POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Study of the neuroprotective effects of terpenes isolated from the red marine alga Sphaerococcus coronopifolius in an in vitro model of Parkinson´s disease

Juan José Córdoba Granados

[2020]

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Estudo do efeito neuroprotetor de terpenos isolados da alga Sphaerococcus coronopifolius num modelo in vitro da doença de Parkinson.

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Dissertation to obtain the Master's Degree in Marine Resources Biotechnology

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Title: Study of the neuroprotective effects of terpenes isolated from the red marine alga *Sphaerococcus coronopifolius* in an *in vitro* model of Parkinson´s disease

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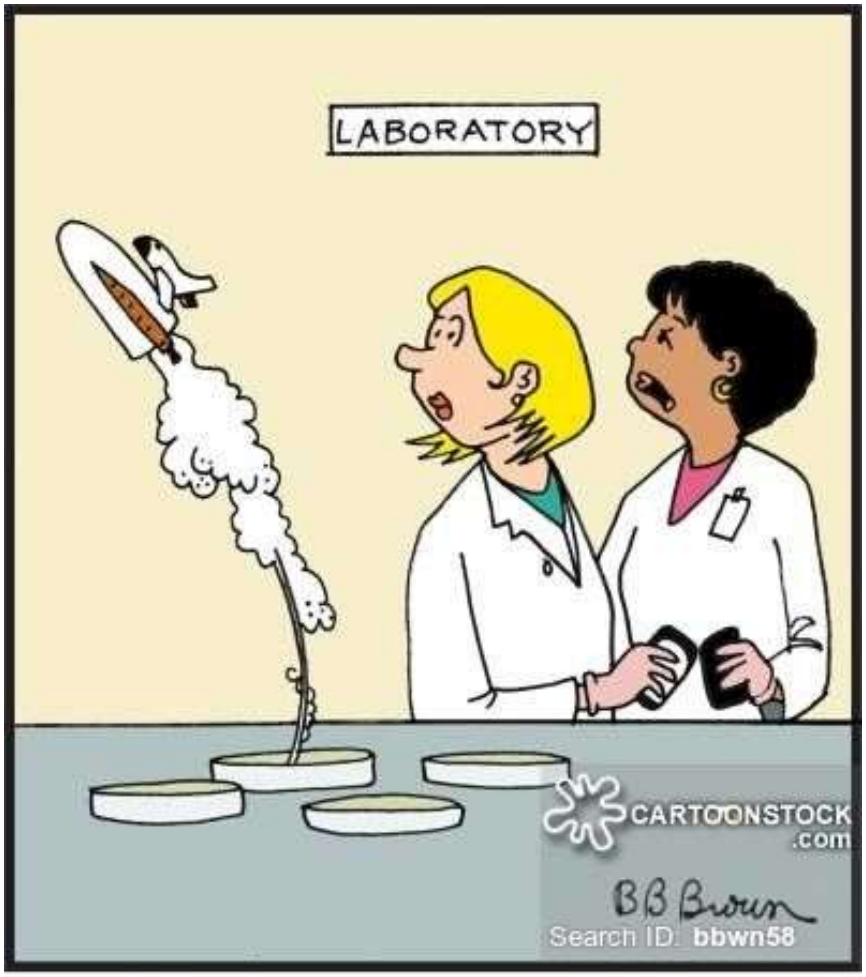

"Now, ***that's*** the sign of a very advanced culture!"

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To my grandfather. The musician.

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Abstract

ABSTRACT

Parkinson´s disease (PD) is a neurodegenerative illness characterized by the progressive loss of dopaminergic cells and the formation of Lewy bodies in the *substantia nigra pars compacta,* leading to motor system dysfunctions such as resting tremor and bradykinesia, affecting autonomy and cognition. Furthermore, following the growing pattern of old-age population, the discovery of new therapeutic agents able to prevent or treat this condition is of utmost importance. In this context, especially when compared with terrestrial environment, marine environment represents a vast area that still remains widely unexplored, constituting a huge source of interesting secondary metabolites with unusual chemical structures with several biological activities, including neuroprotective activities. Therefore, the objective of this dissertation was to isolate compounds from the red seaweed, *Sphaerococcus coronopifolius*, aiming to evaluate their antioxidant and neuroprotective activities on an *in vitro* cellular model of PD.

The isolation and purification of compounds was achieved through preparative chromatographic techniques, namely column chromatography and thin layer chromatography. Structural elucidation of compounds was determined by nuclear magnetic resonance (NMR). Regarding biological activities, antioxidant activity of compounds (1 – 100 µM) was evaluated through the 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) methods. The neuroprotective activity was evaluated on SH-SY5Y human neuroblastoma cellular model, which was exposed to the neurotoxin 6-hydroxy-dopamine (6-OHDA- 100 µM), mimicking PD effects, in absence and/or presence of the compounds (0.3, 1, 3 and 10µM; 24 h). Several biomarkers linked to the loss of dopaminergic neuronal cells, namely production of reactive oxygen species (ROS), alterations of mitochondrial membrane potential (MMP), and Caspase 3 activity were evaluated. The cytotoxicity of compounds was evaluated both on SH-SY5Y neuronal cells and on fibroblasts (3T3 cells).

In our results, four compounds were isolated and when compared to NMR literature data they were identified as the sesquiterpene alloaromadendrene and the brominated diterpenes (**1**)**,** sphaerococcenol A (**2**), 12*S*-hydroxy-bromosphaerol (**3**) and 12*R*-hydroxy-bromosphaerol (**4**). These terpenes revealed weak antioxidant activity compared to ascorbic acid in all methods tested. Through the ORAC assay, 12R-hydroxy-bromosphaerol

revealed the highest capacity to reduce peroxyl radicals compared with the remaining compounds. Regarding the neuroprotective activities, treatment of SH-SY5Y cells with 6-OHDA decreased cell viability in approximately 50%, an effect that was mediated by an increase of ROS production, a depolarization of mitochondrial membrane potential and a stimulation of Caspase-3 activity. On the other hand, when SH-SY5Y cells were exposed to 6-OHDA in the presence of 12*R*-hydroxy-bromosphaerol there was an increase of SH-SY5Y cell viability of 24%, 23% and 18% at 3, 1 and 0.3 μM, respectively. The treatment with 1 μM 12*R*-hydroxy-bromosphaerol promoted a significant decrease of ROS levels (9%) and the treatment with 3, 1, and 0.3 μM concentrations reduced the MMP depolarisation induced by 6-OHDA exposition in 13%, 19% and 35%. Furthermore, the treatment with 0.3 μM 12*R*-Hydroxy-bromosphaerol reduced Caspase-3 activity in 113% to values similar with vehicle situation. Sphaerococcenol A and alloaromadendrene did not revealed capacity to recover the neurotoxicity induced by 6-OHDA.

In conclusion, 12*R*-hydroxy-bromosphaerol exhibited the highest neuroprotective activity that seems to be mediated by the inhibition of ROS production, MMP protection and Caspase-3 activity. Therefore, additional assays should be considered in order to validate the therapeutic potential of this compound in PD.

Keywords: Antioxidant activity; 6-hydroxi-dopamine; SH-SY5Y cells; Apoptosis, Mitochondrial dysfunction; Oxidative stress; Marine Natural Products

Resumo

RESUMO

A doença de Parkinson é uma patologia neurodegenerativa caracterizada pela perda progressiva de células dopaminérgicas e a formação de corpos de Lewy na *substância negra pars compacta*. Manifesta-se em disfunções do sistema nervoso, causando tremores, bradicinesia e consequentemente afetando a autonomia do paciente. Além disso, tendo em conta o padrão de crescimento do envelhecimento da população, a descoberta de novos agentes terapêuticos capazes de prevenir ou tratar esta condição patológica é da maior importância. Neste contexto, especialmente quando comparado com o ambiente terrestre, o ecossistema marinho representa uma vasta área ainda pouco explorada e uma enorme fonte de metabolitos secundários de interesse que têm exibido diferentes atividades biológicas incluindo neuroprotetora. Desta forma, o objetivo desta dissertação consistiu em isolar e purificar compostos a partir da macroalga vermelha *Sphaerococcus coronopifolius*, com a finalidade de avaliar a sua atividade antioxidante e neuroprotetora num modelo celular da doença do Parkinson.

Os compostos foram isolados através da realização de diferentes técnicas cromatográficas, incluindo colunas cromatográficas e cromatografia de capa fina. A elucidação estrutural dos compostos isolados foi determinada através de ressonância magnética nuclear (RMN). A atividade antioxidante (1 – 100 μM) foi determinada por diferentes métodos antioxidantes complementares, nomeadamente através da redução do radical 2,2-difenil-1-picril-hidrazilo (DPPH), da capacidade de absorção dos radicais livres de oxigénio (ORAC) e do poder de redução férrica (FRAP). Por sua vez, o efeito neuroprotetor dos compostos foi avaliado no modelo celular SH-SY5Y derivado de neuroblastoma humano quando exposto à neurotoxina 6-hydroxy-dopamina (6-OHDA – 100 μM; 24 h), que mimetiza a DP, na presença e/ ou ausência dos compostos (0.3, 1, 3 and 10 μM; 24 h). Os efeitos sobre vários biomarcadores associados à DP, nomeadamente, alterações do potencial de membrana mitocondrial (MMP), a produção de espécies reactivas de oxigénio (ROS) e a atividade da Caspase-3 foram avaliados. A atividade citotóxica dos compostos foi avaliada na linha celular 3T3 (fibroblastos), assim como na própria linha dopaminérgica SH-SY5Y.

Nos resultados, quatro compostos foram isolados e comparados com dados da literatura tendo sido possível identificar o sesquiterpeno alloaromadendrene (**1**) e os diterpenos brominados, sphaerococcenol A (**2**), 12*S*-hydroxy-bromosphaerol (**3**) and 12*R*-hydroxy-bromosphaerol (**4**). Os compostos demonstraram fraca atividade antioxidante quando

comparados com o ácido ascórbico. No caso do ensaio de ORAC, o 12*R*-hydroxy-bromosphaerol demonstrou ser o composto com a maior capacidade de reduzir radicais de peroxil quando comparado comos restantes.

Relativamente os resultados do ensaio de neuroprotecção observou-se que exposição das células SH-SY5Y à neurotoxina 6-hidroxi-dopamina (6-OHDA) reduziu a viabilidade celular aproximadamente em 50%, a qual foi mediada por um aumento da produção de ROS, da despolarização da membrana mitocondrial, assim como da atividade da Caspase-3. Contudo, quando as células SH-SY5Y foram expostas à neurotoxina na presença do diterpeno 12*R*-hydroxy-bromosphaerol (**4**), observou-se um aumento significativo da viabilidade celular em 24%, 23% e 18% para as concentrações de 3, 1, e 0.3 μM respetivamente. Também foi possível verificar que o 12*R*-hydroxy-bromosphaerol reduziu significativamente a produção de espécies reativas de oxigénio (1 μM - 9%), a despolarização da membrana mitocondrial (3 μM - 13%, 1 μM - 19%, 0.3 μM - 35%) e a atividade da Caspase-3 (0.3 μM – 113%) induzida pela 6-OHDA. Por sua vez, o sphaerococcenol A e o alloaromadendrene não demostraram capacidade de reduzir os efeitos tóxicos induzidos pela 6-OHDA.

De acordo com os resultados obtidos o12*R*-hydroxy-bromosphaerol foi o composto que revelou maior capacidade neuroprotetora contra a neurotoxicidade induzida pela 6-OHDA. Os efeitos observados foram acompanhados por uma diminuição da produção de ROS, proteção do MMP e da inibição da atividade da Caspase-3. Desta forma, estudos futuros deverão ser desenvolvidos de forma a determinar o real potencial farmacológico deste composto para o tratamento da doença de Parkinson.

Palavras - chave: Atividade antioxidante; 6-hidroxi-dopamina; Células SH-SY5Y; Apoptose; Disfunção mitocondrial; Stress Oxidativo; Produtos naturais marinhos;

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List of Abbreviations

Abbreviations and acronyms

ARE - Antioxidant response element

AD - Alzheimer´s disease

CAT - Catalase

CC - Column chromatography

Complex II - (CII), Succinate: ubiquinone oxidoreductase

Complex III - (CIII), Ubiquinol: cytochrome c oxide-reductase

Complex IV - (CIV), Cytochrome c oxidase

Complex V - (CV) ATP synthase

DAT - Dopamine active transporter

DA - Dopamine

DCF - 2',7' dichlorodihydrofluorescein

DISC - Death-inducing signalling complex

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

DRs - Cell surface death receptors

FADD - Fas-associated death domain

FRAP - Ferric reducing ability power

GPx - Glutathione peroxidase

H_20_2 - Hydrogen peroxide

LRRK2 - Leucine-rich repeat kinase 2

MAOB - Monoamine oxidase-B

MMP - Mitochondrial membrane potential

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

NADH - Ubiquinone oxidoreductase-(Complex I)

NA - Norepinephrine

OH - Hydroxyl radical

0_2^- - Superoxide anion

ORAC - Oxygen radical absorbance capacity.

PD - Parkinson's disease

PETEN - Phosphatase and tensin homolog

Prx - Peroxiredoxin

ROS - Reactive oxygen species

SOD - Superoxidase dismutase

6-OHDA - 6-Hydroxy-dopamine

TPC - Total phenolic content

VMAT2 - Vesicular monoamine transporter 2

Introduction

1. Introduction

1.1. Parkinson's disease

Neurodegenerative diseases include several medical disorders that present a great symptomatology variability, from cognitive deterioration in Alzheimer's disease and Frontal-temporal Lobe Dementia (FTLD), to diminished control of motor functions in Huntington's disease and Parkinson's disease. In most cases, these diseases are related to a progressive process of degeneration of specific neuronal cell populations associated to age, with symptoms developing gradually, usually taking decades to emerge (Linsley *et al*.,2019). As a matter of fact, the raise of these age-dependent disorders in the last decades is directly associated with the elderly population increase, as life expectancy is growing each day (Heemels *et al*.,2016). In this context, the World Health Organization and the Harvard School of Public Health refer to neurodegenerative diseases as one of the greatest challenges to the future of human health, and a problem that will be expected to increase in the coming years (Aarli *et al*., 2006).

Parkinson´s disease (PD) is the second most common neurodegenerative disease, affecting approximately 1% of the society over the age of 60, while in individuals over the age of 85 this prevalence reaches 5% (Table 1). These facts highlight the relevance of advanced age on the risk to develop this condition (Reeve *et al*., 2014; Sherer *et al*.,2012). In Europe, there are estimated 1.2 million people suffering PD (Feigin *et al*., 2017). Due to that, research about PD is becoming of the upmost importance, in fact, in the last decades, several loci and risk variants have been identified and linked to the pathology of familial and sporadic PD in diverse populations worldwide (Singleton *et al*., 2013), including mutations in leucine-rich repeat kinase 2 (LRRK2) (Zimprich *et al*.,2004), or PRKN (parkin, PARK2), PINK1 (PARK6), and DJ-1 (PARK7), that have been reported as a cause autosomal-recessive forms of PD (Bonifati *et al*.,2005).

Table 1.

Prevalence of Parkinson's disease per age and geographical location per 100,000 habitants (Adapted from Pringsheim *et al*., 2014).

	Age range			
Location	**50-59**	**60-69**	**70-79**	**80+**
Europe/North America/ Australia	113	540	1602	2953
Asia	88	376	646	1418
South America	228	637	2180	6095

Symptoms of PD are directly associated with the loss of dopaminergic cells in the *substantia nigra pars compacta* (SNpc), a decline in caudate-putamen dopamine content and progressive accumulation of Lewy bodies, which are cytoplasmic eosinophilic inclusions composed of the presynaptic protein α-synuclein in affected regions of the central nervous system (Johry *et al*., 2012; Duty and Jenner, 2011; Schaphira, 2008). The referred chronic disturbs cause severe motor dysfunction, such as bradykinesia, resting tremor, rigidity and postural instability, affecting autonomic function and cognition (Lesage and Brice, 2009; Poewe, 2008).

1.2. Intracellular signalling pathways underlying Parkinson's disease development

Regarding the molecular processes within PD development, there is evidence suggesting that neurons in the *substantia nigra pars compacta* (SNpc) present differences when compared with the remaining tissues. These neurons exhibit pacemaking activity and increased oxidative stress associated to the metabolism of dopamine and the high levels of iron and calcium (Dias *et al*., 2013, Guzman *et al*., 2009; Jenner *et al*.,2006). In addition, there is also evidence indicating that neurons from SNpc suffer mitochondrial dysfunction due to age. In fact, it has been reported that a great number of mitochondrial DNA deletions related with age occurs in the SNpc when compared with other brain areas, being significantly important in the cytochrome C oxidase translation (Bender *et al*., 2006; Kraytsberg *et al*., 2006).

Furthermore, as previously mentioned, apart from the typical oxidative stress produced in normal mitochondria, it is suggested that SNpc neurons also suffer oxidative stress due to the metabolism of dopamine, since the dopamine metabolization by monoamine oxidase

generates a great level of reactive oxygen species (ROS). Regarding this, SNpc neurons protect themselves using the dopamine transporter (DAT), which takes the dopamine back into the nerve terminal where it can be repacked into synaptic vesicles by the vesicular monoamine transporter 2, (VMAT2) (Reeve *et al*., 2014). However, it has also been reported that DAT expression in the SNpc declines through time, probably resulting in a decrease in the effectiveness of antioxidant mechanisms against oxidative stress (Ma *et al*., 1999).

Following this, there are other endogenous mechanisms involved in the antioxidant protection in neurons that can be divided in two groups, antioxidants enzymes and pathways and low molecular weight antioxidants (LMWA).

The action of the first group is related with the neutralization of reactive oxygen species, like superoxide (O_2^-) which is converted by superoxide dismutase enzyme (SOD) to hydrogen peroxidase (H_2O_2) which in turn is converted into water by peroxidases like glutathione peroxidase (GPx) or peroxiredoxin (Prx), or even transformed to water and oxygen by catalase (CAT) (Martinez *et al*., 2010). Likewise, there is also evidence highlighting the protective role of the Nrf2–ARE transcriptional factor, which encodes detoxification enzymes and antioxidant proteins (Element *et al*.,2004) and activates in neurodegenerative conditions, reducing oxidative stress and neuroinflammation (Buendia *et al*.,2016).

The second group include low weigh molecules like ascorbic acid, vitamin E or uric acid (Ames *et al*.,1993), representing incorporated or metabolized molecules able to donate electrons to reactive oxygen species preventing their interaction and reaction with biomolecules and thus avoiding cellular damages.

1.1.2. Mitochondrial dysfunction in Parkinson's disease

The mitochondrial respiratory electron transport chain is a biological machinery composed by different electron transport complexes embedded in the inner mitochondrial membrane, namely Complex I (CI NADH: ubiquinone, oxidoreductase), Complex II (CII, Succinate: ubiquinone oxidoreductase), Complex III (CIII, ubiquinol: cytochrome c oxide-reductase), Complex IV (CIV, cytochrome c oxidase) and Complex V (CV ATP synthase) (Figure 1). These complexes play a crucial role during mitochondrial respiration, where oxygen is consumed in a process that aims to produce ATP by oxidative phosphorylation.

A mitochondrial dysfunction was identified by the first time in 1989 in SNpc of PD patients (Schapira *et al*., 1990). Posterior evidence pointed out that neuronal cells in SNpc exhibited

around 35% deficiencies in Complex I rates (Johry *et al*., 2012¸Mann *et al*., 1994). Although there are different opinions about the relevance of Complex I deficiency in the pathogenesis of PD, it was also observed that these facts seem to occur exclusively in SNpc, since the mentioned complex I mitochondrial dysfunction was not verified in other brain tissues of patients with PD, including caudate, putamen, globus pallidus, tegmentum, cortex, cerebellum or *substantia innominata* (Schapira *et al*., 1990).

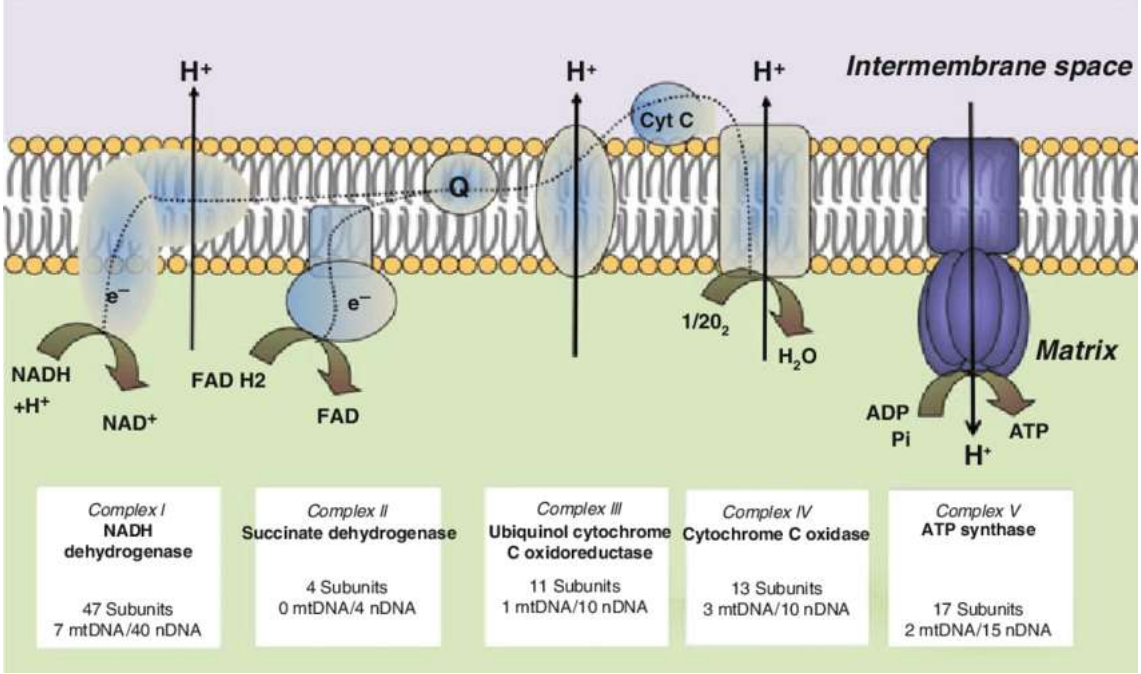


Figure 1. Schematic representation of the mitochondrial respiratory chain (Nadege *et al*., 2009)

Following the reports of complex I deficiency in SNpc in PD, respiratory chain abnormalities were described also from, platelets, lymphocytes and less consistently in muscle tissue from patients with PD (Johry *et al*., 2012; Schapira *et al*., 2008).

On the other hand, mitochondria are dynamic organelles that are transported through the interactions with a variety of cytoskeletal proteins. Furthermore, mitochondrias also fuse and divide (fusion is mediated by OPA1, Mfn1, and Mfn2, and fission is mediated by the proteins fission1 and Drp1). In this context, unbalanced fusion leads to mitochondrial elongation and eventually to excessive mitochondrial fragmentation, both of which impair the function of the mitochondria (Johry *et al*., 2012).

Morevover, several pathways may be involved in the appearance of PD due to impair fragmentation. For instance, phosphatase and tensin homolog (PTEN) enzyme is involved

in the chemical pathway that signals cells to stop dividing and triggers cells to apoptosis, being also important to maintain the cell genetic information (Naredra and Ypule 2011). In addition, there is also evidence that (PETEN)-induce kinase (PINK1) has a direct role in the mitochondrial quality-control pathways, identifying impaired mitochondria with reduced membrane potential and selectively eliminating them from the mitochondrial network by mitophagy (Naredra and Ypule 2011). Furthermore, there is evidence of a direct involvement of PINK1 in abnormal mitochondrial dynamics in fly, rat, and mouse models of PD (Wang *et al*., 2011) and thus, this implicates a failure in mitophagy as an important factor in the pathogenesis of PD (Narendra and Ypule, 2011).

Accordingly, methods able to assess *in vivo* mitochondrial integrity remain as good empiric procedures to evaluate the different effects associated with the increase or decrease of mitochondrial disfunction. For instance, the mitochondrial membrane potential assay (MMP), which allows to detect changes in mitochondrial membrane potential is widely used in apoptosis studies to monitor mitochondrial function status (Lee *et al*.,2015), since mitochondrial membrane permeabilization plays a key role in the mitochondrial apoptotic pathway (Bove *et al*.,2012).

1.1.3. Role of oxidative stress and antioxidants in Parkinson's disease

Oxidative stress has also been proved to play a relevant role in the development of several pathological conditions such as cardiovascular disease, cancer, neurological disorders, and ageing (Dalle-Donne *et al*., 2006). In fact, apart from the already mentioned disfunctions that may be involved in neurodegenerative diseases, and particularly in PD, several studies have also explored the effects of oxidative stress as a factor implicated in the cascade events that lead to degeneration of dopaminergic neuronal cells (Figure 2). The involvement of oxidative stress in PD has been observed in after *post-mortem* studies where the brains of PD patients presented higher levels of ROS in the SNpc (Sarrafchi *et al*., 2016; Valko *et al*., 2007). Furthermore, the expression and activity of monoamine oxidase-B (MAO-B) is also significantly enhanced in this organ, where it is involved in the oxidation of dopamine in the mitochondrial membrane. The pathological elevated activity of (MAO-B) leads to dopamine (DA) oxidation generating deleterious H_20_2 and thus contributing to intensify the oxidative stress condition (Hermida-Ameijeiras *et al*.,2004).

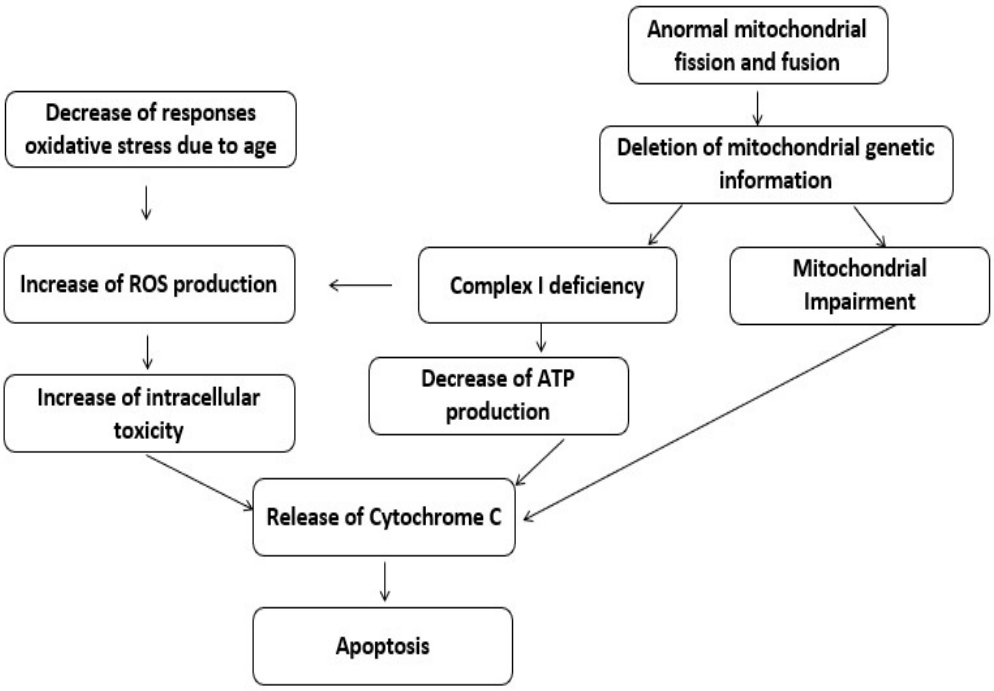


Figure 2. Intracellular events linked to mitochondria in Parkinson's disease**.**

Within this framework, oxidative stress is usually induced by free radicals which include several ROS species produced in the cells. The term ROS encompasses oxygen free radicals, such as superoxide anion radical ($O_2^{\cdot -}$) and hydroxyl radical (OH^-), and nonradical oxidants, such as hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) which can be converted from one to another by enzymatic and nonenzymatic mechanisms inside the cell (Zorov *et al*.,2014). Regarding their harmful effects, free radicals are reactive molecules having an unpaired electron orbital, like hydroxyl radical ($0H^-$) or the carbene molecule (CH_2). This electronic configuration is highly energetic (Pavlin *et al*.,2016), making them unstable and likely to react with nearby molecules such as carbohydrates, lipids or nucleic acids (Ozcan *et al*., 2015; Valko *et al*., 2007). For instance, molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical (O_2^-), which is highly reactive (Miller *et al.,* 1990).

Accordingly, several preventing approaches have been proposed as neuroprotection against those oxidative stress events, based on the endogenous antioxidant defensive mechanisms developed by the biological systems to protect themselves against oxidative stress-induced cell damage, like catalase or superoxide dismutase. These mechanisms

help to maintain the redox homeostasis and thus, a regulation of this mechanisms mediated by exogenous compounds presented in our diet can contribute to prevent the development of chronic diseases (Di Dominico *et al*., 2015). As matter of fact, strategies against neurodegeneration based in antioxidants aim to counteract the damaging effects of ROS production, preventing malfunctions in the neuronal intracellular machinery or reversing and re-regulating already damaged systems like the mitochondrial transport chain of electrons. Those mechanisms include free radical scavenging of reactive species, sequestration of transition metals, stimulation of enzymatic antioxidant defences and/ or inhibition of enzymes involved in the overproduction of ROS (Sheeja Malar *et al*., 2015; Kim *et al*., 2010).

As an example, polyphenols are among the most widely employed natural compounds to protect cellular components from the oxidative damage caused by ROS and transition metals (Losada-Barreiro *et al.,* 2017; Roleira *et al*., 2015; Brewer *et al*., 2015; Vladimir-Knežević *et al*., 2012) due to their well-recognized antioxidant properties and their beneficial effects in controlling aging and related neurodegenerative diseases (Di Dominico *et al*., 2015; Roleira *et al*., 2015). Those compounds have been identified from several sources, revealing great antioxidant activities as well as neuroprotective activities on several PD models (Cha *et al*., 2016; Dutta *et al.,* 2015; Camilleri *et al*., 2013; Vijayabaskar *et al*., 2012).In this context it should be mentioned that a great variety of polyphenols, including catechins, flavonols, and phlorotannins, can be found in marine macroalgae (Murrary et al.,2018; Murugan et al. 2015) and have been reported for their antioxidant and neuroprotective activities (Cha *et al*., 2016; Vijayabaskar *et al*., 2012).

Concerning terpenoids, tanshinone, a diterpenoid isolated from *Salvia* species has been reported in literature for its antioxidant capability through the upregulation of Nrf2/ARE signalling pathway and its anti-inflammatory capability in neuroprotective models, the second one associated to the regulation of anti-inflammatory cytokines (González-Cofrade *et al*.,2019; Park *et al*.,2014). Likewise, carnosic acid and carnosol, abietane diterpenoids isolated from *Rosmarinus officinalis* (rosemary) have also been reported for its antioxidant activity associated with the modulation of the Nrf2/ARE pathway, the major regulator of the cellular redox homeostasis in mammalian animals (González-Cofrade *et al*.,2019; de Oliveira *et al*.,2016 Satoh *et al*.,2013). In fact, carnosic acid and carnosol are not electrophilic molecules, but in response to oxidation they can become electrophilic and then activate the Nrf2/ARE pathway to synthesize endogenous antioxidant phase 2 enzymes (Satoh *et al.,*2013). Altogether, these results highlight the relevance of terpenes as potential therapeutic molecules able to mitigate the harmful effects of ROS production in pathologies like Parkinson's disease.

1.1.4. Apoptosis in Parkinson's disease

Apoptosis or programmed cell death is an evolutionary conserved process of elimination of dysfunctional or damaged cells that may represent a problem to the homeostasis of multicellular organisms (Nagley et al.,2010). Within this framework, regarding PD, enhanced apoptotic cell death in neurons from SNpc has been demonstrated in *post mortem* tissues of PD patients (Tatton *et al*.,2003; Kosel *et al*.,1997), highlighting the relevance of apoptotic pathways in this disease. Following this, apoptosis is a complex mechanism that can be separated in two different processes, intrinsic apoptosis and extrinsic apoptosis.

Intrinsic apoptosis signalling pathway is centred in mitochondria and characterized by the mitochondrial outer membrane permeabilization, triggering the activation of the Bcl-2 family members, Bax and Bak, resulting in the release of pro-apoptotic proteins, including cytochrome C, into the cytosol (Wei *et al*., 2001). This process leads to the formation of the apoptosome complex when cytochrome c binds Apaf-1 activating factor, resulting in the activation of pro-Caspase-9 (Figure 3). Caspase-9 can directly cleave and active pro-Caspase-3 and pro-Caspase-7 (Pandey *et al*., 2000). Those last effector caspases are responsible for initiating the biological processes of the degradation phase of apoptosis, including DNA fragmentation, cell shrinkage and membrane blebbing (Brentnall *et al*., 2013).

On the other hand, the extrinsic apoptosis signalling pathway is activated by pro-apoptotic ligands that interact with specialized cell surface death receptors (DRs) (Ashkenaz *et al*., 2002). After activation, each receptor can independently form the death-inducing signalling complex (DISC) by recruiting the adapter Fas-associated death domain (FADD) and pro-caspases 8 and 10 (Kischkel *et al.,* 2001; Kischkel *et al*., 2000), which in turn, undergo self-processing releasing active caspase molecules into the cytoplasm. These enzymes then cleave the pro-caspases forms and thereby active the effectors Caspase-3, -6, and -7, which ultimately execute apoptosis (LeBlanc *et al*., 2002).

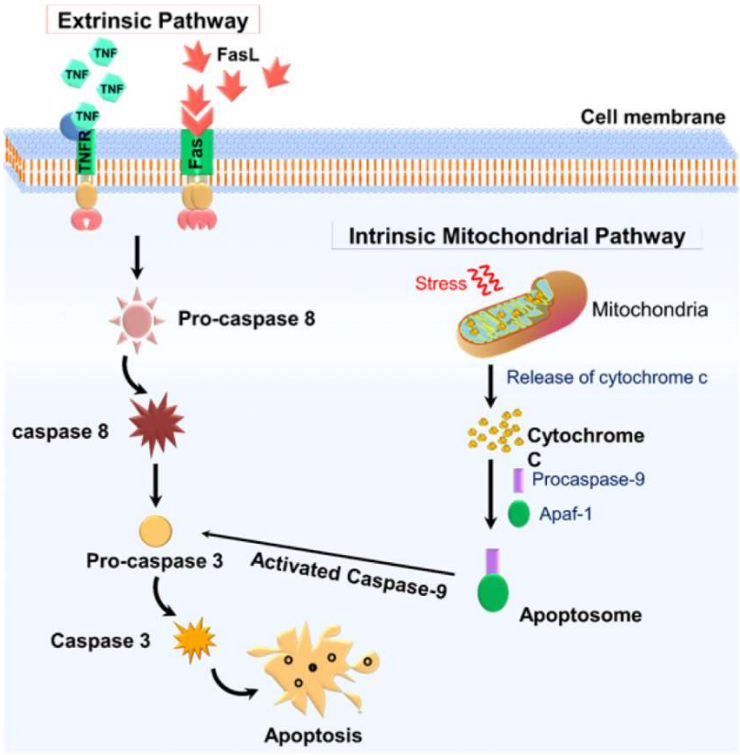


Figure 3. Intrinsic and extrinsic apoptotic signalling pathways (Bhosale *et al*.,2020)

The extrinsic and intrinsic signalling pathways converge at the level of the effector caspases, and cross talk can occur between the two pathways (Sayers *et al*., 2011; Lavrik *et al*., 2005). In fact, the activity of initiator (Casp-9, Casp-8) and the effector caspase (Casp-3) has been reported to increase in SNpc of dopaminergic neurons of PD patients (Hartmann *et al*.,2001; Tatton 2000). Therefore, the assessment of the different apoptotic pathways that may be related with dopaminergic cell loses and thus PD is currently being studied. Furthermore, the search for compounds able to inhibit or diminish apoptosis represent as well, an increasing field of research. For instance, rasagiline, an inhibitor drug of MAO-B currently employed in PD therapeutics, has been reported to mediate a protective effect against apoptotic cell death, stabilizing the mitochondrial membrane potential (Am *et al*., 2004; Jenner *et al*., 2004).

Concerning terpenes, triptolide, a diterpene isolated from Chinese herb *Tripterygium wilfordii,* exhibited ability to inhibit apoptosis in models of neuroprotection against middle cerebral artery occlusion in mouse models (González-Cofrade *et al.,*2019). Likewise, Asiatic acid, a pentacyclic triterpenoid isolated from *Centella asiatica* have also displayed antiapoptotic effects in the rotenone-induced apoptosis cellular model, using SH-SYS5Y cells (Nataraj *et al*.,2017; Xiong *et al*.,2009).

In the same way, the marine derived compound,11-dehydro-sinulariolide, isolated from soft corals, also decrease apoptosis, inhibiting Caspase-3 activation on *in vitro* studies conducted with a human neuroblastoma cells (SH-SY5Y) incubated under the adverse effect of the neurotoxin 6-OHDA (Chen *et al*., 2012).

1.2. Relevance of the neurotoxin 6-OHDA on *in vitro* cellular models of Parkinson's disease

The 6-hydroxydopamine (6-OHDA) neurotoxin has been extensively used in the experimental assays conducted in animal models of Parkinson's disease due of its *in vivo* selectivity over dopaminergic neuronal cells. Furthermore, although 6-OHDA is commonly employed as an exogenous neurotoxin in PD models, previous studies carried out in PD patients have shown an increased amount of 6-OHDA in their urine (Andrew *et al*., 1993). Likewise, it has also been reported that SNpc of PD patients showed an increase in the amount of iron Fe(III) and Fe (II) (Hirsch *et al*.,1991) and thus when free ferric iron is present in addition to H_2O_2 the main product of dopamine oxidation is 6-0HDA (Jameson and Linert, 2000). Following this, there is evidence suggesting that 6-OHDA can be formed *in vivo* from dopamine in PD patients.

As for the assimilation of exogenous 6-OHDA, this toxin is actively incorporated into the cells by the dopamine transporter (Figure 4). Once inside the neurons, 6-OHDA accumulates and undergoes non-enzymatic auto-oxidation, promoting ROS formation such as H_2O_2 and hydroxyl radicals in the presence of iron (Bladini *et al*., 2008). Likewise, it seems to be toxic to the mitochondrial complex I, leading to the formation of superoxide and hydroxyl radicals (Rodriguez-Pallares *et al*., 2007), once again suggesting the relevance of a dysfunction in the mitochondrial complex I as an important factor in PD development. The inhibitory effect over Complex I activity in mitochondria also accounts for the described mechanism of ROS generation mediated by this neurotoxin (Lehmensiek *et al*., 2006).

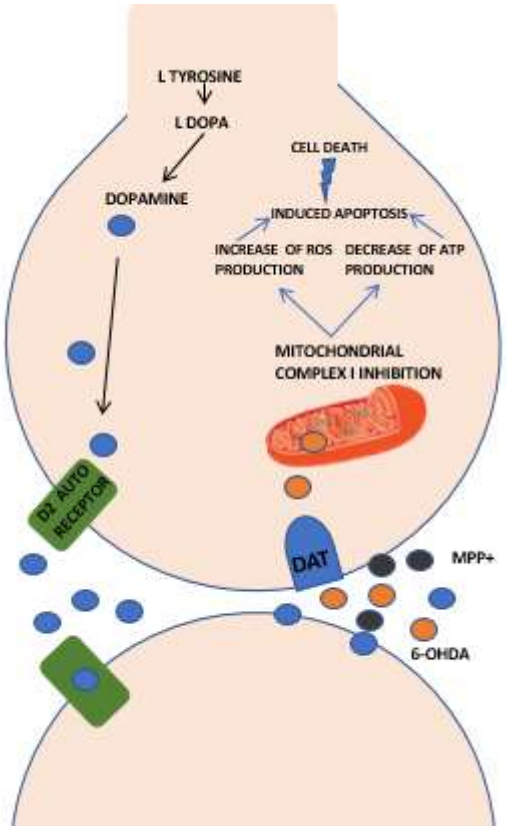


Figure 4. Schematic representation of the intracellular signalling mechanism associated to the neurotoxicity induced by 6-hydroxydopamine and its repercussion inside of SNpc neuron cells that mimics Parkinson's disease effects (DAT - dopamine active transporter).

Different *in vitro* cellular models have been employed to mimic the events associated to Parkinson's disease, like SH-SY5Y cell line (ATCC® CRL-2266™), which is a neuroblastoma cell line derived from a metastatic bone tumor biopsy and are a sub-line of the parental line *SK-N-SH* (ATCC® HTB-11™) which was sub-cloned three times; first to *SH-SY*, then to *SH-SY5* and, finally, to *SH-SY5Y,* in 1970, by June L.Biedler (Kovalevich *et al*., 2013). Since 1980's this cell line has been widely used as a cellular model of neurons for scientific research, mainly because it possesses many biochemical and functional properties.

Due to their characteristics, SH-SY5Y cells have also been used as a dopaminergic neuronal cellular model for Parkinson's disease. As a matter of fact, several characteristics of the SH-SY5Y cell line make it useful for this purpose: firstly, they have capacity to synthesize dopamine (DA) and norepinephrine (NA), since these cells express tyrosine and dopamine hydroxylases. Tyrosine hydroxylase is the rate-limiting enzyme of the catecholamine synthesis pathway and converts tyrosine to L-dopa, the precursor of DA, which is converted to NA by dopamine-B-hydroxylase (Nagatsu *et al*., 2019). They also

express the dopamine transporter (DAT), a protein expressed only in dopaminergic neurons. Likewise, DAT is a pre-requisite for the incorporation into neurons of 1-methyl-4-phenylpyridinium (MPP^+), a toxin that is also used in neurotoxicity models in SH-SY5Y cells to study this mechanism of action (Xie *et al*., 2010).

For instance, gallic acid pre-treatment in SH-SY5Y cells before exposition to 6-OHDA demonstrated neuroprotective activity, resulting in a decrease of the Bax/Blc-2 ratio (Chandrasekhar *et al*., 2018), triggering also an upregulation of antioxidant enzymes like CAT, GPx or SOD. On the other hand, hinokitiol, a tropolone-related compound, have also been tested against 6-OHDA induced neurotoxicity in SH-SY5Y cells and results proved that the pre-treatment with hinokitinol downregulated PTEN and induced kinase PINK1, suggesting an effect in increasing mitochondrial stability (Varier *et al*., 2019).

Furthermore, in recent studies, employing SH-SY5Y cells, several terpenoids extracted from the resins of *Populus euphratica,* an endogenous tree from the Gobi desert, have been reported to display neuroprotective effects against 6-OHDA-induced damage in a SH-SY5Y cellular model (Liu *et al.,*2020). Similar results have been also reported from the terpenois isolated from *Zea mays*, most referred as maize, which protected cells from an induced-oxidative stress condition through the upregulation of the Nrf2 pathway (Song *et al*.,2020).

Regarding marine-derived compounds, 11-Dehydrosinulariolide which as previously mentioned was isolated from cultured soft corals, significantly reduced 6-OHDA induced cytotoxicity and apoptosis on SH-SY5Y cells (Chen *et al*.,2012). In the same way, two flavalins, which are nardosinane-type sesquiterpenoids, isolated from the soft coral *Lemnania flava* promoted a significant reduction 6-hydroxydopamine (6-OHDA) induced cytotoxicity at a concentration of 20 μM in SH-SY5Y cells after preincubation (Lu *et al*.,2011).

1.3. Marine derived secondary metabolites as neuroprotective and antioxidant agents

In the marine environment, especially in sessile organisms like algae, corals or sponges, there is a strong competition for space, a factor that increases competitiveness and thus stimulates species to develop mechanisms to overcome its surroundings or survive predation. Due to that, these species have developed mechanisms to synthesize unique chemical structures through the secondary metabolism, which plays a crucial role in ecological processes. Recent studies have also reported how these chemical compounds

may represent a decisive chemical weapon for the population growth or even for the replacement of native species by invasion into new ecosystems (Svensson *et al*., 2013). These compounds are commonly referred as “secondary metabolites” and have shown great biological activities for pharmacological development (Simmons *et al*., 2005). These secondary metabolites can be unique to a taxonomic family, genus, species or even organism and are characterized by their wide heterogeneity and many of them have been reported for their therapeutic potential for human health as antioxidant (Pinteus *et al*., 2017), anti-inflammatory, (De Souza *et al*., 2009), neuroprotective (Pangestuti and Kim, 2011) and antitumor agents (Alves *et al*., 2016; Rodrigues *et al*., 2015).

As a brief resume, several marine natural compounds have been tested to evaluate their neuroprotective and antioxidant capabilities. For instance, astaxanthin, a keto-carotenoid extracted from the microalgae *Haematococcus pluvialis* and *Chlorella zofingiensis,* has been proved to have anti-apoptotic properties (Grimmig *et al*.,2017; Yuan *et al*., 2011; Ikeda *et al*., 2008). Fucoidan, a sulphated polysaccharide extracted from the macroalgae *Turbinaria decurrens,* showed promising effects too, resulting in an increment of antioxidant enzymes and dopamine levels (Meenakshi *et al*., 2016; Ananthi *et al*., 2010). Also, several compounds isolated from marine fungi have shown neuroprotection, for example xyloketal B, a compound isolated from *Xylaria sp*. (mangrove fungus) exhibited neuroprotection against 1-methyl-4-phenylpyridinium (MPP^+) induced neurotoxicity (Chen *et al*., 2009; Lin *et al*., 2001). In addition, 3-methylorsellinic acid and 8-methoxy-3,5-dimethylisochroman-6-ol, both isolated from *Penicillum* sp., have shown protection against 6-OHDA induced neuronal death (Yurchenko *et al*., 2018; Yurchenko *et al*., 2016).

Regarding macroalga, which is our case of study, in previous reports it has been demonstrated that fractions isolated from the macroalgae *Padina pavonica*, *Saccorhiza polyschides*, *Codium tomentosum*, and *Ulva compressa* are able to diminish the neurotoxic effects of 6-OHDA in SH-SY5Y cells (Silva *et al.,* 2018). Likewise, crude extracts from the brown seaweed *Sargassum hemiphyllum* have been reported for its capability to protect SH-SY5Y cells from 6-OHDA-induced apoptosis (Huang *et al*.,2018). Altogether, considering the above-mentioned results, the search for new neuroprotective and antioxidant compounds derived from marine algae sources currently represents an increasing area of study.

1.3.1. Biological activities of *Sphaerococcus coronopifolius* compounds

The red alga *S. coronopifolius* is a widely distributed cosmopolitan macroalgae that belongs to the family Sphaerococcaceae, phylum Rhodophyta. This alga usually grows on rocky bottoms being found in depths between 20 to 70 metres, although sometimes it can be found in shallow waters too. This specie is distributed in the East Atlantic, the Mediterranean and the Black Sea (Guiry and Guiry, 2010). *Sphaerococcus coronopifolius* has demonstrated to be a great source of interesting terpenes including diterpenes and sesquiterpenes, most of which contain one or more bromine atoms (Rodrigues *et al*., 2015; Etahiri *et al*.,2001), thus representing an unusually prolific source of these kind of metabolites with more than 40 compounds isolated and described from this specie (Smyrniotopoulos *et al*., 2020; Smyrniotopoulos *et al*., 2015; Rodrigues *et al*., 2015; Smyrniotopoulos *et al*., 2010; Smyrniotopoulus *et al*., 2008). Some of these metabolites have proved to possess interesting bioactive properties. In fact, in recent studies it has been demonstrated that some of them possess significant cytotoxic activity and anti-tumor potential (Alves *et al*., 2016; Rodrigues *et al*., 2015; Smyrniotopoulos *et al*., 2008). Likewise, anti-fouling (Piazza *et al*.,2011) and antimicrobial activity have been reported as well (Ethari *et al*.,2001; Smyrniotopoulus *et al*., 2008).

Regarding diterpenes, they are a group of terpenoids found as secondary metabolites in terrestrial and marine organisms (Córdoba-Guerrero *et al*., 2013). They are compounds formed by four isoprene units, possessing a core skeleton of twenty carbons (Hao *et al*., 2015). In marine brown algae, diterpenes are relatively common secondary metabolites; nonetheless, in red algae this balance is less profuse. In Rodophyta, they have been found mainly in species of the genus *Laurentia*, and in the unrelated species *S. coronopifolius* (Erickson *et al*., 1983; Fenical *et al*., 1973). On the other hand, sesquiterpenes are formed by the assembly of three isoprene units, resulting in a core skeleton of 15 carbons. They have been reported to display anti-inflammatory (Kurniasih *et al*.,2018) and antitumoral properties (Alirei *et al.,*2013). The presence of sesquiterpenes in red alga has also been reported for *Laurencia dendroidea* (Da Silva *et al*.,2011*)*, *Laurentia obtuse* (Alirei *et al.,*2012) and *S. coronopifolius* (Smyrnitiopoulos *et al.,*2010).

Considering this, *S. coronopifolius* has been proved as an interesting source of new natural compounds with pharmacology potential, however their antioxidant and neuroprotective potential remains widely unexplored.

Objectives

2. Research objectives

Parkinson's disease is the second most common neurodegenerative disease being characterised by the progressive loss of dopaminergic cells in the *substantia nigra pars compacta* (SNpc), and the formation of Lewy bodies, manifesting in symptoms like resting tremor, bradykinesia and lack of autonomy. Due to the increase of the economic and social impact of this disease and the absence of effective therapeutics, the discovery and development of new therapeutic agents is a matter of utmost importance. Regarding this, the marine environment remains a vastly unexplored area of study and a potential source for new natural compounds with therapeutic ends. For instance, macroalgae currently represents an emerging area of study due to their ability to produce a vast number of bioactive compounds. Therefore, the main goal of this dissertation was to study the antioxidant and neuroprotective activity of compounds isolated from the red seaweed *Sphaerococcus coronopifoliu*s on an *in vitro* human cellular model of Parkinson´s Disease induced by the neurotoxin 6-hydroxydopamine.

2.1. Specific objectives

In order to achieve the general goal, specific objectives were outlined:

1. Extraction, purification, and structural elucidation of compounds from *Sphaerococcus coronopifolius* extracts by chromatographic techniques and NMR spectroscopy techniques.
2. Evaluation of the antioxidant activity of the compounds by different methods (DPPH, FRAP, ORAC).
3. Evaluation of cytotoxicity and neuroprotective activities of the compounds in SH-SY5Y and 3T3 cell lines.
4. Study of the neuroprotective effects of compounds on Parkinson's Disease biomarkers, namely ROS production, MMP and Caspase-3 activity.

Materials and Methods

3. Materials and Methods

3.1. Reagents and solvents

For the fractionation and purification processes, HPLC quality grade solvents (VWR, Fontenay-sous-Bois, France) were used. Column chromatography (CC) was performed using silica Kieselgel (60-200 μm) (VWR, ref.84893.290, Leuven, Belgium) eluting with mixtures of *n*-hexane, ethyl acetate, and methanol. Thin layer chromatography (TLC) was accomplished using Kieselgel 60 F254 aluminium sheets (Merck, Ref. 5554, Darmstadt, Germany).

Concerning the antioxidant assays, the following reagents were used: 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl reagent (Sigma, Steinheim, Germany) was employed for the DPPH radical scavenging assay; Fluorescein (Sigma-Aldrichh, St Louis, MO, USA), α,α′-azodiisobutyramidine dihydrochloride (APPH) (Sigma-Aldrich, St Louis MO, USA), trolox, (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid) and phosphate buffer (Sigma, St Louis, MO, USA) were used in the Oxygen Radical Absorbance Capacity (ORAC) assay;. Iron (II) sulphate (FeSO4) (Sigma-Aldricht,St Louis, MO), 2,3,5-Triphenyltetrazolium chloride (TPTZ), Iron (III) chloride ($FeCl_3$) (Sigma-Aldrich, St Louis, MO, USA) and acetate buffer were employed as main reagents (Sigma, Karkanata, India) in Ferric Reducing Antioxidant Power (FRAP) assay.

Regarding cell culture experiments, fetal bovine serum (FBS), Dulbecco´s modified Eagle medium (DMEM/F12) (Biowest, Riverside, MO, USA), antimicotic solution (Sigma, Rehovot, Israel), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Fisher Bioregents, Geel, Belgium) and sodium carbonate (Na_2CO_3) (Panreac, Barcelona, Spain) were used in cell culture medium. Trypsin 1x (Biowest, Riverside, MO, USA) was employed for cell detachment. Finally, for the cytotoxicity assessment, saponin (Sigma, 47036-50G-F, St Louis,MO, USA) was employed as cellular dead control, while 6-hydroxydopamine (6-OHDA), (Sigma,St Louis,MO,USA) was used in the neuroprotection assays.

3.2. Fractionation, purification and structural elucidation of compounds from *Sphaerococcus coronopifolius* extracts

3.2.1. Isolation and purification of compounds

The red algae *S. coronopifolius* was previously collected between May and June from Peniche coast, Portugal and immediately transported to MARE-Polytechnic of Leiria facilities. Samples were then cleaned and washed to remove epiphytes, encrusting materials and detritus. Posteriorly they were freeze-dried (Scanvac Cool Safe, LaboGene, Lynge Denmark) and stored in dry powder at -80 ºC. Samples to be purified derived from a previously fractionation step of *S. coronopifolius* extracts by vacuum liquid chromatography (VLC) followed by semi-preparative high performance liquid chromatography (HPLC). Preparative column chromatography (CC) was performed aiming to achieve the purification of the major compounds present in the 3 selected fractions (F CC-F8-34, SC-P1 and SC-P2). CC was performed in glass tubes with different diameters and lengths, accordingly with the samples weights that were to be purified. The solid phase used as silica-gel (60-200 µm) and elution performed with mixtures of *n*-Hexane and ethyl acetate by gradient polarity, followed by methanol. Briefly, fraction F CC-F8-34 (87.4 mg) was eluted with mixtures of n-Hexane and ethyl acetate of increasing polarity, with sub-fraction 3/4 (*n*-hexane, 100%) affording compound 1 (34.1 mg), (Table 6, Annex 1). A similar process was performed with fraction SC-P1 (96.0 mg), from which sub-fraction 5/6 (*n*-hexane/ethyl acetate, 90:10, v/v) afforded compound 2 (28.4 mg), and sub-fraction 13/15 (*n*-hexane/ethyl acetate, 75:25, v/v) afforded compound 3 (3.9 mg), (Table 7, Annex I). On the other hand, for the purification of SC-P2, a previous HPLC which rendered seven fractions was performed. After that chromatography column was performed with SC-P2-F5, from which subfractions 5-9 (*n*-hexane/ethyl acetate, 80:20, v/v) afforded compound 4 (1.9 mg). The isolation process is depicted in Figure 5.

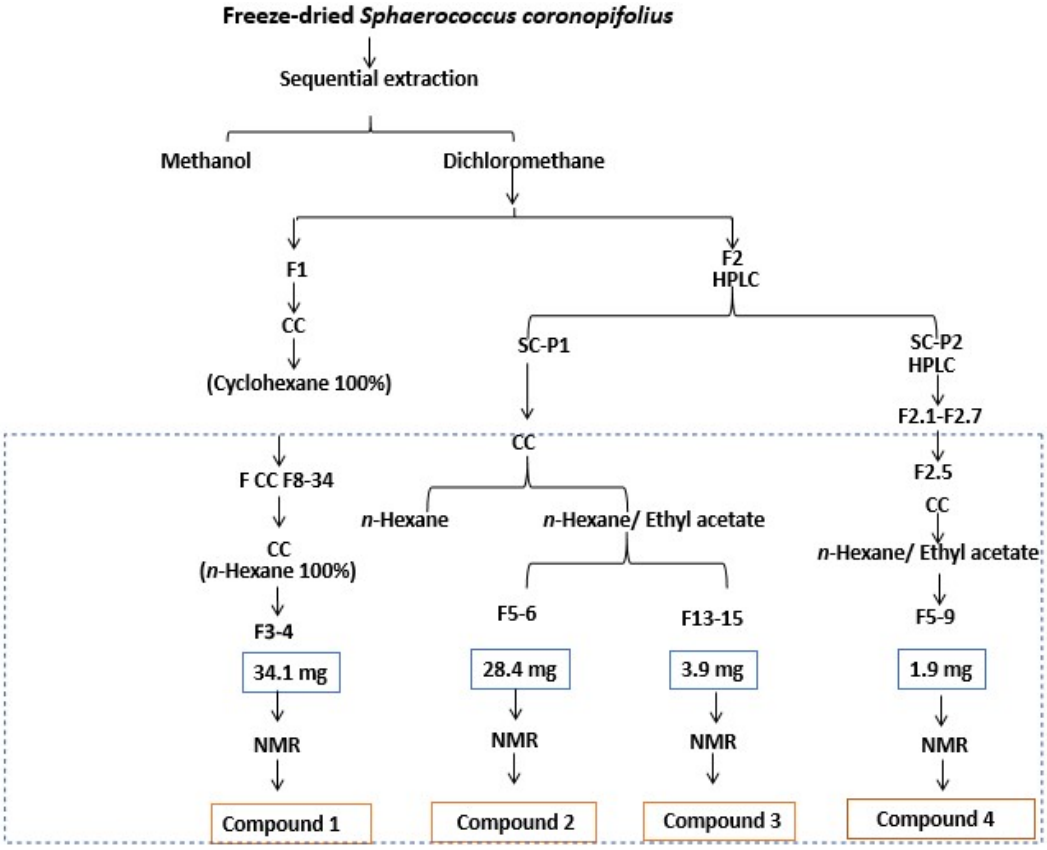


Figure 5. Schematic representation of *Sphaerococcus coronopifolius* compound´s isolation process**.** Processes inside the box represent the work developed in this dissertation. Schematic representation outside the box represents previous isolation work developed.

3.2.2. Structural elucidation of compounds by NMR

The isolated compounds were identified by nuclear magnetic resonance (NMR). NMR spectra were acquired on a Bruker Advance 400 spectrometer with a frequency of 400 MHz for ^{1}H, and 100 MHz for ^{13}C. Samples were dissolved in 500 µL of $CDCl_3$ (Sigma-Aldrich, St. Louis, MO, USA). Chemical shifts were expressed in ppm and reported to the residual solvent signals. Coupling constants (*J*) are expressed in Hertz (Hz).

3.3. Antioxidant activity of compounds isolated from *Sphaerococcus coronopifolius*

3.3.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH Radical Scavenging assay was performed accordingly to Brand-Williams *et al*. (1995) with slight modifications (Pinteus *et al*.,2017). This method is based in the capability of antioxidant compounds to react with free radicals and reduce them. In this case, DPPH was employed as a free radical, stable at room temperature, that may be reduced in the presence of antioxidant molecules, resulting in a change of colour whose absorbance can be measured. DPPH solution was prepared in ethanol in a concentration of 0.1 mM. Following this, 2 µL of each compound (5-150 µM) was added to 198 µL of DPPH solution in a 96-well plate. Mixtures were incubated at room temperature in the dark for 30 min. Absorbance was measured at 517 nm (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA). For blank samples, ethanol (198 µL) was used instead of DPPH solution, while control samples were prepared with 2 µL DMSO plus 198 µL of DPPH solution. The results of the antioxidant capability to scavenge DPPH were estimated in % of reduced DPPH from the control, following the equation:

$$\textit{DPPH radical scavenging capability (\% control)} = \left[\left(\frac{(Ab.sample - Ab.sampleblank)}{Ab.control - Ab.controlblank}\right) * 100\right] \quad \textbf{(eq.1)}$$

3.3.2. Oxygen Radical Absorbance Capacity (ORAC)

ORAC assay was performed following the methodology described by Davalos *et al*. (2004). This assay is based on the fluorescent probe damage, in this case fluorescein, caused by an oxidizing reagent, resulting in a loss of fluorescent intensity over time (Ou *et al*., 2001). A calibration curve was prepared, using trolox (0-80 µM) as antioxidant standard in 75 mM phosphate buffer (pH 7.4). Compounds were diluted in phosphate buffer 0.75 M (pH 7.4). Ascorbic acid was used as a positive control. Briefly, 20 µL of the different calibration curve solutions and the samples were added in a 96-well plate, following this, fluorescein (120 µL, 75 mM) was added. The mixture was then pre-incubated for 15 min at 37 °C. Phosphate buffer was employed for the blanks instead of fluorescein. Ended this

time, APPH solution (60 µL, 20 µM final concentration) was added to both samples and blanks. Fluorescence was measured at $\lambda_{excitation}$: 458 nm and $\lambda_{emission}$: 520 nm wavelengths and recorded every minute for 240 min. Samples were automatically shaken before each read. Oxygen Radical Absorbance Capacity was calculated from the Area Under the Curve (AUC) of the samples and extrapolated from AUC of a trolox calibration curve by the means of the following equations:

$$AUC=\left(\frac{R_1}{R_1}\right)+\left(\frac{R_2}{R_1}\right)+\left(\frac{R_3}{R_1}\right)+\cdots+\left(\frac{R_n}{R_1}\right) \quad \textbf{(eq.2)}$$

Where R_1 is the fluorescence at the starting point of the reaction and R_n is the final measurement. AUC were calculated by GraphPad Prism 5 software using equations 3 and 4, respectively.

$$NET\ AUC = AUCsample - AUCBlank \quad \textbf{(eq.3)}$$

$$Oxygen\ Radical\ Absorbance\ Capacity = \left[\left(\frac{NETAUC.compounds-}{m}\right)\right] \quad \textbf{(eq.4)}$$

Results are presented as µmol of Trolox equivalents per mg of compound (µmol TE/ /mg compound).

3.3.3. Ferric Reducing Antioxidant Power (FRAP)

This method was performed as previously described by Benzie and Strain (1999) with minor modifications (Pinteus *et al.*,2017). The assay is based in the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by the presence of antioxidants molecules. A calibration curve was prepared with $FeSO_4$ 50 µM (0-10 µM). FRAP reagent was prepared in 0.3 M acetate buffer (pH=3.6), using 10 mM of TPTZ prepared in 40 mM HCl and 20 mM ferric solution ($FeCl_3$). The final working FRAP reagent was incubated at 37 °C. Ascorbic acid and acetate buffer were used as positive control and blank, respectively. Samples were diluted in acetate buffer 300 mM (pH 3.6), and 2 µL of that was added to 198 µL of FRAP reagent, remaining in the dark at 37 °C for 30 min. Concluded this time, the absorbance increase was measured in the microplate reader at 593 nm (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA). The FRAP reducing antioxidant power was determined following the equation:

$$FRAP\ reducing\ antioxidant\ power=\left[\left(\frac{(Ab.compound-A\ .blank)-b}{m}\right)\right] \quad \textbf{(eq.5)}$$

Results were presented as µM equivalents of $FeSO_4$ per mg of compound (µM $FeS0_4$ EQ/mg compound)

3.4. Cytotoxic and neuroprotective activities of *Sphaerococcus coronopifolius* compounds

3.4.1. Cell culture maintenance

SH-SY5Y cells (ACC 209) were acquired from DMSZ bank-German collection of microorganisms and cell cultures and cultivated in Dulbecco´s Modified Eagle's Medium (DMEM). Cell medium was supplemented with 10% (v/v) of fetal bovine serum (FBS) (Hyclone, Northumherlan, UK) and 1% of antibiotic/antimycotic commercial solution (Hyclone, Thermoscientific, Waltham, MA, USA). Cells suspension was split in a 1:3 ratio and sub-cultured into cell culture flasks of 25 cm^2 growth area. Medium was replaced every 2-3 days until the cells reached the total confluence (4-5 days of initial seeding). Biological assays were performed after SH-SY5Y cells reached total confluence in 96 well plates. Cells were maintained in controlled conditions: humidified atmosphere, 5% CO_2 and constant temperature of 37 °C.

3.4.2. Cytotoxicity of compounds on 3T3 and SH-SY5Y cells

SH-SY5Y and 3T3 cells were cultured in DMEM medium and treated with different concentrations of the compounds (10,3, 1 and 0.3 µM) during 24 h. Saponin was used as positive control of cell death. Their effects on SH-SY5Y cell viability were estimated by the means of MTT assay. This assay is based on the reduction of a yellow tetrazolium salt by NAD(P)H-dependent oxidoreductase enzymes of viable cells to purple formazan crystals that can be measured spectrophotometrically. Briefly, MTT was dissolved in DMEM medium to a final concentration of 0.5 mg/mL. After cells treatment, the medium was removed, cells were washed with phosphate buffer (PBS X1) and 100 µL of MTT solution were then added to each well. Plates were maintained in the CO_2 chamber during 2 h. MTT solution was then removed, and 100 µL of DMSO were added to each well to dissolve the formazan crystals. Absorbance was read at 570 nm in a microplate reader. Results were expressed in percentage of the control (untreated cells).

3.5. Neuroprotective activity of the compounds

The neuroprotective effect of compounds was assessed on SH-SY5Y cells when exposed to 6-OHDA neurotoxin as previously described by Silva et al. (2018). Two different approaches were carried out. The first one, SH-SY5Y cells were exposed to 6-OHDA (100 μM) in the presence of non-toxic concentrations of compounds (3, 1 and 0.3, μM) for 24 h. In a second approach, SH-SY5Y cells were pre-treated with non-toxic concentrations of compounds (3, 1 and 0.3, μM) for 1 h before exposition to 6-OHDA for 24 h. Cell viability was estimated through the MTT assay as previously described. Results were expressed in percentage of the control (untreated cells).

3.6. Neuroprotective effects of compounds in Parkinson's Disease biomarkers

3.6.1. Production of reactive oxygen species (ROS)

ROS levels were determined using carboxy-H2DCFDA probe. Briefly, the carboxy-H2DCFDA molecule is incorporated into the cells, where it is deacetylated by cellular esterases to a non-fluorescent compound, which in turn may be oxidized by ROS to DCF, a molecule that is highly fluorescent. Cells were treated with 6-OHDA (100 μM) in the presence/ absence of compounds (3, 1 and 0.3, μM) for 6 h. Concluded this time, cell medium was removed and cells were washed with PBS (1x) twice. 100 μL carboxy-H2DCFDA (20 μM) solution, previously dissolved in culture medium, was added and cells were incubated in the dark for 1 h. Finally, the fluorescence was measured at $\lambda_{excitation}$: 498 nm and $\lambda_{emission}$: 522 nm wavelengths. Results were expressed as percentage of control (untreated cells).

3.6.2. Mitochondrial membrane potential (MMP)

MMP was assessed using the JC-1 fluorescence probe (Molecular Probes, T3168, Eugene, Oregon, USA). Firstly, SH-SY5Y cells were exposed to 6-OHDA (100 μM) in the presence/ absence of different concentrations of compounds (3, 1 and 0.3, μM) for 6 h. Concluded this time, culture medium was removed and cells were incubated with JC-1 solution (3 μM) for 15 min at 37 °C. After that, cells were washed with PBS (1x) twice and 100 μL of PBS was added to each well and plate was placed in the plate reader for 30 min. Ending this time, cells were washed again and a FCCP (50 μM) plus oligomycin (1 μg/mL) conjugated solution was added to each well. Formation of JC-1 aggregates, and JC-1

monomers were measured at 490 nm excitation and 590 nm emission and at 490 nm excitation and 530 nm emission wavelengths, respectively. Results were expressed in percentage of control based in the ratio of monomers/aggregates of JC-1.

3.6.3. Caspase-3 activity

Caspase-3 (cysteine-aspartic acid protease) activity was determined by the means of "Caspase-3 activity fluorometric" kit according to manufacture instructions and as detailed by Silva *et al*.,2018. This assay is based in the hydrolysis of the peptide acetil-Asp-Glu-Val-Asp-7-amido-4-metilcumarina (Ac-DEVD-AMC) by the Caspase-3 enzyme resulting in 7-amine-4-metilcumarina (AMC), a high fluorescence product that can be measured. Briefly, cells were exposed to 6-OHDA (100 µM) in 6-well plate in the presence/ absence of compounds (3, 1 and 0.3, µM) for 6 h. Cells were then washed with PBS (x1) and detached for centrifugation at 3,300 *g* for 5 min. After that, 100 µL lysis buffer was added to cells and incubated in ice for 20 min followed by 20 min of centrifugation at 22,500 *g* and supernatant was taken and stored at - 80 °C. Pellet was stored at - 80 °C for further protein quantification by the means of Lowry method (Waterborg 2002). For Caspase-3 activity determination, 5 µL supernatant of each sample was placed into a 96-well plate plus 200 µL substrate solution and fluorescence was measured every minute during 60 min at a wavelength of 360 nm excitation and 460 nm emission. Caspase-3 activity was calculated by the slope of the linear phase of the resulting fluorescence and expressed in arbitrary fluorescence units per mg of protein per minute (Δ fluorescence (u.a).mg^{-1} of protein min^{-1}).

3.7. Data treatment and statistical analysis

One factor analysis of variances (ANOVA) was performed to test the null hypothesis in which values would not vary using the same treatment (p-value > 0.05). Furthermore, ANOVA was also used to analyse differences between measurements with different treatments. Before that, Kolmogorov-Smirnov test was used to test normality distribution and Cochran Test (Cochran, 1951) was used to test homogeneity of variances. When such parameters were not found, data was transformed using $\sqrt{x+1}$ or logx+1. When transformations did not solve the problem α was adjusted to 0.01, considering that ANOVA is sturdy enough specially when the design is well balanced and contains several samples (Underwood, 1997). When significant differences among treatments were found, Dunnett test (Dunnett *et al.,* 1991) and Tukey's (HSD) were employed (Sokal and Rohlf, 1995). Likewise, principal component analysis was employed to obtain a general overview of the

different concentrations and cellular assays accomplished (Hotelling *et al*.,1933). All statistical analyses were performed using R (Team, 2016) and GraphPad 8 (Graphpad Software, Inc. La Jolla, CA, USA) software. When applicable, results are presented as mean standard error of the mean (SEM). At least three independent experiments were carried out in triplicate.

Results

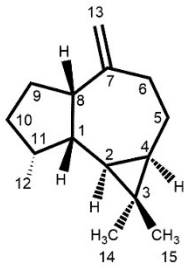
4. Results

4.1. Isolation and purification of *Sphaerococcus coronopifolius* compounds

The isolation and purification of *Sphaerococcus coronopifolius* compounds was performed from fractions previously obtained. The fractions were subjected to chromatography column (CC) and thin layer chromatography techniques using different conditions (Figure 5), allowing to isolate four compounds. The structural identification of those compounds was accomplished NMR spectroscopy and comparison with literature data. Regarding our results, when compared with literature data, it was possible to identify, unambiguously, the following compounds:

NMR data of compound **1** (Table 2) confirmed the presence of a sesquiterpene derivative with 15 carbons, registering two methyl signals forming an isopropyl unit at δ_H 1.00 (*s*, H-14, 3H) and 0.96 (*s*, H-15, 3H) and one methyl group bonded to a tertiary carbon from the five membered carbon ring δ_H 0.94 (*d*, 7.2 Hz, H-12, 3H). In addition, a methylene group of an exocyclic double bond was indicated through the highest chemical shift signals at δ_H 4.70 *m* and 4.73 *m* (m, H-13, 2H). NMR data allowed to identify the gross structure of an aromadendrene skeleton, identifying compound **1** as alloaromadendrene, in agreement with results previously reported (Faure *et al*., 1991, De Rosa *et al*.,1988).

Table 2 – Comparison of NMR data ($CDCl_3$, 400 MHz) of **compound 1** isolated from *Sphaerococcus coronopifolius* and alloaromadenddrene from literature[1],[2].



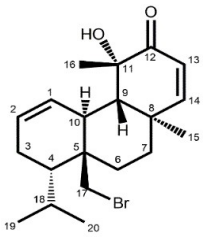
	Compound 1		Alloaromadendrene[1]		Alloaromadendrene [2]		
n°	δ ^{1}H	δ ^{13}C	δ^1H	δ ^{13}C	δ ^{1}H	δ ^{13}C	type
1	1.86 *m*	42.1	1.80-1.90 *m*	42.3	1.86 *m*	42.24	CH
2	0.24 *dd* (10.0, 9.7)	23.5	0.25 *dd* (11.0, 9.6)	23.7	0.24 *dd* (10.9; 9.9)	23.59	CH
3	-	17.21	-	17.3	-	17.23	C
4	0.54 *m*	24.7	0.56 *m*	25.0	0.55 *m*	24.87	CH
5	1.85 *m* 1.24 *m*	22.1	1.80-1.90 *m* 1.26 *m*	22.2	1.84 *m*(α) 1.24 *m*(β)	22.20	CH_2
6	2.31 *m*	35.6	2.32 *m*	35.8	2.34 *m*(α) 2.28 *m*(β)	35.76	CH_2
7	-	152.58	-	152.2	-	152.32	C
8	2.67 *m*	50.8	2.67 *m*	51.0	2.65 *m*	50.84	CH
9	1.89 *m* 1.72 *m*	28.2	1.73 *m*	28.3	1.88 *m* 1.73 *m*	28.27	CH_2
10	1.74 *m* 1.33 *m*	31.17	1.80-1.90 *m*	31.3	1.73 *m*(β) 1.32 *m*(α)	31.26	CH_2
11	2.07 *m*	37.8	2.07 *m*	37.8	2.07 *m*	37.86	CH
12	0.94 *d* (7.2)	16.3	0.94 *d* (7.2)	16.4	0.94 *d* (8.0)	16.44	CH_3
13	4.73 *m* 4.70 *m*	109.6	4.73 *m* 4.71 *m*	109.6	4.74 *m* 4.71 *m*	109.78	CH_2
14	1.00 *s*	28.6	1.00 *s*	28.7	1.01 *m*	28.65	CH_3
15	0.95 *s*	15.9	0.96 *s*	15.8	0.96 *m*	15.89	CH_3

[1] NMR data (500MHz, CDCl3) from De Rosa *et al*. 1988. [2] NMR data (400MHz, CDCl3) from Faure *et al*. 1991. δ^1H (proton chemical shift in ppm, multiplicity, coupling constant in Hz); δ ^{13}C (carbon chemical shift in ppm);

NMR data of compound **2** (Table 3) confirmed the structure of sphaerococcenol A, in agreement with previous results reported in literature (Smyrniotopoulos *et al*., 2008; Etahiri *et al*., 2001; De Rosa *et al*., 1988). NMR spectra showed a presence of a diterpene (C-20) with two methyl doublets at δ_H 0.92 (*d*, 5.1 Hz, H-19, 3H) and 0.94 (*d*, 5.1 Hz, H-20, 3H), and two methyl singlets at δ_H 1.09 (*s*, H-15, 3H) and 1.33 (*s*, H-16, 3H), being the two methyl doublets part of an isopropyl group. Likewise, results showed a heteroatom-substituted secondary carbon at δ_C 39.81 (CH_2, C-17) with two diastererotopic protons (δ_H 3.72*br* and 3.89 *d*, $J_{geminal}$= 10.6 Hz) characteristic of a brominated methylene group. In addition, it also presented two pairs of *cis* vinyl protons indicated by the signals at δ_H 6.04 (*brd*, 10.0 Hz,

H-1, 1H)/ 5.74 (*m*, H-2, 1H) and 6.07 (*d*, 9.7 Hz, H-13, 1H)/6.83 (*d*, 9.7 Hz, H-14, 1H). It is also possible to identify at δ_c 203.27 a signal characteristic of ketone carbon (>C=O) and at δ_c 75.13 of a quaternary carbon link to a hydroxyl group.

Table 3 – Comparison of NMR data ($CDCl_3$, 400 MHz) of **compound 2** isolated from *Sphaerococcus coronopifolius* and sphaerococcenol A from literature[1].



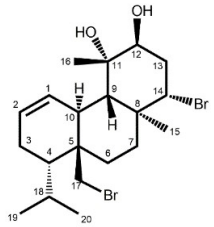
	Compound 2		Sphaerococcenol A[1]		
n°	**δ ^{1}H**	**δ ^{13}C**	**δ ^{1}H**	**δ ^{13}C**	**Type**
1	6.04 *br d* (10.0)	128.18	6.05 *br d* (10.3)	128.31	CH
2	5.74 *m*	127.45	5.74 *dm* (10.3)	127.67	CH
3	2.16 *m*	22.18	2.16 *m*	22.38	CH_2
	1.98 *m*		1.90 – 2.00 *m*		
4	1.77 *brd* (5.6)	41.88	1.78 *m*	42.16	CH
5	-	40.06	-	40.31	C
6	1.59 *m*	24.37	1.46 *m*	33.21	CH_2
	1.85 *m*		1.68 *m*		
7	1.46 *dm* (13.4)	32.87	1.59 *m*	24.61	CH_2
	1.85 br*t* (13.4)		1.76 *m*		
8	-	36.67	-	36.90	C
9	1.93 *d* (11.7)	45.33	1.46-1.54 *m*	45.75	CH
10	2.90 *br d* (11.7)	35.33	2.90 *br d* (13.0)	35.61	CH
11	-	75.13	-	75.36	C
12	-	203.27	-	203.34	C
13	6.07 d (9.7)	124.33	6.07 d (9.8)	124.51	CH
14	6.83 d (9.7)	161.92	6.83 d (9.8)	161.94	CH
15	1.09 *s*	21.29	1.09 *s*	21.07	CH_3
16	1.33 *s*	31.16	1.33 *s*	31.38	CH_3
17	3.72 br*d* (10.6)	39.81	3.72 *d* (10.7)	39.82	CH_2
	3.89 *d* (10.6)		3.89 *d* (10.7)		
18	1.92-2.00 *m*	25.69	1.90 – 2.00 *m*	25.95	CH
19	0.92 *d* (5.1)	19.39	0.92 *d* (6.8)	19.45	CH_3
20	0.97 *d* (5.1)	25.87	0.97 *d* (6.8)	26.00	CH_3
	2.95 *s*	-	-	-	OH

[1] RMN data (500MHz, $CDCl_3$) from de Rosa *et al.* 1988. δ ^{1}H (proton chemical shift in ppm, multiplicity, coupling constant in Hz). δ ^{13}C (carbon chemical shift in ppm).

NMR data of compound **3** (Table 4) confirmed another molecule with a diterpene structure that was identified as 12*S*-hydroxy-bromosphaerol (Smyrniotopoulos *et al.,*2008). Results showed the presence of two methyl singlets at δ_H 1.28 (*s*, H-15, 3H), 1.46 (*s*, H-16, 3H). On the other hand, it was also found the signals at δ_H/δ_C (3.61 *d*, 3.95 *d*, $J_{geminal}$ = 10.5 Hz,

2H/40.55, CH_2) and δ_H/δ_C (4.48 *dm*, 13 Hz, 1H/63.36, CH) that are consistent with the presence of brominated methylene (-CH_2Br) and methine (-CHBr) groups at position C-17 and C-14, respectively. Additionally, it is also possible to identify at δ_H/δ_C (3.43 *m*, 1H/79.40, CH) a presence of methine group link to a hydroxyl group.

Table 4 – Comparison of NMR data ($CDCl_3$, 400 MHz) of **compound 3** isolated from *Sphaerococcus coronopifolius* and 12*S*-hydroxy-bromosphaerol from literature[1].

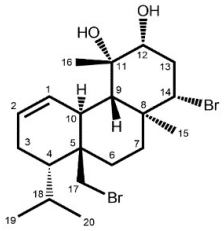


	Compound 3		12*S*-hydroxy-bromosphaerol [1]		
n°	**δ^1H**	**δ^{13}C**	**δ ^{1}H**	**δ^{13}C**	**Type**
1	5.98 *brd* (10.6)	128.46	5.97 *br d* (10.4)	128.5	CH
2	5.72 *m*	127.51	5.69 *ddt* (10.4, 5.0, 2.5)	127.5	CH
3	1.95 *m* 2.14 *m*	21.92	α1.93 *m* β2.10 *m*	21.9	CH_2
4	1.75 *m*	42.60	1.72 *br s*	42.6	CH
5	-	41.05	-	41.1	C
6	1.80 *m* 1.54 *m*	24.86	α 1.75 *m* β1.51 *m*	24.9	CH_2
7	1.84 *m* 1.32 *m*	36.40	α1.81 *m* β1.29 *m*	36.4	CH_2
8	-	41.77	-	41.8	C
9	1.81 *m*	45.93	1.78 *m*	45.9	CH
10	2.99 *dm* (10.4)	36.80	2.97 *dm* (11.2)	36.8	CH
11	-	74.83	-	74.8	C
12	3.47 *m*	79.40	3.45 *br s*	79.4	CH
13	2.16 *dm* (14) 2.72 br*t* (13)	37.36	β 2.14 *dt* (13.7, 3.7) α 2.70 *ddd* (13.7, 12.8, 2.9)	37.4	CH_2
14	4.48 *dm* (13)	63.40	4.46 *dd* (12.8, 3.7)	63.4	CH
15	1.28 *s*	14.85	1.27 *s*	14.9	CH_3
16	1.46 *s*	31.84	1.44 *s*	31.8	CH_3
17	3.61 *d* (10.5) 3.95 *d* (10.5)	40.55	3.60 *dd* (10.8, 2.1) 3.93 *d* (10.8)	40.5	CH_2
18	1.96 *m*	25.89	1.93 *m*	25.9	CH
19	0.91 *d* (5.4)	19.71	0.89 *d* (7.0)	19.7	CH_3
20	0.97 *d* (5.4)	25.93	0.95 *d* (7.0)	25.9	CH_3
					OH

[1] NMR data (500MHz, CDCl3) from Smyrniotopoulos *et al* 2008. δ ^{1}H (proton chemical shift in ppm, multiplicity, coupling constant in Hz). δ ^{13}C (carbon chemical shift in ppm).

NMR data of compound **4** (Table 5) allowed to identify 12*R*-hydroxy-bromosphaerol, the 12-epimer of compound **3**. Both compounds have similar NMR data, being the most significant difference the chemical shifts and coupling constants of the methine proton geminal to the hydroxyl group at C-12 (δ_H 3.37 *dd*, 11.7 and 5.2 Hz, 1H/ δ_C 76.95, -CHOH) and the methylene group at C-13 (δ_H 2.23 *ddd*, 12.4, 5.2 and 3.2 Hz and 2.35 *q*, 12.4 Hz, CH_2). These data are in accordance with previous reports (Smyrniotopoulos *et al*., 2008; Cafieri *et al*,1987) that also isolated 12*R*-hydroxy-bromosphaerol from the red alga *S. coronopifolius.*

Table 5 – Comparison of NMR data ($CDCl_3$, 400 MHz) of **compound 4** isolated from *Sphaerococcus coronopifolius* and 12*R*-hydroxy-bromosphaerol from literature[1].



	Compound 4		12*R*-hydroxy-bromosphaerol[1]		
n°	δ^1H	δ^{13}C	δ^1H	δ^{13}C	Type
1	6.01 *br d* (10.6)	128.79	5.99 *br d* (10.4)	128.8	CH
2	5.69 *m*	126.41	5.67 *ddt* (10.4, 5.8, 2.9)	126.4	CH
3	2.14 *m* 1.93 *m*	21.80	β: 2.12 *m* α : 1.91 *m*	21.8	CH_2
4	1.77 *m*	42.63	1.73 *m*	42.5	CH
5	-	40.47	-	40.5	C
6	1.81 *m* 1.52 *m*	24.94	α : 1.77 *m* β: 1.50 *m*	24.9	CH_2
7	1.82 *m* 1.22 *m*	35.97	α: 1.78 *m* β : 1.18 *m*	36.0	CH_2
8	-	41.97	-	42.0	C
9	1.40 *d* (10.7)	48.71	1.38 *d* (10.8)	48.7	CH
10	3.03 *dm* (10.7)	37.49	3.02 *dm* (10.8)	37.5	CH
11	-	73.46	-	73.5	C
12	3.37 *dd* (11.7, 5.2)	76.94	3. 34 *dt* (11.6, 5.4)	76.9	CH
13	2.23 *ddd* (12.4, 5.2, 3.2) 2.35 *q* (12.4)	38.03	β : 2.21 *ddd* (12.4, 5.4, 3.3) α : 2.33 *ddd* (12.8, 12.4, 11.6)	37.9	CH_2
14	3.90 *dd* (12.8, 3.2)	62.96	3.88 *dd* (12.8, 3.3)	63.0	CH
15	1.26 *s*	13.64	1.25 *s*	13.6	CH_3
16	1.43 *s*	30.86	1.41 *s*	30.9	CH_3
17	3.91 *d* (10.3) 3.60 br*d* (10.3)	40.28	3.89 *d* (10.4) 3.59 *dd* (10.4, 1.7)	40.3	CH_2
18	1.94 *m*	25.83	1.93 *m*	25.8	CH
19*	0.91 *d* (6.8)	19.94	0.89 *d* (7.1)	19.9	CH_3
20*	0.98 *d* (6.8)	26.13	0.95 *d* (7.1)	26.1	CH_3

[1] NMR data (500MHz, CDCl3) from Smyrniotopoulos *et al* 2008. δ^1H (proton chemical shift in ppm, multiplicity, coupling constant in Hz). δ^{13}C (carbon chemical shift in ppm).

Summarizing, the CC and the previous HPLC techniques applied led to the isolation of four compounds identified as alloaromadendrene **(1),** sphaerococcenol A **(2)**, 12*S*-hydroxy-bromosphaerol **(3)** and 12*R*-hydroxy-bromosphaerol **(4)**. Their chemical structures are depicted in Figure 6.

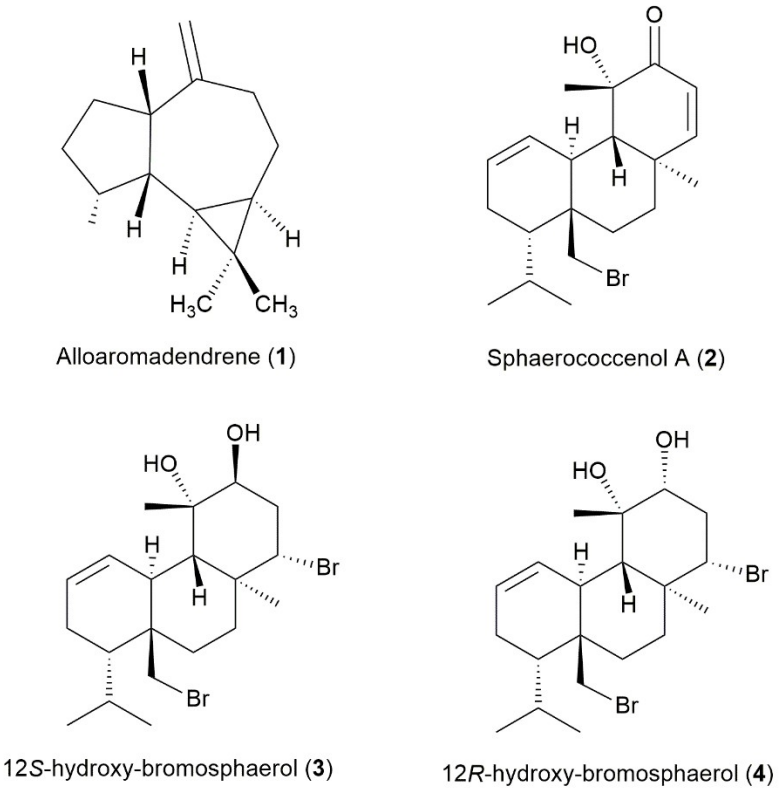


Figure 6. Chemical structures of the terpenes isolated from *Sphaerococcus coronopifolius*, collected in the Berlenga Nature Reserve, Peniche, Portugal.

However, due to the low purity of compound 3, which correspond to 12*S*-hydroxy-bromosphaerol, it was not integrated in the biological assays.

4.2. Antioxidant activity of *Sphaerococcus coronopifolius* compounds

Antioxidant activities of alloaromadendrene**,** sphaerococcenol A and 12*R*-hydroxy-bromosphaerol isolated from the red algae *S. coronopifolius* was evaluated through the following complementary methods: DPPH radical scavenging assay (DPPH), Oxygen Radical Absorbance Capacity (ORAC), and Ferric Reducing Antioxidant Power (FRAP). The results are present in the following sections 4.2.1 - 4.2.3.

4.2.1. DPPH radical scavenging activity

DPPH radical scavenging assay have been widely used to evaluate the antioxidant potential of distinct samples, including marine natural products. Following this, the ability of *S. coronopifolius* terpenes (5-150 µM) to neutralize DPPH radicals was evaluated and the results were presented as percentage (%) of reduced DPPH (Figure 7).

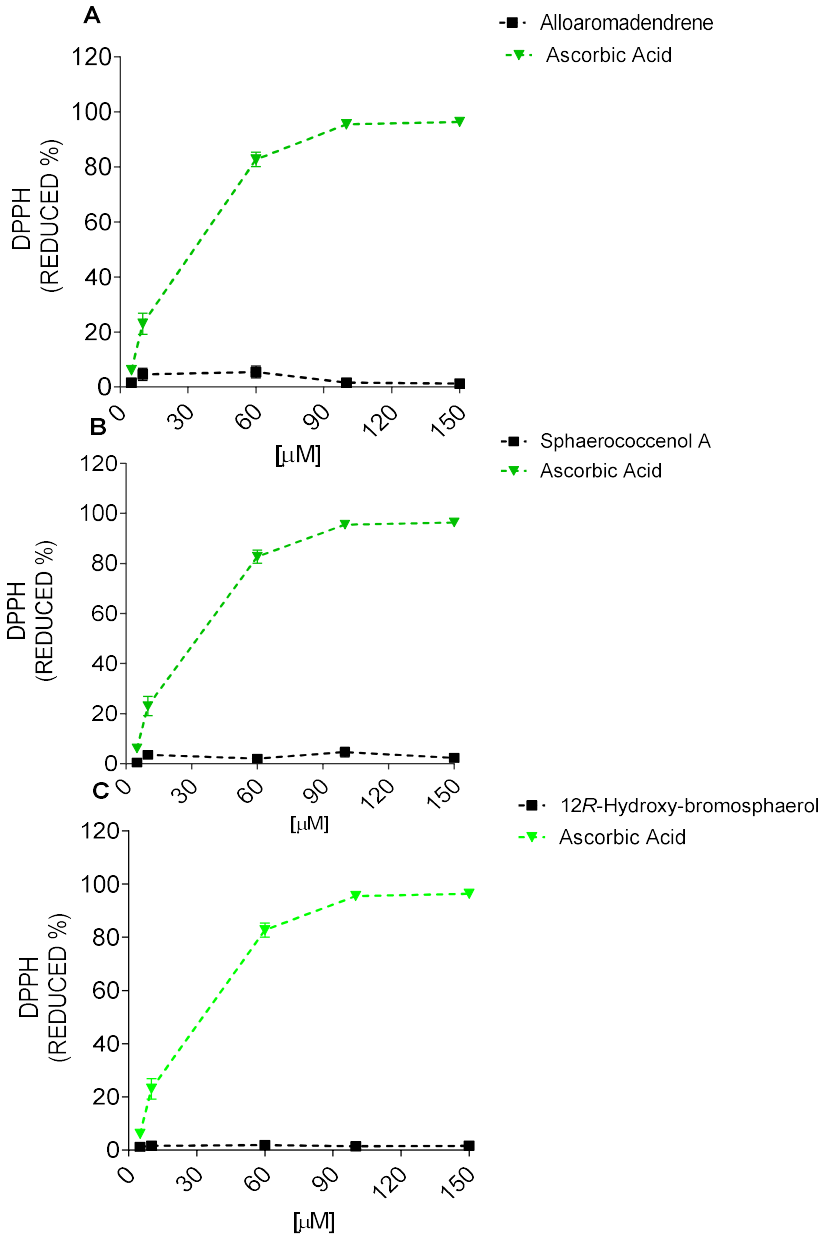


Figure 7. DPPH radical scavenging activity of alloaromadendrene (A), sphaerococcenol A (B), and 12*R*-hydroxy-bromosphaerol (C) isolated from *Sphaerococcus coronopifolius* and ascorbic acid antioxidant standard tested at different concentrations (5 - 150 µM) expressed as percentage of reduced DPPH. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate.

As seen in figure 7, it is possible to observe that the terpenes alloaromadendrene, sphaerococcenol A and 12*R*-hydroxy-bromosphaerol exhibited a weak capacity to reduce DPPH radicals when compared with the ascorbic acid antioxidant standard. The highest capacity of alloaromadendrene, sphaerococcenol A and 12*R*-hydroxy-bromosphaerol was observed at the concentrations of 60 µM, 100 µM and 150 µM, inducing a DPPH reduction of 4.71%, 5.49%, and 1.91% respectively. The highest antioxidant activity was exhibited by the ascorbic acid that neutralize DPPH radicals in 82.70%, 95.51%, 96.38% DPPH when tested at the concentrations of 50 µM, 100 µM, and 150 µM, respectively. Furthermore, significant differences were found for the three terpenes at every concentration when compared with ascorbic acid (ANOVA, Tukey's Test, $p<0.05$).

4.2.2. Oxygen Radical Absorbance Capacity (ORAC)

The ability of *S. coronopifolius* terpenes alloaromadendrene**,** sphaerococcenol A and 12*R*-hydroxy-bromosphaerol to reduce peroxyl radicals was evaluated by means of the ORAC method. Results are presented as µmol of trolox equivalents per mg of compound (µmol Trolox EQ/ mg compound) and depicted in Figure 8.

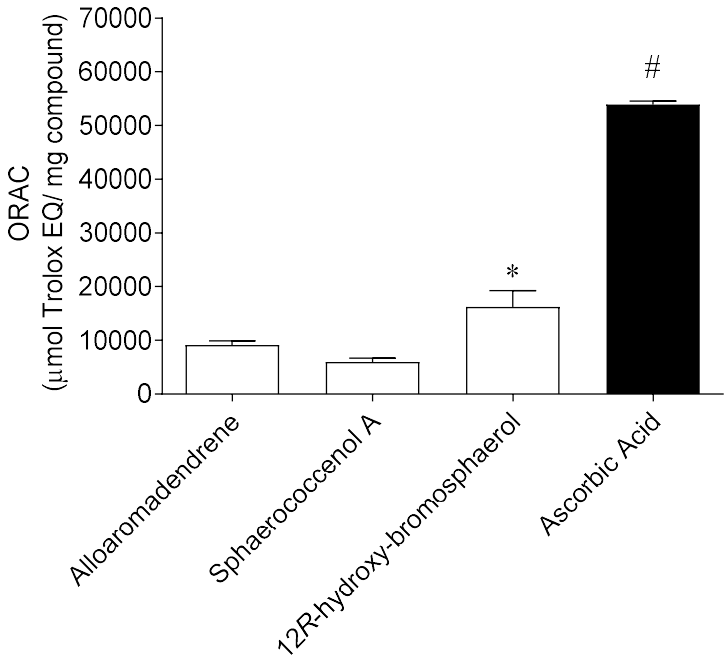


Figure 8. Oxygen Radical Absorbance Capacity of *Sphaerococcus coronopifolius* compounds and ascorbic acid. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent significant differences (ANOVA, Tukey's Test, $p<0.05$) when compared to: * alloaromadendrene and sphaerococcenol A; # when compared with the remaining compounds.

The highest oxygen radical absorbance capacity was exhibited by the ascorbic acid standard antioxidant with a value of 53,887.10 ± 651.86 µmol Trolox EQ/ mg compound (Figure 8). Regarding *S. coronopifolius* terpenes, 12*R*-hydroxy-bromosphaerol (16,208.77 ± 3,048 µmol Trolox EQ/ mg compound) displayed the highest capacity to reduce peroxyl radicals following by alloaromadendrene (9,120.10 ± 816.33 µmol Trolox EQ/ mg compound) and sphaerococcenol A (5,998.35 ± 663.62 µmol Trolox EQ/ mg compound). Moreover, it is possible to observe that 12*R*-hydroxy-bromosphaerol presents significant differences when compared with alloaromadendrene and sphaerococcenol A, standing out as the compound with best capability to reduce peroxyl radicals among the tested terpenes.

4.2.3. Ferric Reducing Antioxidant Power (FRAP)

FRAP method is based in the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) which may be triggered by antioxidants. Thus, the capacity of *S. coronopifolius* terpenes to reduce ferric ions by electron donation was evaluated. Results were expressed in µM of $FeSO_4$ equivalents per mg of compound (µM $FeSO_4$ EQ/ mg compound) and shown in Figure 9.

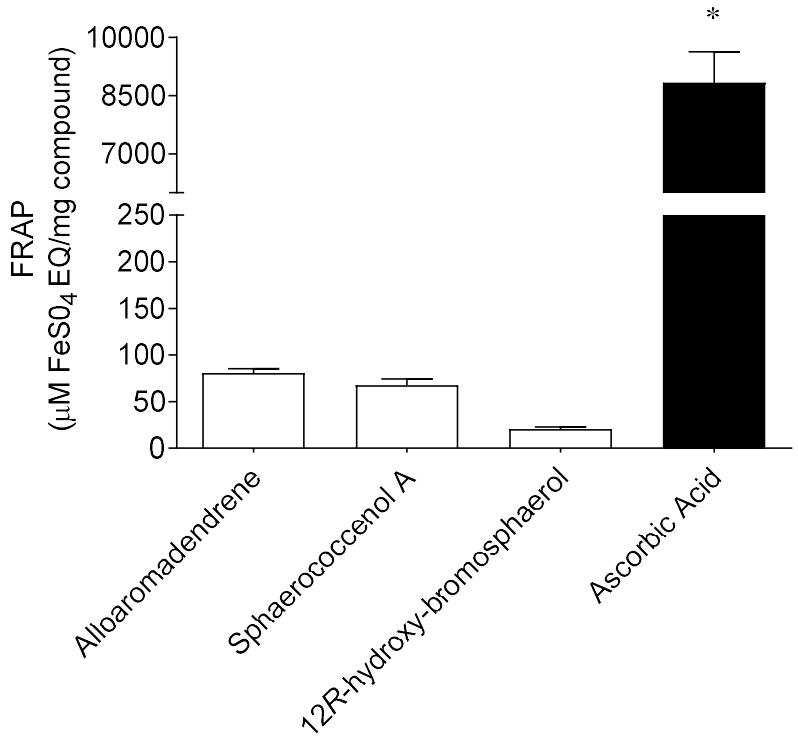


Figure 9. Ferric reducing antioxidant power (FRAP) of *Sphaerococcus coronopifolius* terpenes and ascorbic acid. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate. Symbol (*) represents significant differences (ANOVA, Tukey's Test, $p<0.05$) when compared to other all compounds.

Results presented in Figure 9 showed that the greater capability to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) among *S. coronopifolius* compounds was mediated by alloaromadendrene (80.45 ± 4.91 µM $FeSO_4$ EQ/mg compound). However, no significant differences were found when it was compared with 12*R*-hydroxy-bromosphaerol (67.70 ± 6.77 µM $FeSO_4$ EQ/ mg compound) or sphaerococcenol A (20.35 ± 2.38 µM $FeSO_4$ EQ./ mg compound). On the other hand, the highest antioxidant activity was mediated by ascorbic acid (8,832.52 ± 714.52 µM $FeSO_4$ EQ/ mg compound), which displayed the highest capacity to reduce ferric iron (Fe^{3+}), presenting significant differences when compared with the remaining compounds.

4.3. Biological activities of compounds isolated from *Sphaerococcus coronopifolius* on *in vitro* cellular models

4.3.1. Cytotoxicity of compounds isolated from *Sphaerococcus coronopifolius*

Different concentrations (0.3 – 10 µM) of the terpenes (alloaromadendrene**,** sphaerococcenol A and 12*R*-hydroxy-bromosphaerol) isolated from *S. coronopifolius* were tested on fibroblasts (3T3 cells) and neuronal cells (SH-SY5Y cells) in order to define the non-toxic concentrations to be used in the assessment of their neuroprotective activities. Results were expressed as percentage of control and depicted in Figure 10 and 11.

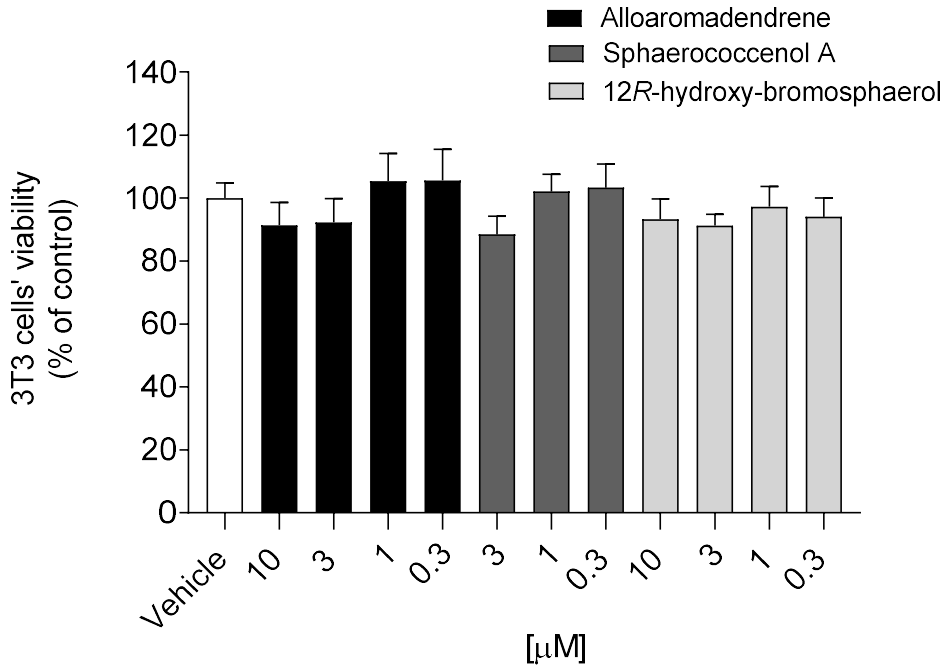


Figure 10. Cytotoxicity of *Sphaerococcus coronopifolius* compounds (0.3 – 10 µM; 24 h) on 3T3 cells' viability. The effects were estimated by the MTT assay. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate.

The treatment accomplished on fibroblasts with the compounds extracted from *S. coronopifolius* did not induced cytotoxicity in all the concentrations tested (Figure 10).

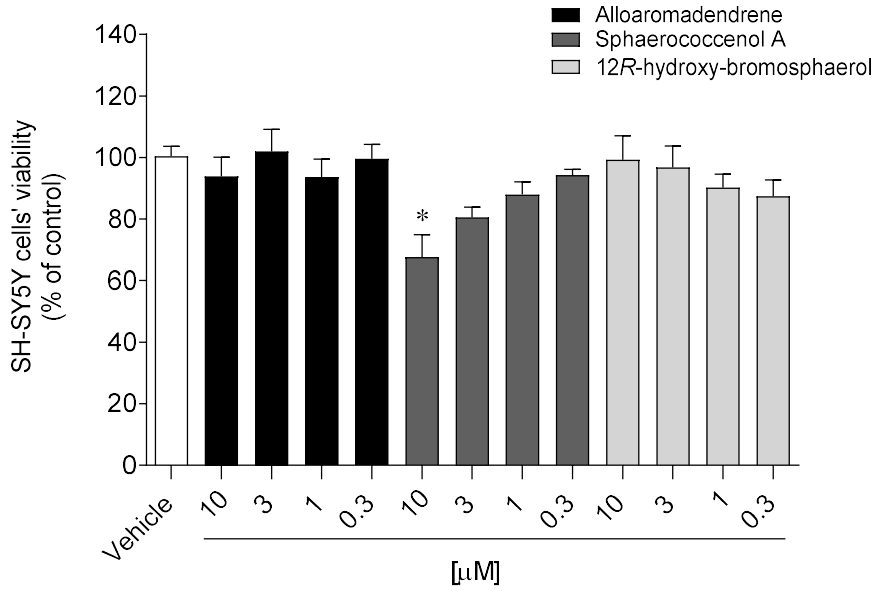


Figure 11. Neurotoxicity of *Sphaerococcus coronopifolius* compounds (0.3 – 10 μM; 24 h) on SH-SY5Y cells' viability. The effects were estimated by the MTT assay. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate. Symbol represents significant differences (ANOVA, Dunnett´s test, $p<0.05$) when compared to: * vehicle.

On the other hand, the exposition of SH-SY5Y cells to alloaromadendrene, 12*R*-hydroxy-bromosphaerol and sphaerococcenol A did not decrease SH-SY5Y cells' viability, except in the case of 10 μM sphaerococcenol A, which decreased cells' viability in 33% when compared with the vehicle**.** Therefore, the mentioned concentration was not used in the following neuroprotective biological assays.

4.3.2. Neuroprotective activity of compounds isolated from *Sphaerococcus coronopifolius*

The neuroprotective activity of *S. coronopifolius* compounds at non-toxic concentrations was evaluated on SH-SY5Y cells exposed to 6-OHDA neurotoxin. Results were expressed as percentage (%) of control and presented in Figure 12.

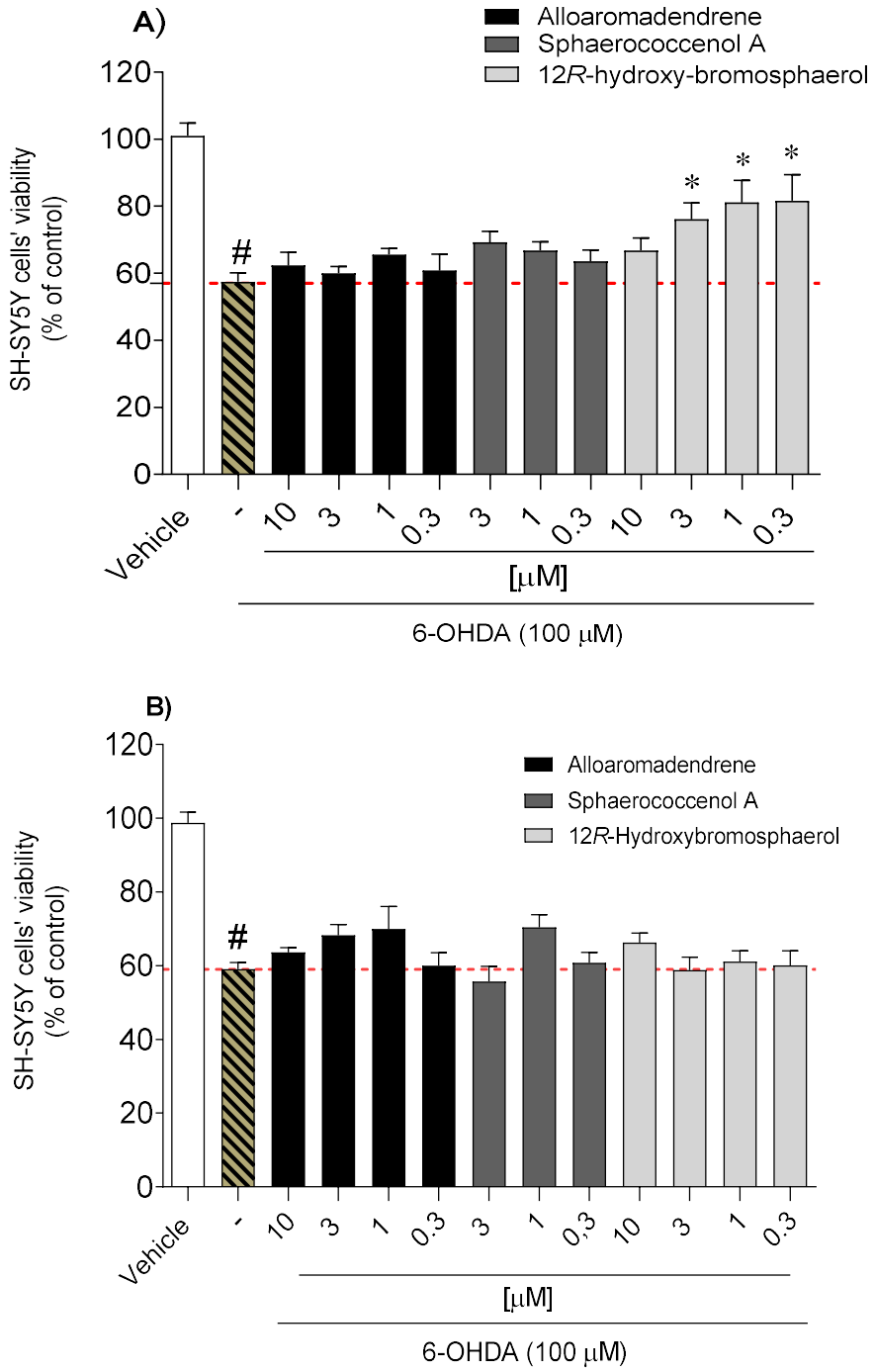


Figure 12. (A) Neuroprotective effects of *Sphaerococcus coronopifolius* compounds (0.3 – 10 μM) against 6-OHDA (100 μM) - induced neurotoxicity on SH-SY5Y cells after 24 h of treatment. (B) Neuroprotective effects of the *Sphaerococcus coronopifolius* compounds pre-incubated for 1 h before 6-OHDA (100 μM) exposition on SH-SY5Y cells. The effects were estimated by the MTT assay. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent significant differences (ANOVA, Dunnett´s test, $p<0.05$) when compared to: # vehicle or *-OHDA.

Treatment with 6-OHDA resulted in a 42% decrease of SH-SY5Y cell viability (Figure 12A). On the other hand, the treatment performed in the presence of 12*R*-hydroxy-bromosphaerol at 3 µM, 1 µM and 0.3 µM exhibited a significant capacity to decrease the neurotoxicity induced by 6-OHDA, increasing SH-SY5Y cell viability in 18.66 ± 4.81%, 23.66 ± 6.58%, and 24.19 ± 7.74%, respectively. Finally, the treatments performed in the presence of alloaromadendrene and sphaerococcenol A did not showed significant differences when compared to 6-0HDA.

On the other hand, pre-incubation with compounds on SH-SY5Y cells for 1 h did not exhibited capacity to neutralize the 6-OHDA - induced neurotoxicity (Figure 12B).

4.3.3. Neuroprotective effects of compounds on Parkinson's disease biomarkers

According to the results described above, 12*R*-hydroxy-bromosphaerol revealed the highest neuroprotective potential. Therefore, the mechanism of action underlying its activities and related to Parkinson's disease development, namely oxidative stress, mitochondrial disfunction and Caspase-3 activity were evaluated. The results are presented in the following sections 4.3.3.1 to 4.3.3.3.

4.3.3.1. Production of reactive oxygen species (ROS)

The levels of reactive oxygen species produced by SH-SY5Y cells after exposition to 6-OHDA in presence/ absence of 12*R*-hydroxy-bromosphaerol were measured. Results were presented as percentage (%) of control (Figures 13).

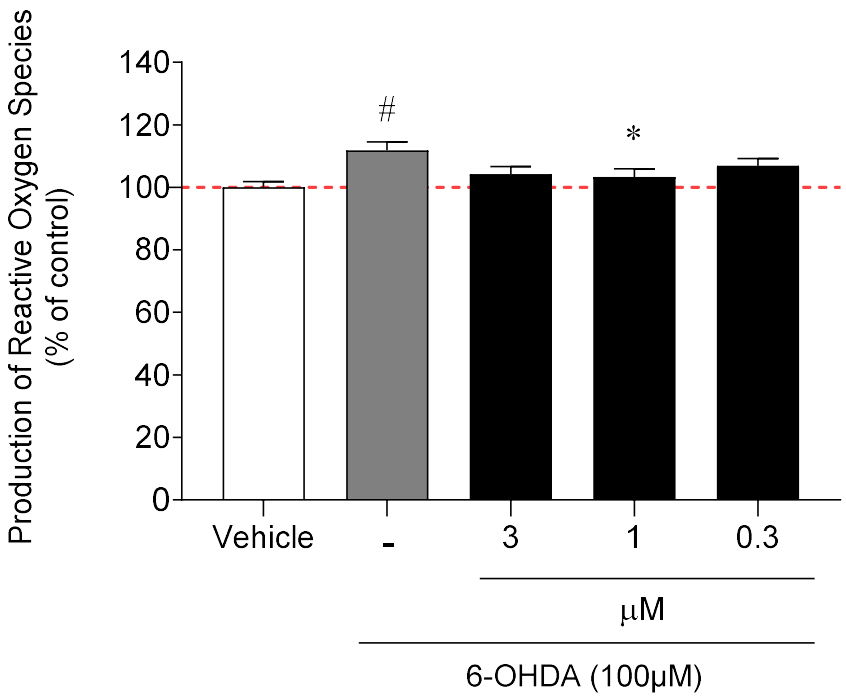


Figure 13. Production of reactive oxygen species on SH-SY5Y cells treated with 6-OHDA (100 μM) in the presence/ absence of 12*R*-hydroxy-bromosphaerol (0.3 – 3 μM) after 6 h of incubation. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent significant differences (ANOVA, Dunnett´s test, $p<0.05$) when compared to: # vehicle or *6-OHDA.

The exposition of SH-SY5Y cells to 6-OHDA significantly increased the production of ROS in 11% (111.9±2.6 %) when compared to vehicle situation (Figure 13). On the other hand, the treatment performed in the presence of 12*R*-hydroxy-bromosphaerol, when tested at 1 μM, promoted a significant decrease of ROS levels produced by SH-SY5Y cells when compared with 6-OHDA treatment (103.1 ± 3.26%).

Furthermore, no significant differences were observed when cells were treated with 12*R*-hydroxy-bromosphaerol at 3 μM and 0.3 μM, although results showed a decrease of 6.89% and 4.53%, respectively in ROS levels.

4.3.3.2. Mitochondrial membrane potential

Changes in the mitochondrial membrane potential of SH-SY5Y cells exposed to 6-OHDA in presence/ absence of 12*R*-hydroxy-bromosphaerol were evaluated after 6 h of treatment. Results were expressed as percentage of control (ratio of monomers/ aggregates JC-1) and presented in Figure 14.

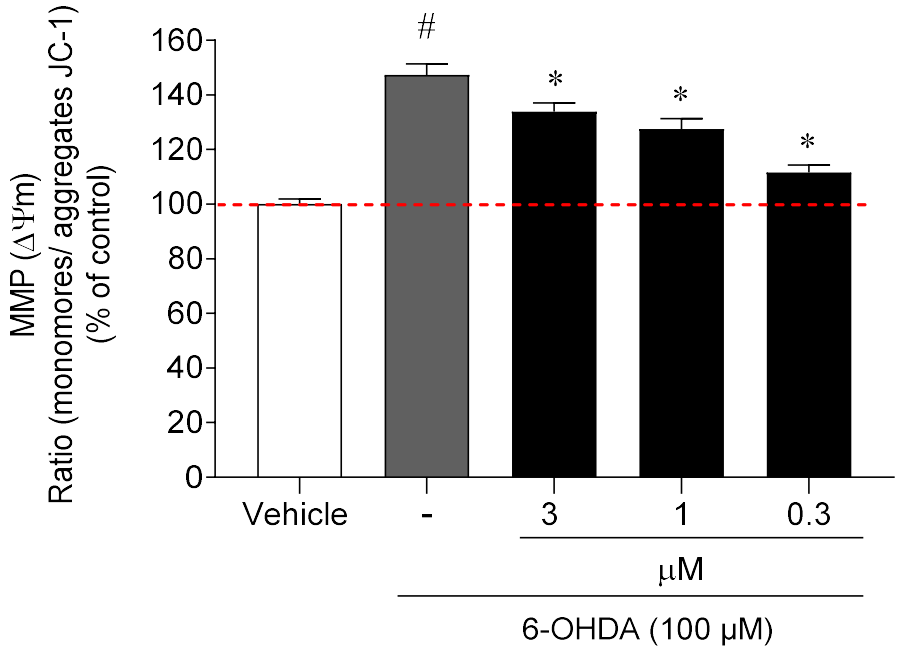


Figure 14. Mitochondrial membrane potential of SH-SY5Y cells treated with 6-OHDA (100 μM) in the presence/ absence of 12*R*-hydroxy-bromosphaerol (0.3 – 3 μM) after 6 h of incubation. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent significant differences (ANOVA, Dunnett´s test, $p<0.05$) when compared to: # vehicle or *6-OHDA.

The treatment of SH-SY5Y cells in the presence of 6-OHDA neurotoxin (147.25 ± 4.09%) induced a significant depolarization of the mitochondrial membrane potential when compared with the vehicle situation (100.00 ± 1.85%). However, when SH-SH5Y cells were incubated in the presence of 12*R*-hydroxy-bromosphaerol, it was possible to observe a significant protective effect on MMP mediated by the compound when tested at 3, 1, and 0.3 μM, reducing the depolarization promoted by 6-OHDA in 13.47%, 19.85%, and 35.67%, respectively when compared to vehicle situation

4.3.3.3. Caspase-3 activity

Caspase-3 activity of SH-SY5Y cells when exposed to 6-OHDA in presence/ absence of 12*R*-hydroxy-bromosphaerol was evaluated after 6 h of treatment. Results were expressed as percentage of control and displayed in Figure 15.

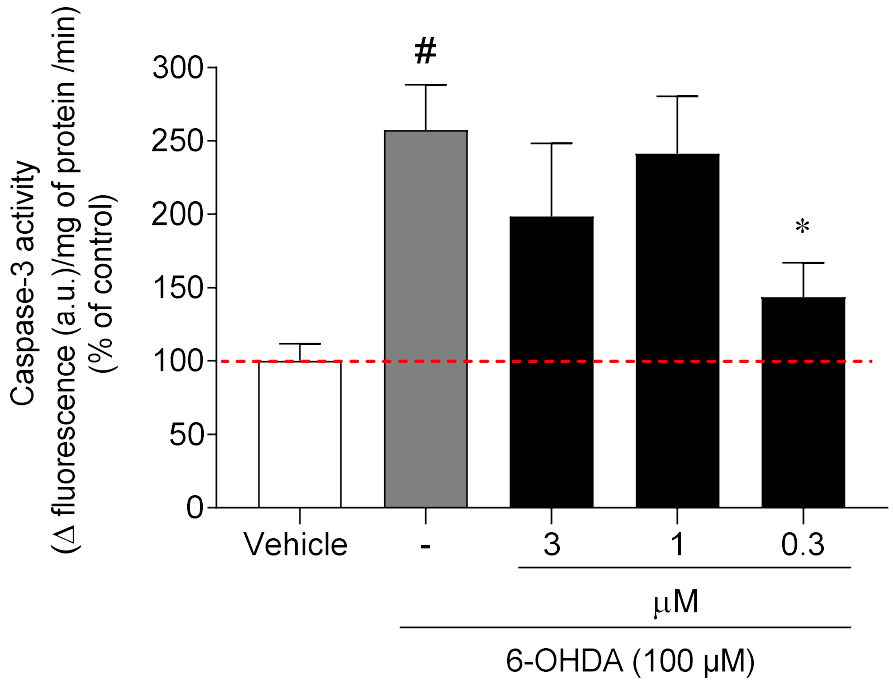


Figure 15. Caspase-3 activity of SH-SY5Y cells when exposed to 6-OHDA in the presence/absence of 12*R*-hydroxy-bromosphaerol (0.3 – 3 μM) for 6 h. Values correspond to mean ± SEM of at least three independent experiments carried out in triplicate. Symbols represent significant differences (ANOVA, Dunnett´s test, $p<0.05$) when compared to: # vehicle or *6-OHDA.

SH-SY5Y cell exposure to 6-OHDA induced a marked increase of Caspase 3 activity around 157% (257.31 ± 30.92%) when compared with the vehicle situation. On the other hand, when SH-SY5Y cells were treated in the presence of 12*R*-hydroxy-bromosphaerol at 0.3 μM a significant decrease of Caspase-3 activity was observed when compared with the 6-OHDA treatment.

4.4. Principal Component Analysis (PCA)

The PCA was carried out in order to have an overview of the similarities and differences among de three different concentrations tested of 12*R*-hydroxy-bromosphaerol and to determine their relationship with the distinct assays developed to evaluate the neuroprotective potential, namely, ROS production, MMP, and Caspase-3 activity (Figure 16).

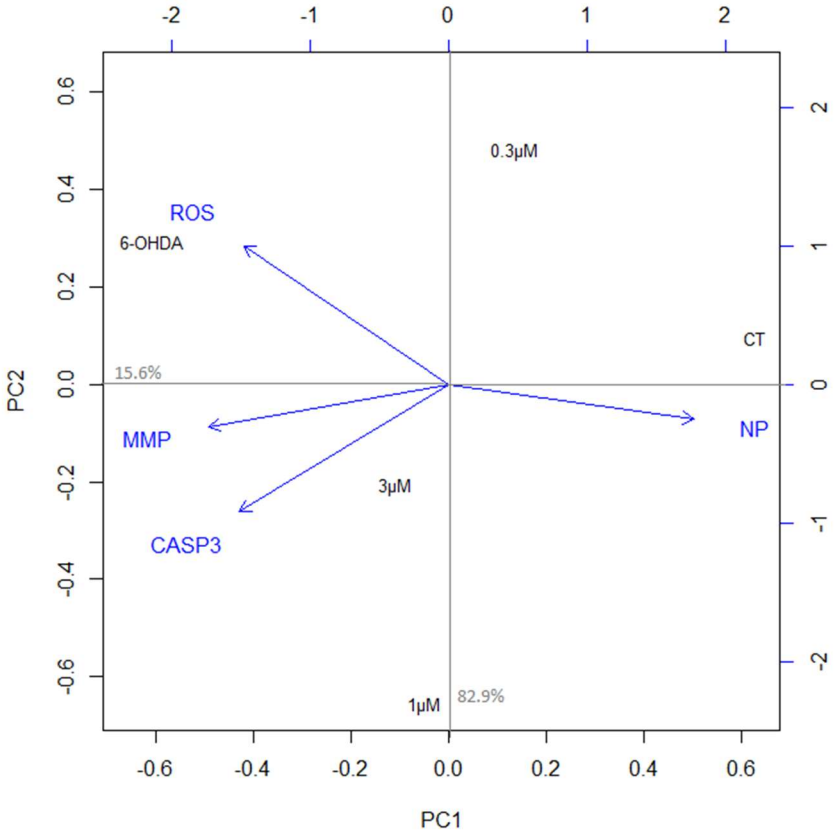


Figure 16. Principal component analysis (PCA) of the neuroprotective activities (NP)and the Parkinson's Disease biomarkers, namely oxidative stress (ROS), mitochondrial dysfunction (MMP) and apoptosis (CASP3). Vectors represent the variances between the different assays (Neuroprotection, ROS, MMP, CASP3).

PC1, first principal component, and PC2, second principal component, represent 82.9% and 15,6% of the data total variance, respectively. Results showed that there is a negative correlation for the neuroprotective assay (right) when compared with the biomarkers studied (left) which is clearly verified by the PC1 axes. Likewise, there is a strong negative correlation between 6-OHDA (left) and control (right). Moreover, in agreement with the previous results, 0.3 µM 12*R*-hydroxy-bromosphaerol concentration was the closest one to the neuroprotection and CT values (left), pointing out the protection of mitochondrial membrane potential, decrease of ROS production and inhibition of Caspase-3 activity.

Discussion and Conclusion

5. Discussion and Conclusions

Parkinson´s disease is the second most common neurodegenerative disease after Alzheimer´s disease, affecting a significant percentage of the old-age population in the world, with symptoms that manifest in tremor, posture rigidity and lack of autonomy. In this context, the search for new bioactive compounds with neuroprotective activities, currently represents an increasing area of study. Nonetheless, considering that the terrestrial environment is widely studied, the discovery of novel natural compounds becomes more difficult. On the contrary, the marine ecosystem represents an almost unexplored environment and thus, is currently focusing new attention as a source of new compounds with distinct chemical features and mechanisms of action when compared to the terrestrial compounds. In fact, as marine environment has been explored, more marine drugs have been reported, representing nonetheless a small indication of a larger possibility, especially since marine environment constitutes a unique medium to produce a great amount of secondary metabolites with great biological activities.

Accordingly, the red alga *S. coronopifolius* has been proved to be an interesting source of new compounds with different biological activities, like anti-fouling (Piazza *et al*.,2011), anti-microbial (Smyrniotopoulus *et al*., 2008;Ethari *et al*.,2001;) and anti-tumor potentials (Alves *et al*., 2016; Rodrigues *et al*., 2015; Smyrniotopoulos *et al*., 2008), and thus, the aim of this study was to isolate compounds from the mentioned alga and characterize their biological activities, namely antioxidant and neuroprotective.

In this report, by means of chromatography techniques it was possible to isolate four compounds belonging to chemical class of terpenes. The compounds were identified as alloaromadendrene (**1**), sphaerococcenol A (**2**) 12*S*-hydroxy-bromosphaerol (**3**) and 12*R*-hydroxy-bromosphaerol (**4**)**,** as evidenced by the NMR spectral data. These compounds are commonly found in *S. coronopifolius* and they have been previously isolated from this species as early reported; (Smyrniotopoulos *et al*.,2008; Etahiri *et al*., 2001; Faure *et al*., 1991; De Rosa *et al*., 1988; Cafieri *et al*,1987). The biological activities of compounds **1-4** have been evaluated for their antitumour properties against several human cell lines, displaying as well antimicrobial, cytotoxic, and anti-tumour activities (Alves *et al*., 2016; Rodrigues *et al*., 2015; Smyrniotopoulos *et al*., 2015; Smyrniotopoulos *et al*., 2008; Sawan *et al*.,2007; Smyrniotopoulos *et al*., 2010). However, to our best knowledge, this is the first report regarding their neuroprotective activities.

As previously mentioned, PD is a neurodegenerative illness associated with the loss of neuronal dopaminergic cells. Due to that, compounds able to reverse, or minimize neuronal

death represent interesting pharmacological assets to be used as neuroprotective agents. Moreover, different mechanisms may be associated with neuroprotective effects and thus, it is vital to employ different approaches aiming to achieve a general overview of the different processes occurring inside the cell.

As reported in literature, oxidative stress plays a significative role in PD (Sarrafchi *et al*., 2016¸Valko *et al*., 2007). In fact, the inability caused by cellular machine disfunction leads to an increase of ROS production, which are highly unstable and tend to react with nearby molecules causing damage and triggering cell death (Pavlin *et al*.,2016). Due to that, the search for new sources of antioxidant compounds represents an increasing area of study. Considering this and aiming to determine its antioxidant potential, the antioxidant capability of the isolated compounds was assessed by three complementary methods: DPPH, FRAP, and ORAC.

DPPH is a stable nitrogen centred free radical which can be effectively scavenged by antioxidants (Villaño *et al*., 2007) and is also considered as a good kinetic model for peroxyl radicals (Rackova *et al*., 2007). In the present study, when the compounds were tested in the DPPH assay, they showed a weak antioxidant potential (Figure 7). Nonetheless, antioxidant activity of *S. coronopifolius* non-purified extracts has been previously reported (Pinteus *et al.,*2017). Effectively, these authors observed that the highest scavenging capacity was displayed by *S. coronopifolius* methanol extract when it was compared with other five methanolic extracts derived from other seaweeds. Moreover, similar antioxidant capacity of *S. coronopifolius* methanol extracts has been reported by Rhimou *et al*. (2013), where aqueous fractions obtained from this alga also showed capacity to reduce DPPH radicals, being associated to the presence of polysaccharides.

The above-mentioned results suggest that the antioxidant-scavenging potential of this algae may not be related with the three terpenes studied in the present work, since those were isolated from a dichloromethane crude extract. Moreover, the antioxidant capacity of the methanolic extracts from *S. coronopifolius* could actually be associated to the presence of phenolic compounds. In fact, high levels of phenolic compounds have been reported from *S.coronopifolius* before, where this macroalgae demonstrated the highest total phenolic content (TPC), both in summer and in winter, when it was compared with two brown seaweeds, *Halopteris scoparia and Zonaria tournefortii* (Fellah *et al*.,2017).Additionally, there is a great correlation between TPC and antioxidant activity that has been reported by many researchers (Rajauría *et al*., 2010; Wang *et al*., 2009; Chew *et al*., 2008), since algae derived polyphenols have been proved to scavenge peroxyl radicals (Wang *et al*., 2009) and chelate ferrous ions (Chew *et al*., 2008). On the other hand, previous reports suggest

that in the case of the aqueous extracts from *S. coronopifolius* in which no phenolic compounds were found, the radical scavenging ability was associated with the presence of polysaccharides (Rhimou *et al*., 2013).

The FRAP assay determines the capability of antioxidant compounds to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in a colorimetric oxidation-reduction reaction based on electron transfer (Murugan *et al*., 2012). In the present work, even if the compounds isolated displayed a slight capability to reduce ferric iron (III), the effects were not significant when compared with ascorbic acid (Figure 8). A weak, reducing power has also been reported before from red algae extracts (Mellouk *et al*., 2017; Allencar *et al*.,2014) being associated with the presence of polyphenols too, although in this reports it was concluded that phenolic compounds only serve a minimum role in the scavenging of metallic ions and cannot represent the principal reducing agent of ferric ions (Meenakshi *et al*., 2011) suggesting that if anything its reducing power was associated with hydroxyl groups present in polyphenols, which may act as electron donors (Mellouk *et al*., 2017; Kumaran et al.,2007).

Regarding ORAC assay, it was used to evaluate the capability of compounds to scavenge peroxyl radicals that induce the oxidation of fluorescein (Mounir *et al*., 2014;Kindleysides *et al*., 2012;). Our results (Figure 9) demonstrated their capability to scavenge peroxyl radicals but no significant antioxidant capacity was found when compared with ascorbic acid for any of the three compounds assessed in this study.

Concerning assessment of neuroprotective activity, it was evaluated the capacity of compounds to protect SH-SY5Y cells from the neurotoxicity induced by 6-OHDA treatment, which mimics the neurodegeneration process observed in PD. In order to do that, first, a cytotoxicity assessment was carried out to determine the non-cytotoxic concentrations that were to be employed. In this case, the cytotoxicity assays (Figure 10) revealed that only 10 µM sphaerococcenol A induced a significant reduction of SH-SY5Y cell viability. Likewise, cytotoxicity assessment for treatments with both, 12*R*-hydroxy-bromosphaerol and alloaromadendrene, did not exhibited significant toxicity in all tested concentrations. On the other hand, the treatment with the compounds accomplished in 3T3 fibroblasts did not induced any cytotoxic effect (Figure 11).

As for the cytotoxic effects obtained in this study for sphaerococcenol A, which resulted in a 33% decrease of cell viability, our results are in agreement with previous results obtained with sphaerococcenol A 10 µM, which induced a significant reduction (55%) in non-malignant human lung fibroblasts cells (HBF) (Alves *et al*.,2020).

Regarding neuroprotection, the protective effects were evaluated in SH-SY5Y dopaminergic cell line exposed to 6-OHDA. As previously mentioned, this neurotoxin has been reported to induce neurodegeneration through the processing of hydrogen peroxide and hydroxyl radicals in the presence of iron, since once inside the neurons, the toxin accumulates and undergoes non-enzymatic auto-oxidation, leading to the formation of ROS (Bladini *et al*., 2008). In this study, the exposition of SH-SY5Y cells to 6-OHDA neurotoxin led to a reduction of cell viability around 50%. It also resulted in a significant increase of ROS levels (11%), mitochondrial membrane potential depolarization (47%) and Caspase 3 activity (157%). On the other hand, through the incubation of SH-SY5Y cells with 6-OHDA in the presence of non-toxic concentrations of compounds, it was possible to observe a protective effect against the adverse effects of 6-OHDA mediated by 12*R*-hydroxy-bromosphaerol at 3, 1, and 0.3 μM concentrations (Figures 12).

The treatment accomplished with alloaromadendrene and sphaerococcenol A did not showed any recover in the neurotoxicity induced by 6-OHDA. Similar results were obtained for all compounds when they were pre-incubated for 1 h before exposition to 6-OHDA. No significant differences were observed when they were compared with 6-OHDA treatment.

Regarding the results attained for 12*R*-hydroxy-bromosphaerol concentrations, they are in agreement with previous results reported to tanshionone, an abietane diterpenoid, obtained from *Salvia miltiorrhiza*, which demonstrated a significant neuroprotection of SH-SY5Y cells against 6-OHDA - induced neurotoxicity (Wang *et al*., ,2015). Likewise, eleganolone, a diterpene isolated from the brown seaweed *Bifurcaria bifurcata* has been reported to exhibit neuroprotective effects, when incubated in presence of 6-OHDA in SH-SY5Y cells, resulting in an increase of cell viability of 18.28% and 25.53%, when tested at 0.5 and 1 μM, respectively (Silva *et al*.,2019).

Concerning the results obtained for the pre-incubation approach with the *S. coronopifolius* compounds prior to 6-OHDA exposition, no significant neuroprotection was observed. Nonetheless, these results should be interpretated carefully. In this aspect further assays with longer periods of pre-incubation should be considered, aiming to determine if pre-incubation time plays a significant role in the neuroprotective effects. Moreover, upregulation of intrinsic antioxidant pathways may be induced only in an oxidative stress conditions. As a matter of fact, it has been reported that the expression of representative phase II detoxifying enzymes such as GPx or SOD, results mainly from the activation mediated by Nrf2 transcription factor and its interaction with the antioxidant response element (ARE) (Na *et al*.,2008;Kong et al.,2001). Furthermore, Nrf2 transcription factor is also related with the expression of antioxidant enzymes caused by oxidative stress (Ishii *et*

al., 2000). Thus, an interesting question that should be assessed in further studies is to understand the mechanisms involved in pre-incubation with our compounds before 6-OHDA exposition, for an external regulation of antioxidant mechanism may occur only after stress conditions.

Nonetheless, as 12*R*-hydroxy-bromosphaerol was the only compound that demonstrated neuroprotective potential, its effects on the different mechanisms related with PD development were studied. As earlier discussed, oxidative stress is believed to play an important role in neurodegenerative diseases like PD, since mitochondrial disfunctions like complex I deficiencies, leads to ROS production and thus stimulates several processes leading to cell death. Furthermore, auto-oxidation of 6-OHDA produce p-quinone, leading to oxidative stress condition, triggering apoptotic mediated cell death (Izumi *et al*.,2005). Thus, considering these facts and aiming to understand if the effects mediated by 12*R*-hydroxy-bromosphaerol are related with those intracellular signalling pathways, the ROS levels were measured. As previously shown in Figure 15, when ROS production was assessed on SH-SY5Y cells exposed to 6-OHDA in the presence of 1 μM 12*R*-hydroxy-bromosphaerol, it was possible to observe a significative reduction (8.8%) of ROS levels when compared to 6-OHDA. Since the compounds exhibited weak antioxidant capacity by the chemical methods directly related with the neutralization of radicals, the results observed suggest that 12*R*-hydroxy-bromosphaerol neuroprotective activities may be related with the upregulation of inner antioxidant pathways like Nrf2/ARE, SOD, CAT and GPx, preventing the increase of ROS levels. In literature, similar results were reported for the diterpene tanshinone (Wang et al.,2015), where its antioxidant activity was associated with the regulation of the Nrf2/ARE pathway, which plays an important role in the resistance to oxidative stress condition (Ma, 2013). In fact, quite recently isolated terpenoids from *Zea mays*, have been reported to decrease induced-oxidative stress by the upregulation of the Nrf2 pathway, a neuroprotective effect that was reversed when Nfr2 was silenced (Song *et al*.,2020). Furthermore, although using gallic acid instead of terpenes, similar results and conclusions have been reported with SH-SY5Y cells treated with 6-OHDA, where gallic acid was able to reduce the levels of ROS production and upregulated Nrf2 levels (Chandrasekhar *et al*.,2018).

As for the mitochondrial membrane potential assay, which aimed to determine mitochondrial membrane depolarisation of SH-SY5Y cells after 6-OHDA exposition, results showed that 0.3, 1, and 3 μM 12*R*-hydroxy-bromosphaerol induced a significant reduction of the mitochondrial membrane potential of 24.23%, 13.53% and 5.56% respectively when compared with 6-OHDA treatment. These results are particularly interesting in the case of

1 μM 12*R*-hydroxy-bromosphaerol, which also presented capacity to reduce the production of ROS, thus manifesting a likely mitochondrial membrane potential protection from 6-OHDA neurotoxicity. Evidence of a relation between ROS production and membrane depolarisation has been reported before, since mitochondrial ROS production is particularly sensitive to changes in mitochondrial membrane potential (Starkov *et al*.,2003; Korshubov *et al*.,1997). Furthermore, mitochondrial depolarisation also compromises oxidative phosphorylation and consequently the production of ATP. All those events prolonged in the time can lead to cellular death (Zorova *et al* 2018), thus justifying the relevance of this biomarker as one the first key points leading to cell death by intrinsic apoptosis pathway. Moreover, similar results were also reported for the fractions extracted from *B. bifurcata* seaweed (Silva *et al.,*2019), and extracts from *C. tomentosum* and *U. compressa*, that displayed a preventive effect in the mitochondrial depolarisation induced by 6-OHDA on SH-SY5Y cells (Silva *et al*.,2018).

Following this, several reports have demonstrated that neuronal cell death in PD is associated with the release of cytrochome C and consequently, with the activation of Caspase 3, which plays an essential role in the process of cell death by apoptosis (Kristian *et al*.,2011; Latchoumycandane *et al*., 2011). Concerning Caspase-3 activity, 0.3 μM 12*R*-hydroxy-bromosphaerol was the concentration that exhibited the highest capability to reduce in 44.35% the stimulating effects of 6-OHDA on Caspase-3 activity. Moreover, even if no significant, concentrations 3 and 1 μM also showed a relevant decrease of Caspase-3 activity of 23.56 and 7.52%, respectively. Moreover, as previously discussed, 6-OHDA treatment has been widely used for studies on PD due to its effects, which include, upregulating the intrinsic apoptotic pathways (Li *et al.,*2011), and thus, assessing the activity of Caspase-3 combined with MMP and ROS production allows to obtain relevant data to understand the ability of compounds to act in those mechanisms that seems to play a critical role in the PD disease development. These results are in agreement with previous reports of marine extracts from *C. tomentosum, S. polyschides, P. pavonica and U. compressa* seaweeds which were able to inhibit the activity of Caspase-3 activity stimulated by 6-OHDA treatment (Silva *et al*.,2018), pointing out the interesting potential of seaweeds components as antiapoptotic agents. Furthermore, our results are also in agreement with results reported for the eleganolone diterpene isolated from *B. bifurcaria* brown seaweed, which also exhibited ability to decrease Caspase-3 activity in 76% and 75% when tested at 0.5 μM and 1 μM, respectively. According with results attained the hypothesized mechanism of action of 12*R*-hydroxy-bromosphaerol was illustrated in the Figure 17.

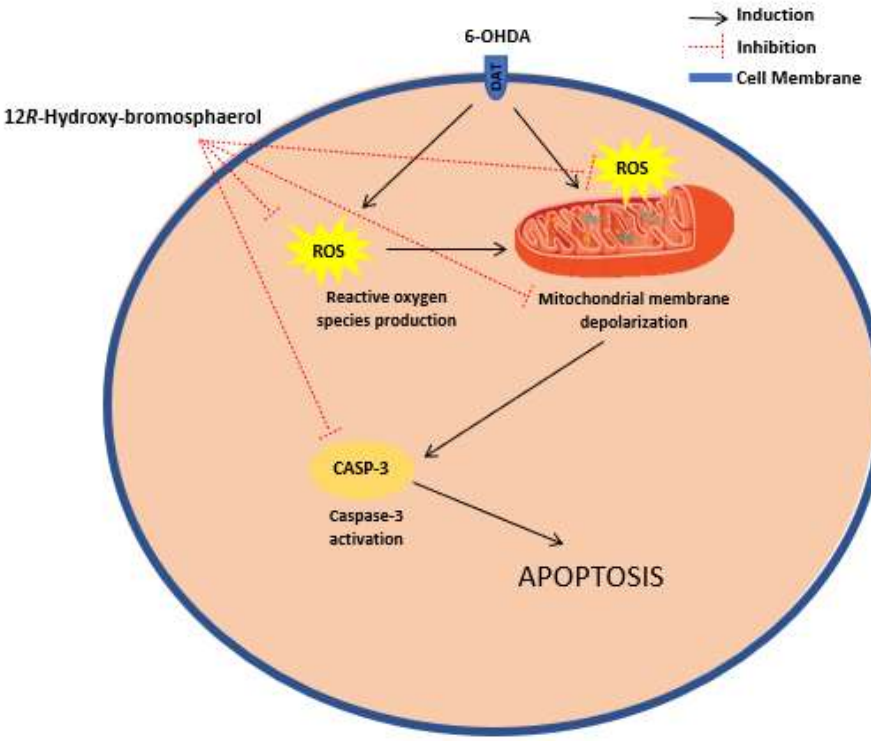


Figure 17. Possible mechanism of action underlying the neuroprotective effect of the 12*R*-hydroxy-bromosphaerol isolated from the red alga *Sphaerococcus coronopifolius* against the 6-OHDA - induced neurotoxicity on SH-SY5Y cells.

In conclusion, the work developed in the present thesis allowed to isolate four compounds already described in the literature from *Sphaerococcus coronopifolius* red seaweed, namely alloaromadendrene, sphaerococcenol A, 12*S*-hydroxy-bromosphaerol and 12*R*-hydroxy-bromosphaerol**.**

Regarding biological activities, 12*R*-hydroxy-bromosphaerol, alloaromadendrene, and sphaerococcenol A exhibited a weak antioxidant activity determined by DPPH, ORAC and FRAP chemical methods. Concerning neuroprotection, among the three terpenes studied, only 12*R*-hydroxy-bromosphaerol displayed ability to protect SH-SY5Y cells viability against neurotoxicity induced by the 6-OHDA neurotoxin.

In view of the already known adverse effects of 6-OHDA treatment in neuronal dopaminergic cells, the neuroprotective effects of 12*R*-hydroxy-bromosphaerol seem to be related with the prevention of ROS production, the prevention of mitochondrial membrane depolarization and the inhibition of Caspase-3 activity. Accordingly, further experimental

assays should be considered to understand the actual therapeutic potential of 12*R*-hydroxy-bromosphaerol as neuroprotective agent to Parkinson's Disease treatment.

Future Perspectives

6. Future Perspectives

Nowadays, marine derived secondary metabolites proceeding from seaweeds have aroused the interest of the scientific community due to their therapeutic potential, which may represent new opportunities to inspire the development of new drugs to treat human diseases. Accordingly, the present study was based on the assessment of the antioxidant and neuroprotective capabilities of three terpenes isolated from the red alga *S. coronopifolius*. Nonetheless this study represents one the first steps in a longer way aimed to understand the actual potential of these compounds as effective therapeutic agents in the treatment of neurodegenerative diseases like PD.

Likewise, as due to timing and low purity the neuroprotective and antioxidant activities of the compound 12*S*-hydroxy-bromosphaerol were not assessed in this report, another interesting study should be considered to determine its biological activities. Furthermore, regarding the great potential displayed by *S. coronopifolius* as a source of bioactive compounds and considering that most of them have never been tested for their neuroprotective capabilities the isolation of more molecules from this red seaweed remains as an interesting area of study that should be assessed in the near future.

On the other hand, it should also be relevant to assess other mechanisms related with PD that have not been evaluated in this report, for instance, downregulation and upregulation of other apoptotic biomarkers like the Bax/BcL-2 ratio. Likewise, considering the antioxidant assay results, one proposition could be the use of different antioxidants assays with more relevancy inside the cells. In this aspect, biological assays more able to represent the intracellular mechanisms involved in the inner antioxidant machinery should be performed. For instance, the upregulation and downregulation of antioxidant pathways like SOD, CAT, GPx and Nfr2 transcriptional factor should be interesting to evaluate both at mRNA and protein expression. In fact, the response of the mentioned pathways represents an important asset against ROS production and thus, external molecules able to modulate their activity may be interesting assets to fight neurodegeneration triggered by oxidative stress.

On the other hand, regarding PD models, a first step to determine its effectiveness on more alike *in vitro* assays would be to differentiate the SH-SYSY cells with retinoic acid, mimicking the neuronal cells phenotype that occurs *in vivo*. Likewise, another interesting approach would be to move forward from monoculture cell systems to 3D cellular models. *In vitro* assays in 3D cellular models represent a more alike condition to that happening in

the human brain, where cells are not necessarily flattened and are able to grow in 3D shape, establishing connections with other cells and been completely surrounded by the culture medium, resulting in a physiologically model more relevant to validate the therapeutic potential of the compounds. A third step would be to use organoids cultures, a more complex system able to accurately represent the complex interdomains between the intraneuronal system and its connections with other elements and tissues of the body.

Likewise, it is important to assess if our compounds are able to pass through the blood brain barrier, which prevents molecules circulating in the blood to enter the central nervous system where dopaminergic cells are located. For instance, In vitro studies with brain endothelial cell lines co-cultured with astrocytes have reported the successful diffusion of many flavonoids (Solanki *et al*.,2015). On the other hand, if the compound were not able to pass through the blood brain barrier, the development of nanoparticles carriers, like polymeric nanoparticles, which enables to encapsulate a wide variety of therapeutics including chemotherapeutic drugs represents an interesting focus of drug delivery research, particularly to the brain (Hersh et al.,2016; Alan *et al*.,2010).

Finally, as a “proof-of-concept”, rat models treated with the 6-OHDA toxin, should be considered to obtain direct knowledge of the different cognitive enhancements that can be related with the potential neuroprotective activity of the compounds evaluated. For instance, behavioural *in vivo* assays like the open-field test and the rotarod-test could be performed.

References

7.References

Aarli J. A., Dua T., Janca A., Muscetta A., (2006). Neurological Disorders. Public Health Challenges. World Health Organization.

Alam, M. I., Beg, S., Samad, A., Baboota, S., Kohli, K., Ali, J., ... & Akbar, M. (2010). Strategy for effective brain drug delivery. *European Journal of Pharmaceutical Sciences*, 40(5), 385-403. Doi: 10.1016/j.ejps.2010.05.003

Alarif, W. M., Al-Lihaibi, S. S., Ayyad, S. E. N., Abdel-Rhman, M. H., & Badria, F. A. (2012). Laurene-type sesquiterpenes from the Red Sea red alga *Laurencia obtusa* as potential antitumor–antimicrobial agents. *European Journal of Medicinal chemistry*, *55*, 462-466. Doi: 10.1016/j.ejmech.2012.06.060.

ALENCAR, D. B., SILVA, S. R., Pires-Cavalcante, K., LIMA, R. L., Pereira Junior, F. N., SOUSA, M. B., ... & Sampaio, A. H. (2014). Antioxidant potential and cytotoxic activity of two red seaweed species, *Amansia multifida* and *Meristiella echinocarpa*, from the coast of Northeastern Brazil. *Anais da Academia Brasileira de Ciências*, *86*(1), 251-263.Doi: 10.1590/0001-37652014116312.

Alves, C., Serrano, E., Silva, J., Rodrigues, C., Pinteus, S., Gaspar, H., ... & Pedrosa, R. (2020). *Sphaerococcus coronopifolius* bromoterpenes as potential cancer stem cell-targeting agents. *Biomedicine & Pharmacotherapy*, *128*, 110275.Doi: 10.1016/j.biopha.2020.110275

Alves, C., Pinteus, S., Horta, A., & Pedrosa, R. (2016). High cytotoxicity and anti-proliferative activity of algae extracts on an *in vitro* model of human hepatocellular carcinoma. *Springer Plus*, *5*(1), 1339. Doi: 10.1186/s40064-016-2938-2

Am, O. B., Amit, T., & Youdim, M. B. (2004). Contrasting neuroprotective and neurotoxic actions of respective metabolites of anti-Parkinson drugs rasagiline and selegiline. *Neuroscience Letters*, 355(3), 169-172. Doi: 10.1016/j.neulet.2003.10.067.Ananthi, S., Raghavendran, H. R. B.,

Sunil, A. G., Gayathri, V., Ramakrishnan, G., & Vasanthi, H. R. (2010). *In vitro* antioxidant and in vivo anti-inflammatory potential of crude polysaccharide from *Turbinaria ornata* (Marine Brown Alga). *Food and Chemical Toxicology*, *48*(1), 187-192. Doi: 10.1016/j.fct.2009.09.036.

Ames, B. N., Shigenaga, M. T., & Hagen, M. (1993). Oxidants, antioxidants and the degenerative and pharmacological evidence for the involvement of oxygen radicals and lipid peroxidation. Free Radical Biology and Medicine, *6*, 303-313.

Andrew, R., Watson, D. G., Best, S. A., Midgley, J. M., Wenlong, H., & Petty, R. K. H. (1993). The determination of hydroxydopamines and other trace amines in the urine of Parkinsonian patients and normal controls. *Neurochemical Research*, *18*(11), 1175-1177.Doi: 10.1007/BF00978370.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nature Reviews Cancer*, *2*(6), 420. Doi: 10.1038/nrc821.

Bender, A., Krishnan, K. J., Morris, C. M., Taylor, G. A., Reeve, A. K., Perry, R. H., ... & Taylor, R. W. (2006). High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nature Genetics, 38(5), 515*. Doi: 10.1038/ng1769.

Bentayeb, K., Vera, P., Rubio, C., & Nerín, C. (2014). The additive properties of Oxygen Radical Absorbance Capacity (ORAC) assay: The case of essential oils. *Food Chemistry, 148, 204-208*. Doi: 10.1016/j.foodchem.2013.10.037.

Benzie, I. F., & Strain, J. J. (1999). [2] Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *In Methods in Enzymology (Vol. 299, pp. 15-27)*. Academic press.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. L. W. T. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology*, *28*(1), 25-30. Doi: 10.1016/S0076-6879(99)99005-5.

Bhosale, P. B., Ha, S. E., Vetrivel, P., Kim, H. H., Kim, J. A., Park, K. I., ... & Kim, G. S. (2020). Flavonoid-induced apoptotic cell death in human cancer cells and its mechanisms. *Journal of Biomedical and Translational Research (JBTR*), 21(2), 50-58. Doi: 10.12729/jbtr.2020.21.2.050

Bonifati, V., Rohe, C. F., Breedveld, G. J., Fabrizio, E., De Mari, M., Tassorelli, C., ... & Fincati, E. (2005). Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes. *Neurology*, *65*(1), 87-95.Doi: 10.1212/01.wnl.0000167546.39375.82.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. L. W. T. (1995). Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology, *28*(1), 25-30.

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Cepero, E., & Boise, L. H. (2013). Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Mollecular and Cell Biology, *14*(1), 32. Doi: 10.1186/1471-2121-14-32.

Brewer, M. S. (2011). Natural antioxidants: sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety*, *10*(4), 221-247. Doi: 10.1111/j.1541-4337.2011.00156.x.

Bové, J., & Perier, C. (2012). Neurotoxin-based models of Parkinson's disease. *Neuroscience*, *211*, 51-76. Doi: 10.1016/j.neuroscience.2011.10.057.

Buendia, I., Michalska, P., Navarro, E., Gameiro, I., Egea, J., & León, R. (2016). Nrf2–ARE pathway: an emerging target against oxidative stress and neuroinflammation in neurodegenerative diseases. *Pharmacology & therapeutics*, *157*, 84-104. Doi: 10.1016/j.pharmthera.2015.11.003.

Cafieri, F., De Napoli, L., Fattorusso, E., & Santacroce, C. (1987). Diterpenes from the red alga *Sphaerococcus coronopifolius*. *Phytochemistry*, *26*(2), 471-473.Doi:10.1016/S0031-9422(00)81435-1

Carradori, S., Secci, D., Bolasco, A., Chimenti, P., & D'Ascenzio, M. (2012). Patent-related survey on new monoamine oxidase inhibitors and their therapeutic potential. *Expert Opinion on Therapeutic Patents*, *22*(7), 759-801. Doi: 10.1517/13543776.2012.698613.

Camilleri, A., Zarb, C., Caruana, M., Ostermeier, U., Ghio, S., Högen, T., ... & Vassallo, N. (2013). Mitochondrial membrane permeabilisation by amyloid aggregates and protection by polyphenols. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1828*(11), 2532-2543. Doi: 10.1016/j.bbamem.2013.06.026.

Cha, S. H., Heo, S. J., Jeon, Y. J., & Park, S. M. (2016). Dieckol, an edible seaweed polyphenol, retards rotenone-induced neurotoxicity and α-synuclein aggregation in human dopaminergic neuronal cells. *RSC Advances*, *6*(111), 110040-110046. Doi: 10.1039/C6RA21697H.

Castell, J. V., & Gmez-Lechn, M. J. (Eds.). (1996). In vitro methods in pharmaceutical research. Elsevier.

Chandrasekhar, Y., Kumar, G. P., Ramya, E. M., & Anilakumar, K. R. (2018). Gallic acid protects 6-OHDA induced neurotoxicity by attenuating oxidative stress in human dopaminergic cell line. *Neurochemical Research*, *43*(6), 1150-1160. Doi: 10.1007/s11064-018-2530-y.

Chen, W. L., Qian, Y., Meng, W. F., Pang, J. Y., Lin, Y. C., Guan, Y. Y., ... & Wang, G. L. (2009). A novel marine compound xyloketal B protects against oxidized LDL-induced cell injury *in vitro*. *Biochemical Pharmacology*, *78*(8), 941-950. Doi: 10.1016/j.bcp.2009.05.029.

Chen, W. F., Chakraborty, C., Sung, C. S., Feng, C. W., Jean, Y. H., Lin, Y. Y., ... & Sung, P. J. (2012). Neuroprotection by marine-derived compound, 11-dehydrosinulariolide, in an *in vitro* Parkinson's model: A promising candidate for the treatment of Parkinson's disease. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *385*(3), 265-275.Doi: 10.1007/s00210-011-0710-2.

Chew, Y. L., Lim, Y. Y., Omar, M., & Khoo, K. S. (2008). Antioxidant activity of three edible seaweeds from two areas in South East Asia. *LWT-Food Science and Technology*, *41*(6), 1067-1072. Doi: 10.1016/j.lwt.2007.06.013.

Cochran, W. G. (1951). Testing a linear relation among variances. *Biometrics*, 7(1), 17-32. Doi: 10.2307/3001601.

Córdova-Guerrero, I., San Andrés, L., Leal-Orozco, A. E., Padrón, J. M., Cornejo-Bravo, J. M., & León, F. (2013). New strategy toward the diverted synthesis of oxidized abietane diterpenes via oxidation of 6, 7-dehydroferruginol methyl ether with dimethyldioxirane. *Tetrahedron Letters*, *54*(33), 4479-4482. Doi: 10.1016/j.tetlet.2013.06.048.

da Silva Machado, F. L., Pacienza-Lima, W., Rossi-Bergmann, B., de Souza Gestinari, L. M., Fujii, M. T., de Paula, J. C., ... & Soares, A. R. (2011). Antileishmanial sesquiterpenes from the Brazilian red alga *Laurencia dendroidea*. *Planta médica*, *77*(07), 733-735.

Dalle-Donne, I., Rossi, R., Colombo, R., Giustarini, D., & Milzani, A. (2006). Biomarkers of oxidative damage in human disease. *Clinical Chemistry*, *52*(4), 601-623.Doi: 10.1373/clinchem.2005.061408.

Dávalos, A., Gómez-Cordovés, C., & Bartolomé, B. (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC− fluorescein) assay. *Journal of Agricultural and Food Chemistry*, *52*(1), 48-54. Doi: 10.1021/jf0305231.

de Oliveira, M. R. (2016). The dietary components carnosic acid and carnosol as neuroprotective agents: a mechanistic view. *Molecular neurobiology*, *53*(9), 6155-6168. Doi: 10.1007/s12035-015-9519-1.

de Rosa, S., de Stefano, S., Scarpelli, P., & Zavodnik, N. (1988). Terpenes from the red alga *Sphaerococcus coronopifolius* of the north Adriatic Sea. *Phytochemistry*, *27*(6), 1875-1878. Doi: 10.1016/0031-9422(88)80468-0.

De Souza, É. T., Pereira de Lira, D., Cavalcanti de Queiroz, A., Costa da Silva, D. J., Bezerra de Aquino, A., Mella, C., ... & Xavier, J. (2009). The antinociceptive and anti-inflammatory activities of caulerpin, a bisindole alkaloid isolated from seaweeds of the genus *Caulerpa*. *Marine Drugs*, *7*(4), 689-704. Doi: 10.3390/md7040689.

Devi, K. P., Suganthy, N., Kesika, P., & Pandian, S. K. (2008). Bioprotective properties of seaweeds: in vitro evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. *BMC Complementary and Alternative Medicine*, *8*(1), 38. Doi: 10.1186/1472-6882-8-38.

Dias, V., Junn, E., & Mouradian, M. M. (2013). The role of oxidative stress in Parkinson's disease. Journal of Parkinson's disease, 3(4), 461-491. Doi: 10.3233/JPD-130230

Di Domenico, F., Barone, E., Perluigi, M., & Butterfield, D. A. (2015). Strategy to reduce free radical species in Alzheimer's disease: an update of selected antioxidants. *Expert Review of Neurotherapeutics*, *15*(1), 19-40. Doi: 10.1586/14737175.2015.955853.

Dolk, V. S., Borgers, D. P., & Heemels, W. P. M. H. (2016). Output-Based and Decentralized Dynamic Event-Triggered Control With Guaranteed Gain Performance and Zeno-Freeness. *IEEE Transactions on Automatic Control*, *62*(1), 34-49. Doi: 10.1109/TAC.2016.2536707.

Dunnett, C. W., & Tamhane, A. C. (1991). Step-down multiple tests for comparing treatments with a control in unbalanced one-way layouts. *Statistics in Medicine*, *10*(6), 939-947. Doi: 10.1002/sim.4780100614.

Dutta, D., & Mohanakumar, K. P. (2015). Tea and Parkinson's disease: Constituents of tea synergize with antiparkinsonian drugs to provide better therapeutic benefits. *Neurochemistry International*, *89*, 181-190. Doi: 10.1016/j.neuint.2015.08.005.

Duty, S., & Jenner, P. (2011). Animal models of Parkinson's disease: a source of novel treatments and clues to the cause of the disease. *British Journal of Pharmacology*, *164*(4), 1357-1391. Doi: 10.1111/j.1476-5381.2011.01426.x.

Element, A. R. (2004). An important role of Nrf2-ARE pathway in the cellular defence mechanism. J Biochem Mol Biol, 37(2), 139-143.

Erickson, K. L., & Scheuer, P. J. (1983). Marine natural products: chemical and biological perspectives.

Etahiri, S., Bultel-Poncé, V., Caux, C., & Guyot, M. (2001). New bromoditerpenes from the red alga *Sphaerococcus coronopifolius*. *Journal of natural products*, *64*(8), 1024-1027.Doi: 10.1021/np0002684

Fahn, S. (2008). The history of dopamine and levodopa in the treatment of Parkinson's disease. *Movement Disorders: Official Journal of the Movement Disorder Society*, *23*(S3), S497-S508. Doi: 10.1002/mds.22028.

Faure, R., Ramanoelina, A. R., Rakotonirainy, O., Bianchini, J. P., & Gaydou, E. M. (1991). Two-dimensional nuclear magnetic resonance of sesquiterpenes. Application to complete assignment of 1H and 13C NMR spectra of some aromadendrane derivatives. *Magnetic Resonance in Chemistry*, *29*(9), 969-971. Doi: 10.1002/mrc.1260290920.

Fellah, F., Louaileche, H., Dehbi-Zebboudj, A., & Touati, N. (2017). Seasonal variations in the phenolic compound content and antioxidant activities of three selected species of seaweeds from Tiskerth islet, Bejaia, Algeria. *J. Mater. Environ. Sci*, *8*(12), 4451-4456.

Feigin, V. L., Abajobir, A. A., Abate, K. H., Abd-Allah, F., Abdulle, A. M., Abera, S. F., ... & Aichour, M. T. E. (2017). Global, regional, and national burden of neurological disorders during 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet Neurology*, *16*(11), 877-897. Doi: 10.1016/S1474-4422(17)30299-5.

Fenical, W., Sims, J. J., Squatrito, D., Wing, R. M., & Radlick, P. (1973). Marine natural products. VII. Zonarol and isozonarol, fungitoxic hydroquinones from the brown seaweed *Dictyopteris zonarioides.* The Journal of Organic Chemistry, 38(13), 2383-2386. Doi: 10.1021/jo00953a022.

González-Cofrade, L., de Las Heras, B., Ticona, L. A., & Palomino, O. M. (2019). Molecular targets involved in the neuroprotection mediated by terpenoids. *Planta Medica*, *85*(17), 1304-1315.

Grimmig, B., Kim, S. H., Nash, K., Bickford, P. C., & Shytle, R. D. (2017). Neuroprotective mechanisms of astaxanthin: a potential therapeutic role in preserving cognitive function in age and neurodegeneration. *Geroscience*, *39*(1), 19-32. Doi: 10.1007/s11357-017-9958-x.

Guiry, M. D. (2010). AlgaeBase. Worldwide electronic publication, National University of Ireland, Galway. *http://www. algaebase. org/*.

Guzman, J. N., Sánchez-Padilla, J., Chan, C. S., & Surmeier, D. J. (2009). Robust pacemaking in substantia nigra dopaminergic neurons. *Journal of Neuroscience*, 29(35), 11011-11019. Doi: 10.1523/JNEUROSCI.2519-09.2009

Hartmann, A., Michel, P. P., Troadec, J. D., Mouatt-Prigent, A., Faucheux, B. A., Ruberg, M., ... & Hirsch, E. C. (2001). Is Bax a mitochondrial mediator in apoptotic death of dopaminergic neurons in Parkinson's disease?. *Journal of Neurochemistry*, *76*(6), 1785-1793.

Hartmann, A., Troadec, J. D., Hunot, S., Kikly, K., Faucheux, B. A., Mouatt-Prigent, A., ... & Hirsch, E. C. (2001). Caspase-8 is an effector in apoptotic death of dopaminergic neurons in Parkinson's disease, but pathway inhibition results in neuronal necrosis. *Journal of Neuroscience*, *21*(7), 2247-2255.

Hermida-Ameijeiras, Á., Méndez-Álvarez, E., Sánchez-Iglesias, S., Sanmartín-Suárez, C., & Soto-Otero, R. (2004). Autoxidation and MAO-mediated metabolism of dopamine as a potential cause of oxidative stress: role of ferrous and ferric ions. Neurochemistry International, 45(1), 103-116.

Hersh, D., S Wadajkar, A., B Roberts, N., G Perez, J., P Connolly, N., Frenkel, V., ... & J Kim, A. (2016). Evolving drug delivery strategies to overcome the blood brain barrier. *Current Pharmaceutical Design*, *22*(9), 1177-1193.

Hirsch, E. C., Brandel, J. P., Galle, P., Javoy-Agid, F., & Agid, Y. (1991). Iron and aluminum increase in the *substantia nigra* of patients with Parkinson's disease: an X-ray microanalysis. *Journal of Neurochemistry*, *56*(2), 446-451.Ikeda, Y., Tsuji, S., Satoh, A., Ishikura, M., Shirasawa, T., & Shimizu, T. (2008). Protective effects of astaxanthin on 6-hydroxydopamine-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Journal of Neurochemistry*, *107*(6), 1730-1740. Doi: 10.1111/j.1471-4159.1991.tb08170.x.

Hotelling, H. (1933). Analysis of a complex of statistical variables into principal components. *Journal of educational psychology*, *24*(6), 417.

Huang, C. Y., Kuo, C. H., & Chen, P. W. (2018). Compressional-puffing pretreatment enhances neuroprotective effects of fucoidans from the brown seaweed *Sargassum hemiphyllum* on 6-hydroxydopamine-induced apoptosis in SH-SY5Y cells. *Molecules*, *23*(1), 78. Doi: 10.3390/molecules23010078

Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., ... & Yamamoto, M. (2000). Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *Journal of Biological Chemistry*, *275*(21), 16023-16029.Doi: 10.1074/jbc.275.21.16023

Izumi, Y., Sawada, H., Sakka, N., Yamamoto, N., Kume, T., Katsuki, H., ... & Akaike, A. (2005). p-quinone mediates 6-hydroxydopamine-induced dopaminergic neuronal death and ferrous iron accelerates the conversion of p-quinone into melanin extracellularly. *Journal of neuroscience research*, *79*(6), 849-860. Doi: 10.1002/jnr.20282.

Jaisin, Y., Thampithak, A., Meesarapee, B., Ratanachamnong, P., Suksamrarn, A., Phivthong-ngam, L., ... & Sanvarinda, Y. (2011). Curcumin I protects the dopaminergic cell line SH-SY5Y from 6-hydroxydopamine-induced neurotoxicity through attenuation of p53-mediated apoptosis. *Neuroscience Letters*, *489*(3), 192-196. Doi: 10.1016/j.neulet.2010.12.014.

Jameson, G. N., & Linert, W. (2000). 6-Hydroxydopamine, Dopamine, and Ferritin: A Cycle of Reactions Sustaining Parkinson's Disease? *Oxidative Stress and Disease*, *5*, 247-272.

Jenner, P., & Olanow, C. W. (2006). The pathogenesis of cell death in Parkinson's disease. Neurology, 66(10 suppl 4), S24-S36. Doi: 10.1212/WNL.66.10_suppl_4.S24Jenner, P. (2004). Preclinical evidence for neuroprotection with monoamine oxidase-B inhibitors in Parkinson's disease. *Neurology*, *63*(7 suppl 2), S13-S22. Doi: 10.1212/WNL.63.7_suppl_2.S13.

Johri, A., & Beal, M. F. (2012). Mitochondrial dysfunction in neurodegenerative diseases. *Journal of Pharmacology and Experimental Therapeutics*, *342*(3), 619-630. Doi: 10.1124/jpet.112.192138.

Kim, J., Lee, H. J., & Lee, K. W. (2010). Naturally occurring phytochemicals for the prevention of Alzheimer's disease. *Journal of Neurochemistry*, *112*(6), 1415-1430. Doi: 10.1111/j.1471-4159.2009.06562.x.

Kindleysides, S., Quek, S. Y., & Miller, M. R. (2012). Inhibition of fish oil oxidation and the radical scavenging activity of New Zealand seaweed extracts. *Food Chemistry*, *133*(4), 1624-1631. Doi: 10.1016/j.foodchem.2012.02.068.

Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J., & Ashkenazi, A. (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity*, *12*(6), 611-620. Doi: 10.1016/S1074-7613(00)80212-5.

Kischkel, F. C., Lawrence, D. A., Tinel, A., LeBlanc, H., Virmani, A., Schow, P., & Ashkenazi, A. (2001). Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. *Journal of Biological Chemistry*, *276*(49), 46639-46646. Doi: 10.1074/jbc.M105102200.

Kong, A. N. T., Owuor, E., Yu, R., Hebbar, V., Chen, C., Hu, R., & Mandlekar, S. (2001). Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). *Drug Metabolism Reviews*, *33*(3-4), 255-271. Doi : 10.1081/DMR-120000652.

Korshunov, S. S., Skulachev, V. P., & Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS letters*, *416*(1), 15-18. Doi: 10.1016/S0014-5793(97)01159-9.

Kösel, S., Egensperger, R., von Eitzen, U., Mehraein, P., & Graeber, M. B. (1997). On the question of apoptosis in the parkinsonian substantia nigra. Acta neuropathologica, 93(2), 105-108.Doi:

Kovalevich, J., & Langford, D. (2013). Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. In *Neuronal Cell Culture* (pp. 9-21). Humana Press, Totowa, NJ. Doi: 10.1007/978-1-62703-640-5_2.

Kraytsberg, Y., Kudryavtseva, E., McKee, A. C., Geula, C., Kowall, N. W., & Khrapko, K. (2006). Mitochondrial DNA deletions are abundant and cause functional impairment in aged human *substantia nigra* neurons. *Nature Genetics*, *38*(5), 518. Doi: 10.1038/ng1778.

Kristian, T., Balan, I., Schuh, R., & Onken, M. (2011). Mitochondrial dysfunction and nicotinamide dinucleotide catabolism as mechanisms of cell death and promising targets for neuroprotection. *Journal of Neuroscience Research*, *89*(12), 1946-1955.

Kumaran, A., & Karunakaran, R. J. (2007). In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India. *LWT-Food Science and Technology*, *40*(2), 344-352.Doi: 10.1016/j.lwt.2005.09.011

Kurniasih, N., Milawati, H., Fajar, M., Hidayat, A. T., Abdulah, R., Harneti, D., ... & Azmi, M. N. (2018). Sesquiterpenoid compounds from the stembark of Aglaia minahassae (Meliaceae). *Molekul*, *13*(1), 56-62. Doi:10.20884/1.jm.2018.12.1.410

Latchoumycandane, C., Anantharam, V., Jin, H., Kanthasamy, A., & Kanthasamy, A. (2011). Dopaminergic neurotoxicant 6-OHDA induces oxidative damage through proteolytic activation of PKCδ in cell culture and animal models of Parkinson's disease. *Toxicology and applied pharmacology*, *256*(3), 314-323.Doi: 10.1016/j.taap.2011.07.021.

Lavrik, I. N., Golks, A., & Krammer, P. H. (2005). Caspases: pharmacological manipulation of cell death. *The Journal of Clinical Investigation*, *115*(10), 2665-2672. Doi: 10.1172/JCI26252.

LeBlanc, H., Lawrence, D., Varfolomeev, E., Totpal, K., Morlan, J., Schow, P., & Ashkenazi, A. (2002). Tumor-cell resistance to death receptor–induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nature Medicine*, *8*(3), 274. Doi: 10.1038/nm0302-274.

Lee, Y., Park, H. R., Chun, H. J., & Lee, J. (2015). Silibinin prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease via mitochondrial stabilization. *Journal of Neuroscience Research*, *93*(5), 755-765.

Lehmensiek, V., Tan, E. M., Liebau, S., Lenk, T., Zettlmeisl, H., Schwarz, J., & Storch, A. (2006). Dopamine transporter-mediated cytotoxicity of 6-hydroxydopamine in vitro depends on expression of mutant α-synucleins related to Parkinson's disease. *Neurochemistry International*, *48*(5), 329-340. Doi: 10.1016/j.neuint.2005.11.008.

Lesage, S., & Brice, A. (2009). Parkinson's disease: from monogenic forms to genetic susceptibility factors. *Human Molecular Genetics*, *18*(R1), R48-R59. Doi: 10.1093/hmg/ddp012.

Li, Y., Luo, F., Wei, L., Liu, Z., & Xu, P. (2011). Knockdown of glycogen synthase kinase 3 beta attenuates 6-hydroxydopamine-induced apoptosis in SH-SY5Y cells. *Neuroscience letters*, *487*(1), 41-46. Doi: 10.1016/j.neulet.2010.09.070.

Lin, Y., Wu, X., Feng, S., Jiang, G., Luo, J., Zhou, S., & Zsila, F. (2001). Five unique compounds: xyloketals from mangrove fungus *Xylaria sp*. from the South China Sea coast. *The Journal of Organic Chemistry*, *66*(19), 6252-6256. Doi: 10.1021/jo015522r.

Linsley, J. W., Reisine, T., & Finkbeiner, S. (2019). Cell death assays for neurodegenerative disease drug discovery. *Expert Opinion on Drug Discovery*, *14*(9), 901-913. Doi: 10.1080/17460441.2019.1623784.

Liu, Y. Y., Qin, F. Y., He, T. C., Xiong, Y. P., Yan, Y. M., & Cheng, Y. X. (2020). Structurally diverse terpenoids with neuroprotective activities from the resins of *Populus euphratica*. *Fitoterapia*, 104560.

Losada-Barreiro, S., & Bravo-Diaz, C. (2017). Free radicals and polyphenols: The redox chemistry of neurodegenerative diseases. *European Journal of Medicinal Chemistry*, *133*, 379-402. Doi: 10.1016/j.ejmech.2017.03.061.

Lu, Y., Li, P. J., Hung, W. Y., Su, J. H., Wen, Z. H., Hsu, C. H., ... & Sheu, J. H. (2011). Nardosinane sesquiterpenoids from the Formosan soft coral *Lemnalia flava*. *Journal of natural products*, *74*(2), 169-174. Doi: 10.1021/np100541a

Ma, S. Y., Ciliax, B. J., Stebbins, G., Jaffar, S., Joyce, J. N., Cochran, E. J. & Mufson, E. J. (1999). Dopamine transporter-immunoreactive neurons decrease with age in the human *substantia nigra*. *Journal of Comparative Neurology*, *409*(1), 25-37. Doi: 10.1002/(SICI)1096-9861(19990621)409:1<25::AID-CNE3>3.0.CO;2-E.

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annual review of pharmacology and toxicology*, *53*, 401-426.

Martínez-Lazcano, J. C., Boll-Woehrlen, M. C., Hernández-Melesio, M. A., Rubio-Osornio, M., Sánchez-Mendoza, M. A., & Ríos, C. (2010). Radicales libres y estrés oxidativo en las enfermedades neurodegenerativas. *Mensaje Bioquimico*, *34*, 43-59. Doi:

Mann, V. M., Cooper, J. M., Daniel, S. E., Srai, K., Jenner, P., Marsden, C. D., & Schapira, A. H. V. (1994). Complex I, iron, and ferritin in Parkinson's disease *substantia nigra*. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *36*(6), 876-881. Doi: 10.1002/ana.410360612.

Meenakshi, S., Umayaparvathi, S., Arumugam, M., & Balasubramanian, T. (2011). *In vitro* antioxidant properties and FTIR analysis of two seaweeds of Gulf of Mannar. *Asian Pacific Journal of Tropical Biomedicine*, *1*(1), S66-S70. Doi: 10.1016/S2221-1691(11)60126-3.

Meenakshi, S., Umayaparvathi, S., Saravanan, R., Manivasagam, T., & Balasubramanian, T. (2016). Neuroprotective effect of fucoidan from Turbinaria decurrens in MPTP intoxicated Parkinsonic mice. *International Journal of Biological Macromolecules*, *86*, 425-433. Doi: 10.1016/S2221-1691(11)60126-3.

Mellouk, Z., Benammar, I., Krouf, D., Goudjil, M., Okbi, M., & Malaisse, W. (2017). Antioxidant properties of the red alga Asparagopsis taxiformis collected on the North West Algerian coast. *Experimental and Therapeutic Medicine*, *13*(6), 3281-3290. Doi: 10.3892/etm.2017.4413.

Miller, D. M., Buettner, G. R., & Aust, S. D. (1990). Transition metals as catalysts of “autoxidation” reactions. *Free Radical Biology and Medicine*, *8*(1), 95-108. Doi: 10.1016/0891-5849(90)90148-C.

Mounir, M., & Salma, L. (2014). Evaluation of antioxidant capacity of methanol extract and its solvent fractions obtained from four Moroccan macro algae species*. European Science Journal* , *10*, 10-15.

Murray, M., Dordevic, A. L., Ryan, L., & Bonham, M. P. (2018). An emerging trend in functional foods for the prevention of cardiovascular disease and diabetes: Marine algal polyphenols. *Critical Reviews in Food Science and Nutrition*, *58*(8), 1342-1358.Doi: 10.1080/10408398.2016.1259209

Murugan, A. C., Karim, M. R., Yusoff, M. B. M., Tan, S. H., Asras, M. F. B. F., & Rashid, S. S. (2015). New insights into seaweed polyphenols on glucose homeostasis. *Pharmaceutical biology*, *53*(8), 1087-1097.Doi: 10.3109/13880209.2014.959615

Murugan, K., & Iyer, V. V. (2012). Antioxidant and antiproliferative activities of Marine Algae, *Gracilaria edulis* and *Enteromorpha lingulata*, from Chennai Coast. *International Journal of Cancer Research 8* (1), 15-26. Doi: 10.3923/ ijer.2012.15.26.

Na, H. K., & Surh, Y. J. (2008). Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. *Food and Chemical Toxicology*, *46*(4), 1271-1278. Doi: 10.1016/j.fct.2007.10.006

Nadege, B., Patrick, L., & Rodrigue, R. (2009). Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis. *Frontiers in Bioscience*, *14*(11), 4015-4034. Falta Doi

Nagatsu, T., Nakashima, A., Ichinose, H., & Kobayashi, K. (2019). Human tyrosine hydroxylase in Parkinson’s disease and in related disorders. *Journal of Neural Transmission*, *126*(4), 397-409. Doi: 10.1007/s00702-018-1903-3.

Nagley, P., Higgins, G. C., Atkin, J. D., & Beart, P. M. (2010). Multifaceted deaths orchestrated by mitochondria in neurones. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1802(1), 167-185. Doi: 10.1016/j.bbadis.2009.09.004

Narendra, D. P., & Youle, R. J. (2011). Targeting mitochondrial dysfunction: role for PINK1 and Parkin in mitochondrial quality control. *Antioxidants & Redox Signaling*, *14* (10), 1929-1938. Doi: 10.1089/ars.2010.3799.

Nataraj, J., Manivasagam, T., Justin Thenmozhi, A., & Essa, M. M. (2017). Neuroprotective effect of asiatic acid on rotenone-induced mitochondrial dysfunction and oxidative stress-mediated apoptosis in differentiated SH-SYS5Y cells. *Nutritional Neuroscience*, *20*(6), 351-359. Doi: 10.1080/1028415X.2015.1135559.

Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry*, *49*(10), 4619-4626. Doi: 10.1021/jf010586o.

Ozcan, A., & Ogun, M. (2015). Biochemistry of reactive oxygen and nitrogen species. *Basic Principles and Clinical Significance of Oxidative Stress*, *3*, 37-58. Doi: 10.5772/ 61193.

Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. M., Kumar, V., & Kharbanda, S. (2000). Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of procaspase-9 by heat shock protein 90. *The EMBO Journal*, *19*(16), 4310-4322. Doi: 10.1093/emboj/19.16.4310.

Pangestuti, R., & Kim, S. K. (2011). Neuroprotective effects of marine algae. *Marine Drugs*, *9*(5), 803-818. Doi: 10.1016/j.jff.2011.07.001.

Park, J. H., kyu Park, O., Cho, J. H., Chen, B. H., Kim, I. H., Ahn, J. H., ... & Hwang, I. K. (2014). Anti-inflammatory effect of tanshinone I in neuroprotection against cerebral ischemia–reperfusion injury in the gerbil hippocampus. *Neurochemical research*, *39*(7), 1300-1312.Doi: 10.1007/s11064-014-1312-4.

Pavlin, M., Repič, M., Vianello, R., & Mavri, J. (2016). The chemistry of neurodegeneration: kinetic data and their implications. *Molecular Neurobiology*, *53*(5), 3400-3415. Doi: 10.1007/s12035-015-9284-1.

Piazza, V., Roussis, V., Garaventa, F., Greco, G., Smyrniotopoulos, V., Vagias, C., & Faimali, M. (2011). Terpenes from the red alga *Sphaerococcus coronopifolius* inhibit the settlement of barnacles. *Marine Biotechnology*, 13(4), 764-772. Doi: 10.1007/s10126-010-9337-4

Pinteus, S., Silva, J., Alves, C., Horta, A., Fino, N., Rodrigues, A. I., ... & Pedrosa, R. (2017). Cytoprotective effect of seaweeds with high antioxidant activity from the Peniche coast (Portugal). *Food Chemistry*, *218*, 591-599. Doi: 10.1016/j.foodchem.2016.09.067.

Pringsheim, T., Jette, N., Frolkis, A., & Steeves, T. D. (2014). The prevalence of Parkinson's disease: a systematic review and meta-analysis. *Movement Disorders*, *29*(13), 1583-1590. Doi: 10.1002/mds.25945.

Poewe, W. (2008). Non-motor symptoms in Parkinson's disease. *European Journal of Neurology*, *15*, 14-20. Doi: 10.1111/j.1468-1331.2008.02056.x.

Rackova, L., Oblozinsky, M., Kostalova, D., Kettmann, V., & Bezakova, L. (2007). Free radical scavenging activity and lipoxygenase inhibition of *Mahonia aquifolium* extract and isoquinoline alkaloids. *Journal of Inflammation*, *4*(1), 15. Doi: 10.1186/1476-9255-4-15.

Rajauria, G., Jaiswal, A. K., Abu-Ghannam, N., & Gupta, S. (2010). Effect of hydrothermal processing on colour, antioxidant and free radical scavenging capacities of edible Irish brown seaweeds. *International Journal of Food Science & Technology*, *45*(12), 2485-2493. Doi: 10.1111/j.1365-2621.2010.02449.x.

Reeve, A., Simcox, E., & Turnbull, D. (2014). Ageing and Parkinson's disease: why is advancing age the biggest risk factor?*Ageing Research Reviews*, *14*, 19-30. Doi: 10.1016/j.arr.2014.01.004.

Rhimou, B., Hassane, R., & Nathalie, B. (2013). Antioxidant activity of Rhodophyceae extracts from Atlantic and Mediterranean Coasts of Morocco. *African Journal of Plant Science*, *7*(3), 110-117. Doi: 10.5897/AJPS12.048.

Rodrigues, D., Alves, C., Horta, A., Pinteus, S., Silva, J., Culioli, G., ... & Pedrosa, R. (2015). Antitumor and antimicrobial potential of bromoditerpenes isolated from the red alga, *Sphaerococcus coronopifolius*. *Marine Drugs*, *13*(2), 713-726. Doi: 10.3390/md13020713.

Rodriguez-Pallares, J., Parga, J. A., Munoz, A., Rey, P., Guerra, M. J., & Labandeira-Garcia, J. L. (2007). Mechanism of 6-hydroxydopamine neurotoxicity: the role of NADPH oxidase and microglial activation in 6-hydroxydopamine-induced degeneration of dopaminergic neurons. *Journal of Neurochemistry*, *103*(1), 145-156. Doi: 10.1111/j.1471-4159.2007.04699.x.

Rodrigues, D., Alves, C., Horta, A., Thomas, O. P., & Pedrosa, R. (2013). Antibacterial and antitumor activity of isolated fractions of *Sphaerococcus coronopifolius* extracts. *Current Opinion in Biotechnology*, (24), S115-S116. Doi: 10.1016/j.copbio.2013.05.360.

Roleira, F. M., Tavares-da-Silva, E. J., Varela, C. L., Costa, S. C., Silva, T., Garrido, J., & Borges, F. (2015). Plant derived and dietary phenolic antioxidants: Anticancer properties. *Food Chemistry*, *183*, 235-258. Doi: 10.1016/j.foodchem.2015.03.039.

Rotter, B. A., & Oh, Y. N. (1996). MTS/PMS colorimetric assay is unsuitable for measuring mitogenic responses in porcine blood lymphocytes. *Journal of Immunological Methods*, *199*(2), 205-209. Doi: 10.1016/S0022-1759(96)00183-4.

Rotter, B. A., Thompson, B. K., Clarkin, S., & Owen, T. C. (1993). Rapid colorimetric bioassay for screening of Fusarium mycotoxins. Natural Toxins, 1(5), 303-307. Doi: 10.1002/nt.2620010509.

Sarrafchi, A., Bahmani, M., Shirzad, H., & Rafieian-Kopaei, M. (2016). Oxidative stress and Parkinson's disease: new hopes in treatment with herbal antioxidants. *Current Pharmaceutical Design*, *22*(2), 238-246.

Satoh, T., McKercher, S. R., & Lipton, S. A. (2013). Nrf2/ARE-mediated antioxidant actions of pro-electrophilic drugs. *Free Radical Biology and Medicine*, *65*, 645-657.

Sawant, S. S., Youssef, D. T., Sylvester, P. W., Wali, V., & Sayed, K. A. E. (2007). Antiproliferative sesquiterpenes from the Red Sea soft coral Sarcophyton glaucum. *Natural Product Communications*, *2*(2), 1934578X0700200201. Doi: 10.1177/1934578X0700200201.

Sayers, T. J. (2011). Targeting the extrinsic apoptosis signalling pathway for cancer therapy. *Cancer Immunology and Immunotherapy*, *60*(8), 1173-1180. Doi: 10.1007/s00262-011-1008-4.

Schapira, A. H. V., Cooper, J. M., Dexter, D., Clark, J. B., Jenner, P., & Marsden, C. D. (1990). Mitochondrial complex I deficiency in Parkinson's disease. *Journal of Neurochemistry*, *54*(3), 823-827. Doi: 10.1111/j.1471-4159.1990.tb02325.x.

Schapira, A. H. V., Mann, V. M., Cooper, J. M., Dexter, D., Daniel, S. E., Jenner, P., ... & Marsden, C. D. (1990). Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. *Journal of Neurochemistry*, *55*(6), 2142-2145. Doi: 10.1111/j.1471-4159.1990.tb05809.x.Schapira, A. H. (2008). Mitochondrial dysfunction in

neurodegenerative diseases. *Neurochemical Research*, *33*(12), 2502-2509. Doi: 10.1007/s11064-008-9855-x.

Sheeja Malar, D., & Pandima Devi, K. (2014). Dietary polyphenols for treatment of Alzheimer's disease–future research and development. *Current Pharmaceutical Biotechnology*, *15*(4), 330-342.

Sherer, T. B., Chowdhury, S., Peabody, K., & Brooks, D. W. (2012). Overcoming obstacles in Parkinson's disease. *Movement Disorders*, *27*(13), 1606-1611. Doi: 10.1002/mds.25260.

Silva, J., Alves, C., Pinteus, S., Mendes, S., & Pedrosa, R. (2018). Neuroprotective effects of seaweeds against 6-hydroxidopamine-induced cell death on an *in vitro* human neuroblastoma model. *BMC Complementary and Alternative Medicine*, *18*(1), 58. Doi: 10.1186/s12906-018-2103-2.

Silva, J., Alves, C., Freitas, R., Martins, A., Pinteus, S., Ribeiro, J., ... & Pedrosa, R. (2019). Antioxidant and Neuroprotective Potential of the Brown Seaweed *Bifurcaria bifurcata* in an in vitro Parkinson's Disease Model. *Marine drugs*, *17*(2), 85. Doi: 10.3390/md17020085.

Simmons, T. L., Andrianasolo, E., McPhail, K., Flatt, P., & Gerwick, W. H. (2005). Marine natural products as anticancer drugs. *Molecular Cancer Therapeutics*, *4*(2), 333-342.Falta Doi

Singleton, A. B., Farrer, M. J., & Bonifati, V. (2013). The genetics of Parkinson's disease: Progress and therapeutic implications. *Movement Disorders*, *28*(1), 14-23. Doi: 10.1002/mds.25249.

Smith, E. S., Clark, M. E., Hardy, G. A., Kraan, D. J., Biondo, E., Gonzalez-Lima, F., ... & Lee, H. J. (2017). Daily consumption of methylene blue reduces attentional deficits and dopamine reduction in a 6-OHDA model of Parkinson's disease. *Neuroscience*, *359*, 8-16. Doi: 10.1016/j.neuroscience.2017.07.001

Smyrniotopoulos, V., Quesada, A., Vagias, C., Moreau, D., Roussakis, C., & Roussis, V. (2008). Cytotoxic bromoditerpenes from the red alga *Sphaerococcus coronopifolius*. *Tetrahedron*, *64*(22), 5184-5190. Doi: 10.1016/j.tet.2008.03.042.

Smyrniotopoulos, V., Vagias, C., & Roussis, V. (2009). Sphaeroane and neodolabellane diterpenes from the red alga *Sphaerococcus coronopifolius. Marine Drugs*, *7*(2), 184-195. Doi: 10.3390/md7020184

Smyrniotopoulos, V., Vagias, C., Bruyère, C., Lamoral-Theys, D., Kiss, R., & Roussis, V. (2010). Structure and i*n vitro* antitumor activity evaluation of brominated diterpenes from the red alga *Sphaerococcus coronopifolius. Bioorganic & Medicinal Chemistry*, *18*(3), 1321-1330. Doi: 10.3390/md7020184.

Smyrniotopoulos, V., Vagias, C., Rahman, M. M., Gibbons, S., & Roussis, V. (2010). Structure and antibacterial activity of brominated diterpenes from the red alga *Sphaerococcus coronopifolius. Chemistry & Biodiversity*, *7*(1), 186-195. Doi: 10.1002/cbdv.200800309.

Smyrniotopoulos, V., Kiss, R., Mathieu, V., Vagias, C., & Roussis, V. (2015). Diterpenes with unprecedented skeletons from the red alga *Sphaerococcus coronopifolius*. *European Journal of Organic Chemistry*, *2015*(13), 2848-2853. Doi: 10.1002/ejoc.201500133.

Smyrniotopoulos, V., de Andrade Tomaz, A. C., Vanderlei de Souza, M. D. F., Leitão da Cunha, E. V., Kiss, R., Mathieu, V., ... & Roussis, V. (2020). Halogenated Diterpenes with *In Vitro* Antitumor Activity from the Red Alga *Sphaerococcus coronopifolius*. *Marine drugs*, *18*(1), 29.Doi: 10.3390/md18010029

Sokal, R. R., & Rohlf, F. J. (1995). The principles and practice of statistics in biological research. New York: Edition, 3.

Song, X. Y., Guo, R., Qi, X. L., Han, F. Y., Lin, B., Huang, X. X., ... & Song, S. J. (2020). Terpenoids from stigma maydis (Zea mays L.) alleviate hydrogen peroxide-induced SH-SY5Y cell injury by activating Nrf2. *Bioorganic Chemistry*, *102*, 104131.

Starkov, A. A., & Fiskum, G. (2003). Regulation of brain mitochondrial H2O2 production by membrane potential and NAD (P) H redox state. *Journal of neurochemistry*, *86*(5), 1101-1107. Doi: 10.1046/j.1471-4159.2003.01908.x.

Svensson, J. R., Nylund, G. M., Cervin, G., Toth, G. B., & Pavia, H. (2013). Novel chemical weapon of an exotic macroalga inhibits recruitment of native competitors in the invaded range. *Journal of Ecology*, *101*(1), 140-148. Doi: 10.1111/1365-2745.12028.

Tatton, W. G., Chalmers-Redman, R., Brown, D., & Tatton, N. (2003). Apoptosis in Parkinson's disease: signals for neuronal degradation. Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 53(S3), S61-S72.Doi: 10.1002/ana.10489

Tatton, N. A. (2000). Increased caspase 3 and Bax immunoreactivity accompany nuclear GAPDH translocation and neuronal apoptosis in Parkinson's disease. *Experimental neurology*, *166*(1), 29-43.

Team, R. C. (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2014. Teo, K. C., & Ho, S. L. (2013). Monoamine oxidase-B (MAO-B) inhibitors: implications for disease-modification in Parkinson's disease. *Translational Neurodegeneration*, *2*(1), 19. Doi: 10.1186/2047-9158-2-19.

Yuan, J. P., Peng, J., Yin, K., & Wang, J. H. (2011). Potential health-promoting effects of astaxanthin: a high-value carotenoid mostly from microalgae. *Molecular Nutrition & Food Research*, *55*(1), 150-165. Doi: 10.1002/mnfr.201000414.

Yurchenko, A. N., Smetanina, O. F., Ivanets, E. V., Kalinovsky, A. I., Khudyakova, Y. V., Kirichuk, N. N., ... & Afiyatullov, S. S. (2016). Pretrichodermamides D–F from a marine algicolous fungus *Penicillium* sp. KMM 4672. *Marine Drugs*, *14*(7), 122. Doi: 10.3390/md14070122.

Yurchenko, E. A., Menchinskaya, E. S., Pislyagin, E. A., Trinh, P. T. H., Ivanets, E. V., Smetanina, O. F., & Yurchenko, A. N. (2018). Neuroprotective activity of some marine fungal metabolites in the 6-hydroxydopamin-and paraquat-induced Parkinson's disease models. *Marine Drugs*, *16*(11), 457. Doi: 10.3390/md14070122.

Underwood, A. J. (1997). Experiments in ecology: their logical design and interpretation using analysis of variance. Cambridge University Press.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The international Journal of Biochemistry & Cell Biology*, *39*(1), 44-84. Doi: 10.1016/j.biocel.2006.07.001.

Varier, K. M., & Sumathi, T. (2019). Hinokitiol Offers Neuroprotection Against 6-OHDA-Induced Toxicity in SH-SY5Y Neuroblastoma Cells by Downregulating mRNA Expression of MAO/α-Synuclein/LRRK2/PARK7/PINK1/PTEN Genes. *Neurotoxicity Research*, *35*(4), 945-954.

Vijayabaskar, P., & Shiyamala, V. (2012). Antioxidant properties of seaweed polyphenol from *Turbinaria ornata* (Turner) J. Agardh, 1848. *Asian Pacific Journal of Tropical Biomedicine*, *2*(1), S90-S98. Doi: 10.1007/s12640-018-9988-x.

Villaño, D., Fernández-Pachón, M. S., Moyá, M. L., Troncoso, A. M., & García-Parrilla, M. C. (2007). Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta*, *71*(1), 230-235. Doi: 10.1016/j.talanta.2006.03.050.

Vladimir-Knežević, S., Blažeković, B., Bival Štefan, M., & Babac, M. (2012). Plant polyphenols as antioxidants influencing the human health. *Phytochemicals as Nutraceuticals–Global approaches to their role in nutrition and health*, 155-180.

Wang, X., Winter, D., Ashrafi, G., Schlehe, J., Wong, Y. L., Selkoe, D., & Schwarz, T. L. (2011). PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell*, *147*(4), 893-906. Doi: 10.1016/j.cell.2011.10.018.

Wang, Y., Xu, Z., Bach, S. J., & McAllister, T. A. (2009). Sensitivity of *Escherichia coli* to seaweed (*Ascophyllum nodosum*) phlorotannins and terrestrial tannins. *Asian-Australasian Journal of Animal Sciences*, *22*(2), 238-245. Doi: 10.5713/ajas.2009.80213

Wang, S., Jing, H., Yang, H., Liu, Z., Guo, H., Chai, L., & Hu, L. (2015). Tanshinone I selectively suppresses pro-inflammatory genes expression in activated microglia and prevents nigrostriatal dopaminergic neurodegeneration in a mouse model of Parkinson′ s disease. *Journal of Ethnopharmacology*, *164*, 247-255.

Waterborg (2002). The Lowry Method for Protein Quantitation – The Protein Protocols Handbook. Springer, Ed. 7º, pp: 7-9.

Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., & Korsmeyer, S. J. (2000). tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome C. *Genes & Development*, *14*(16), 2060-2071. Doi: 10.1101/gad.14.16.2060.

Xie, H. R., Hu, L. S., & Li, G. Y. (2010). SH-SY5Y human neuroblastoma cell line: *in vitro* cell model of dopaminergic neurons in Parkinson's disease. *Chinese Medical ournal*, *123*(8), 1086-1092. Doi: 10.3760/cma.j.issn.0366-6999.2010.08.021.

Xiong, Y., Ding, H., Xu, M., & Gao, J. (2009). Protective effects of asiatic acid on rotenone-or H 2 O 2-induced injury in SH-SY5Y cells. *Neurochemical Research*, *34*(4), 746-754. Doi: 10.1007/s11064-008-9844-0.

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., ... & Stoessl, A. J. (2004). Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron*, *44*(4), 601-607.

Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., ... & Sollott, S. J. (2018). Mitochondrial membrane potential. *Analytical biochemistry*, *552*, 50-59. Doi:10.1016/j.ab.2017.07.009.

Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. Physiological reviews, 94(3), 909-950. Doi: 10.1152/physrev.00026.2013

Annexes

8. Annexes

Annex I: Column chromatography data.

Table 6

Elution programme and recovered fractions from sample F CC-F8-34

Solvent	Recover Fractions	Mass recovered (mg)
Hexane (100%)	F1-2	-
	F3-4	34,1
	F5-7	17,6
Hexane/ Ethil Acetate	F8-9 (9/2)	-
	F10-12 (9/4)	-
	F13-16 (9/6)	-
Dichloromethane (100%)	F17-18	-

Table 7

Elution programme and recovered fractions from sample SC-P1

Solvent	Recover Fractions	Mass recovered (mg)
Hexane (100%)	F1-4	-
		-
		-
Hexane/ Ethil Acetate	F5-8 (9/1)	28,4
	F9-13 (8/2)	-
	F14-18 (7/3)	3,9
	F19-23 (6/4)	-
	F24-28(5/5)	-
		-
Ethil Acetate (100%)	F29-36	-
	F37-39 (9/2)	-
Dichloromethane (100%)	F40-43 (8/3)	-
	F44-45(7/4)	-