as percentage of control.

Determination of nitric oxide (NO) production

The inflammatory and anti-inflammatory effects of *B. bifurcata* fractions were estimated through the nitric oxide (NO) production, according to Freitas et al. (2020). RAW 264.7 cells (5 x 10⁴ cells/mL) were pretreated (1 h) with seaweed fractions (100 μ g/mL) and stimulated with LPS (1 μ g/mL) for 24 h. NO levels were determined by measuring nitrite levels in the culture media using the Griess reagent (1% sulphanilamide in phosphoric acid (2.5%) + 0.1% naphthylethylenediamine dihydrochloride). The results are expressed in percentage of control.

Measurement of IL-6 and TNF- α

RAW 264.7 cells (1×10^5 cells/mL) were pre-treated for 1 h with seaweeds fractions and stimulated with LPS (1 μ g/mL) for 18 h. The concentration of IL-6 and TNF- α were determined using ELISA kits according to the manufacturer's instructions.

2.4. Chemical Characterization

The chemical profile of the *B. bifurcata* most bioactive fractions were evaluated by NMR, HPLC-DAD and GC-MS techniques.

2.4.1. Nuclear Magnetic Resonance (NMR) analysis

NMR spectra were acquired on a Bruker Avance 400 spectrometer with a frequency of 400 MHz for ^1H , and 100 MHz for ^{13}C . Samples were dissolved in 500 μ L of CDCl_3 or D_2O (Sigma-Aldrich, St. Louis, MO, USA). Chemical shifts were expressed in ppm and reported to the residual solvent signals. Coupling constants (J) were expressed in Hertz (Hz).

2.4.2. High Performance Liquid Chromatography (HPLC) analysis

NMR spectra were acquired on a Bruker Avance 400 spectrometer with a frequency of 400 MHz for ^1H , and 100 MHz for ^{13}C . Samples were dissolved in 500 μ L of CDCl_3 or D_2O (Sigma-Aldrich, St. Louis, MO, USA). Chemical shifts were expressed in ppm and reported to the residual solvent signals. Coupling constants (J) were expressed in Hertz (Hz).

2.4.3. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS qualitative analysis was performed in a Shimadzu QP2010-Plus GC/MS system equipped with a TRB5MS (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μ m film thickness) capillary column (Teknokroma, Barcelona, Spain) operating in the linear velocity mode. The carrier gas was helium 5.0 (Linde, Portugal), at a constant flow of 1 mL/min. Samples were dissolved in dichloromethane and automatically injected. Injections were performed in split mode, with a ratio of 1/9. The injector port was heated to 280°C. The initial column temperature of 60°C was held for 2 min, followed by a temperature ramp of 30°C/min to 300°C held for 15 min. All mass spectra were acquired in electron impact (EI) mode at 70 eV. The operate temperatures were 200°C for MS ion source, and 250°C for the liner interface. The analysis was performed in full scan mode with mass ranging from 10 to 800 m/z . Compounds were identified by matching the mass fragmentation patterns with those stored in the GC-MS mass spectral databases (Wiley 229 and NIST-National Institute of Standards and Technology libraries).

2.5. Data and statistical analysis

When applicable, results are presented as mean $\pm$ standard error of the mean (SEM). The determination of EC_{50} was attained from sigmoidal dose-response variable-slope curves using the GraphPad Prism V.8

software (GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) with Dunnett’s multiple comparison of group means was employed to determine significant differences ($p < 0.05$) relatively to the control treatment. All other *post- hoc* analyses were conducted using Tukey’s test. All data were checked for normality and homoscedasticity. Comparisons concerning variables, which did not meet variance or distributional assumptions, were carried out with Kruskal–Wallis non-parametric tests. At least three independent experiments were carried out in triplicate.

3. Results

3.1. Antioxidant activity

In order to evaluate the antioxidant potential of *B. bifurcata* fractions (MF1–MF7; MC18F1-MC18F5; AEF1 – AEF11) different approaches have been outlined, including the determination of total phenolic content (TPC) by the Folin–Ciocalteu method, DPPH radical scavenging activity, oxygen radical absorbance capacity (ORAC), and ferric reducing antioxidant power (FRAP). The results are shown in **Table 1**.

Table 1. Antioxidant potential of *Bifurcaria bifurcata* fractions collected off Peniche coast, Portugal.

Extraction	Fractions	Extraction Yield (%)	TPC ^a	DPPH ^b	ORAC ^c	FRAP ^d
MeOH	F1	0.26	0.05 ± 0.01	>100	46.96 ± 6.30	447.18 ± 7.35
	F2	3.00	14.94 ± 0.98	>100	635.94 ± 9.94	547.96 ± 2.75
	F3	5.71	11.24 ± 1.78	>100	398.15 ± 21.28	603.99 ± 29.82
	F4	3.47	8.16 ± 2.81	>100	1747.20 ± 164.13	512.60 ± 19.61
	F5	1.14	12.89 ± 0.28	>100	6945.81 ± 301.40	561.66 ± 8.20
	F6	0.47	5.56 ± 0.62	>100	1216.21 ± 139.04	682.62 ± 7.21
	F7	4.96	7.75 ± 0.17	>100	591.81 ± 29.79	115.06 ± 7.83
MeOH C18	F1	32.18	8.17 ± 0.34	>100	588.26 ± 39.61	299.95 ± 1.67
	F2	5.48	63.33 ± 2.95	>100	6631.05 ± 108.96	3431.22 ± 23.15
	F3	4.94	10.17 ± 0.51	> 100	768.65 ± 45.94	400.84 ± 4.86
	F4	1.89	144.76 ± 5.91	>100	683.49 ± 30.31	412.74 ± 5.49
	F5	1.45	9.11 ± 0.29	>100	2532.34 ± 113.47	329.29 ± 3.02
Ethyl acetate	F1	3.41	12.79 ± 5.02	> 100	2054.83 ± 31.96	537.13 ± 3.37
	F2	0.54	12.03 ± 1.34	> 100	3245.26 ± 30.75	540.50 ± 4.88
	F3	0.26	10.23 ± 1.87	> 100	6944.25 ± 126.71	498.17 ± 6.78
	F4	0.30	16.40 ± 1.38	> 100	1934.54 ± 53.29	470.27 ± 10.43
	F5	0.44	7.08 ± 1.37	> 100	2405.159 ± 45.68	523.18 ± 3.93
	F6	11.59	17.07 ± 1.54	> 100	18 951.24 ± 571.14	577.29 ± 1.27

F7	15.52	24.12 ± 2.54	> 100	16 896.72 ± 645.48	455.36 ± 10.18
F8	0.82	74.79 ± 3.25	23.58 (11.40 – 48.77)	16 901.58 ± 323.58	3442.03 ± 40.88
F9	1.88	72.02 ± 2.40	> 100	7419.54 ± 368.96	937.56 ± 13.69
F10	0.93	76.22 ± 1.59	> 100	10 942.03 ± 472.20	985.43 ± 1.44
F11	0.54	12.79 ± 5.02	> 100	10 772.03 ± 472.20	922.89 ± 23.15
- Phloroglucinol	-	-	> 100	9 567.93 ± 521.86	-

^a mg phloroglucinol equivalents/g extract (mg PE /g extract); ^b radical scavenging activity (EC₅₀ µg/mL);

^c µmol Trolox equivalents/ g extract (µmol TE /g extract); ^d µM FeSO₄/g extract. MeOH – Methanol.

As shown in Table 1, the best extraction yields were achieved with water and a mixture of MeOH and DCM (MC18F1 – 32.18%; AEF7 – 15.52%; AEF6 – 11.59% fractions), while lower yields were obtained with less polar solvents (MF1 – 0.26%, AEF3 – 0.26%, and AEF4– 0.30% fractions).

Regarding the TPC, fractions MC18F4 (144.76 ± 5.91 mg PE /g extract), AEF8 (74.79 ± 3.25 mg PE/g extract), AEF9 (72.02 ± 2.40 mg PE/g extract), AEF10 (76.22 ± 1.59 mg PE/g extract), and MC18F2 (63.33 ± 2.95 mg PE/g extract) presented the highest phenolic content, when compared to other fractions. Concerning the DPPH radical scavenging ability, AEF8 fraction demonstrated the highest potential to reduce the DPPH radical (EC₅₀ = 23.58 µg/ mL (11.40 – 48.77)) when compared with other fractions. In the ORAC method, fractions AEF6 (18 951.24 ± 571.14 µmol TE/g extract), AEF7 (16 896.72 ± 645.48 µmol TE/g extract) and AEF8 (16 901.58 ± 323.58 µmol TE/g extract) showed the highest antioxidant activity. Fractions MC18F2 (3431.22 ± 23.15 µM of FeSO₄/g of extract) and AEF8 (3442.03 ± 40.88 µM of FeSO₄/g of extract) also revealed to be effective in reducing ferric ions when compared with other fractions, for example MF7 (115.06 ± 7.83 µM of FeSO₄/g of extract) and MC18F1 (299.95 ± 1.67 µM of FeSO₄/g of extract) (Table 1).

The main components analysis (PCA) carried out in this study allowed us to correlate the antioxidant capacity with the total phenolic content. It is possible to observe a clear arrangement of two groups that differ according to their antioxidant capacity (**Figure 2**)

Figure 2 shows the ordination of the different antioxidant methods using PCA analysis of the fractions obtained from *B. bifurcata*. PC1 accounted for 80.7% and PC2 13.4% of the total variance. In PC1, the horizontal axis expresses an opposition between DPPH (right), and antioxidant methods FRAP and TPC (Left). Moreover, FRAP and TPC present a negative correlation with DPPH (Figure 2). Since DPPH radical

scavenging activity is expressed by EC₅₀, it is possible to verify that fractions which presented high phenolic content and potential to reduce iron ions also exhibited high DPPH radical scavenging activity (Group I). On the other hand, the fractions present in group I, mainly MF7 and MF1, showed low levels of TPC, and exhibited low DPPH radical scavenging activity. Moreover, fractions MC18F2, MC18F4, AEF8, AEF9 and AEF10 showed high FRAP and TPC values when compared with the samples present in group II.

3.1 Cytotoxicity and neuroprotective activity of *Bifurcaria bifurcata* fractions on SH-SY5Y cells

The cytotoxic and neuroprotective effects of *B. bifurcata* fractions on SH-SY5Y cells were evaluated. Firstly, SH-SY5Y cells were treated with different *B. bifurcata* fractions (100 µg/mL) for 24 h. Fractions with no cytotoxicity were further tested for their neuroprotective potential. The neuroprotective effects were evaluated on SH-SY5Y cells treated with 6-OHDA in the presence/ absence of *B. bifurcata* fractions (100 µg/mL) after 24 h. The results are presented in **Figures 3A and 3B**.

In Figure 3A it is possible to observe that only MF7, MC18F1, MC18F2 and AEF8-AEF11 fractions showed no cytotoxic effects when compared with the vehicle situation. Thus, fractions that did not exhibit cytotoxicity were evaluated for their neuroprotective activity and associated mechanisms on SH-SY5Y cells treated with 6-OHDA (100 µM) in the presence of *B. bifurcata* fractions (100 µg/mL) for 24 h. Observing Figure 3B, it is possible to verify that the treatment accomplished with 6-OHDA ($61.86 \pm 1.88\%$) led to a decrease of mitochondrial function about $38.14 \pm 1.88\%$ when compared with vehicle ($100.00 \pm 3.15\%$). On the other hand, fractions MC18F1 ($86.67 \pm 3.95\%$), MC18F2 ($81.68 \pm 5.08\%$), AEF9 ($98.53 \pm 4.82\%$), AEF10 ($87.38 \pm 6.17\%$) and AEF11 ($105.90 \pm 5.05\%$) showed neuroprotective effect recovering the mitochondrial function loss induced by 6-OHDA treatment ($24.81 \pm 3.95\%$; $19.82 \pm 5.08\%$; $36.07 \pm 4.82\%$; $25.52 \pm 6.16\%$ and $44.04 \pm 5.05\%$, , respectively).

3.2 Study of the effects on Parkinson's Disease biomarkers

In order to better understand which cellular mechanisms are associated with the neuroprotective effects mediated by *B. bifurcata* fractions on SH-SY5Y cells, different hallmarks linked to PD development were studied, namely MMP, ROS production and Caspase-3 activity. The results are presented as percentage of control and depicted in **Figure 4**.

The treatment with seaweed fractions did not induce effects on the mitochondrial membrane potential, ROS production and Caspase-3 activity when compared with the vehicle (data not shown). SH-SY5Y cells were treated with 6-OHDA (100 µM) in the presence of *B. bifurcata* fractions for 6 h. Cells exposed to 6-OHDA increase ROS levels about $65.6 \pm 4.19\%$ when compared with the vehicle situation (Figure 4A). In the other hand, MC18F2, AEF9 and AEF10 fractions reduced ROS production in $52.90 \pm 15.05\%$, $40.7 \pm 1.78\%$, and $41.7 \pm 4.19\%$, respectively. The effects on mitochondrial membrane potential ($\Delta\psi_m$) were evaluated using the JC-1 probe. SH-SY5Y cells exposed to 6-OHDA induced a depolarization of $\Delta\psi_m$ ($192.70 \pm 16.86\%$) when compared to the vehicle. By the other side, fractions AEF9, AEF10 and AEF11 exhibited ability to restore the mitochondrial membrane potential loss promoted by 6-OHDA treatment

($156.00 \pm 12.53\%$, $108.80\% \pm 24.29\%$ and $145.10 \pm 18.55\%$, respectively) (Figure 4B). Regarding, Caspase-3 activity, the treatment of SH-SY5Y cells with 6-OHDA ($239.90 \pm 11.30\%$) led to a significant increased of the Caspase-3 activity when compared with vehicle ($100.00 \pm 33.69\%$). On the other hand, fractions MC18F2 ($179.1\% \pm 5.23\%$) and AEF9 ($22.24\% \pm 1.54\%$) exhibited a significant decrease of Caspase-3 activity promoted by 6-OHDA (Figure 4C).

3.3 Anti – inflammatory activity of *Bifurcaria bifurcata* fractions on RAW 264.7 cells

For the *B. bifurcata* fractions that displayed neuroprotective activities, their anti-inflammatory effects were studied on LPS - induced RAW264.7 cells. Firstly, the cytotoxicity of seaweed fractions ($100\text{ }\mu\text{g/mL}$; 24 h) was evaluated on RAW264.7 cells and the effects were estimated by the MTT method. Fractions without cytotoxicity were further tested for their inflammatory and anti-inflammatory potential on RAW264.7 cells. The inflammatory and anti-inflammatory effects of seaweed fractions was estimated through the NO production. The results are presented in **Figures 5A-C**

Bifurcaria bifurcata fractions at $100\text{ }\mu\text{g/mL}$ did not induce cytotoxicity on RAW 264.7 cells (Figure 5A). After cells' treatment with LPS and fractions, it was possible to observe that only LPS stimulated the NO production (Figure 5B). On the other hand, fractions MC18F1 ($286.80 \pm 38.85\%$), MC18F2 ($98.89 \pm 7.34\%$), AEF8 ($122.20 \pm 6.57\%$), AEF10 ($153.50 \pm 15.81\%$) and AEF11 ($122.10 \pm 6.57\%$) decreased the NO levels in the LPS-stimulated RAW 264.7 cells when compared with LPS situation ($390.10 \pm 17.08\%$) (Figure 5C).

3.4 Effects of *Bifurcaria bifurcata* fractions on the pro-inflammatory cytokine's levels

The effects of of pre-treatment with *B. bifurcata* polar fractions ($100\text{ }\mu\text{g/mL}$; 1h) on LPS - induced RAW264.7 cells was evaluated in the levels of TNF- α and IL-6 cytokines after 18 h (**Figure 6**).

LPS-induced RAW 264.7 cells induced a significant increase of TNF- α ($703.40 \pm 22.30\%$), IL-6 ($329.20 \pm 52.47\%$) production as compared to vehicle situation ($100.00 \pm 18.94\%$ and $100.00 \pm 2.52\%$, respectively). On the other hand, all fractions induced a significant decrease of TNF- α and IL-6 levels as compared with LPS situation.

A general overview of the antioxidant, neuroprotective and anti-inflammatory activities of *B. bifurcaria* fractions is presented in **Table 2**.

In the Table II is possible to observe that AEF8 fraction showed high antioxidant capacity in all the evaluated methods when compared with other fractions. On the other hand, this fraction did not exhibit neuroprotective potential. However, fractions AEF9, AEF10, AEF11, MC18F1 and MC18F2 displayed neuroprotective activity and capacity to decrease ROS production, mitochondrial dysfunction and Caspase-3 activity against 6-OHDA damage. Regarding the anti-inflammatory activity, all fractions, excepting fraction AEF9 decrease NO production induced by LPS treatment. Furthermore, seaweed fractions also revealed capacity to decreased IL-6 and TNF- α cytokines release. Accordingly, these five fractions that exhibited the best bioactive potential i were studied regarding their chemical profile.

3.5 Chemical characterization of *Bifurcaria bifurcata* bioactive fractions

The chemical profile of the five most bioactive fractions (MC18F2, AEF8, AEF9, AEF10, and AEF11) from *B. bifurcata* were analyzed by NMR spectroscopy. The ^1H NMR spectra of MC18F2 fraction revealed signals (3.6-3.9 ppm) attributed to mannitol, the major component, being spectral data in full accordance with the available standard (Figure S1). Spectral data of fraction AE1F11 showed several chemical shifts ranges (7.8-7.6, 7.6-7.4, 4.4-4.1, 2.4-0.8 ppm) compatible with phthalate derivatives, while HPLC-DAD analysis evidenced a major peak (RT= 27.09—min) supported by a UV-Vis spectra characteristic of a phthalate (Figure S2). Additionally, a GC-MS analysis has shown fragmentation peaks (m/z 149, 167, 261, 279) attributed to di-*n*-octyl phthalate, which is in full accordance with literature data (Yin et al. 2014). The complexity of fractions AEF8, AEF9 and AEF10 evidenced by ^1H NMR spectroscopy (Figure S3) was also confirmed by HPLC-DAD, showing that they were mixtures of many compounds (Figure S4). More specifically, NMR spectra of those fractions (Figure S5) evidenced some similarities between them having shown chemical shifts ranges (6.4-5.9, 5.6-4.9, 4.4-3.5, 2.2-0.8 ppm) compatible with the structures of linear diterpenes commonly found in *Bifurcaria* species (Pais et al. 2019). Indeed, overlapped small intensity signals in the 6.0-6.4 region can also be attributed to phenolic compounds, which may suggest the presence of minor amounts of phlorotannins (Roy 2020).

(Figures S1 – S4 are available at Supporting Information.)

3.6 Evaluation of the neuroprotective activity of mannitol

The neuroprotective activity of mannitol, identified by NMR spectroscopy as the major compound of the bioactive fraction MC18F2, was also evaluated. SH-SY5Y cells were exposed to several concentrations (1 – 100 μM ; 24 h) of mannitol to define the non-neurotoxic concentrations, being that 1-100 μM concentrations no revealed cytotoxicity. Thus, neuroprotective activity was tested in SH-SY5Y cells exposed to 6-OHDA (100 μM) in the presence/ absence of mannitol, for 24 h. The effect on cells' viability and the hallmarks associated to PD, namely ROS production, mitochondrial membrane potential and Caspase-3 were studied. The results are presented in **Figure 7**.

The exposure of SH-SY5Y cells to 6-OHDA reduced cells' viability in about 32% ($67.64 \pm 3.33\%$) when compared to vehicle ($100.00 \pm 5.57\%$) after 24 h treatment. However, when 6-OHDA was incubated in the presence of 50 and 100 μM of mannitol, cells' viability increased in about 30 - 35% (Figure 7A). At these concentrations, mannitol also showed capacity to reduce ROS levels (50 μM : $109.4 \pm 3.00\%$ and 100 μM : $100.7 \pm 1.55\%$) (Figure 8B) and, at 100 μM , mannitol displayed ability to inhibit Caspase-3 activity ($177.30 \pm 6.67\%$) (Figure 7D) when compared with 6-OHDA situation ($119.60 \pm 3.27\%$ and $227.60 \pm 4.45\%$, respectively). Concerning the MMP depolarization, none of the assayed concentrations prevented the effects induced by 6-OHDA treatment (Figure 7C).

4. Discussion

The prevalence of neurodegenerative disorders is growing worldwide. In parallel, there is an urgent need to find new compounds for the treatment of such impairments, in which oxidative stress is a common hallmark, being suggested to play a causative role in the etiology and progression of Parkinson's disease (PD) (Uttara et al. 2009). Several lines of evidence have shown that brains of PD patients have low levels of endogenous antioxidants, increased dopamine oxidation, and high levels of iron, suggesting that oxidative stress is a significant target to counteract the progression of the disease. Natural and endogenous antioxidants such as polyphenols, Coenzyme Q10 and vitamins A, C and E, have been proposed as therapeutic agents for preventing and delaying the development of PD (Tan et al. 2018). However, more efficient approaches to counteract this disease are necessary. Although some current drugs are effective in the early stages of the disease, long-term therapy has been associated with serious adverse effects. Over the last decade, in an attempt to search new alternative therapies for PD, basic science has focused on the discovery of marine natural products as a source of potent and effective agents in the treatment of this devastating pathology. In the present study, the antioxidant, neuroprotective and anti-inflammatory properties of several fractions from the brown seaweed *B. bifurcata* were evaluated. Regarding their antioxidant capacity, three complementary methods, DPPH, FRAP and ORAC, were assayed and the results correlated with their total phenolic content (TPC). Amongst the twenty-three evaluated fractions, the most polar ones revealed to have the best antioxidant potential. This capacity can be mediated by a great variety of compounds including phlorotannins, linear diterpenes and mannitol, commonly found in brown seaweeds and also identified in *B. bifurcata*. Molecules with great antioxidant capacity due to their redox properties, acting as reducing agents (Costa et al. 2011; Farasat et al. 2014) and hydrogen (H⁺) donors, contribute to the production of less reactive radicals and for the removal of metal ions (Kasote et al. 2015).

Effectively, oxidative stress is related with several diseases, including neurodegenerative disorders like PD. Several studies have reported that marine-derived compounds exhibit neuroprotective effects against 6-OHDA neurotoxicity (Chen et al. 2012; Souza et al. 2018; Ye et al. 2019). So, in this work the 6-OHDA-induced neurotoxicity model in SH-SY5Y cell line was used, and it was verified that 6-OHDA *per se* reduced cells' viability by about 40%. Regarding the neuroprotection assay, *B. bifurcata* fractions exhibited capacity to promote cells' recovery (20 – 40%) from the 6-OHDA - induced neurotoxicity. Tancheva et al. (2020) (Tancheva et al. 2020) evaluated the neuroprotective action of three natural antioxidants, ellagic acid, α -lipoic acid and myrtenal, in an experimental model of PD that was induced in male Wistar rats through an intrastriatal injection of 6-OHDA. These authors verified that the three compounds improved learning and memory performances as well as neuromuscular coordination. Additionally, in the biochemical assays, all the three compounds substantially decreased lipid peroxidation (LPO) levels and restored Catalase (CAT) activity and DA levels that were impaired by the challenge with 6-OHDA. On the other hand, the decrease of neurotoxicity associated with 6-OHDA by marine-derived compounds has already been reported in previous scientific works. Our results are consistent with other studies in which methanolic extracts from the brown seaweeds *Sargassum muticum* and *Saccorhiza polyschides* increased neuronal cells' viability in about 35 - 40%, blocking the toxic effects of 6-OHDA (Silva et al. 2018). Chen and collaborators (2012) (Chen et al. 2012) also

evaluated the protective effect of the macrolide 11-dehydrosinulariolide, isolated from the cultured soft coral *Sinularia flexibilis*, against 6-OHDA – induced neurotoxicity in SH-SY5Y cell lines.

The mechanisms involved in the neuroprotective effects exhibited by seaweed fractions on the SH-SY5Y cells' viability were studied on different hallmarks associated to PD development. For this purpose, different *in vitro* assays were performed (mitochondrial membrane potential, production of ROS, and Caspase-3 activity) on cells treated with the neurotoxin 6-OHDA, in the presence/absence of fractions. Oxidative stress has been intimately linked to mitochondrial dysfunction and mitochondria are responsible for more than 90% of cellular ROS production (Zorov et al. 2014), being completely dependent of an efficient antioxidant machinery to prevent oxidative stress conditions. In fact, mitochondrial dysfunctions play a central role in the neurodegeneration, especially in PD, leading to a disruption of the respiratory chain and, consequently, to an increase of ROS production, a decrease of mitochondrial Complex I enzyme activity and Caspase-3 activation (Zorov et al. 2014; Redza-Dutordoir and Averill-Bates 2016). Based on the results obtained in the present work, it was observed that *B. bifurcata* ethyl acetate fractions (AEF9, AEF10 and AEF11) were able to prevent ROS production and mitochondrial dysfunction promoted by 6-OHDA treatment. These properties can be related with the presence of linear diterpenes in fractions AEF9 and AEF10, which biological properties were recently reported by Pais et al. (2019) (Pais et al. 2019). Di-*n*-octyl phthalate was the major component identified in fraction AEF11 and, although phthalate-derived compounds are normally regarded as synthetic contaminants, they can also be biosynthesized by several organisms. These group of compounds were recently reported to have a broad range of bioactivities, including antioxidant and anti-inflammatory properties (Roy 2020).

Since the Caspase-3 enzyme is a key player in cell death by apoptosis, its activity was also determined to understand if the neuroprotective effects of seaweed fractions were mediated by apoptotic pathways. The exposure of SH-SY5Y cells to 6-OHDA increased its activity, and the treatment with *B. bifurcata* fractions MC18F2 and AEF9 led to a marked decrease of Caspase-3 activity.

Microglial activation in the SNpc is another hallmark of PD that leads to a progression of inflammation, being the main cause of this disease condition, triggering the overproduction of pro-inflammatory mediators (Kany et al. 2019; Baek et al. 2020). The macrophages are known to cross the leaky blood-brain barrier in PD to interact with microglia and stimulate the secretion of inflammatory cytokines causing brain damage via neuroinflammation. These are important immune regulatory cells that play essential roles in inflammatory responses by producing various inflammatory mediators, such as nitric oxide (NO), as well as different pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 (Kany et al. 2019). LPS is the main component of endotoxins from Gram-negative bacteria, inducing a host inflammatory response that results in increased production of chemokines, cytokines, and pro-inflammatory mediators by the immune system (Dickson and Lehmann 2019). This way, macrophages exposed to LPS during microorganisms' infections stimulate the immune system to produce cytokines and chemokines, followed by inflammatory events (Monguió-Tortajada et al. 2018; Kany et al. 2019). Thus, inhibiting macrophage activation by LPS is an important focus for therapeutic strategies aiming

the treatment of diseases where inflammation is involved, including in PD (Ji et al. 2020). In this study, macrophages were exposed to LPS to induce inflammation in the presence of the most promising *B. bifurcata* fractions in previous trials. Based on the results presented in Figures 5 and 6, it is possible to observe that all fractions, excepting AEF9, prevented inflammatory conditions by decreasing NO production. On the other hand, all fractions prevented inflammation by reducing TNF- α and IL-6 levels. The anti-inflammatory properties of linear diterpenes (Pais et al. 2019) and phthalate derivatives (Roy 2020) can explain, at least in part, the positive effect of those fractions. Nevertheless, fraction MC18F2 seems to be the most prominent in a great part of the *in vitro* assays here performed and as described above, mannitol was the major metabolite present in this fraction. Mannitol, a sugar alcohol that is used as a sweetener in diabetic food, is present in many seaweeds, such as *Sargassum mangarevense*, *Turbinaria ornata*, *Ascophyllum nodosum* and *Laminaria hyperborean* and is reported to have a broad range of applications namely in the pharmaceutical sector (Zubia et al. 2008; Gomez-Zavaglia et al. 2019). Clinically, mannitol can act as a diuretic, promoting the urinary excretion of toxic substances. Additionally, it elevates blood plasma osmolality, resulting in enhanced flow of water from tissues, including the brain and cerebrospinal fluid, into interstitial fluid and plasma. As a result, cerebral edema, elevated intracranial pressure, and cerebrospinal fluid volume and pressure are reduced.

Mannitol was identified as a major component the promising fraction, suggesting that the evidence bioactivities can be mediated by this compound. Therefore, in this work, the neuroprotective effect of mannitol was evaluated for the first time in an *in vitro* model of PD induced by 6-OHDA, and it showed capacity to increase cellular viability, to decrease ROS production and Caspase-3 activity. There are reports referring that the neuroprotective effects of mannitol can be related to its antioxidant properties (Conrozier et al. 2014; Andr 2017), improvement of the blood brain barrier permeability (Cerri et al. 2014; Choi et al. 2018) and also to its capacity to interact with key proteins involved in PD (Shaltiel-Karyo et al. 2013). Additionally, the ability of mannitol to interfere with the aggregation process of α -synuclein on *in vitro* and *in vivo* models of PD, and its blood-brain barrier-disrupting properties were also previously verified (Shaltiel-Karyo et al. 2013). Specifically, high concentrations of mannitol significantly decreased the formation of α -synuclein fibril tetramers and high molecular weight oligomers and shifted the secondary structure of α -synuclein, suggesting potential alternative pathways for aggregation. When administered to a transgenic mice model, mannitol evidenced a general neuroprotective effect, which included the dopaminergic system, whereas no adverse effects were observed in control animals. Based on these findings, mannitol (Shaltiel-Karyo et al. 2013) and mannitol-based molecules (Paul et al. 2019) can be regarded as a basis for a dual mechanism therapeutic agent for the treatment of Parkinson's disease.

Conclusion

In conclusion, the current lack of studies on seaweed-derived neuroprotective agents can open new research lines in the near future. This work has shown that *B. bifurcata* bioactive fractions markedly inhibited 6-OHDA neurotoxicity induced in human neuroblastoma SH-SY5Y cells, suggesting that the neuroprotective effects are mediated by the mitigation of ROS generation and mitochondrial

dysfunctions, together with the reduction of Caspase-3 activity. In addition, this work also showed that fractions inhibited inflammation processes promoted by LPS on macrophages, through a decrease of NO production and a reduction of pro-inflammatory interleukins, such as TNF- α and IL-6. Mannitol was identified as a major component of the most promising fraction, suggesting that the evidenced bioactivities can be mediated by this compound. However, the hypothesis that other minor components and their synergistic effects may also be involved in those properties cannot be discarded. Studies are ongoing aiming a more detailed chemical characterization of bioactive samples as well as the isolation and structural characterization of the compounds responsible for the neuroprotective and anti-inflammatory properties.

Abbreviations

ROS: reactive oxygen species; PD: Parkinson's disease; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; ORAC: oxygen radical absorbance capacity; 6-OHDA: 6-hydroxydopamine; IL-6: interleukin - 6; TNF- α : Tumor necrosis factor; NO: nitric oxide; eNOS: endothelial NOS; iNOS: inducible NOS; IL-1 β : interleukin 1 β ; NF- κ B: transcription nuclear factor kappa B; MMP: metalloproteinases; RNS: reactive nitrogen species; JNK: c-Jun N-terminal kinase; ERK: Extracellular signal-regulated kinase; MeOH: methanol; H₂O: water; DCM: dichloromethane; TPC: Total phenolic content; TPTZ: 2,4,6-tri(2-pyridyl)-s-triazine; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; carboxy-H2DCFDA: carboxy-2',7'-dichlorodihydrofluorescein diacetate; ATCC: American Type Culture Collection; NMR: Nuclear Magnetic Resonance; HPLC-DAD: High Performance Liquid Chromatography; GC-MS: Gas Chromatography-Mass Spectrometry; PCA: Principal component analysis; LPS: Lipopolysaccharides.

Declarations

Acknowledgments: Authors are very grateful for the financial support of the Projects and Programmes described in the funding section. FCT is also acknowledged for the grants attributed to JS (SFRH/BD/103255/2014).

Author Contributions: JS did main experiments (antioxidant, cytotoxicity, neuroprotective, signal pathway mechanisms). JS and PS were involved in the evaluation of anti-inflammatory activity. AM and HG were involved in the chemical characterization procedures. JS, AM, CA, SP and RP conceived the research topic and design the study. RP and AA coordinated the study. All authors read and approved the final manuscript and are accountable for the integrity of this manuscript.

Funding: This work was supported by the Portuguese Foundation for Science and Technology (FCT) through the strategic project UID/04292/2020 granted to MARE—Marine and Environmental Sciences Centre and UIDP/04046/2020 and UIDB/04046/2020 granted to BioISI—BioSystems and Integrative Sciences Institute, through POINT4PAC project (Oncologia de Precisão: Terapias e Tecnologias Inovadoras, SAICTPAC/0019/ 2015-LISBOA- 01-0145-FEDER-016405), through CROSS-ATLANTIC project (PTDC/BIA-OUT/29250/2017), co-financed by COMPETE (POCI-01-0145-FEDER-029250) and through

Molecules for Health project (PTDC/ BIA-BQM/28355/2017). This work was also funded by the Integrated Programme of SR&TD Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate (Centro-01-0145-FEDER-000018), co-funded by Centro 2020 Programme, Portugal 2020, European Union, through the European Regional Development Fund.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Declarations Conflict of interest

The authors declare that they have no conflict of interest.

Consent for publication

All authors have read and approved the manuscript before submitting it to *Bioresources and Bioprocessing*.

Ethics approval and consent to participate

Not applicable

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Tables

Due to technical limitations, Table 2 is only available as a download in the Supplemental Files section.

Supplementary

Supplemental Figures S1-S4 are not available with this version

Figures

Figure 1

Schematic representation of the extraction and fractionation process of *Bifurcaria bifurcata*. VLC - vacuum liquid chromatography; SPE - solid phase extraction; CC - column chromatography. MeOH – methanol; EA – Ethyl acetate.

Figure 2

Principal component analysis (PCA) of total phenolic content (TPC) and antioxidant activities (DPPH, ORAC, FRAP) of *Bifurcaria bifurcata* fractions.

Figure 3

Neurotoxicity (100 $\mu\text{g/mL}$) (A) and neuroprotective effects of non-toxic *Bifurcaria bifurcata* fractions (100 $\mu\text{g/mL}$, 24 h) on SH-SY5Y cells exposed to 6-OHDA (100 μM). (-) 6-OHDA (B). Values in each column represent the mean $\pm$ SEM of 3 or 4 independent experiments. Symbols represent significant differences (ANOVA, Dunnett's test, $p < 0.05$) when compared to: * vehicle; # to; 6-OHDA.

Figure 4

Effects on PD hallmarks on SH-SY5Y cells exposed to 6-OHDA (100 μ M) in the presence of *Bifurcaria bifurcata* fractions (100 μ g/mL; 6 h). (A) ROS production; (B) changes in mitochondrial membrane potential; (C) Caspase-3 activity. (-) 6-OHDA. Values in each column represent the mean $\pm$ SEM of 3 or 4 independent experiments. Symbols represent significant differences (ANOVA, Dunnett's test, $p < 0.05$) when compared to: * vehicle; # to: 6-OHDA.

Figure 5

Cytotoxicity and anti-inflammatory activities of *Bifurcaria bifurcata* on RAW 264.7 cells. A) RAW 264.7 cells' viability following 24 h of exposure to *B. bifurcata* fractions (100 µg/mL); B) nitric oxide (NO) production by RAW 264.7 cells when exposed to *B. bifurcata* fractions during 24 h, at sub-toxic concentrations; C) NO production by LPS-stimulated RAW 264.7 cells in the presence of *B. bifurcata* fractions. (-) LPS. Values in each column represent the mean $\pm$ SEM of 3 or 4 independent experiments.

Symbols represent significant differences (ANOVA, Dunnett's test, $p < 0.05$) when compared to: * vehicle; # to: LPS.

Figure 6

Levels of pro-inflammatory cytokines (A) TNF- α and (B) IL-6 on LPS – stimulated RAW 264.7 cells in the presence/ absence of Bifurcaria bifurcata fractions. (-) LPS. Values in each column represent the mean $\pm$ SEM of 3 or 4 independent experiments. Symbols represent significant differences (ANOVA, Dunnett's test, $p < 0.05$) when compared to: * vehicle; # to: LPS.

Figure 7

A) Neuroprotective potential of mannitol (1 – 100 μ M) on SH-SY5Y cells exposed to 6-OHDA (100 μ M). PD hallmarks evaluation on SH-SY5Y cells when exposed to 6-OHDA (100 μ M) and mannitol (50 – 100 μ M; 6 h); B) Levels of ROS production; C) Changes in mitochondrial membrane potential; D) Caspase-3 activity. (-) 6-OHDA. Values in each column represent the mean $\pm$ SEM of 3 or 4 independent experiments.

Symbols represent significant differences (ANOVA, Dunnett's test, $p < 0.05$) when compared to: * vehicle; # to: 6-OHDA.

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