TESE DE DOUTORAMENTO

NEUROPROTECTIVE EFFECTS OF POTENT ANTIOXIDANT MOLECULES ISOLATED FROM *BIFURCARIA BIFURCATA* AND *CODIUM TOMENTOSUM* IN AN *IN VITRO* AND *IN VIVO* PARKINSON DISEASE MODEL

[Joana Rita Martins da Silva]

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NEUROPROTECTIVE EFFECTS OF POTENT ANTIOXIDANT MOLECULES ISOLATED FROM BIFURCARIA BIFURCATA AND CODIUM TOMENTOSUM IN AN IN VITRO AND IN VIVO PARKINSON DISEASE MODEL

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En Santiago de Compostela, 19 de Marzo de 2021

“Vivo sempre o presente.

O futuro, não o conheço.

O passado, já o não tenho.”

Fernando pessoa

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ABSTRACT

Keyworks: Marine natural products, Neurodegenerative disease, 6-OHDA, SH-SY5Y cells, RAW 264.7 cells, Oxidative stress, Mitochondrial dysfunction, Apoptosis, Inflammation.

The increase of life expectancy average has led to a growing of the elderly population, and thus leading to a prevalence of neurodegenerative disorders, such as Parkinson's disease (PD). Presently, there is no effective treatment for this disease becoming urgent to find new solutions to overcome the deleterious effects of neurodegeneration. Marine environment has proven to be a source of unique and diverse chemical structures with great therapeutical potential to be used in the treatment of several pathologies, including neurodegenerative impairments. Thus, the main objective of this thesis was to study the neuroprotective and anti-inflammatory bioactive potential of two macroalgae, *Bifurcaria bifurcata* and *Codium tomentosum,* collected from Peniche coastline (Portugal). Extracts and fractions of both seaweeds were submitted to a bio-guided chemical screening to access the most bioactive samples. These last ones were then subjected to further purification steps by preparative chromatographic methods (TLC, CC, HPLC) aiming the isolation of bioactive compounds. The structural elucidation was attained by nuclear magnetic resonance (NMR) and mass spectrometry (GC-MS) techniques. The cytotoxicity of samples (extracts, fractions, compounds) was estimated by the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Neuroprotection was evaluated in an *in vitro* model of PD induced by the 6-hydroxydopamine (6-OHDA) neurotoxin in human neuroblastoma cells (SH-SY5Y cells), while an inflammatory model induced by lipopolysaccharide (LPS) in macrophages RAW264.7 was used to evaluate the anti-inflammatory potential. In order to understand the intracellular signaling pathways involved in the neuroprotective activity, biomarkers associated with the oxidative stress, namely production of reactive oxygen species (ROS), production of hydrogen peroxide (H_2O_2) in real time, Catalase activity and translocation of the protein nuclear factor kappa B (NF-κB factor) to nucleus, as well mitochondrial dysfunction (mitochondrial membrane potential and ATP levels), and apoptosis (Caspase-3 activity and DNA condensation and /or fragmentation) were studied in SH-SY5Y exposed to 6-OHDA in the presence/ absence of seaweed-derived samples. Regarding the anti-inflammatory activity, the nitric oxide (NO) levels and the expression of proinflammatory (TNF-α, IL-6) and anti-inflammatory (IL-10)

cytokines was studied in RAW264.7 macrophages exposed to 6-OHDA in the presence/ absence of seaweed-derived samples.

Concerning the results, seven compounds were isolated and identified as eleganolone (**1**), eleganonal (**2**), bibiolone (**3**), 14,15-dihydro-eleganolone (**4**), fucosterol (**5**), mannitol (**6**) from *B. bifurcata***,** and loliolide (**7**) from *C. tomentosum*. However, only compounds with the best extraction yields (**1**, **2**, **5**, **6**, **7**) were evaluated regarding their neuroprotective and anti-inflammatory potential.

With the exception of compound **1**, all compounds (**2**, **5**, **6** and **7**) were not cytotoxic below 100 μM. Thus, compounds **2**, **5**, **6** and **7** were studied regarding their neuroprotective potential at a concentration range of 1-100 μM and compound **1** between 0.05 - 1 μM. Regarding the neuroprotection assay, only compounds **1** (0.1,0.5 and 1 μM; 24 h), **6** (50 and 100 μM; 24 h) and **7** (50 and 100 μM; 24 h) exhibited neuroprotective activity. Relating to the involved mechanisms of action, the treatment with the compounds eleganolone (**1**) and loliolide (**7**) prevented the increase of ROS production, increased Catalase activity, inhibited of the activation of the transcription of the NF-κB factor from the cytoplasm to the nucleus, decreased the mitochondrial dysfunction, and increased Caspase-3 activity promoted by 6-OHDA treatment. Additionally, they also exhibited anti-inflammatory activity decreasing the production of NO and pro-inflammatory interleukins (TNF-α, IL-6) levels promoted by the LPS treatment.

In summary, eleganolone (1 μM) and loliolide (100 μM) compounds isolated from the *Bifurcaria bifurcata* and *Codium tomentosum* seaweeds, respectively, exhibited neuroprotective activity, which seems to be mediated by a reduction of the oxidative stress condition, mitochondrial dysfunction and apoptosis. On the other hand, these compounds also displayed anti-inflammatory activity, that seems mediated by a decrease of NO production and pro-inflammatory interleukins (TNF-α and IL-6) levels. The results here presented constitute the first evidence of the neuroprotective potential of both seaweed metabolites. Therefore, further studies in more complex *in vitro* and *in vivo* models should be considered to validate their effectiveness as potential therapeutic agents for the prevention/treatment of Parkinson's disease.

RESUMO

Palavras – chave: Produtos naturais marinhos, Doenças neurodegenerativas, 6-OHDA, Células SH-SY5Y, Células RAW 264.7, Stress oxidativo, Disfunção mitocondrial, Apoptose, Atividade anti-inflamatória.

O aumento da esperança média de vida tem levado a um aumento da população idosa, originando uma prevalência de várias doenças neurodegenerativas, incluindo a doença de Parkinson (DP). Até ao momento, não foi encontrado um tratamento eficaz contra esta doença e, por isso, é de extrema importância a procura de novas soluções terapêuticas capazes de combater os efeitos nefastos da neurodegeneração.

O ambiente marinho tem-se revelado um recurso de grande valor como fonte de novos compostos bioativos, caraterizados por uma grande diversidade e especificidade estrutural, e que provaram ser eficazes no tratamento de diversas doenças, incluindo as neurodegenerativas. Assim, o principal objetivo desta tese foi o estudo do potencial bioativo de duas macroalgas, *Bifurcaria bifurcata* e *Codium tomentosum*, recolhidas na costa de Peniche (Portugal).

Extratos e frações obtidos de ambas as espécies foram objeto de um estudo químico guiado por bioensaios, de modo a selecionar as amostras com maior potencial bioativo. Estas últimas foram posteriormente submetidas a vários processos de purificação através de diferentes métodos de cromatografia preparativa (TLC, CC, HPLC) com vista ao isolamento de compostos puros cujas as estruturas foram elucidadas por técnicas de ressonância magnética nuclear (RMN) e espetrometria de massa (GC-MS). A citotoxicidade das diferentes amostras (extratos, frações, compostos isolados) foi avaliada pelo método de MTT (brometo de 3-(4,5-dimetil-tiazol-2-il)-2,5-difeniltetrazólio. Posteriormente, o potencial neuroprotetor foi estudado num modelo *in vitro* da doença de Parkinson induzido pela neurotoxina 6-hidroxidopamina (6-OHDA) na linha celular de neuroblastoma humano (SH-SY5Y). Paralelamente, avaliou-se o potencial anti-inflamatório através de um modelo de inflamação induzido por lipopolissacarídeos (LPS) em macrófagos RAW 264.7. De modo a compreender as vias de sinalização intracelular envolvidas na atividade neuroprotetora, biomarcadores associados ao stress oxidativo, nomeadamente a produção de espécies reativas de oxigénio (ROS), produção de peróxido de hidrogénio (H_2O_2) em tempo real, atividade da catalase, e inibição da ativação do fator de transcrição NF-κB, a disfunção mitocondrial (potencial mitocondrial membranar e níveis de ATP) e a apoptose (Caspase-3 e condensação do ADN e/ou fragmentação) foram

estudados nas células SH-SY5Y tratadas com 6-OHDA na presença e na ausência dos compostos derivados das algas. Atividade anti-inflamatória, o óxido nítrico (NO) e a expressão de citocinas pró-inflamatórias (TNF-α, IL-6) e anti-inflamatórias (IL-10) foram estudadas nos macrófagos RAW264.7 tratados com LPS na presença e na ausência dos compostos derivados das algas.

Foram isolados sete compostos identificados como sendo: eleganolone **(1)**, eleganonal **(2)**, bibiolone **(3)**, 14,15 – di-hidro-eleganolone **(4)**, fucosterol **(5)** e manitol **(6)** isolados a partir da alga *B. bifurcata* e loliolide **(7)** a partir da alga C. *tomentosum*. Contudo, apenas foi avaliado o potencial bioativo dos compostos com maior rendimento **(1**, **2**, **5**, **6**, **7)**. Com exceção do composto **1,** todos os restantes compostos (**2**, **5**, **6**, **7**) não revelaram efeitos citotóxicos a concentrações inferiores a 100 μM. Por este motivo, os ensaios de neuroprotecção foram efetuados na gama de concentrações de 1-100 μM, exceto o composto **1**, que foi testado no intervalo de concentrações de 0.05 - 1 μM.

No ensaio de neuroprotecção, apenas os compostos **1** (0.1, 0.5 e 1 μM; 24 h), **6** (50 e 100 μM; 24 h) e **7** (50 e 100 μM; 24 h) exibiram atividade neuroprotetora. No que respeita ao estudo dos mecanismos de ação envolvidos, verificou-se que o tratamento das células com os compostos eleganolone (**1**) e loliolide (**7**) preveniram o aumento da produção de ROS, a diminuição da ativação da catalase, a transcrição do factor NF-κB para o núcleo, e a diminuição da disfunção mitocondrial, prevenindo igualmente o aumento da atividade da Caspase-3 promovida pela neurotoxina 6-OHDA. Adicionalmente, o tratamento com estes compostos preveniu o aumento da produção NO e de interleucinas pró-inflamatórias promovidas pelo LPS, o que sugere o potencial anti-inflamatório destas moléculas.

Em suma, os compostos eleganolone (1 μM) e loliolide (100 μM) isolados das macroalgas *Bifurcaria bifurcata* e *Codium tomentosum*, respetivamente, exibiram atividade neuroprotetora relevante no modelo *in vitro* estudado associada à redução do stress oxidativo, da disfunção mitocondrial e da apoptose. Estes compostos revelaram igualmente propriedades anti-inflamatórias significativas, que se traduziram no decréscimo da produção de NO e das interleucinas pró-inflamatórias (TNF-α e IL-6). Os resultados obtidos com os modelos *in vitro* aqui estudados constituem uma primeira abordagem ao potencial neuroprotetor de ambos os metabolitos de origem marinha. Contudo, estudos adicionais em modelos *in vitro* e *in vivo* de maior complexidade serão necessários para validar a sua eficácia como novos agentes terapêuticos para a prevenção / tratamento da doença de Parkinson.

RESUMEN

Palabras clave: Produtos naturais mariños, algas, enfermidades neurodexenerativas, 6-OHDA, células SH-SY5Y, células RAW 264.7, estrés oxidativo, disfunción mitocondrial, apoptose, actividade anti-inflamatoria

Có aumento do envellecemento da poboación mundial, a prevalenza de enfermidades neurodexenerativas, coma a enfermidade de Párkinson (EP), está medrando rapidamente e, de acordo coa Fundación da enfermidade de Párkinson, esta doenza afecta arredor de 10 millóns de persoas no mundo. Sábese que este desorde neurolóxico prodúcese pola diminución das células neuronais responsables da produción de dopamina na *substantia nigra pars compacta*, unha estrutura nos ganglios basais do cerebro. Os medicamentos dispoñibles actualmente reducen os síntomas da EP, pero non impiden a progresión da doenza.

No final da década dos 60, a introdución da levodopa na terapéutica da enfermidade de Párkinson revolucionou o tratamento sintomático, e ata hoxe, os tratamentos utilizados no manexo da EP só compensan a perda de dopamina e da función dopaminérxica. Sen embargo, o seu uso prolongado tende a desenvolver unha serie de complicacións motoras e potenciais efectos secundarios ao longo do tempo coma trastornos do control do impulso, somnolencia, sono repentino e síndrome de desregulación da dopamina. Ademais, a enfermidade continúa a progresar e os síntomas non sensibles á dopamina coma a disfunción cognitiva e o desequilibrio fanse máis prominentes e levan a unha discapacidade a longo prazo. Polo tanto, é necesario investigar e desenvolver axentes terapéuticos máis efectivos para esta enfermidade neurodexenerativa, con menos efectos secundarios que os medicamentos dispoñibles actualmente.

Aínda que a etioloxía da EP é descoñecida, crese que hai moitos mecanismos implicados na aparición desta patoloxía, como o estrés oxidativo, a disfunción mitocondrial, a acumulación de alfa-sinucleina ou a neuroinflamación, entre outros. O estrés oxidativo é unha condición resultante do desequilibrio entre as defensas antioxidantes e a xeración de especies oxidativas. Debido a que o estrés oxidativo activa numerosas rutas metabólicas que producen a perda de estruturas e funcións neuronais, un proceso denominado neurodexeneración, este mecanismo considérase un importante factor nesta doenza neurolóxica.

O principal produtor de especies reactivas é a mitocondria a través da fosforilación oxidativa para xerar ATP. As especies reactivas de osíxeno son moléculas altamente reactivas que resultan da transferencia de electróns dun donante redox ao osíxeno molecular. O primeiro produto resultante desta reacción é o anión superóxido (O_2^-) que é convertido en peróxido de hidróxeno (H_2O_2), o que á súa vez xera o radical máis reactivo, o hidroxilo ($HO^\bullet$). Aínda que son esenciais para a vida, xogando un papel indispensable na homeostase e fisioloxía celular e participando en señais intracelulares e respostas adaptativas como a síntese de hormonas, a homeostase do calcio e a remodelación do citoesqueleto, entre outras, estas moléculas oxidantes deben ser balanceadas, xa que senón producirán danos significativos nas proteínas e o ADN, peroxidación lipídica, morte celular e enfermidade. En condicións saudables, o corpo humano ten unha maquinaria antioxidante altamente efectiva para manter ás especies reactivas a niveis basais. Sen embargo, diversos factores biolóxicos como a idade e a herdanza xénica, ou axentes físicos externos como o tabaquismo, a contaminación, a radiación e a dieta desequilibrada poden promover a sobreprodución de especies reactivas.

O cerebro ten un sistema de defensa antioxidante endóxeno relativamente débil en comparación con outros tecidos e é susceptible ao desequilibrio da homeostase redox. A catalasa ten un importante papel na protección das células, detoxificando o H_2O_2 xerado, por exemplo durante o metabolismo da dopamina, ou incluso na fosforilación oxidativa durante a cadea de transporte electrónico. Unha inhibición da actividade da catalasa pode resultar nun aumento da citotoxicidade e a produción de especies reactivas de osíxeno, conducindo a disfuncións neurolóxicas como a EP.

A enfermidade de Párkinson tamén está caracterizada pola inflamación crónica, intimamente relacionada co estrés oxidativo. Nun intento de manexar o dano producido polo estrés oxidativo, actívanse diversas rutas defensivas secundarias, que inclúen a liberación de mediadores inflamatorios como as citocinas TNF-α, IL-1β, e IL-6, resultando en inflamación. Aínda que a inflamación é una estratexia protectora para a reparación dos tecidos, a inflamación crónica causa dano celular que, cando ocorre no cerebro, produce a perda de neuronas. A cascada inflamatoria é unha complexa rede con múltiples participantes. O factor nuclear NF-κB é un complexo proteico cun papel crucial na regulación da producción de citocinas, polo que tamén regula a inflamación e, en definitiva, a apoptose. Como resultado, este factor está implicado na programación cerebral do envellecemento sistémico, pero tamén na patoxénese de moitas enfermidades neurodexenerativas, incluíndo a enfermidade de Párkinson.

Neste sentido, moitos investigadores teñen estudiado os efectos neuroprotectores dos antioxidantes a través da administración de moléculas antioxidantes esóxenas, debido a que se demostrou que son fundamentais na prevención do dano inducido polo estrés oxidativo, xa que teñen como diana ás moléculas oxidantes e reducen a produción de especies reactivas de osíxeno e especies reactivas de nitróxeno. En consecuencia, o interese nas potenciais aplicacións terapéuticas das moléculas antioxidantes ten aumentado. Sen embargo, a maioría dos antioxidantes desenvolvidos para uso clínico foron inefectivos. Como resultado, é de crítica importancia a procura de novas alternativas terapéuticas para contrarrestar a EP.

Dende tempos ancestrais, a natureza proporcionou á humanidade substancias para o tratamento de innumerables enfermidades, sendo vital para a supervivencia humana e a evolución. Sen embargo, co aumento na esperanza de vida, as enfermidades volvéronse un reto, e máis liñas de investigación necesitan ser exploradas urxentemente. Os océanos representan arredor do 70% da superficie da Terra, e albergan aproximadamente o 80% das especies coñecidas. Aínda que os océanos teñen unha gran riqueza biolóxica, a maioría das especies atópanse na pequena interface terra-océano, que ten unha gran concentración de especies e está entre os ecosistemas máis diversos e produtivos do planeta. A intensa concentración de especies que coexisten neses limitados hábitats, xunto coas arduas condicións do océano, derivadas do continúo cambio nos factores abióticos como a salinidade, a presión, a temperatura, a luz e a dispoñibilidade de nutrientes, fai que estes ecosistemas sexan altamente competitivos e complexos. Como consecuencia desta intensa competición, un alto porcentaxe de especies desenvolveu sinais químicas como estratexias de defensa, respostas á cadea alimentaria ou sinais de comunicación co medio ambiente. Estas sinais químicas normalmente adoitan a forma de moléculas, denomínanse metabolitos secundarios, e son unha fonte de importantes produtos naturais bioactivos. Desta forma, nas últimas décadas, demostrouse que o medio mariño é unha fonte de estruturas químicas únicas e diversas cun gran potencial terapéutico para ser usadas no tratamento de moitas patoloxías, incluíndo as doenzas neurodexenerativas. Entre os recursos mariños, as algas reveláronse como unha prometedora fonte de novas e interesantes moléculas, principalmente debido á súa gran produtividade, ampla diversidade, e composición heteroxénea, cunha gran abundancia de compostos bioactivos.

Nesta tese, dúas abundantes algas da costa portuguesa foron investigadas en relación ao seu potencial como produtoras de moléculas bioactivas con relevancia para a terapéutica contra a EP, *Bifurcaria bifurcata* e *Codium tomentosum*.

A alga parda *Bifurcacria bifurcata* (Ochrophyta) é unha especie que se atopa na costa atlántica de Francia, España e Portugal e esténdese cara as costas sur e oeste de Inglaterra, sendo ás veces frecuente na costa oeste de Irlanda. Esta especie gañou interese nos últimos anos debido ao seu gran contido de diterpenos lineares bioactivos que, en comparación cos seus conxéneres cíclicos, son moi raros na natureza, comprendendo unha gran variedade de actividades biolóxicas, como antioxidante, antibacteriana, antitumoral, antifouling, antiadhesiva, antiprotozoo ou antimalaria. *Codium tomentosum* Stackhouse (Chlorophyta) é unha alga verde pertencente á familia Codiaceae. Esta alga é orixinaria do nordeste do océano Atlántico, dende as Illas Británicas ate as Azores e Cabo Verde, e pode atoparse en rochas expostas e en pozas profundas na costa baixa. Aínda que é un alga común en moitas costas arredor do mundo, os estudios cos metabolitos de *C.tomentosum* están limitados aos ácidos graxos, ácidos orgánicos e a súa composición fenólica e volátil, e o seu potencial bioactivo, principalmente usando extractos, inclúe efectos antioxidantes, antixenotóxicos, antimicrobianos, antitumorais, neuroprotectores e hipoglicémicos. No xénero *Codium* xa foron identificados numerosos esterois, a saber, decortinol, sodecortinol, decorticona, clerosterol e 3-*O*-β-D-galacto-piranosil clerosterol. Que se saiba, os estudos sobre o potencial neuroprotector de ambas algas son practicamente inexistentes.

Polo tanto, o principal obxectivo desta tese doutoral foi o estudo do perfil químico da alga parda *Bifurcaria bifurcata* e da alga verde *Codium tomentosum*, recollidas na costa de Peniche (Portugal) e a avaliación da actividade antioxidante, neuroprotectora e anti-inflamatoria dos seus extractos, fraccións e principais metabolitos, aplicando unha estratexia de cribado bio-guiada.

Xurdindo dos obxectivos xerais, a presente tese tivo como obxectivo responder a preguntas específicas:

1) ¿É o perfil químico dos espécimes das macroalgas *Bifurcaria bifurcata* e *Codium tomentosum* recollidas na costa portuguesa similar ao de espécimes recollidos na costa irlandesa e o Mar Mediterráneo? ¿Poden as distintas condicións de cada localización promover a produción de metabolitos secundarios diferentes por estas especies?
2) ¿Cal é o potencial neuroprotector dos compostos derivados de *Bifurcaria bifurcata* e *Codium tomentosum*? ¿Poden estes compostos mostrar tamén actividades anti-inflamatorias?

3) ¿Que rutas de sinalización intracelular son subxacentes ás actividades neuroprotectoras e anti-inflamatorias mostradas por estes compostos?
4) ¿Os compostos illados terán a capacidade de actuar nas rutas de sinalización intracelulares implicadas na progresiva dexeneración das neuronas dopaminérxicas na EP?

As respostas a estas preguntas contribuirán a comprender o potencial neuroprotector e anti-inflamatorio dos compostos illados da alga parda *Bifurcaria bifurcata* e da alga verde *Codium tomentosum* para inspirar o desenvolvemento de novos axentes terapéuticos para a enfermidade de Párkinson. A relevancia deste estudo aínda é maior polo feito de que existen poucos estudos sobre as actividades neuroprotectoras de compostos naturais mariños derivados de algas para ser aplicados na terapéutica da EP. Para cumprir cos obxectivos propostos, os extractos e fraccións de ambas algas sometéronse a unha selección química bio-guiada para acceder ás mostras máis bioactivas.

Debido á complexidade dos extractos mariños evidenciada pola cromatografía analítica en capa fina, realizáronse novas técnicas de separación e purificación para obter fraccións activas e/ou produtos naturais puros. A cromatografía, especialmente a cromatografía preparatoria en columna, foi o principal método empregado neste traballo para obter fraccións activas e compostos mariños puros a partir de mesturas complexas. A separación baséase nas diferenzas entre as afinidades de adsorción dos produtos naturais para a superficie dos adsorbentes (fase estacionaria). A elección do adsorbente e a fase móbil é crucial para lograr a mellor separación dos compostos diana e evitar a súa adsorción irreversible á fase estacionaria. Neste traballo, empregouse o xel de sílice como adsorbente tanto en métodos cromatográficos analíticos como preparativos. Xeralmente, a separación de compostos menos polares realizouse mediante cromatografía en columna de fase normal, mentres que os máis polares separáronse mediante cromatografía en fase inversa usando columnas C_{18} nun equipo de cromatografía líquida de alto rendemento (HPLC). A técnica de cromatografía de gases acoplada a espectrometría de masas (GC-MS) utilizouse na separación e identificación de compostos volátiles en función dos seus espectros de masas. Xunto coa HPLC-DAD, esas técnicas tamén dan información sobre a complexidade das fraccións e sobre a pureza dos compostos illados. A espectroscopía de resonancia magnética nuclear mono

e bidimensional (1D RMN, 2D RMN), utilizáronse para examinar a composición química das fraccións bioactivas así como para dilucidar a estrutura dos compostos illados.

Para avaliar o potencial bioactivo das algas seleccionadas, previamente optimizáronse unha serie de ensaios *in vitro*.

En primeiro lugar, en termos de avaliación da capacidade antioxidante, realizáronse tres ensaios complementarios: actividade de eliminación radical 2,2-difenil-1-picril-hidrazilo, capacidade de absorción do radical osíxeno (ORAC-fluoresceína) e poder antioxidante redutor férrico e os resultados correlacionáronse co contido fenólico total de ambas algas.

En segundo lugar, para comprender o potencial neuroprotector de todos os extractos, fraccións e compostos, estimouse a súa citotoxicidade mediante o ensaio de 3-(4,5-dimetitiazol-2-il) -2,5-difenil tetrazolio (MTT). Despois, os efectos neuroprotectores de todas as mostras non tóxicas foron avaliadas nun modelo *in vitro* de EP no que o estado neurotóxico foi inducido pola 6-hidroxidopamina (6-OHDA) nas células SH-SY5Y. Ademais, utilizouse un modelo inflamatorio inducido con lipopolisacárido nos macrófagos RAW 264.7 para avaliar o potencial anti-inflamatorio de ambas especies de algas.

Co fin de comprender as vías de sinalización intracelular implicadas na actividade neuroprotectora, estudáronse varios biomarcadores asociados ao estrés oxidativo en células SH-SY5Y expostas a 6-OHDA en presenza/ausencia das mostras derivadas das algas, a saber: i) produción de especies reactivas de osíxeno, ii) produción de peróxido de hidróxeno (H_2O_2) en tempo real, iii) actividade da catalasa, iv) translocación do factor NF-kB, v) disfunción mitocondrial (potencial da membrana mitocondrial e niveis de ATP), vi) apoptose , vii) actividade da caspasa-3 e viii) condensación e/ou fragmentación do ADN. En canto á actividade anti-inflamatoria, estudáronse os niveis de óxido nítrico (NO) e a expresión de citocinas pro-inflamatorias (TNF-α, IL-6) e anti-inflamatorias (IL-10) nos macrófagos RAW 264.7 expostos a 6-OHDA en presenza/ausencia das mostras derivadas de algas.

Os resultados dos ensaios bioguiados das actividades antioxidantes e neuroprotectoras de *B. bifurcata* leváronse a cabo e preséntanse no Capítulo 3. As fraccións obtidas de *B. bifurcata* (DF5, MC18F2, MC18F4, AEF8, AEF9, AEF10)

mostraron un potencial antioxidante significativo, que pode correlacionarse cos seus altos niveis de contido fenólico total, verificándose tamén que as fraccións máis polares mostraban unha actividade antioxidante importante. Aínda que os compostos fenólicos, as florotaninas, que normalmente se atopan en especies de algas pardas, están totalmente descritos polo seu potencial antioxidante, a presenza doutros compoñentes químicos nas fraccións bioactivas, incluso en cantidades menores, xunto cos seus efectos sinérxicos, tamén poden contribuír ás propiedades antioxidantes.

No Capítulo 4 recóllense os ensaios bioguiados das actividades antioxidantes e neuroprotectoras de *C. tomentosum*. Ningunha das fraccións obtidas mostrou un potencial antioxidante significativo cando se compara coas fraccións de *B. bifurcata*. O potencial neuroprotector de *B. bifurcata* e os mecanismos de acción asociados foron avaliados no modelo *in vitro* de EP anteriormente mencionado e recollido no Capítulo 3. Neste estudo, demostrouse que varias fraccións de *B. bifurcata* (DF3, DF4, DF5, MC18F1, MC18F2, AEF9, AEF10, AEF11) foron capaces de promover a recuperación das células da toxicidade inducida pola exposición a 6-OHDA. Efectivamente, o tratamento das células SH-SY5Y con 6-OHDA reduciu a viabilidade das células nun 35-40% e, en presenza das fraccións de *B. bifurcata*, houbo un aumento da viabilidade das células SH-SY5Y dun 20 a un 25%. Por outra banda, no Capítulo 4 demostrouse que varias fraccións de *C. tomentosum* (MF2, DF1, DF2 e DF5) foron capaces de promover a recuperación das células da toxicidade inducida polo 6-OHDA, nun 30-50%. Comparando ambas algas, *C. tomentosum* revelou o maior potencial neuroprotector.

Os mecanismos implicados nos efectos neuroprotectores sobre a viabilidade das células SH-SY5Y que presentan as fraccións das algas tamén se estudaron en diferentes sinais distintivas asociadas ao desenvolvemento da EP, comprobándose que as fraccións de *B. bifurcata* (DF4, AEF9, AEF10 e AEF11), como se recolle no Capítulo 3, e as fraccións de *C. tomentosum* (MF2, DF1, DF2 e DF5), como se mostra no Capítulo 4, foron capaces de previr a produción de especies reactivas de osíxeno e a disfunción mitocondrial promovida polo tratamento con 6-OHDA. Por outra banda, a exposición das células SH-SY5Y a 6-OHDA aumentou a actividade da caspasa-3, e o tratamento con fraccións de *B. bifurcata* (DF3, DF4, MC18F2, AEF9) e con fraccións de *C.*

tomentosum (MF2, DF2, DF5) provocou unha marcada diminución da actividade da caspasa-3.

As fraccións que mostraron o mellor potencial neuroprotector foron sometidas a etapas de fraccionamento e purificación máis refinadas mediante técnicas cromatográficas preparativas (TLC, CC, HPLC) co obxectivo de illar as posibles moléculas bioactivas responsables das actividades observadas. As fraccións de *B. bifurcata* proporcionaron tres compostos principais: eleganolona (**1**), eleganonal (**2**) e manitol (**6**) e, en cantidades menores, os compostos bibiolona (**3**), 14,15 - dihidroxi-eleganolona (**4**) e fucosterol (**5**). A partir da fracción metanólica de *C. tomentosum* foi posible illar o composto bioactivo loliolida (**7**). Entre os sete compostos illados e identificados como eleganolona (**1**), eleganonal (**2**), bibiolona (**3**), 14,15 - dihidroxi-eleganolona (**4**), fucosterol (**5**), manitol (**6**) e loliolida (**7**), só se avaliaron os compostos cos mellores rendementos de extracción (**1, 2, 5, 6, 7**) respecto ao seu potencial neuroprotector e anti-inflamatorio.

Nos Capítulos 3 e 5 observouse que, coa excepción do composto **1**, todos os compostos (**2, 5** e **6**) non eran citotóxicos por debaixo de 100 μM. Así, estudáronse os compostos **2, 5** e **6** con respecto ao seu potencial neuroprotector nun rango de concentración de 1-100 μM e o composto **1** entre 0,05-1 μM. En canto ao ensaio de neuroprotección, só os compostos **1** (0,1, 0,5 e 1 μM; 24 h) e **6** (50 e 100 μM; 24 h) presentaron actividade neuroprotectora nas células SH-SY5Y con neurotoxicidade inducida por 6-OHDA. Non obstante, o composto **1** foi o máis prometedor ao comparalo co composto **6** e, deste xeito, só se avaliaron os mecanismos implicados para o composto **1**. En canto aos mecanismos de acción implicados na enfermidade de Párkinson, o tratamento co composto eleganolona (**1**) evitou as alteracións celulares promovidas polo tratamento 6-OHDA: aumento da produción de especies reactivas de osíxeno, diminución da actividade da catalasa, bloqueo da translocación do factor NF-kB do citoplasma ao núcleo, aumento da disfunción mitocondrial, diminución dos niveis de ATP e aumento da actividade da caspasa-3. Ademais, a eleganolona (**1**) tamén mostrou actividade anti-inflamatoria nas células RAW 264.7 con inflamación inducida por lipopolisacárido, diminuíndo a produción de NO e interleuquinas pro-inflamatorias (TNF-α, IL-6) promovidas polo tratamento con lipopolisacárido. Non obstante, este composto non aumentou os niveis da interleucina anti-inflamatoria IL-10.

No Capítulo 6 observouse que o composto **7**, identificado como loliolida, non era citotóxico por debaixo de 100 μM. Así, estudouse o potencial neuroprotector da loliolida nun rango de concentración de 1-100 μM. Nos ensaios de neuroprotección, este composto (50 e 100 μM; 24 h) exhibiu actividade neuroprotectora nas células SH-SY5Y cando se expuxeron á neurotoxicidade inducida por 6-OHDA. En relación cos mecanismos de acción implicados na EP, o tratamento con loliolida (**7**) evitou o aumento da produción de especies reactivas de osíxeno, a diminución da actividade da catalasa, o bloqueo da translocación do factor NF-kB do citoplasma ao núcleo, o aumento da disfunción mitocondrial e o aumento da actividade da Caspasa-3 promovida polo tratamento con 6-OHDA. Ademais, a loliolida tamén mostrou actividade anti-inflamatoria nas células RAW 264.7 con inflamación inducida por lipopolisacárido, ao diminuír a produción de NO e de interleucinas pro-inflamatorias (TNF-α, IL-6) promovida polo tratamento con lipopolisacárido. Non obstante, a loliolide (**7**) non revelou un aumento da interleucina anti-inflamatoria (IL-10).

En resumo, os compostos eleganolona (1 μM) e loliolida (100 μM) illados das algas *B. bifurcata* e *C. tomentosum*, respectivamente, presentaron actividade neuroprotectora, que parece estar mediada por unha redución da condición de estrés oxidativo, evitando a disfunción mitocondrial e a apoptose. Por outra banda, estes compostos tamén presentaron actividade anti-inflamatoria, que parece estar mediada por unha diminución da produción de NO e dos niveis de interleucinas proinflamatorias (TNF-α e IL-6). Os resultados aquí presentados constitúen a primeira evidencia do potencial neuroprotector dos compostos derivados de *C. tomentosum* e *B. bifurcata*. Polo tanto, debería considerarse a realización de máis estudos en modelos *in vitro* e *in vivo* máis complexos para validar a súa eficacia como potenciais axentes terapéuticos para a prevención/tratamento da EP.

ABBREVIATIONS AND ACRONYMS

6-OHDA – 6-Hydroxydopamine

AI – Aging Index

ALDH – Aldehyde dehydrogenase

ATP – Adenosine triphosphate

BDNF – Brain-derived neurotrophic factor

cGMP – Cyclic guanosine monophosphate

CH_2Cl_2 – Dichrolomethane

CNS – Central Nervous System

COMT – Catechol-*O*-methyltransferase

CoQ10 – Coenzima Q10

CSF – Cerebrospinal fluid

D1 – Dopaminergic receptor -1

D2 – Dopaminergic receptor -2

DA – Dopamine

DAT – Dopamine transporter

DMEM – Dulbecco's Modified Eagle Medium

DMEM:F12 – Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12

DMSO – Dimethyl sulfoxide

DOPAC – Dihydroxyphenyl acetic acid

DSMZ – German Collection of Microorganisms and Cell Cultures GmbH

EA – Ethyl acetate

EDTA – Ethylenediamine tetraacetic acid

Fe (II) – Iron (II)

GABA – Gamma-aminobutyric acid

GABA – Gamma-Aminobutyric acid

GPe – Globus pallidus external

GPi – Globus pallidus internal

GSH – Glutathione

GSSG – Glutathione disulfide

H_2O_2 – Hydrogen peroxide

HVA – Homovanilic acid

IL-10 – Interleukin 10

IL-1β – Interleukin 1β

IL-6 – Interleukin 6

JC-1 – 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

JNK – c-Jun N-terminal kinases

LBs – Lewy Bodies

LC-MS – Liquid chromatography – Mass spectrometry

L-DOPA – Levodopa

LI – Longevity Index

LPS – Lipopolysaccharide

MAO – Monoamine oxidase

MAPK-p38 – Mitogen-activated protein kinase

MeOH – Methanol

MMP – Mitochondrial membrane potential

MNP – Marine natural products

MPP^+ – 1-Methyl-4-phenylpyridinium

MPTP – 1-Metyl-4-phenyl-1,2,3,6-tetrahyidropyridine

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

NF-κB – Nuclear factor-Kappa B

NMR – Nuclear magnetic resonance

NO – Nitric oxide

$NO\cdot$ – Nitric oxide radical

NP – Natural products

Nrf2 – Nuclear factor erythroid 2-related factor 2

O_2 – Oxygen

·O_2^- – Oxygen radical

·OH – Hydroxyl radical

Parkin – 465-Residue E3 ubiquitin ligase

PBS – Phosphate-buffered saline

PCA – Principal component analysis

PD – Parkinson's disease

PI3K – Phosphoinositide 3-kinase

PINK1 – PTEN-induced kinase 1

Q – Quinones

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

SDS – Sodium dodecyl sulfate

SEM – Standard error of the mean

SNpc – *Substantia nigra pars compacta*

SNpr – *Substantia nigra pars reticulata*

STN – Subthalmic nucleus

TH – Tyrosine hydroxilase

TNF–α – Tumor necrosis factor

TPC – Total phenolic content

Trolox – 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

WHO – World Health Organization

α-syn – α-synuclein

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CHAPTER 1
GENERAL INTRODUCTION

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1. INTRODUCTION

Neurodegenerative diseases are one of the major concerns among aging population across the world takes a devastating effect on the life quality and severe socioeconomic impacts. These pathologies are often associated with a progressive and substantial loss of neuronal cells leading to the development of several neurological impairments such as motor and cognitive decline and dementia [1,2]. According to the World Health Organization (WHO) the percentage of the global population over 60 years age will double reaching almost 20 billion in 2050 [3]. This reality will be more marked in the developed countries where the prevalence of neurodegenerative diseases has been growing due to the increase of life expectancy [4].

Among neurodegenerative diseases, Parkinson's Disease (PD) is the second most common affecting about 2-3% of the world's population aged ≥ 65 years old. This disease is characterized by the progressive loss of dopaminergic neurons of the *substantia nigra pars compacta* that project to the striatum and by the presence of Lewy bodies [5,6]. Previous studies have reported that dopaminergic cell death in PD is related with the high levels of apoptosis, and several evidences suggest that oxidative stress, mitochondrial dysfunction and neuroinflammation events are also involved [7,8].

In the last decades, several efforts have been made to understand the PD etiology and pathogenesis allowing identifying several cellular targets that can be used in the development of new therapeutic approaches. Furthermore, despite the knowledge achieved in the last years, a better understanding of the mechanisms linked to PD neurodegeneration is crucial to develop effective therapies [9]. Until now, there are not effective drugs for PD treatment since the medicines available in the market only bring symptomatic benefits over a short period of time. Furthermore, due to a lacking therapeutic option, most of the clinical trials in development associated to PD have as main aim to improve the efficacy of drugs currently in the market. Therefore, the search for innovative PD therapeutic agents is of utmost importance.

In this area, natural products have played a crucial role in the medicine over the last decades, exhibiting relevant pharmacological properties for the treatment and prevention of several pathologies [10]. This potential has aroused the interest of the scientific community to explore nature in order to find new chemical entities with therapeutic activities. Within this context, marine organisms have revealed to be a promising source of new molecules with great antioxidant, antimicrobial, anti-tumor, and neuroprotective activities, among others [11,12].

Regarding neuroprotective activities, twenty-nine marine natural products shown to be effective in experimental *in vivo* models of neurological disorders, particularly in PD models. However, most of them exhibited weak therapeutically efficacy, and only five marine natural products are currently undergoing clinical trials [11]. Therefore, it is extremely important to pursue new chemical structures that may contribute to inspire the development of new effective therapeutic agents for PD treatment.

1.1. CHALLENGES OF AGEING SOCIETY: WHY IS ADVANCING AGE OF MAJOR RISK FACTOR IN THE PARKINSON'S DISEASE DEVELOPMENT

Demographic trends are a key factor for the World future. The numbers and proportion of older people in the global population are growing essential due of the birth rates decrease and life expectancy increase [13]. According to the WHO, this increase will tend to continue throughout this century being expected in 2050 that the number of older people (> 60 years) reach the number of young people (< 15 years) [3]. Therefore, the population aging represents nowadays one of the most worrying demographic phenomena in modern societies. According to the "World Statistics", European population aged over 65 in 2017 was about 18% of the global population, having observed a significant increase since 2010 (**Figure 1.1**). On the other hand, in 2016, the Asian and African continents presented a lower percentage of aged population over 65, around 5% and 3%, respectively. However, those countries also observed that the percentage of population over 65 increased between 2010 and 2017, being more evident in Asia. The life expectancy variation between continents is also influenced by the health-care-related features, such as lack of proper and widespread recognition of the disorder, difficulties in accessing to health care systems, as well as genetic, environmental and methodological differences.

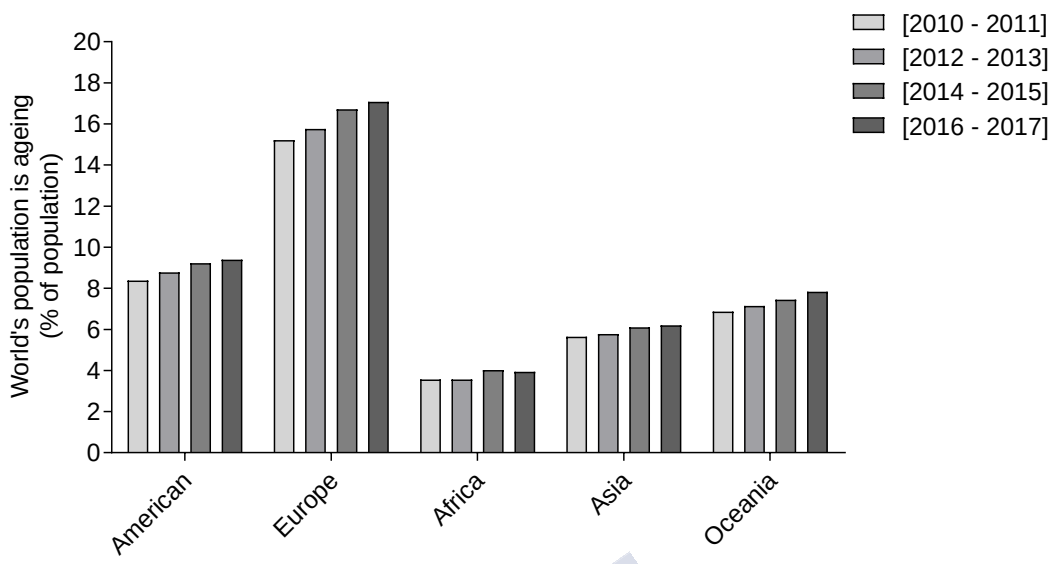


Figure 1.1. Percentage of global population with age above 65 years old. Adapted from the "World Statistic" data platform in 2019.

Based in the aging scenario of world population, especially in Europe, the elderly is the most vulnerable population to develop neurodegenerative diseases being the aging a critical risk factor. This scenario is supported by the aging index (AI) that has been growing in Europe in the last decade, being accompanied by increased longevity index (LI) (**Figure 1.2**).

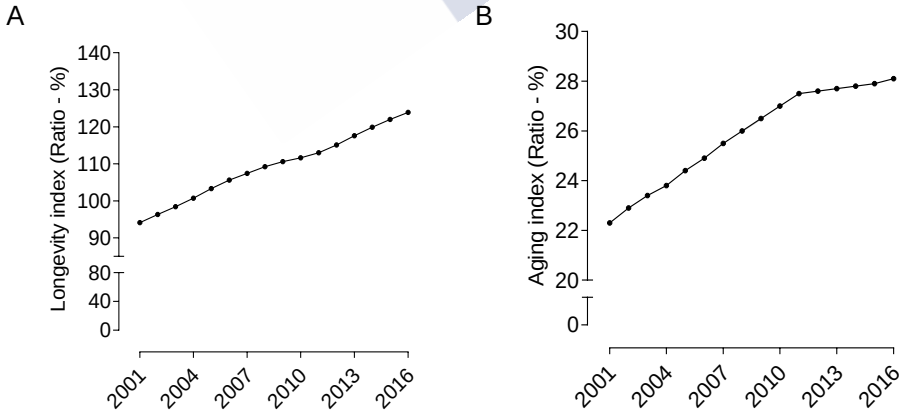


Figure 1.2. A) Longevity index (ratio - %) in Europe (>80 years) between 2001 and 2018; **B)** Aging index in Europe (ratio - %) between 2001 and 2016, (Adapted from Statics National Institute (Prodata), 2020) [14].

In Europe, according to the National Institute of Statistics (Prodata), the aging index has increased (**Figure 1.2B**), reaching in 2016, 124 elderly people per 100 young people compared to 2001, which was 94 elderly people per 100 young people. However, this increase in the number of elderlies is also reflected in the increase longevity index (**Figure 1.2A**) that reached in 2016, 28 elderly people with more than 75 years-old per 100 elderly compared to the 2001, which reached 22 elderly.

Parkinson' Disease prevalence is about 2-3% in the world population aging over 65 years old and 4% in the population over 80s and rare exceptions may occur in young people between 20 and 50 years [15–17]. However, with advancing age, several essential processes related to the neurons functions in the substantia nigra per compacta, including dopamine metabolism, the number of mitochondrial DNA (mtDNA) copies and the decline of protein degradation (Figure 1.3) can be altered. For instance, dopamine metabolism generates a significant amount of reactive oxygen species that can affect different cellular processes in the neurons. On the other hand, a decline in mtDNA copies number can lead to a decrease in the ATP production and a reduction of the protein's degradation affecting the functioning of neurons [18]. The accumulation of melanin, the ability of neurons and mitochondria to cope with calcium and the levels of iron within the cells will be also affected, so that additional insults such as mitochondrial complex deficiencies I and IV and α-synuclein aggregation cause the loss of vulnerable neurons, leading to the occurrence of symptoms associated to PD [17]. As age advances and the accumulation of several cellular defects become SNpc neurons more vulnerable. Therefore, the effects of aging can contribute to trigger a cascade of stressors events within the substantia nigra per compacta weakening the neurons response capacity to new insults that are seen as part of the disease process [19].

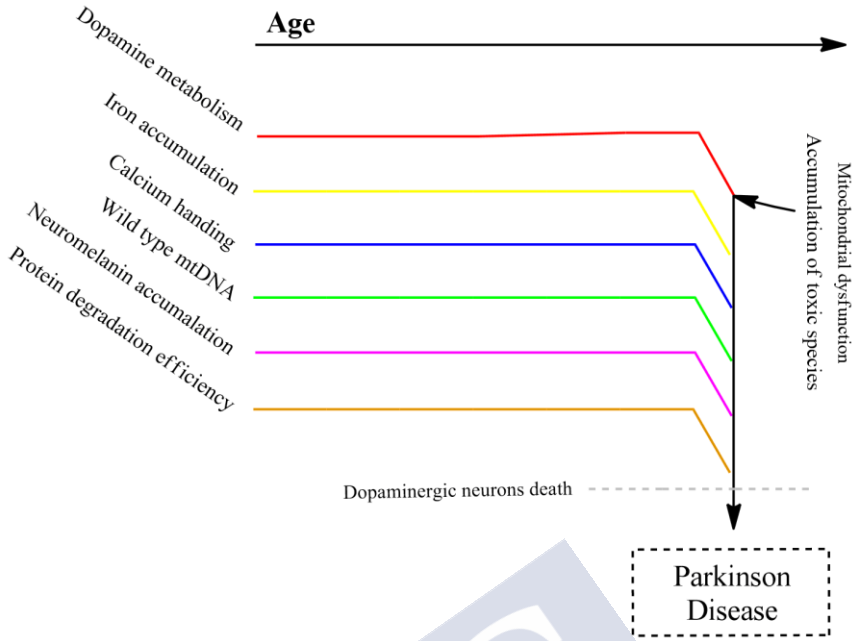


Figure 1.3. Age-related changes mediated by distinct processes that led to the death of dopaminergic neurons in the substantia *nigra per compacta* (Adapted from Reeve *et al*. 2014) [17].

1.2. GENERAL OVERVIEW OF PARKINSON'S DISEASE

Parkinson's Disease (PD) is a neurological syndrome described by James Parkinson in 1847 at the first time. It is a progressive neurodegenerative disease of the central nervous system (CNS) of unknown etiology and neuropathologically characterized by the loss of midbrain pigmented neurons in the substantia *nigra pars compacta* [20,21]. This disease presents an incidence of 187 cases in 100 thousand people and it is clinically characterized by the onset of motor symptoms, such as bradykinesia, muscular rigidity, resting wobbly, posture and gait disturbances, as well as non-motorized symptoms such as sleep disturbances, depression, and cognitive impairment [22–24]. Under normal conditions, the dopaminergic neurons produce the dopamine (DA) neurotransmitter to striatum responsible to coordinate the functionality of the body muscles and movement through the signal transmission [25]. In the absence of dopamine (<80%) receptors are not stimulated [20] leading to a disruption in the basal ganglia circuits [23,26] (**Figure 1.4**). It is estimated that at the onset of PD symptoms approximately 50-60% of dopaminergic neurons in the SNpc are lost and, at the same time, there is a 70-80% loss of dopamine in the striatum [27].

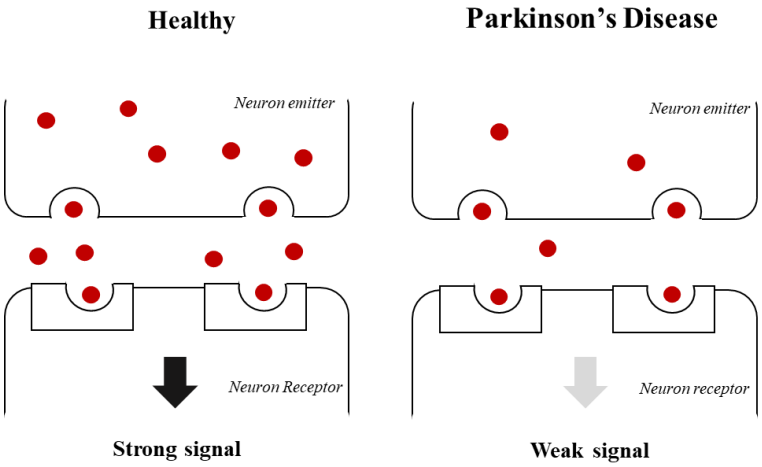


Figure 1.4. Dopamine levels (red spots) in the brain of healthy people and Parkinson's Disease patients.

Dopaminergic neuronal cells loss lead to an irreversible degeneration of the nigrostriatal pathway, followed by striatal dopaminergic denervation, causing pathological changes in the neurotransmission of basal ganglia motor circuit, resulting in characteristic Parkinsonian symptoms [28]. Another pathological hallmark of the PD is the presence of protein inclusions in the cytoplasm designed by Lewy bodies (LBs) corresponding to abnormal intracellular α-synuclein (α-syn) aggregates that lead neuronal cells undergo neurodegenerative processes and subsequently die [29].

Despite PD etiology is mostly unknown, several factors have been associated with its development, including aging, genetic and environmental, which can contribute to trigger a cascade of cellular responses, such as excessive production of reactive oxygen species, mitochondrial dysfunction, neuroinflammation, incorrect "winding" of α-syn protein, responsible for the formation of LBs, leading to dopaminergic neuronal cell death, and thus to DA levels decrease [23,30,31].

DA neurotransmitter is synthesized in the brain through the activity of the tyrosine hydroxylase (TH) enzyme, which converts the amino acid tyrosine, captured in the adrenergic terminal, into L-DOPA which, in turn, is decarboxylated into DA and later into norepinephrine (**Figure 1.5**).

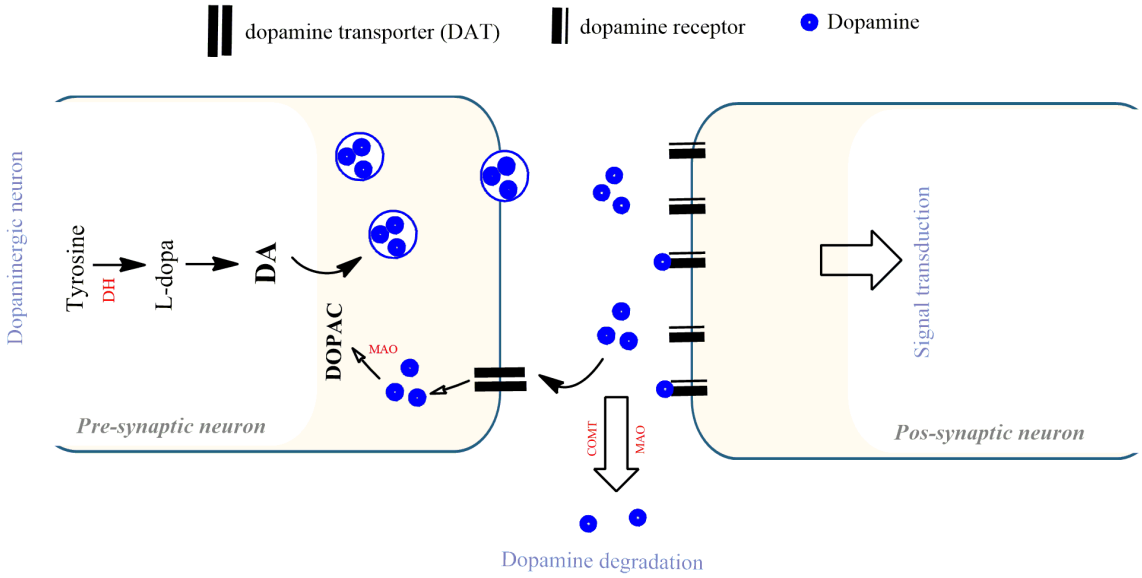


Figure 1.5. Dopamine synthesis from L-dopa in dopaminergic neurons in substantia *nigra pars compact*.

The rate of DA synthesis is modulated by the TH activity and can be attenuated by the activation of auto receptors located at presynaptic terminals [32]. DA is then stored in the synaptic vesicles and released by exocytosis stimulated by the nervous impulse that act on specific receptors present in the central nervous system (CNS) known as dopaminergic receptors (D1 and D2). At the postsynaptic level, DA can act on the receptors of D1 (D1 and D5) or D2 (D2, D3 and D4) families [33]. In this way, the decrease of mesencephalic DA levels in PD is accompanied by a loss of dopamine transports (DAT) and an increase of the levels of both D1 and D2 receptors [32]. After DA release, this its converted to 3,4-dihydroxyphenylacetaldehyde (DOPAL), then in dihydroxyphenylacetic acid (DOPAC) and finally in homovanillic acid (HVA) by monoaminooxidase (MAO A or B) enzymes, aldehyde dehydrogenase (ALDH) and catechol-O-methyl transferase (COMT), acting in sequence [32]. Main end-product is homovanillic acid (HVA) enter the cerebral spinal fluid from which they are actively transported out of the brain and then, into the bloodstream wheres filtered out by the kidneys and then excreted in the urine. This way, measurements of homovanillic acid in plasma have been used to estimate levels of DA activity in the brain [34]. Very although DA is normally broken by oxidoreductase enzyme, it is also susceptible to oxidation by direct reaction with oxygen leading free radicals production such as products. The mechanism of oxidation of DA can be increased by the presence of ferric

iron that reacting with H_2O_2 can lead to the formation of the OH radical. This mechanism may contribute to the death cell that occurs in PD.

Inside the brain, DA plays important a role in executive functions, such as motor control, motivation and others. The nigrostriatal pathway (**Figure 1.6**) is a main dopaminergic neurons (DAergic) pathway that connects the SNpc to the caudate and putamen nucleus of dorsal striatum involved in the motor control [35], concentrating about 80% of the DA in the brain.

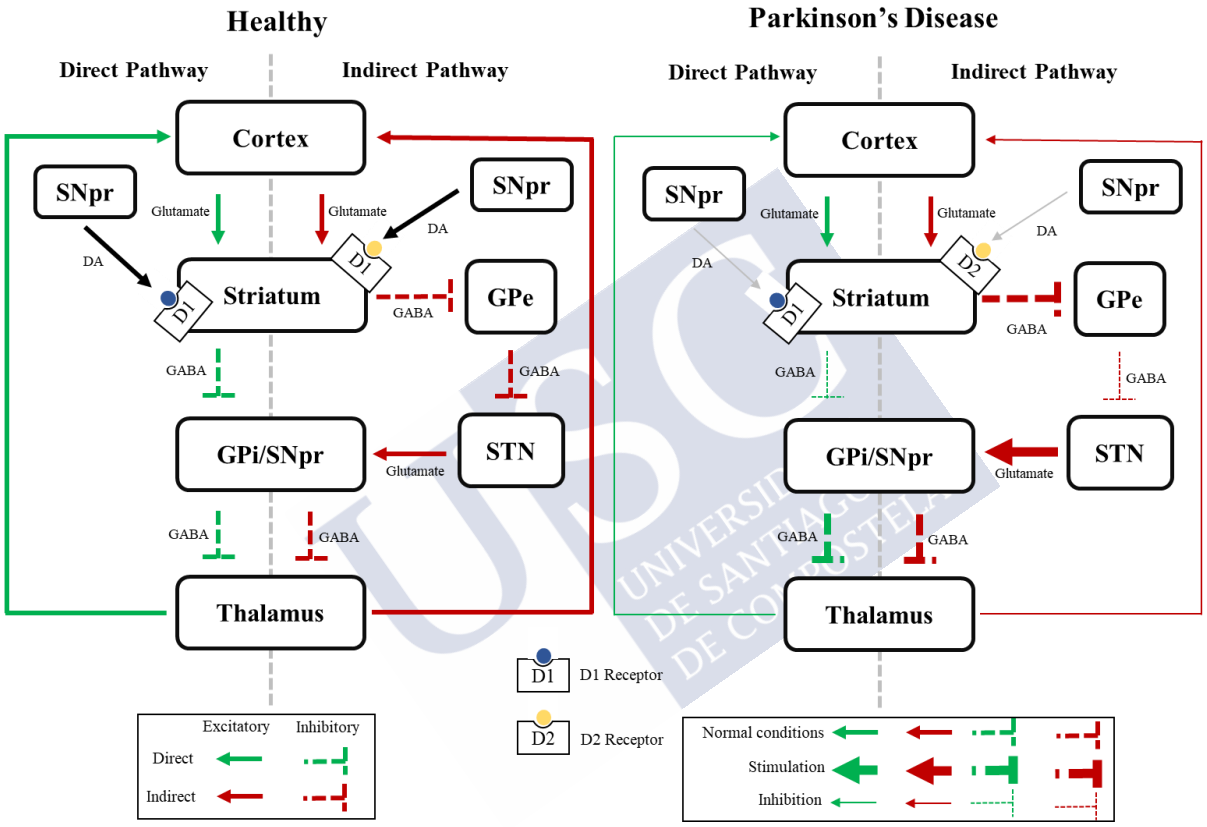


Figure 1.6. Representative diagram of the nigrostriatal pathways, direct and indirect, through the cortex and basal ganglia. The direct pathway is represented by the green color; the indirect pathway is represented by the red color. GPi - globus pallidus internal; GPe - external; STN - Subthalamic nucleus; SNpr -Substantia *nigra par reticulata*; SNpc -Substantia *nigra par compacta*.

The main function of the nigrostriatal pathway is to influence the voluntary movement through the DA release in the basal ganglia [20,21]. However, it is necessary that Cortex provides excitatory glutamatergic projections to the putamen (a part of striatum), which sends GABAergic inhibitory projections to the globus pallidus internal GPi and the substantia *nigra par reticulata* (SNpr) through two pathways: On the direct route, the released DA will act

mainly on the type D1 DAergic receptors, thus stimulating neurons that release GABA, which will later inhibit GPi and SNpr, disinhibiting the thalamus and releasing movements. In the indirect pathway, the DA released in the striatum will act mainly on the type D2 receptors, causing the inhibition of neurons that release GABA in the globus pallidus external (GPe), which in turn instead it will inhibit the subthalmic nucleus (STN) ceasing to stimulate GPi and SNpr, thus disinhibiting the thalamus and later the movements [26,36]. Therefore, the stimulation of the direct pathway and inhibition of the indirect pathway by DA facilitate the occurrence of movements [37]. Due to the loss of dopaminergic neurons, and thus, the decrease of DA levels leads to an imbalance in the activity of the direct and indirect pathways, promoting motor deficits that are observed in PD. Therefore, currently, dopamine replacement is the main therapeutic approach for the treatment of motor symptoms in PD. To date, there is no available an effective therapy, being the treatments only symptomatic based on the L-DOPA drug being its action associated with the inhibitors of the metabolism of dopamine and dopaminergic agonists [38]. These therapies do not prevent the disease progression and cause undesirable side effects, and thus the development of alternative treatments for PD is mandatory [39].

1.3. INTRACELLULAR MECHANISMS UNDERLYNG PARKINSON'S DISEASE DEVELOPMENT

Dopaminergic neuronal loss in the SNpc, and depletion of DA levels in the striatum represent hallmarks of PD pathology [40]. Despite the exact mechanism of the dopaminergic neuronal cell death in the SNpc is not well understood, the oxidative stress, mitochondrial damage, energy failure, excitotoxicity, neuroinflammation, gene mutations, protein misfolding and aggregation seem to be involved in the onset and progression of PD (**Figure 1.7**) [5,23,41,42].

1.3.1.1. Metabolism of Dopamine Neurotransmitter and Oxidative Stress

The brain is an organ particularly susceptible to oxidative damage essential due of its high metabolic activity and consequent high rate of O_2 consumption promoting a basal production of ROS, high lipid content and relative scarcity of antioxidant enzymes compared to other organs [4,43,44]. Due of those factors a unbalance between the production of ROS and

antioxidant defenses may result in the development of a oxidative stress condition that can play a critical role in mitochondrial dysfunction, since damages in the mitochondrial Complex I of dopaminergic neuronal cells lead to a decrease of ATP levels and thus to cell death [45]. Furthermore, molecular damage caused by ROS is considered one of the major causes of aging and a precursor in the development of PD [46,47]. All these unfavorable events contribute to altered antioxidant defenses suggesting that oxidative stress may play an important role in PD. Thus, therapeutic strategies that prevent oxidative stress may be a key to PD treatment [43,48,49]. Dopamine neurons are especially exposed to oxidative stress due to the metabolism of DA that lead to the production of several molecules that can act as endogenous toxins if not eliminated correctly. The oxidation of DA can occur by two pathways: (1) metabolized by MAO in DOPAC (a non-toxic metabolism) and in hydrogen peroxide (H_2O_2); or (2) undergo self-oxidation by reacting with oxygen (O_2) at neutral pH leading to toxic dopamine-quinone species and superoxide radical's formation [8]. Since cytoplasmic dopamine can originate ROS very quickly, it must be synthesized or transported from the extracellular space into the cell and stored inside the synaptic vesicles [50]. Thus, several circumstances may contribute to an increase of oxidative stress condition and consequent cellular damage in the CNS, including (a) an increase of DA levels; (b) an increase of the iron deposit; (c) glutathione (GSH) deficiency causing a decrease of brain capacity to eliminate H_2O_2; (d) DNA damage; and (e) lipid peroxidation [15,51,52]. According to *post-mortem* studies with brains of individuals PD patients, all the neurons in the SNpc seemed to be under a high oxidative stress state, presenting high levels of iron, decrease of GSH levels and oxidative damages in lipids, proteins, and DNA [52–54]. Therefore, due of the critical role of oxidative stress in the neuronal cell death in PD, the antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase) that represent the main defense system against oxidative lesions, activated by the transcription of Nrf2 factor have been seen as one of the pharmacological strategies to attenuate the oxidative damages induced by ROS [55].

One of the major changes in antioxidant defenses observed in PD is related with the decrease of GSH concentration [4,56] that, according to *post mortem* studies, PD patients presented lowest levels (60%) compared to healthy individuals, while the oxidized glutathione (GSSG) levels were slightly higher [4,54]. Although GSH is not the only antioxidant molecule altered in PD patients, it is thought that the magnitude of its depletion is the first indicator of nigrostriatal degeneration [53]. Furthermore, previous reports have described that the decrease

of GSH levels in the PD patients may be accompanied by a reduction of mitochondrial activity, which is regionally selective in SNpc of PD patients, and it is not associated with other neurodegenerative diseases related with basal ganglia [4,53]. This evidence suggests that the decrease of nigrostriatal GSH levels may thus initiate or facilitate the occurrence of an oxidative stress condition triggering the degeneration of dopaminergic neurons in PD [4,57,58]. Moreover, other evidence of the involvement of oxidative stress in PD arises from the studies with 1-metil-4-fenil-1,2,3,6-tetraidropiridina (MPTP)-induced parkinsonism, which through the redistribution of DA in the cytoplasm led to an increase of oxidative stress condition [59,60]. For instance, pesticides, such as rotenoids and paraquat, have shown to induce changes in cytoplasm DA levels leading to a stress condition [60]. These evidences suggest that environmental factors may also be involved in the PD oxidative stress mechanisms. Therefore, according to these evidences, oxidative stress seems to be one of the mechanisms that play a critical role in the arise and progression of neurodegeneration [20,61].

1.3.1.2. Mitochondrial Dysfunction

Mitochondria is an essential cytoplasmic organelle that plays various cellular functions including regulation of ROS levels, calcium homeostasis, control of apoptosis activation, and adenosine-triphosphate (ATP) production [62–64]. Mitochondria is bounded by a double-membrane system, consisting of inner and outer mitochondrial membranes separated by an intermembrane space, which have different functions. Together they create so two separate mitochondrial compartments: the internal matrix and intermembrane space. In the inner mitochondrial membrane is located the electron transport chain, constituted by five complexes (I-V), responsible by the energy production through the electron flow along of the complexes and consequently oxidative phosphorylation [45,65]. During this biological process due of the electron transport chain occurs the formation of extremely reactive oxygen species, including the superoxide radicals (O_2^-) generated in the Complex I. Therefore, the inhibition of this complex leads to a decrease of ATP production, damaging all cellular mechanisms dependent of ATP, and also promoting the formation of free radicals leading to a development of oxidative stress condition [75–79]. The occurrence of these events promotes the mitochondrial dysfunction that is characterized by changes in mitochondrial membrane potential, and by insufficient production of ATP [66–68].

Previous studies carried out in PD demonstrated the existence of a deficiency in the Complex I of respiratory chain limited to the substantia *nigra pars compacta*. These studies evidenced the role of mitochondria in the etiology and pathology of this disease, as well as the relationship between oxidative phosphorylation and free radical production [50,67,69] pointing out the role of mitochondria as the main source of ROS that contribute for the development of an intracellular oxidative stress condition [70]. Furthermore, Lang and Lozano (1998) [71] also observed a significant decrease (30 - 40%) of the Complex I activity in the SNpc of PD patients. These facts support the evidence that mitochondrial dysfunction, and especially Complex I deficiency that is present in PD patients can be an important cause involved in the development of this disease. Additionally, to Complex I deficiency, changes in genes related to mitochondria, such as, PTEN-induced kinase 1 (PINK1) and 465-residue E3 ubiquitin ligase (Parkin) seem to be also associated to mitochondrial dysfunction [72]. PINK1 plays functions related with the control of mitochondrial turnover and it has protective functions against oxidative stress. On the other hand, Parkin function is related with the external mitochondrial membrane delaying the mitochondrial swelling, and consequently, the release of cytochrome C, caspase cascade activation, and finally the degeneration of dopaminergic neurons [73,74]. According these facts, mitochondria reveals to be an interesting target for the development of new therapeutic strategies with the purpose to prevent or delay the neurodegeneration observed in PD.

1.3.1.3. Neuroinflammation

In the last years, several studies have reported the involvement of neuroinflammatory mechanisms that trigger a cascade of events leading to the degeneration of neuronal cells [98]. Several *post-mortem* studies carried out on *in vivo* models have highlighted the role of neuroinflammation in Parkinson's disease, which seems to be closely linked to the neuronal cell damage and death through distinct biological mechanisms, such as elevated oxidative stress and glial cells (astrocyte and microglia) activation [75–77]. Chronic neuroinflammation is one of the hallmarks of PD pathophysiology and, under inflammatory condition, activated glial cells release pro-inflammatory factors such as cytokines and prostaglandins, as well as neurotoxic factors such as reactive oxygen species (ROS)/reactive nitrogen species (RNS), and complement proteins, that can exacerbate the neuronal cell damage leading to neurodegeneration [75,78]. Furthermore, previous reports have observed that high levels of

proteins and the presence of Lewy bodies that occur in PD patients activate microglia contributing also to the increase of pro-inflammatory cytokines levels [77,79,80].

The first evidence of inflammation in PD was reported by McGerr and collaborators (1988) that observed activated cGMP in *post-mortem* PD patients mainly in the SNpc and in the putamen, two of the areas most affected in this disease. Moreover, this condition has also detected in the hippocampus and other cortical areas where neuronal cell loss is important [81,82]. In fact, neuroinflammation that is primarily controlled by microglia seems to play a critical role in the neuronal cell loss observed in PD [23,83].

Microglia are immunocompetent cells in the CNS that are responsible to protect the brain against invading pathogens, and capable to stimulate an immune-adaptive response [84,85]. Therefore, several acute lesions that may occur in the CNS led to microglial activation, triggering a series of microglia alterations, including the increase of proliferation and the production of inflammatory mediators [86]. However, this inflammatory process can be considered as beneficial for the neuronal tissue since it promotes the elimination of cellular debris and the secretion of neurotrophic factors. During the PD neuroinflammation processes, microglia releases pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. In fact, these cells act on the endothelium of the blood-brain barrier (BBB) leading to the increase of its permeability and stimulating the expression of adhesion molecules and chemokines that recruit peripheral blood mononuclear cells, such as macrophages, monocytes and T lymphocytes, contributing to the development of neuroinflammation condition through the production and secretion of pro-inflammatory mediators above described [87,88].

Cytokines are glycoproteins produced by several cell types, which model various physiological and pathological functions. Among cytokines, the pro-inflammatory play an important role in the neural and behavioral modulation during aging. Proliferative cytokines such as TNF-α or interleukin 1β (IL-1β) have been detected at high levels in the striatum of PD patients [8]. Moreover, the increase of TNF-α, IL-1β, and IL-6 production has also been implicated in the nitric oxide (NO) levels in several animal models, which contributes to the induction of oxidative stress and, consequently, to the neurodegenerative process in PD [77,79,89,90]. Chinta *et al.* (2013) [91] demonstrated that daidzein, an isoflavone isolated from soybean, attenuated the release of inflammatory mediators in microglial cells (BV-2), namely NO and IL-6. Zhang and Xu (2018) [92] induced a systemic treatment with LPS in PD mice and observing that an induction of an neuroinflammatory process by LPS, leading through the

release of several proinflammatory cytokines, including IL-1β, TNF-α, IL-18, cyclooxygenase and, activating TLR4/nuclear factor NF-κB pathway. In the presence of juglanin natural compound extracted from *Polygonum aviculare*, the authors observed that juglanin significantly reduced LPS-induced production of pro-inflammatory cytokines and blocked TLR4/NF-KB pathway, suggesting that juglanin, ameliorated neuroinflammation-releted memory impairment, and neurodegeneration through impeding TLR4/NF-kB, indicating its potential for PD prevention. Goes and collaborators (2018) [93] also observed that mice injected with 6-hydroxydopamine (6-OHDA) neurotoxin exhibited high levels of TNF-α, IL-1β, IL-2, IL-6 and low levels of DA. The changes induced by 6-OHDA exposition were reverted by the treatment with chrysin, a natural flavonoid isolated from bee propolis [117]. Sanchez-Guajardo and colleagues (2013) also observed that rats with high levels of pro-inflammatory cytokines in the SNpc of the midbrain were more susceptible to death of dopaminergic neurons in response to the neurotoxic damage. Therefore, several studies suggest that inflammatory process mediated by the activation of microglia and subsequent release of neurotoxic and pro-inflammatory factors can play an relevant role in the neuronal cell death associated to PD [91,92].

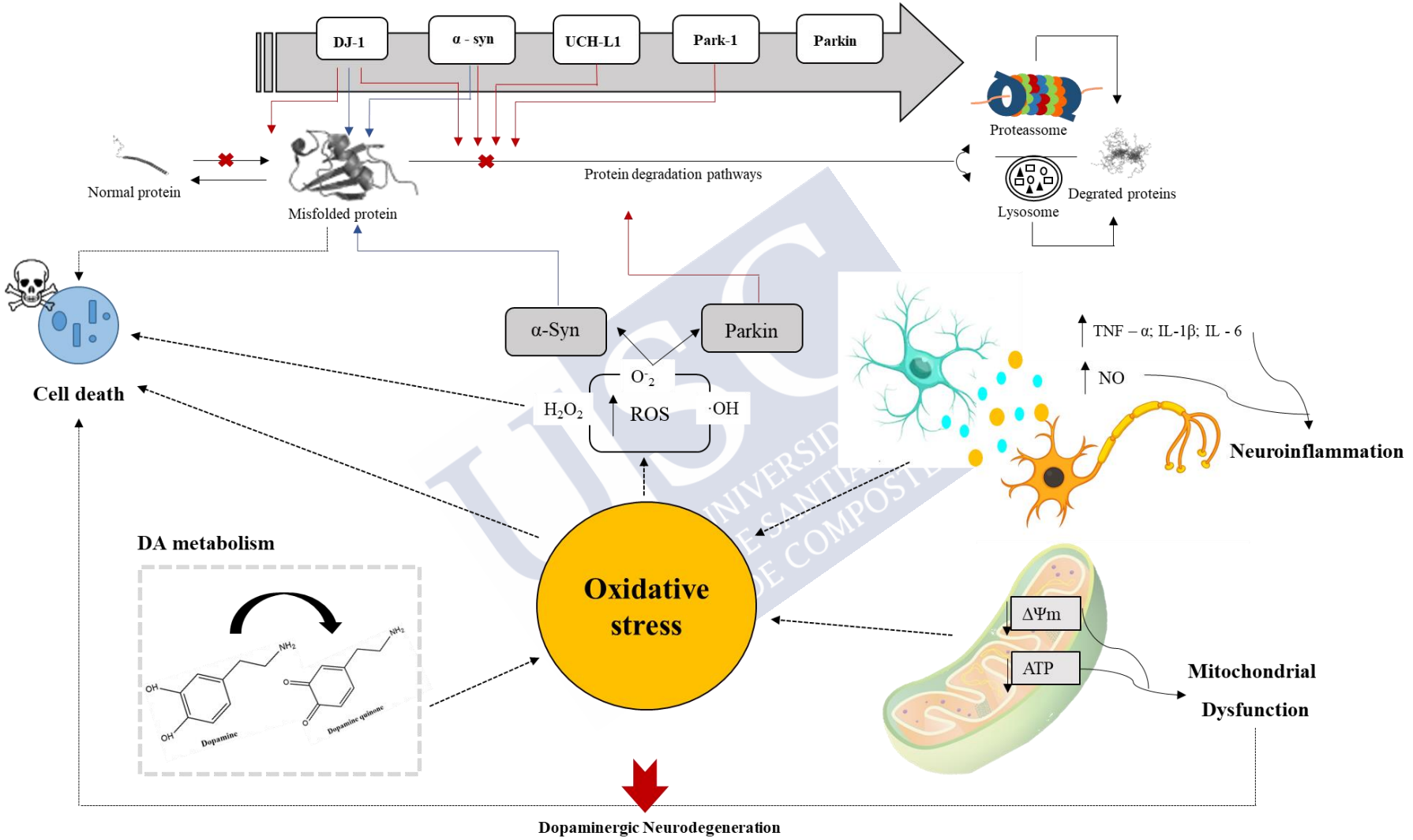


Figure 1.7. Physiological processes related to Parkinson's disease pathogenesis.

1.4. PD CELLULAR MODELS AND PHARMACOLOGYCAL THERAPEUTIC STRATEGIES

According to the Parkinson's Disease (PD) European Association, in 2011, the annual costs of Europe related to this illness were estimated approximately in 13.9 billion euros. Furthermore, as was mentioned previously, due to the aging of European population, and life expectancy increase will be expected to observe an increase of these values, being estimated that the number of people suffering of this pathology will double in the next 20 years. Although the etiology, pathology and molecular mechanisms are not well understood in PD, a better understanding has been obtained in the recent years through the use of several cellular and animal models exposed to neurotoxic agents such as 6-OHDA which cause a selective lesion in the *nigra* - striatal pathway [23,94]. The neurotoxin 6-OHDA (**Figure 1.8**) was the first chemical used to produce specific neurotoxic effects in the catecholaminergic pathways, namely in the dopaminergic neurons, being widely used on PD models, both *in vitro* and *in vivo* [95–97].

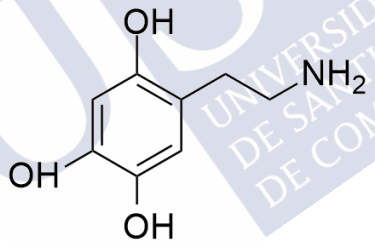


Figure 1.8. Chemical structure of the 6-hydroxidopamine (6-OHDA) neurotoxin.

However, there are evidences that 6-OHDA may be an endogenous and neurotoxic metabolite of dopamine (DA) that causes and / or contributes to Parkinson's disease, once dopaminergic pathway neurons contain high levels of DA, H_2O_2 and Fe (II), that may lead to the formation of 6-OHDA through non-enzymatic reactions.

The generation of this metabolite may also be favored by the presence of melanine increasing Fe (II) levels, and thus amplifying the reaction between DA and Fe ions. Several *in vitro* and *in vivo* studies have demonstrated that 6-OHDA accumulates in the cytoplasm inducing oxidative stress by boosting ROS formation, such as the hydroxyl radical (·OH), simultaneously increasing H_2O_2 levels, thus overwhelming antioxidant defenses [95,97]. The

6-OHDA -induced neurotoxicity also interacts directly with the mitochondrial respiratory chain, inhibiting the Complex I. However, this neurotoxin does not cross the blood-brain barrier and it cannot be administered systemically *in vivo*, being administered intra-cerebrally by stereotactic injection. Jesús-Cortés and Co-Workers (2015) [98] showed a decrease in the DA levels when mice were injected with 6-OHDA. The treatment with P7C3-S243 drug, a recovery of DA levels was observed demonstrating the neuroprotective effects of P7C3-S243 in a PD model induced by 6-OHDA. Therefore, Kim and Co-Workers (2015) [99] also studied the neuroprotective effect of the 5,7-dihydroxychromone (DHC) compound isolated from the plant *Cudrania tricuspidata* on SH-SY5Y cells when exposed to 6-OHDA and observed that the compound was able to reverse the effect induced by the neutoxin. This compound exhibited capacity to activate the Nrf2 / ARE signaling pathway, promoting neuroprotective effects against oxidative stress and apoptosis induced by 6-OHDA treatment, suggesting that the activation of this signaling pathway may be a new route for the PD therapeutics.

To date, there is no way to prevent, stop, or cure PD, and the available therapies only reduce the motor symptoms [100]. Currently, there are only two main therapeutic approaches: pharmacological, which focuses on the research to improve the characteristics of existing drugs, and the surgical, as a traditional alternative. The currently available drugs for PD treatment allow to control the symptoms and prevent progressive neuronal cell degeneration of the dopaminergic system, maintaining the functional capacity and improving the life quality of patients [101]. However, these therapeutic agents have not shown to be effective over the course of treatment presenting some side-effects as toxicity after long use.

Levadopa (L-dopa) is a mainstay therapy widely used in PD treatment that compensate the loss of DA and dopaminergic function [102]. However, its efficacy decreases gradually as the disease progresses developing medication-related complications such as motor fluctuations and dyskinesia, potential side effects such as impulse control disorders, sleepiness or sudden-onset sleep, and DA dysregulation syndrome [103]. Prasad and co-workers (1999) [104] observed that the administration of L-dopa may increase the generation of free radicals *in vivo*, evidencing severe side effects in five years of therapy. The effects observed are possibly due to the increase of free radical production resulting from oxidative metabolism of L -dopa and DA. Accordingly, several researchers continue to investigate the development of new drugs that may mitigate the adverse effects induced by the current drugs.

The involvement of free radicals and, consequently, the oxidative stress mechanism has been widely accepted as a trigger mechanism in several pathologies, especially in PD, leading to cell death [56,101,104]. Consequently, the interest in the development of therapeutic applications based in the use of antioxidant compounds has increased. In the last years, several works have carried out exhaustive studies on *in vitro* and *in vivo* models evaluating the possible therapeutic properties of antioxidant compounds [56]. Xu *et al.* (2017) [105] showed that epigallocatechin gallate (EGCG), the most abundant green tea polyphenol with high antioxidant actvity was capacity to restore MPTP-induced functional and neurochemical deficits in mouse PD model, but also showed capacity regulate the iron-export protein ferroprotein in the substantia *nigra* and reduce oxidative stress.

Coenzyme Q10 (CoQ10) is a compound found naturally in virtually all cells of the human body, recognized for its key role in mitochondrial bioenergetics and its antioxidant properties [106]. Thus, several investigations have been performed with CoQ10, demonstrating its neuroprotective properties in various *in vitro* and *in vivo* models [101], with special focus in the mitochondrial dysfunction induced by paraquat and rotenone and in neurodegeneration in rat mesencephalic primary neurons [101,106,107]. Somayajulu *et al.* (2005) [108] also demonstrated the neuroprotective effects of pre-treatment performed with CoQ10 on SH-SY5Y cells exposed to H_2O_2 that contributed to maintain the mitochondrial membrane potential and reduce the generation of ROS in mitochondria. In addition to antioxidant compounds, other compounds have also revealed a neuroprotective potential in models of PD, such as diterpenes, alkaloids, polysaccharides and sterols. Xu *et al.* (2011) [109] observed a neuroprotective effect of two diterpenes isolated from bulbs of *Fritillaria ebeiensis* againt MPTP-induced neurotoxicity on SH-SY5Ycells. Furthermore, Liu *et al.* (2018) [110] isolated six alkaloids from leaves of *Isatis indigotica fortune* and the results showed that in H_2O_2-induced SH-SY5Y cell injury model, the alkaloids indiforine A and B exhibited promising neuroprotective activities. In addition, the authors verified that the neuroprotective effect was mediated by inhibiting cell apoptosis H_2O_2 – induced.

1.5. MARINE NATURAL PRODUCTS AS NEW PARKINSON'S DISEASE POTENTIAL THERAPEUTIC

From a very early age, humans began to use the oceans to navigate and to search food and new territories, but it was only in the middle of the 19th century that they began to explore the seabed. In the last decades, marine environment has proved to be a unique source of new chemical entities with potential application in different areas such as pharmaceutical, cosmetic, food and agrochemical, revealing itself as a vital factor for the discovery of new products [111].

The number of marine species that inhabit the world's oceans is not truly known, however, experts estimate a number that approaches 1 to 2 million species [112]. These organisms survive and live in a competitive and exigent environment, constituted by complex communities, establishing close associations with other organisms. These interactions subject organisms to several ecological challenges such as competition for space, predation, competition, tidal variations, among others, leading marine organisms to develop distinct strategies, including survival and behavioral, physical and/ or chemical [113,114]. In fact, marine organisms along of their evolution developed capacity to produce secondary metabolites with unusual chemical structures that act as chemical weapons against those ecological threats.

Over the past few decades, several compounds have been isolated from marine organisms and many of them have exhibited great biological activities, including anticancer, antimicrobial, antifungal, antiviral, anti-inflammatory, and others [114–116]. These compounds presented great pharmacological potential can contribute to inspire the development of new therapeutic agents [117]. According to Faulkner and Blunt and their collaborators, between 1977 and 2016, sponges (30.7%), followed by microorganisms & phytoplankton (21.63%) and seaweeds (11.06%) were the marine organisms from which the largest number of new marine natural compounds were isolated (**Figure 1.9**).

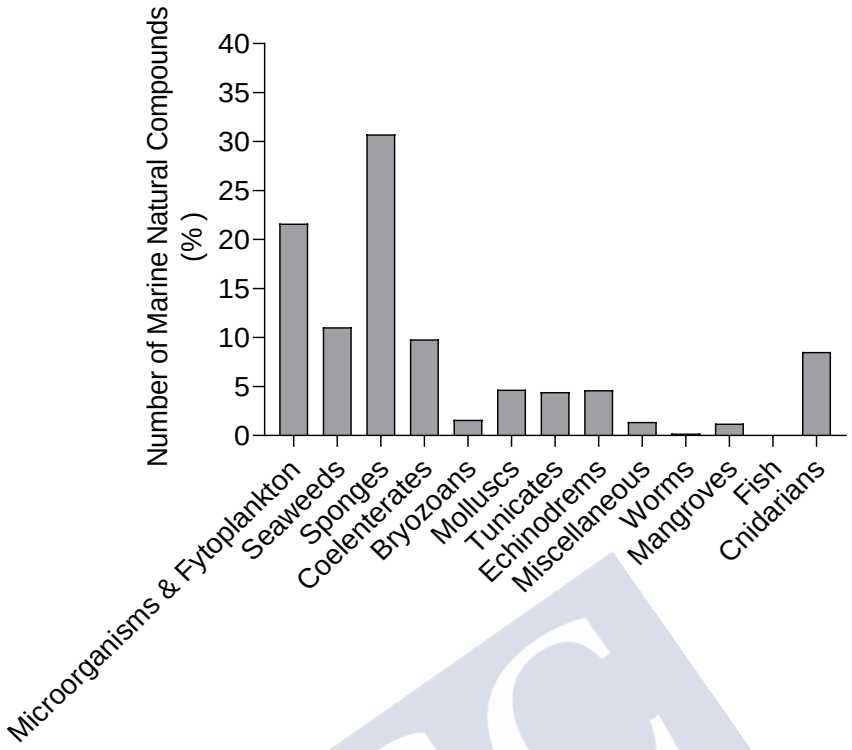


Figure 1.9. Number of new marine natural products (%) isolated from marine organisms between 1977 and 2016 (Adapted from Faulkner, 1984 – 2002; Blunt *et al*. 2003-2018) [118–133].

Due to the urgent needed by discovery and develop new effective therapeutic agents to neurodegenerative diseases treatments, including Parkinson's Disease, as well as the ability of marine organisms biosynthesize compounds with distinct chemical structures, biological activities and mechanisms of action several scientists have explored their ability as neuroprotective agents [10]. Analyzing the compounds with neuroprotective activities isolated from marine organisms (**Table 1.1**), except seaweeds (**Table 1.2**), it is possible to observe that most of them were obtained from sponges, fungi and corals, followed by starfish, cnidarians, and marine bacteria.

Table 1.1. Neuroprotective activities of compounds isolated from marine organisms, except seaweeds, and their mode of action in Parkinson's disease cellular models.

Marine organism	Species	Compound	Mode of action	Reference
Sponge	*Spongionella gracilis*	Gracilin A **(1)**	Inhibition of H_2O_2 - induced neurotoxicity on SH-SY5Y cells; Activation of Nrf2/ARE pathways	[134]
		Gracilin H **(2)**		
		Gracilin J **(3)**		
		Gracilin K **(4)**		
		Gracilin L **(5)**		
		Tetrahydroaplysuphurin-1 **(6)**		
	Haliclona (Rhizoniera) sarai	Sarain A **(7)**	Inhibition of H_2O_2 - induced neurotoxicity on SH-SY5Y cells; Block the mPTP and enhance the Nrf2 pathway	[135]
	-	Hymenin **(8)**	Inhibition of H_2O_2 - induced neurotoxic in primary cortical neurons; Decrease of ROS production; Increased GSH levels; Induces the translocation of the NRf2 factor to the nucleus	[136]
		Hymenialdisine **(9)**		
	Psammaplysilla sp.	Psammaplysene A **(10)**	Neuroprotective effects in the HEK293 cells and Drosophila *in vivo* model mediated by modifying processes dependent on heterogeneous nuclear ribonucleoprotein (HNRNPK) protein that controls biological aspects of RNA	[137]
	Xestospongia testudinaria	24-Hydroperoxy-24-vinylcholesterol **(11)**	Inhibition of NF-kB factor transcription activation	[138,139]
		29-Hydroperoxystigmasta-5.24(28)-dien-3-ol **(12)**	Inhibition of H_2O_2 - induced neurotoxicity on SH-SY5Y cells	
	Iotrochota sp.	Iotrochotazine A **(13)**	Act on the early endosome and lysosome markers in idiopathic PD patients	[140]
	Jaspis splendens	Jaspamycin **(14)**	Increase of lysosomal staining and decrease of the EEA-1 number of that are phenotypic responses on a hONS cellular model of PD	[141]

Soft Coral	*Cladiella australis*	Austrasulfone (15)	Neuroprotective effect on SH-SY5Y cells against 6-OHDA – induced neurotoxicity	[142]
	Sinularia flexibilis	11- Dehydrosinulariolide **(16)**	Neuroprotective effect on SH-SY5Y cells against 6-OHDA – induced neurotoxicity mediated by anti-apoptotic and anti-inflammatory actions via PI3K signaling pathway	[143]
	Sarcophyton glaucum	Sarcophytolide **(17)**	Neuroprotective effect on primary cortical cells from rat embryos against glutamate – induced neurotoxicity mediating an increase of the proto-oncogenebcl-2 (BCl-2) expression	[144]
	Cladiella australis	1-Tosylpentan-3-one **(18)**	Neuroprotective effect on SH-SY5Y cells against 6-OHDA – induced neurotoxicity mediated by activation of both p38 mitogen-activated protein kinase (MAPK), and an increase of Caspase-3 and nuclear factor erythroid 2-related factor 2 (Nrf2) expression	[145]
Fungi	*Eurotium rubrum* *Microsporum* sp *Aspergillus* sp.	Neoechinulin A **(19)**	Inhibition of MPP^+/ rotenone - induced neurotoxicity on PC12 cells; Inhibition of mitochondrial Complex I dysfunction	[146,147]
	Xylaria sp.	Xyloketal B **(20)**	Neuroprotective effects on PC12 cells against MPP^+ -induced neurotoxicity mediated by a increase of viability cellular, reduction of intracellular ROS accumulation, loss of mitochondrial membrane potential and restoration of total GSH level	[148]
	Aspergillus ochraceus Paecilomyces sp.	Secalonic acid A **(21)**	Neuroprotective effects on SH-SY5Y cells against MPP^+ -induced neurotoxicity mediated by an increase viability cellular, reduction of BAX expression and a increase of Caspase – 3;Capacity of increased the number of dopaminergic neurons and upregulated striatal dopamine in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease mice	[149]
	Penicillium sp.	6-Hydroxy-N-acetyl-ß-oxotryptamine **(22)**	Neuroprotective effects against 6-OHDA-induced neuronal cell death	[150]
		3-Methylorsellinic acid **(23)**		
		8-Methoxy-3,5-dimethylisochroman-6-ol **(24)**		
	Aspergillus sp.	Candidusin A **(25)**		
		4" - Dehydroxycandidusin A **(26)**		

Marine Fungi	*Aspergillus flocculosus*	Diketopiperazine mactanamide **(27)**	Neuroprotective effects against 6-OHDA-induced neuronal cell death	[150]
Sea Cucumber	*Cucumaria frondosa*	Glucocerebrosides -1 **(28)**	Neuritogenic effects on PC12 cells in a dose-dependent and structure-selective manner, increasing the ratio of neurite-bearing cells and expression of axonal (GAP-43) and synaptic (synaptophysin) proteins	[151]
		Glucocerebrosides- 2 **(29)**		
		Glucocerebrosides- 3 **(30)**		
Bacteria	*Streptomyces* sp.	NP7 **(31)**	Inhibition of H_2O_2-induced neurotoxicity on Neuronal-enriched and glial mesencephalic cultures; Prevented the increase of ERK and AKT phosphorylation induced by H_2O_2	[152]
	-	Mannosylglycerate **(32)**	Inhibition of α – synuclein aggregation on *Saccharomyces cerevisiae* cells	[153]
	Streptomyces sp	Piloquinones A **(33)**	Inhibition of monoamine oxidase (A and B) activity	[154]
		Piloquinones B **(34)**		
Sea snails	*Conus textile*	α-Conotoxin **(35)**	Regulation of DA release by blocking α6/α3β2β3 nAChR revealin	[155]
Starfish	*Asterias rollestoni*	Glucan **(36)**	Inhibition of 6-OHDA – induced neurotoxicity on MES cells 23.5	[156]
		Mannoglucan sulfate **(37)**		

The chemical structures and mechanisms of action of the marine natural produts presented in the **Table 1.1** are deeply described in the following sections **1.5.1 – 1.5.5**.

1.5.1. Sponge – derived neuroprotective compounds

The gracilins A, H, K, J, L **(1 – 5)** and tetrahydroaplysulphurin -1 **(6)** are a group of diterpernes previously isolated from *Spongionella gracilis* that exhibited capacity to act on tyrosine kinases. Moreover, all compounds demonstrated ability to protect cortical neurons against oxidative damage induced by H_2O_2 mediated by restoring of mitochondrial membrane potential, scavenging of intracellular reactive oxygen species (ROS), increase of glutathione levels, increase of Caspase-3 expression and activation of the Nrf2/ARE pathway [134]. On the other hand, the alkaloid sarain A **(7)** and two bromopyrroloalkaloids, hymenin **(8)** and hymenialdisine **(9)** isolated from *Haliclona (Rhizoniera) sarai* an unidentified sponge, respectively, exhibited the same effects previously described [135,136]. Boccito and co-workers (2017) isolated psammaplysene A bromotyrosine alkaloid **(10)** from the *Psammaplysilla* sp. sponge that displayed neuroprotective effects in the HEK293 cells and *Drosophila in vivo* model that seem mediated by modifying processes dependent on heterogeneous nuclear ribonucleoprotein (HNRNPK) protein that controls biological aspects of RNA [7]. Two sterols 29-hydroperoxystigmasta-5.24(28)-dien-3-ol **(12)** and 24-hydroperoxy-24-vinylcholesterol **(11)** isolated from *Xestospongia testudinaria* also inhibited H_2O_2 - induced neurotoxicity and activated NF-KB transcription factor, respectively [138]. Iotrochotazine A **(13)** derived from the *Iotrochota sp*. sponge, was used as a chemical probe in a phenotypic assay panel based on human olfactory neurosphere-derived cells (hONS) from idiopathic PD patients. The authors verified that Iotrochotazine A did not exhibited cytotoxicity but, affected the morphology and the cellular distribution of lysosomes and endossomes in olfactory neurosphere-derived cells. These results showed that the compound may be a useful tool to investigate the molecular mechanisms underlying PD [140]. Also, Wang *et al.* (2016) isolated jaspamycin **(14)**, a alkaloid from *Jaspis splendens*, which revealed ability to increase lysosomal staining and decrease the number of EEA-1 characteristic of phenotypic response in a hONS neurosphere-derived cell model of Parkinson's Disease [141].

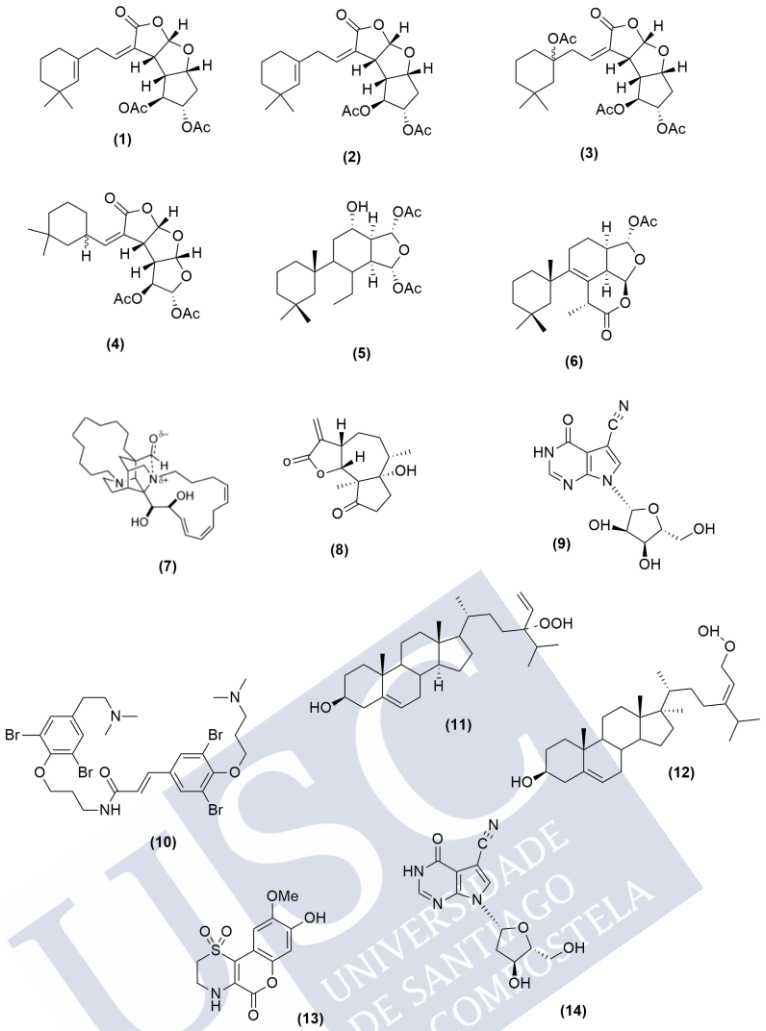


Figure 1.10. Chemical structures of marine natural products isolated from sponges: gracilins (A, H, K, J and L) (1-5), tetrahydroaplysulphurin-1 (6), sarain (7), hymenin (8), hymenialdisine (9), 24-hydroperoxy-24-vinylcholesterol (11), 29-hydroperoxystigmasta-5.24(28)-dien-3-ol (12), iotrochotazine (13) and jaspamycin (14).

1.5.2. Soft Coral – derived neuroprotective compounds

Austrasulfone **(15)**, 11-dehydrosinulariolide **(16**), and 1-tosylpentan-3-one (**18)** compounds isolated from soft corals have shown to protect SH-SY5Y cells from neurotoxicity induced by 6-OHDA acting in different intracellular signaling pathways. Chen and collaborators (2012) [143] isolated 11-dehydrosinulariolide **(16)** from *Sinularia flexibilis,* wich exhibited anti-inflammatory and neuroprotective activities. Regarding the anti-inflammatory effect, the 11-dehydrosinulariolide reduced the expression of two major pro-inflammatory mediators, NO and cyclooxygenase on lipopolysaccharide-stimulated macrophages. On the

other hand, the neuroprotective activity was evaluated on SH-SY5Y cells exposed to 6-OHDA neurotoxin. The compound exhibited ability to recovery the 6-OHDA – induced neurotoxicity on SH-SY5Y cells. This protective effect was accompanied by a significant inhibition of Caspase-3/7 activities as well as by the mediation of via PI3K signaling activating phosphorylated - AKT. Feng and co-workers (2016) [157] also evaluated the neuroprotective effects of 11-dehydrosinulariolide on different *in vitro* and *in vivo* models exposed to 6-OHDA neurotoxin, namely SH-SY5Y cells, rats and zebrafish. The authors observed that the compound enhanced the DJ-1 expression in the SH-SY5Y cells cytoplasm and activated the AKT / PI3K, Nrf2 / HO-1 intracellular signaling pathways. In addition, also revealed a 6-OHDA-induced attenuation on tyrosine hydroxylase (TH), a dopaminergic neuronal marker in zebrafish and rat models of PD could also be reversed by treatment with 11-dehydrosinulariolide.

The metabolite 1-tosilpentan-3-one **(18)** isolated by Kao *et al.* (2017) [145] from *Cladiella australis* exhibited neuroprotective effects in SH-SY5Y cells treated with 6-OHDA mediated by MAPK-p38 activation, inhibition of Caspase-3 activity, and increased expression of factor Nrf2 and oxygenase-1 (HO-1), through the intracellular signaling pathway PI3 / AKT. In addition, the authors also tested the neuroprotective effects of 1-tosilpentan-3-one in a zebrafish model, reporting that, a decrease in locomotive capacity and an increase in 6-OHDA-induced TNF-α was inhibited by this metabolite (**Table 1.1**). Bradia *et al.* (1998) [144] isolated a new diterpene sarcophytolide **(17)** from *Sarcophytom glaucum*, which exhibited to cytoprotective effect against glutamate-induced neurotoxicity in primary cortical cells from rat embryos resulting in a significant increase of percentage of viable cells. The authors observed that cytoprotective effect presented by sarcophytolide was mediated by a decrease of Ca^{2+} levels and an increase of BCl-2 pro-oncogene expression levels.

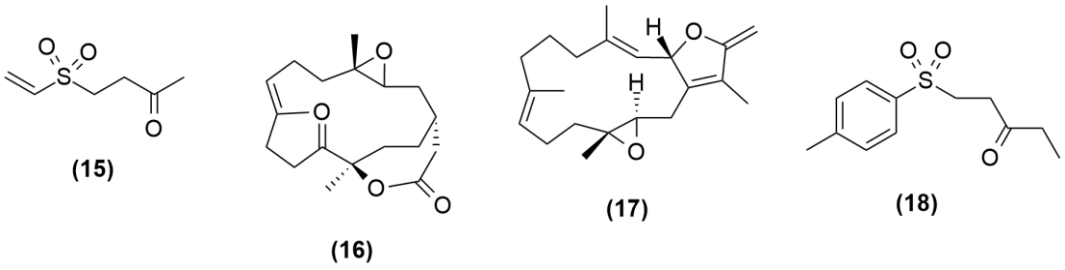


Figure 1.11. Chemical structures of marine natural products isolated from soft corals: austrasulfone **(15)**, 11-dehydrosinulariolide **(16)**, sarcophytolide **(17),** and 1-tosylpentan-3-one **(18)**.

1.5.3. Marine fungi – derived neuroprotective compounds

Neoechinulin A **(19)** an alkaloid isolated from different species of marine fungi, demonstrated ability to decrease the neurotoxic effects induced by 1-methyl-4-phenylpyridinium (MPP+) toxin and rotenone on PC12 cells. Kajimura *et al.* (2008) [146] observed that Neoechinulin A could protect PC12 cells from MPP^+ - induced neurotoxicity mediated by inhibition of mitochondrial dysfunction in Complex I, suggesting that alkaloid can ameliorate downstream events of mitochondrial failure. Furthermore, Akashi *et al.* (2011) [147] also observed that Neoechinulin A could protect PC12 cells from rotenone-induced neurotoxicity. The authors verified that pre-treatment with Neoechinulin A, significantly impeded cell death and decreased ATP levels promoted by rotenone. Lu and co-workers (2010) [148] isolated xyloketal B **(20)** from the fungus *Xylaria* sp., which decrease the neurotoxic effects induced by MMP^+ *in vitro* and *in vivo* models, namely PC12 cells and *Caenorhabditis elegans*, respectively. Xyloketal A attenuated the MMP^+ toxin effects through the decrease of the intracellular ROS levels and restore of the GSH levels. Secalonic acid A **(21)** obtained from the fungus *Aspergillus ochraceus* inhibited the neurotoxicity induced by the MMP^+ toxin on rat cortical neurons. The compound promoted the activation of c-jun N-terminal Kinases (JNK) and MAPK-p38, and the inhibition of Caspase-3 activity decreasing apoptosis triggered by MPP^+ [149]. 6-hydroxy-N-acetyl-β-oxotryptamine (**22**) isolated from *Penicillium* sp. protected Neuro2A cells against the damage induced by 6-OHDA and Paraquat (PQ) neurotoxins, mediating a reduction of ROS production more marked than the antioxidant standard melatonin. Finally, the 3-methylorsellinic acid **(23)** and 8-methoxy-3,5-dimethylisochroman-6-ol **(24)** compounds isolated from *Penicillium* sp. and candidusin A **(25)** and 4" – dehydroxycandidusin **(26)** compounds attained from *Aspergillus* sp. 4" – dehydroxycandidusin also exhibited protective effects against 6-OHDA and PQ neurotoxins treatments on Neuro2A cells decreasing the production of ROS.

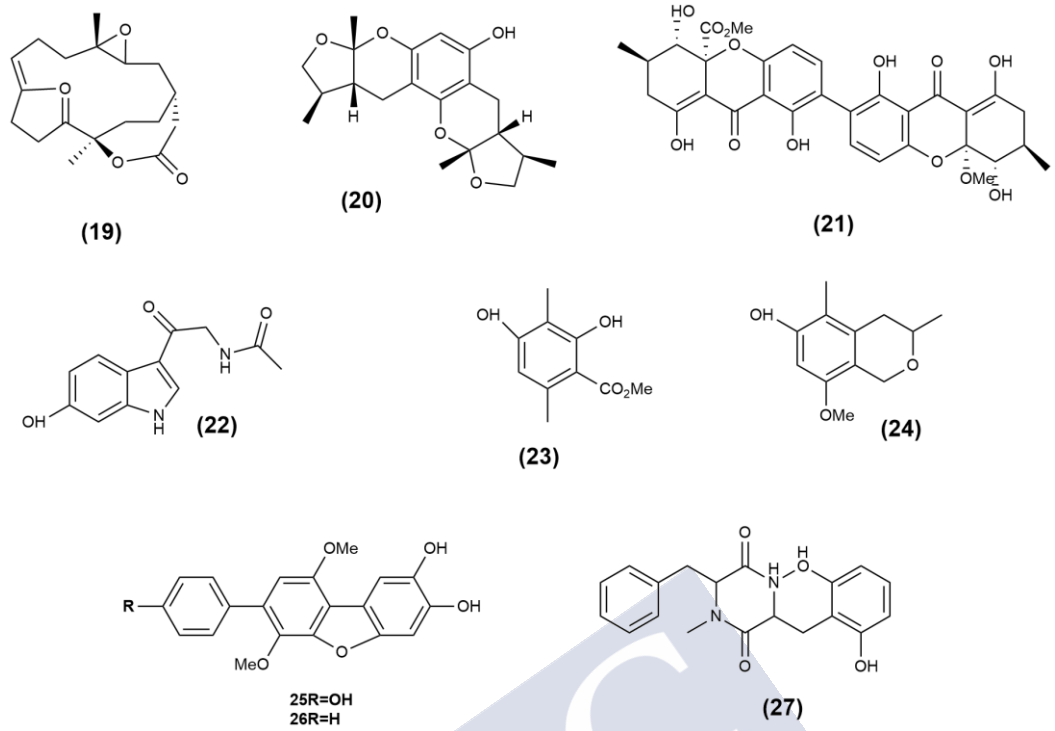
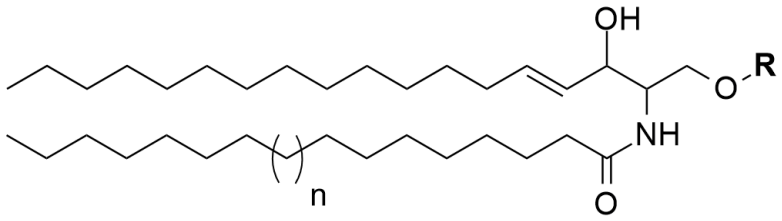


Figure 1.12. Chemical structures of marine natural products isolated from fungi: neoechinulin A **(19)**, xyloketal B **(20)**, sacalonic acid A **(21)**, 6-hydroxy-*N*-acetyl-b-oxotryptamine **(22)**, 3- methylorsellinic acid **(23)**, 8-methoxy-3,5-dimethylisochroman-6-ol **(24)**, candidusin A **(25),** 4``- dehydroxycandidusin A **(26),** and diketopiperazine mactanamide **(27)**.

1.5.4. Sea cucumber – derived neuroprotective compounds

Glucocerebrosides, SCG-1 **(28)**, SCG-2 **(29)** and SCG-3 **(30)** isolated from *Cucumaria frondosa* are important sphingolipids. Wang *et al.* (2018) [151] studied in an nerve growth factor (NGF)-induced PC12 model the neuritogenic effect and signalling pathways of SCGs. Neuritogenesis is a dynamic phenomenon associated with neuronal differentiation, and it is important for neuronal development and regeneration. Neurotrophic factors including NGF, brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4/5 (NT3 and NT4/5, respectively), can stimulate neurite growth, maintain viability, and induce differentiation. This way, the authors observed that SCGs exhibited significant neuritogenic effects in a dose-dependent and structure-selective manner, which increased the ratio of neurite-bearing cells and expression of axonal (GAP-43) and synaptic (synaptophysin) proteins. In addition, mechanistic studies suggested that SCGs reinforced the NGF-induced phosphorylation of TrkA, coupled

with ERK1/2 activation, triggering the activation of CREB, that result in a marked increase of BDNF expression.



28 R=H
29 R=Gal
30 R=Glc

Figure 1.13. Chemical structures of marine natural products isolated from sea cucumber: SCG-1 (**28**), SCG-2 (**29**) and SCG – 3 (**30**).

1.5.5. Bacteria, sea snail and starfish-derived neuroprotective compounds

Several researchers have been searching for new compounds with neuroprotective activity from organisms still poorly explored, such as bacteria, sea snails, and starfish [9,11,15,16]. NP7 **(31)** is a marine-derived compound from *Streptomyces* sp. that prevent apoptosis and necrosis induced by H_2O_2 treatment on neurons and glial cells. Furthermore, NP7 also displayed ability to inhibit the microglial activation and prevent the increase of ERK phosphorylation induced by H_2O_2 exposition [152]. Mannosylglycerate **(32)** isolated by Faria and co-workers (2013) [153] from aggregation / expression thermophilic bacteria reduced the number of *Saccharomyces cerevisiae* cells that presented α-syn as well as inhibited the fibrillation of this protein. These results are a relevant evidence, since α-syn is the main component of the intraneuronal inclusions found in the PD patients' brains. Piloquinone A **(33)** and piloquinone B **(34)** compounds obtained from *Streptomyces* sp. shown to be potent inhibitors of MAO-A (IC_{50}= 6.47 μM) and MAO-B (IC_{50}= 1.21 μM) suggesting their therapeutic potential in PD as MAO inhibitors. α-conotoxin **(35)** isolated from *Conus textile* [155] exhibited ability to regulate DA release by blocking α6/α3β2β3 nAChR revealing to be a promising candidate for new PD therapeutics. From the starfish *Asterias rollestoni*, Zhang and co-workers (2013) [156] isolated two polysaccharides, glucan **(36)** and mannoglucan sulfate

(37) which were able to inhibit the neurotoxic effects induced by 6-OHDA in mouse embryonic stem cells (MES 23.5 cells).

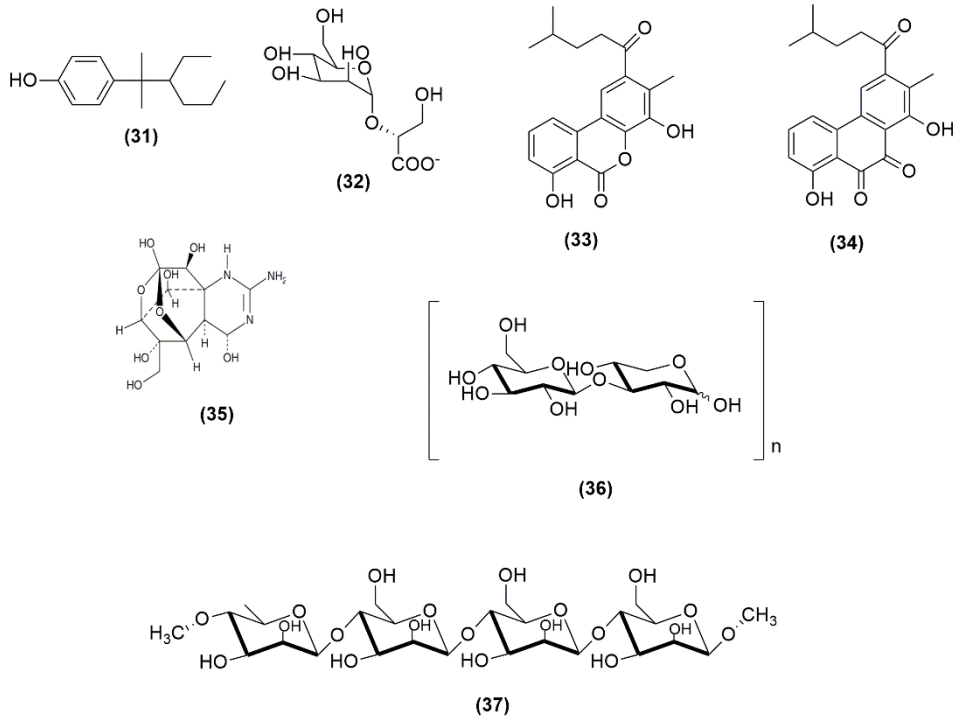


Figure 1.14. Chemical structures of marine natural products derived from bacteria, sea snails and starfish: NP7 **(31)**, mannosylglycerate **(32)**, piloquinone A **(33)**, piloquinone B **(34)**, α-conotoxin **(35),** glucan **(36),** and mannoglucan sulfate **(37).**

1.6. SEAWEEDS AS POTENTIAL SOURCE OF NOVEL COMPOUNDS TO PARKINSON'S DISEASE THERAPEUTICS

Seaweeds have revealed to be a huge source of new chemical entities with great biological activities, being widely used for nutritional or medicinal purposes since many years ago [158]. Seaweeds are defined as marine macroscopic eukaryotic photosynthetic organisms organized into three groups according to their pigments: red (Rhodophyceae), green (Chlorophyceae) and brown (Ochrophyta) seaweeds [159]. Over the last decades, several researchers have studied the chemical composition of seaweeds, which revealed to be rich in various compounds like phytopigments (xanthophylls and carotenoids), polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA), phenolic compounds, tannins, peptides, lipids, enzymes, vitamins, carbohydrates, terpenoids, among others [114,160,161]. The chemical diversity

observed is directly related with the fact that these organisms are widely distributed in diverse and extreme environments. Due of their ecology, seaweeds along of their evolution developed several strategies to survey in the environment where are inserted, including the ability to synthesize complex bioactive substances. These compounds act as chemical defense against distinct ecological and physical pressures, such as predators, competitors, UV light exposure, salinity and temperature variations, among others. Those substances that derived from their primary and secondary metabolism have been reported to possess a wide range of biological properties as antioxidant, antimicrobial, anti-tumoral, neuroprotective, antiviral, anti-inflammatory, among others [116,160,162–164]. Accordingly, seaweeds have aroused the researchers interest as a source of biological active molecules with a huge potential for application in food, cosmetic and pharmacological industries, not only because of their complexity but also for their diversity and the availability of resources [160]. Along of the last five decades is estimated that more 31 000 NPs have been isolated from marine organisms and 11% corresponding to compounds attained from seaweeds. Among of them, the NPs isolated were mostly derived from red seaweeds (53.59%), followed by brown (37.06%) and green (9.35%) seaweeds (**Figure 1.15**).

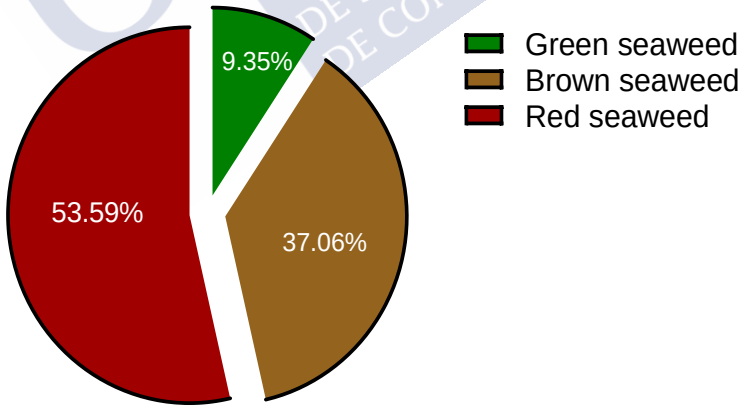


Figure 1.15. Percentage (%) of the new natural products isolated from red, brown and green seaweeds between 1977 and 2016 (Adapted from Faulkner, 1984 – 2002; Blunt *et al*. 2003 – 2018).

Due to the limitations of the currently therapeutics, such as prolonged administration and consequently adverse side-effects there is an exhaustive effort of the scientific community in order to explore the nature as source of new neuroprotective therapeutic agents, as described before. In this area, seaweeds have emerged as potential source of compounds with neuroprotective activities for Parkinson's Disease treatment. The studies already carried out with seaweeds - derived compounds on *in vitro* and *in vivo* PD models are presented **Table 1.2**.

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Table 1.2. Neuroprotective activities of seaweeds - derived compounds and their mode of action on *in vitro* and *in vivo* Parkinson's disease models.

Source	Specie	Compound	Mode of action	Reference
Brown seaweeds	*Sargassum integerrimum* *Sargassum naozhouense* *Sargassum fusiforme*	Heteropolysaccharides **(38)**	Neuroprotective effect on Mes 23.5 cells against 6-OHDA – induced neurotoxicity	[165]
	Saccharina japonica	Fucoidan **(39)**	Neuroprotective effect on Mes 23.5 and SH-SY5Y cells against 6-OHDA – induced neurotoxicity	[166]
	Laminaria japonica	Fucoidan **(39)**	Neuroprotective effect on dopaminergic cell line (MN9D) against MPP+ - induced neurotoxicity and also on MPTP-induced animal model of Parkinsonism (C57/BL mice); Fucoidan attenuates MPTP-induced neurotoxicity on mice; Neuroprotective effect in Mes23.5 cells against H_2O_2 – induced neurotoxicity mediated by apoptosis inhibition increasing Bcl-2 / Bax ratio and decreasing the expression of Caspase-3 in the PI3K / Akt signaling pathway	[167–169]
	Porphyra haitanensis	Porphyran **(40)**	Neuroprotective effects in MES 23.5 cells against 6-OHDA-induced neurotoxicity	[170]
Red seaweeds	-	κ-Carrageenan **(41)**	Protection of microglial cells through the reduction of TNF-α and arginase levels	[171]
	Hypnea musciformis	κ-Carrageenan **(41)**	Neuroprotective effect on SH- SY5Y cells against 6-OHDA – induced neurotoxicity protecting mitochondrial membrane potential and decreasing Caspase -3 activity	[172]
	Gracilaria cornea	Agaran **(42)**	Neuroprotective effect on rat *in vivo* model against 6-OHDA – induced neurotoxicity mediated by reduced the oxidative/nitroactive stress and through alteration in the MAO contents induced by 6-OHDA.	[173]
	Turbinaria decurrens	Fucoidan (**39**)	Neuroprotective effect on rat *in vivo* model against MPTP – induced neurotoxicity	[168]

Jin and *et al.* (2014) [165] studied the neuroprotective effects of six heteropolysaccharides **(38)** isolated from the genus *Sargassum*, namely *Sargassum integerrimum, Sargassum nozhouense* and *Sargassum fusiformis*. On the other hand, Wang and co-workers (2016) isolated the porphyrin **(40)** polysaccharide from the *Porphyra haitanensis* seaweed together with two of its derivatives [170]. All of these compounds inhibited the neurotoxicity induced by 6-OHDA on Mes23.5 dopaminergic neuron cell.

Fucoidan is a sulfated polysaccharide mainly present in the brown seaweeds, having its neuroprotective activity already evaluated on different cellular models [166,167,169,170]. Jin and co-workers (2013) [166] described the neuroprotective potential of fucoidan **(39)** isolated from *Saccharina japonica* on Mes23.5 and SH-SY5Y cells against the toxicity induced by 6-OHDA neurotoxin. On the other hand, Gao and co-workers (2012) [167] evaluated the neuroprotective effect of fucoidan **(39)** isolated from the brown seaweed *Laminaria japonica* on PC12 cells when exposed to H_2O_2. The polysaccharide treatment increased PC12 cells' viability reducing the H_2O_2-induced toxicity, and reducing the ROS generation and lactate dehydrogenase (LDH) [167]. Fucoidan also inhibited apoptosis increasing Bcl-2 / Bax ratio and decreasing the expression of Caspase-3, as well as enhancing the PI3K / Akt signaling pathway. Furthermore, *in vivo* assays were also conducted with fucoidan. Meenakshi and co-workers (2016) [168] reported the neuroprotective effects of fucoidan **(39)** on rats, observing that in the presence of fucoidan occurred an increase of antioxidant defenses and DA levels when compared to MPTP treatment. Moreover, the authors also observed an increase in tyrosine hydroxylase (TH) positive cells in the fucoidan treated group, which correlates with levels of TH protein in the substantia *nigra* and corpus striatum. In addition, verified that the increase is greater than the content of dopamine and DOPAC, which may be explained that the dopaminergic terminals are more sensitive to MPTP toxicity and therefore are more severely damaged that the dopaminergic cell bodies. Souza and co-workers (2017) [173] demonstrated the neuroprotective effect of agaran **(42)**, a polysaccharide isolated from the seaweed *Gracilaria cornea*, on rats when subject to 6-OHDA neurotoxin. The authors observed that agaran promoted neuroprotective effects *in vivo* through reducing the oxidative/nitroactive stress and through alteration in the MAO contents induced by 6-OHDA.

The occurrence of an inflammatory response in the central nervous system have been associated with the development of neurodegenerative diseases, such as Parkinson's disease, that is characterized by activation of microglial cells and an increase of inflammatory cytokines

expression. Accordingly, Yao and *et al.* (2014) [171] evaluated the neuroprotective effects of κ-carrageenan **(41)** isolated from a red seaweed, which inhibited the activation of microglial cells and decreased TNF-α and arginase expression, treated with LPS. In its turn, the κ-carrageenan **(41)** attained from the *Hypnea musciforms* red seaweed [172] protected SH-SY5Y cells against the neurotoxicity induced by 6-OHDA, reducing H_2O_2 production, Caspase-3 activity and MMP depolarization. Souza and co-workers (2017) [173] demonstrated that the sulphated polysaccharide isolated from the *Gracilaria cornea* red seaweed had capacity to revert the damage induced by 6-OHDA treatment changing monoamine content and reducing oxidative stress condition.

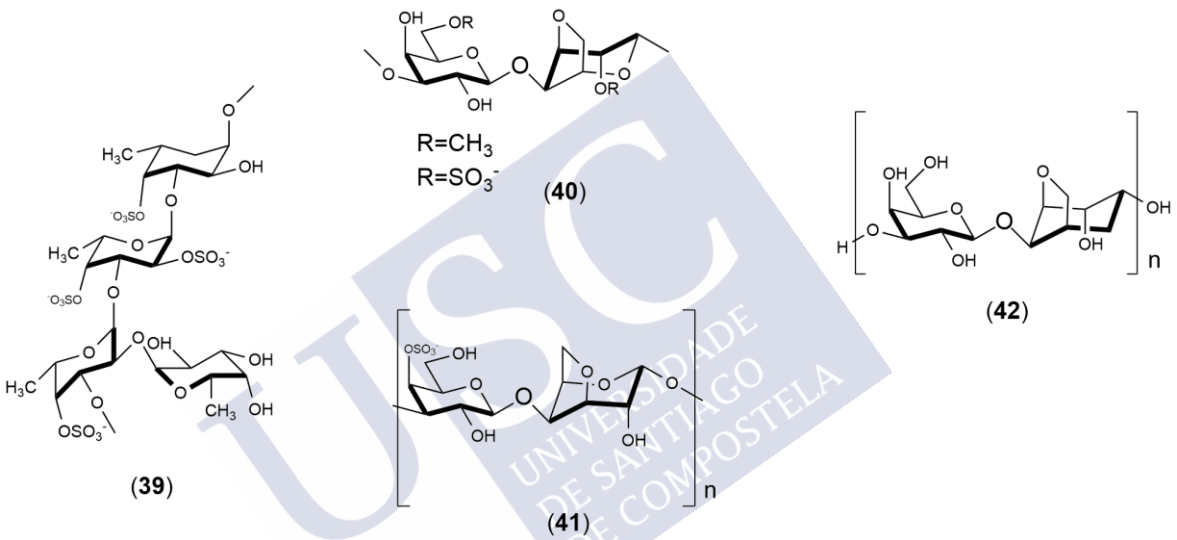


Figure 1.16. Chemical structures of the marine natural products derived from seaweeds: fucoidan **(39)**, phorphyran A **(40)**, κ-carrageenan **(41)** and agaran **(42)**.

Actually, the characterization of neuroprotective activities of seaweeds-derived compounds is limited compared with other biological activities already described for those compounds. Therefore, there is a window of opportunity to evaluate the potential of seaweed - derived compounds as potential innovative drugs for the treatment of neurodegenerative diseases like PD.

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CHAPTER 2
GOALS AND OUTLINE OF THE THESIS

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2. GENERAL GOALS

With the increasing aging of the world population, neurodegenerative diseases have become increasingly common, specifically Parkinson´s Disease (PD), represented about 10 million of the world population according to the Parkinson's Foundation. It is known that PD results from the depletion of the neuronal cells responsible for dopamine (DA) production in the substance *nigra pars compacta*, a structure in the basal ganglia of the brain. However, its exact etiology and associated mechanisms remain poorly understood. Although several drugs are currently used to treat the symptoms of the disease, and to slow down its progression, the treatment is not effective. Furthermore, these drugs have presented undesirable secondary effects, and with the time, the patient's organism stops responding to treatment, becoming of utmost urgency to develop new therapeutic agents.

In the searching for new bioactive molecules from natural origin, marine organisms have shown to be a unique source of compounds with distinct chemical structures, pharmacological activities and mechanisms of action. Among them, seaweeds, sessile organisms, can produce bioactive substances as chemical defenses as a response to ecological pressures such as natural predators. Those compounds have been demonstrated as promising source of innovative therapeutic agents.

Accordingly, the main goal of this PhD thesis was to study the chemical profile of the brown algae *Bifurcaria bifurcata* and green algae *Codium tomentosum* collect from the Peniche coast (Portugal) and to evaluate the antioxidant, neuroprotective and anti-inflammatory activities of its major metabolites applying a bio-guided screening strategy.

The relevance of this study is still highlighted by the fact that there are few studies regarding the neuroprotective activities of marine natural compounds isolated from macroalgae to be applied in Parkinson's disease therapeutics.

2.1. SPECIFIC GOALS

Emerging from the general goals, the present thesis aimed to answer to specific questions:

1) Is the chemical profile of *Bifurcaria bifurcata* and *Codium tomentosum* macroalgae specimens collect in the Portuguese coast similar to the specimens collected in the Irish

2) coast and Mediterranean sea, respectively? Can the distinct conditions of each localization promote the production of distinct secondary metabolites?

3) What is the neuroprotective potential of *Bifurcaria bifurcata* and *Codium tomentosum* derived compounds? Can these compounds also exhibit anti-inflammatory activities?

4) What kind of intracellular signaling pathways are underlying their neuroprotective and anti-inflammatory activities?

5) The isolated compounds will have ability to act in the intracellular signaling pathways involved in the progressive degeneration of dopaminergic neurons in the Parkinson's disease?

The answers to these questions will contribute to understand the neuroprotective and anti-inflammatory potential of the compounds isolated from the brown alga *Bifurcaria bifurcata* and green alga *Codium tomentosum* to inspire the development of new therapeutic agents for Parkinson's Disease.

2.2. OUTLINE OF THE THESIS

The present thesis is divided into seven chapters. Chapter 1 includes the General Introduction; Chapter 2 comprises the goals and outline of this thesis; Chapter 3 describes bio-guided procedures aiming the isolation of *Bifurcaria bifurcata* compounds; Chapter 4 refers to a bio-guided isolation of *Codium tomentosum* compounds; Chapters 5 and 6 describe the laboratory experiments conducted to assess the neuroprotective activities and intracellular signaling pathways activated by the compounds. Chapter 7 discusses, summarizes, and integrates the general conclusions of this thesis.

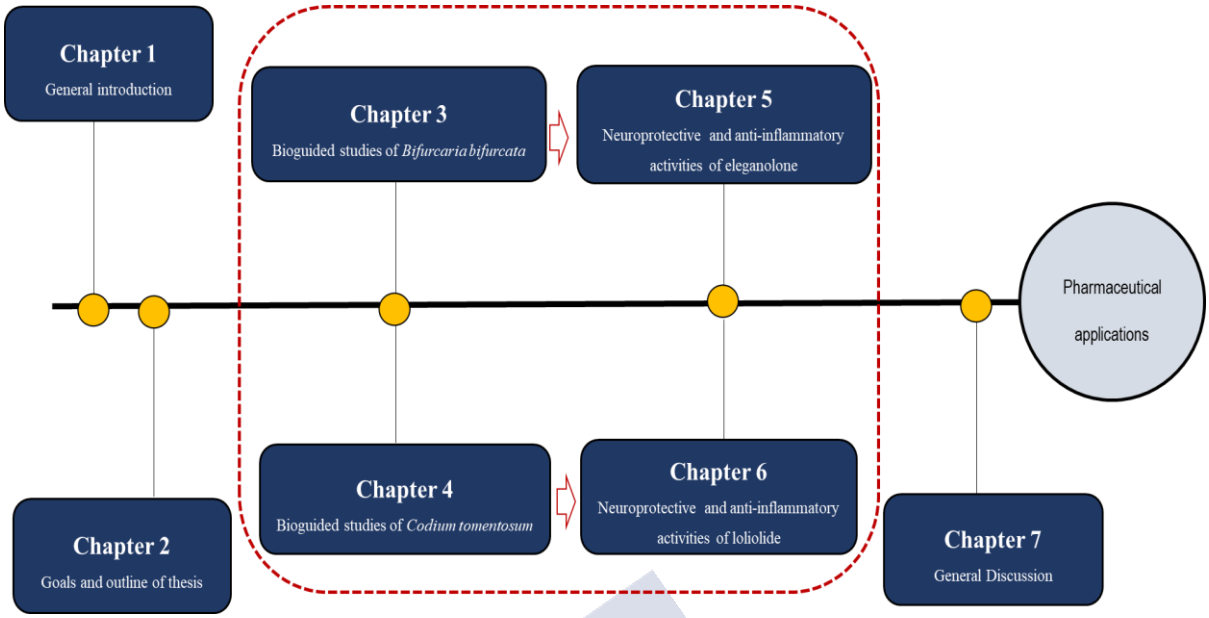


Figure 2.1. Outline of the PhD thesis to evaluate the neuroprotective and anti-inflammatory potential of compounds derived from *Bifurcaria bifurcata* and *Codium tomentosum*.

In detail:

Chapter 3, entitled "Bioguided studies of *Bifurcaria bifurcata"* describes all procedures performed, from the samples collection to compounds isolation, which were driven through the accomplishment of neuroprotective assays on SH-SY5Y cells exposed to 6-OHDA neurotoxicity.

Chapter 4, entitled "Bioguided studies of *Codium tomentosum*" describes all procedures performed, from the samples collection to compounds isolation, which were driven through the accomplishment of neuroprotective assays on SH-SY5Y cells exposed to 6-OHDA neurotoxicity.

Chapter 5, entitled "Neuroprotective and anti – inflammatory activities of eleganolone" describes the experiments performed on capacity of eleganolone to prevent neuronal cells' death induced by 6-OHDA on SH-SY5Y cells and its anti-inflammatory effects in LPS – induced RAW 264.7 macrophages. The mechanism of action underlying its neuroprotective and anti-inflammatory activities linked to oxidative stress, mitochondrial dysfunction, apoptosis, activation of NF-κB pathway, releases of pro-inflammatory and anti-inflammatory cytokines was also studied.

Chapter 6, entitled “Neuroprotective and anti – inflammatory activities of loliolide” death induced by 6-OHDA on SH-SY5Y cells and its anti-inflammatory effects on LPS - induced RAW 264.7 macrophages. The mechanism of action underlying its neuroprotective and anti-inflammatory activities linked to oxidative stress, mitochondrial dysfunction, apoptosis, activation of NF-κB pathway, release of pro-inflammatory and anti-inflammatory cytokines was also studied.

Chapter 7, entitled “General discussion and concluding remarks” discusses the main findings of Chapters 3, 4, 5 and 6, and summarizes the results attained.

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CHAPTER 3

BIOGUIDED STUDIES OF *BIFURCARIA BIFURCATA*

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3. BIOGUIDED STUDIES OF *BIFURCARIA BIFURCATA*

In **chapter 3**, are reported the data obtained in this doctoral thesis concerning the bioguided-assays of *Bifurcaria bifurcata* seaweed. These data resulted in **2 publications**:[1]

1st – publication: Antioxidant and Neuroprotective Potential of the Brown Seaweed *Bifurcaria bifurcata* in an *in vitro* Parkinson's Disease Model - **Published**

Journal: *Marine Drugs*

Impact Factor: 4.073

CiteScore 2019: 5.1 **CiteScoretracker 2020:** 6.3

SJR: 0.71

Quartile: Q2

Area: Drug Discovery (41/143)

2nd – publication: Natural approaches to overcome Parkinson's disease – the neuroprotective and anti-inflammatory potential of the brown seaweed *Bifurcaria bifurcata* – **Submitted** (under review a 2.5 months)

Journal: Algal Research

Impact Factor: 4.008

CiteScore 2019: 6.7 **CiteScoretracker 2020:** 6.7

SJR: 1.257

Quartile: Q1

Area: Biotechnology & Applied microbiology (38/156)

[1] The data provided for the two journals were obtained using the Scopus and Simago platforms in 2021.

Chapter 3 focuses on the extraction and fractionation of *Bifurcata bifurcata* seaweed by applying different extraction solvents and chromatographic techniques, such as preparative columns chromatography and semi-preparative HPLC. The bioactive potential of the obtained fractions was evaluated through a series of previously optimized *in vitro* assays. The antioxidant capacity was evaluated by different biochemical methods, such as QTP, DPPH, FRAP and ORAC, while the neuroprotective activity was evaluated using the 6-OHDA-induced neurotoxicity model in the SH-SY5Y cell line. PD hallmarks, including oxidative stress and mitochondrial dysfunction, were also evaluated.

All results are available in the following publications:

3.1. Antioxidant and Neuroprotective Potential of the Brown Seaweed *Bifurcaria bifurcata* in an *in vitro* Parkinson's Disease Model

3.2. Natural approaches to overcome Parkinson's Disease – the neuroprotective and anti-inflammatory potential of the brown seaweed *Bifurcaria bifurcata*

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3.1. Antioxidant and Neuroprotective Potential of the Brown Seaweed *Bifurcaria bifurcata* in an *in vitro* Parkinson`s Disease Model

Joana Silva [1,2,*], Celso Alves[1] , Rafaela Freitas[1] , Alice Martins[1] , Susete Pinteus[1] , Joana Ribeiro[1] , Helena Gaspar[1,3] , Amparo Alfonso[2] and Rui Pedrosa [1,*]

[1] MARE—Marine and Environmental Sciences Centre, ESTM, Instituto Politécnico de Leiria, 2520-641 Peniche, Portugal;

[2] Department of Pharmacology, Faculty of Veterinary, University of Santiago de Compostela, 27002 Lugo, Spain;

[3] BioISI-Biosystems & Integrative Sciences Institute, Faculty of Sciences, University of Lisboa, Campo Grande, C8, 1749-016 Lisboa, Portugal

Received: 17 December 2018; Accepted: 23 January 2019; Published: 1 February 2019

Abstract: *Bifurcaria bifurcata* is a marine brown seaweed mainly found on the Atlantic coast. Herein, we report the antioxidant and neuroprotective activities of seven fractions (F1–F7) obtained by normal phase chromatography from the *B. bifurcata* dichloromethane extract, as well as of its two major isolated diterpenes. Total phenolic content of fractions was determined by the Folin–Ciocalteu method, while antioxidant activity was evaluated by the DPPH, ORAC, and FRAP assays. Neuroprotective effects were evaluated in a neurotoxic model induced by 6-hydroxydopamine (6-OHDA) in a human neuroblastoma cell line (SH-SY5Y), while the mechanisms associated to neuroprotection were investigated by the determination of mitochondrial membrane potential, H_2O_2 production, Caspase-3 activity, and by observation of DNA fragmentation. Fractions F4 and F5 exhibited the best neuroprotective and antioxidant activities, respectively. F4 fraction prevented changes in mitochondrial potential, and induced a reduction of H_2O_2 levels production and an increase in cell viability, suggesting that it may contain multi-target compounds acting on different pathways. Hence, this fraction was subjected to purification steps, affording the known diterpenes eleganolone and eleganonal. Both compounds exhibited antioxidant potential, being interesting candidates for further neuroprotective studies.

Keywords: 6-hydroxydopamine; seaweeds; SH-SY5Y cells; neuroprotection; mitochondrial membrane potential; Caspase-3 activity; eleganolone; eleganonal; neurodegenerative diseases

Reference: **Joana Silva**, Celso Alves, Rafaela Freitas, Alice Martins, Susete Pinteus, Joana Ribeiro, Helena Gaspar, Amparo Alfonso and Rui Pedrosa (2019). Antioxidant and Neuroprotective Potential of the Brown Seaweed *Bifurcaria bifurcata* in an *in vitro* Parkinson's Disease Model. Marine drugs, 17(85): 1 -16. https://doi.org/10.3390/md17020085

Doctoral student contributions:

The doctoral student, Joana Silva, has been involved in the development of all experimental activities carried out in this work, namely: extraction, fractionation and isolation processes, accomplishment of biological assays (cytotoxicity, antioxidant, and neuroprotective evaluation) as well as the characterization of intracellular signalling pathways to depict the mechanisms of action underlying the observed activities. Furthermore, the doctoral student was responsible for the treatment of results obtained along the experimental activities, and also for the manuscript draft. However, during the experimental work development, the doctoral student had the support of several researchers, which contribution is described in the following Table:

Table 3.1. Description of the contributions of the co-authors.

Co-authors	Co-author contributions
Celso Alves	Participated in the activities related to cytotoxic, neuroprotective assays, and characterization of mechanisms of action;
Rafaela Freitas	Participated in the activities related to the extraction and fractionation of *B. bifurcata* seaweed;
Alice Martins	Participated in the activities related to the isolation and chemical characterization of compounds;
Susete Pinteus	Participated in the activities related to antioxidant assays;
Joana Ribeiro	Participated in the activities related to the extraction and fractionation of *B. bifurcata* seaweed;
Helena Gaspar	Participated in the activities related to the isolation and chemical characterization of compounds;
Amparo Alfonso	Participated in the conception and coordination of the study;
Rui Pedrosa	Participated in the conception and coordination of the study;

All authors read, revised and approved the final manuscript and are accountable for its integrity.

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

Joana Silva[1,2*], Celso Alves[1], Patricia Susano[1], Alice Martins[1], Helena Gaspar[1,3], Susete Pinteus[1], Amparo Alfonso[2] and Rui Pedrosa[4]*

[1] MARE – Marine and Environmental Sciences Centre, Polytechnic of Leiria, 2520-630 Peniche, Portugal.

[2] Department of Pharmacology, Faculty of Veterinary, University of Santiago de Compostela, Lugo 27002, Spain.

[3] University of Lisboa, Faculty of Sciences, BioISI - Biosystems & Integrative Sciences Institute, Campo Grande, C8, 1749-016 Lisboa, Portugal

[1] MARE – Marine and Environmental Sciences Centre, ESTM, Polytechnic of Leiria, 2520-614, Peniche, Portugal.

Abstract: Oxidative stress is strongly involved in the pathogenesis of different neurodegenerative diseases, such as Parkinson´s disease (PD). Particularly, an excess of reactive oxygen species (ROS) released by the cells promotes an oxidative stress condition, which is a main cause of tissue injury leading to nervous system in the dysfunction. In this work, the antioxidant, neuroprotective and anti-inflammatory activities of different fractions from *Bifurcaria bifurcata* are presented and related with their chemical profile. The total phenolic content (TPC) was evaluated by the Folin-Ciocalteu method, while three complementary *in vitro* assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) were used to evaluate the antioxidant potential. The neuroprotective capacity was evaluated against 6-hydroxydopamine (6-OHDA)-induced toxicity on SH-SY5Y cells, while the anti-inflammatory activity was evaluated on an LPS-induced inflammation model on RAW 264.7 cells. The results suggest that ethyl acetate fractions (100 µg/mL) exhibited significant antioxidant and neuroprotective activities in the *in vitro* models assayed. Additionally, at the same concentration, two of the most polar fractions obtained with methanol and water, also evidenced a significant neuroprotective potential. The treatment with *B. bifurcata* fractions decreased ROS production, mitochondrial dysfunction, and Caspase-3 activity. Regarding the anti-inflammatory potential, five fractions (100 µg/mL) inhibited nitric oxide (NO) production, as well as reduced the levels of interleukin – 6 (IL-6) and tumor necrosis factor (TNF-α), thus revealing high anti-inflammatory properties. Mannitol, identified as the major component of the most bioactive fraction, protected SH-SY5Y cells against the toxicity induced by 6-OHDA.

The neuroprotective effects of this compound (50 -100 µM) were mediated by the mitigation of ROS generation, mitochondrial dysfunction, and DNA damage, together with the reduction of Caspase-3 activity. The results suggest that *B. bifurcata* is a relevant source of neuroprotective agents, with particular interest for preventive therapeutics.

Keywords: Antioxidants; Mitochondrial dysfunction; Neuroinflammation; Parkinson´s Disease; SH-SY5Y cells; RAW 264.7 cells.

Reference: **Joana Silva**, Celso Alves, Patricia Susano, Alice Martins, Helena Gaspar, Susete Pinteus, Amparo Alfonso and Rui Pedrosa (XXX). Exploring marine resources against neurological disorders– the neuroprotective and anti-inflammatory potential of the brown seaweed *Bifurcaria bifurcata*. *Algal Research*, xxx: ISSN:2211-9264.

Doctoral student contributions:

The doctoral student, Joana Silva, has been involved in the development of all experimental activities carried out in this work, namely: extraction, fractionation and isolation processes, accomplishment of biological assays (cytotoxicity, antioxidant, neuroprotective and, anti-inflammatory evaluation) as well as the characterization of intracellular signalling pathways to depict the mechanisms of action underlying the observed activities. Furthermore, the doctoral student was responsible for the treatment of results obtained along the experimental activities, and also for the manuscript draft. However, during the experimental work development, the doctoral student had the support of several researchers, which contribution is described in the following Table:

Table 3.2. Description of the contributions of the co-authors.

Co-authors	Co-author contributions
Celso Alves	Participated in the activities related to cytotoxic and neuroprotective assays, and characterization of mechanisms of action;
Patricia Susano	Participated in the activities related to anti-inflammatory assays;
Alice Martins	Participated in the activities related to the isolation and chemical characterization of compounds;
Helena Gaspar	Participated in the activities related to the isolation and chemical characterization of compounds;
Susete pinteus	Participated in the activities related to antioxidants assays;
Amparo Alfonso	Participated in the conception and coordination of the study;
Rui Pedrosa	Participated in the conception and coordination of the study;

All authors read and approved the final manuscript and are accountable for the integrity of this manuscript.

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CHAPTER 4

BIOGUIDED STUDIES OF *CODIUM TOMENTOSUM*

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4. BIOGUIDED STUDIES *OF CODIUM TOMENTOSUM*

In **chapter 4**, the data obtained in this doctoral thesis concerning the bioguided-assays of *Codium tomentosum* seaweed are reported. These data resulted in **1 publication:**

1st – publication: Natural Approaches for Neurological Disorders – The Neuroprotective of *Codium tomentosum* – **Published**

Journal: *Molecules journal* **Impact Factor:** 3.267

CiteScore 2019: 4.1 **CiteScoretracker 2020:** 4.6

SJR: 0.698 **Quartile:** Q2

Area: Pharmaceutical Science (48/169)

Chapter 4 focuses on the extraction and fractionation of *Codium tomentosum* seaweed by applying different extraction solvents and chromatographic techniques, such as vacumn liquid chromatography and chromatography column. The chemical characterization was evaluated through GC-MS. The bioactive potential of the obtained fractions was evaluated through a series of previously optimized *in vitro* assays. The antioxidant capacity was evaluated by different biochemical methods, such as QTP, DPPH, FRAP and ORAC, while the neuroprotective activity was evaluated using the 6-OHDA-induced neurotoxicity model in the SH-SY5Y cell line. PD hallmarks, including oxidative stress and mitochondrial dysfunction, were also evaluated

All results resulted in the following publication:

4.1. Natural Approaches for Neurological Disorders—The Neuroprotective Potential of *Codium tomentosum*

4.1. Natural Approaches for Neurological Disorders—The Neuroprotective Potential of *Codium tomentosum*

Joana Silva [1,2,*], Alice Martins[1], Celso Alves[1] , Susete Pinteus[1], Helena Gaspar[1,4], Amparo Alfonso[2] and Rui Pedrosa [1,*]

[1] MARE—Marine and Environmental Sciences Centre, Instituto Politécnico de Leiria, 2520-641 Peniche, Portugal;

[2] Department of Pharmacology, Faculty of Veterinary, University of Santiago de Compostela, 27002 Lugo, Spain;

[3] BioISI-Biosystems & Integrative Sciences Institute, Faculty of Sciences, University of Lisboa, Campo Grande, C8, 1749-016 Lisboa, Portugal;

[4] MARE—Marine and Environmental Sciences Centre, ESTM, Instituto Politécnico de Leiria, 2520-641 Peniche, Portugal.

Received: 3 November 2020; Accepted: 19 November 2020; Published: 23 November 2020

Abstract: Parkinson's disease (PD) is the second most common neurodegenerative disorder, and is characterized by a progressive degeneration of the dopaminergic neurons in the *substantia nigra*. Although not completely understood, several abnormal cellular events are known to be related with PD progression, such as oxidative stress, mitochondrial dysfunction and apoptosis. Accordingly, the aim of this study was to evaluate the neuroprotective effects of *Codium tomentosum* enriched fractions in a neurotoxicity model mediated by 6-hydroxydopamine (6-OHDA) on SH-SY5Y human cells, and the disclosure of their mechanisms of action. Additionally, a preliminary chemical screening of the most promising bioactive fractions of *C. tomentosum* was carried out by GC-MS analysis. Among the tested fractions, four samples exhibited the capacity to revert the neurotoxicity induced by 6-OHDA to values higher or similar to the vitamin E (90.11 ± 3.74% of viable cells). The neuroprotective effects were mediated by the mitigation of reactive oxygen species (ROS) generation, mitochondrial dysfunctions and DNA damage, together with the reduction of Caspase-3 activity. Compounds belonging to different chemical classes, such as terpenes, alcohols, carboxylic acids, aldehydes, esters, ketones, saturated and unsaturated hydrocarbons were tentatively identified by GC-MS. The results show that *C. tomentosum* is a relevant source of neuroprotective agents, with particular interest for preventive therapeutics.

Keywords: seaweeds; Marine natural products; neurodegenerative disease; SH-SY5Y cells; oxidative stress; mitochondrial dysfunction

Reference: **Joana Silva**, Alice Martins, Celso Alves, Susete Pinteus, Helena Gaspar, Amparo Alfonso and Rui Pedrosa (2020). Natural Approaches for Neurological Disorders—The Neuroprotective Potential of *Codium tomentosum*. *Molecules*, 25(5478): 1 -16. https://doi.org/10.3390/molecules25225478

Doctoral student contributions:

The doctoral student, Joana Silva, has been involved in the development of all experimental activities carried out in this work, namely: extraction, fractionation and isolation processes, accomplishment of biological assays (cytotoxicity, antioxidant, and neuroprotective evaluation) as well as the characterization of intracellular signalling pathways to depict the mechanisms of action underlying the observed activities. Furthermore, the doctoral student was responsible for the treatment of results obtained along the experimental activities, and also for the manuscript draft. However, during the experimental work development, the doctoral student had the support of several researchers, which contribution is described in the following Table

Table 4.1. Description of the contributions of the co-authors

Co-authors	Co-author contributions
Alice Martins	Participated in the activities related to the extraction and fractionation of *Codium tomentosum* seaweed;
Celso Alves	Participated in the activities related to cytotoxic and neuroprotective assays, and characterization of mechanisms of action;
Susete Pinteus	Participated in the activities related to antioxidant assays;
Helena Gaspar	Participated in the activities related to the chemical characterization of *Codium tomentosum* seaweed;
Amparo Alfonso	Participated in the conception and coordination of the study;
Rui pedrosa	Participated in the conception and coordination of the study;

All authors read and approved the final manuscript and are accountable for the integrity of this manuscript.

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CHAPTER 5

NEUROPROTECTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF ELEGANOLONE

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5. NEUROPROTECTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF ELEGANOLONE

In **chapter 5**, the data obtained in this doctoral thesis concerning the neuroprotective and anti-inflammatory potential of isolated compounds from *Bifurcaria bifurcata* seaweed are reported. These data resulted in **1 publications**:

1st – publication: Disclosing the potential of eleganolone for Parkinson's Disease therapeutics: Neuroprotective and anti-inflammatory activities – **Published**

Journal: Pharmacology Research
Impact Factor: 5.893

CiteScore 2019: 9.9 **CiteScoretracker 2020:** 9.6

SJR: 1.609 **Quartile:** Q1

Area: Pharmacology (15/301)

Chapter 5 focuses on the isolation and chemical characterization of compounds from *Bifurcaria bifurcata* seaweed by applying different chromatographic techniques, such as preparative columns chromatography and semi-preparative HPLC. Structural elucidation was performed by NMR spectroscopy. The neuroprotective activity of compounds was evaluated using the 6-OHDA-induced neurotoxicity model in the SH-SY5Y cell line. The anti-inflammatory activity of eleganolone was evaluated using the LPS-induced inflammation model in the RAW 264.7 cell line. The signalling pathaways mechanisms related to the neuroprotective and the anti-inflammatory effects were also evaluated.

All results resulted in the following publications:

5.1. Disclosing the potential of eleganolone for Parkinson's Disease therapeutics: Neuroprotective and anti-inflammatory activities

5.1. Disclosing the potential of eleganolone for Parkinson's Disease therapeutics: Neuroprotective and anti-inflammatory activities

Joana Silva [a,b,*], Celso Alves [a] , Susete Pinteus [a], Patricia Susano [a], Marco Simões [a], Miguel Guedes [a], Alice Martins [a] , Stephanie Rehfeldt [c], Helena Gaspar [a,d], Márcia Goetter [c], Amparo Alfonso [b] and Rui Pedrosa [a,*]

[a] MARE—Marine and Environmental Sciences Centre, Instituto Politécnico de Leiria, 2520-641 Peniche, Portugal;

[b] Department of Pharmacology, Faculty of Veterinary, University of Santiago de Compostela, 27002 Lugo, Spain;

[c] Cell culture Laboratory, Graduated Program in Biotechnology, University of Vale do Taquari – UNIVATES, 95901-120, Lajeado, RS, Brazil;

[d] BioISI-Biosystems & Integrative Sciences Institute, Faculty of Sciences, University of Lisboa, Campo Grande, C8, 1749-016 Lisboa, Portugal

[e] MARE − Marine and Environmental Sciences Centre, ESTM, Polytechnic of Leiria, 2520-614 Peniche, Portugal

Abstract: The treatment of Parkinson´s disease (PD) has benefited from significant advances resulting from the increasing research efforts focused on new therapeutics. However, the current treatments for PD are mostly symptomatic, alleviating disease symptoms without reversing or retarding disease progression. Thus, it is critical to find new molecules that can result in more effective treatments. Within this framework, this study aims to evaluate the neuroprotective and anti-inflammatory effects of three compounds (eleganolone, eleganonal and fucosterol) isolated from the brown seaweed *Bifurcaria bifurcata. In vitro* neuroprotective effects were evaluated on a PD cellular model induced by the neurotoxin 6-hydroxydopamine (6-OHDA) on SH-SY5Y human cells, while lipopolysaccharide (LPS) − stimulated RAW 264.7 macrophages were used to evaluate the anti-inflammatory potential. Additionally, the underlying mechanisms of action were also investigated. Compounds were isolated by preparative chromatographic methods and their structural elucidation attained by NMR spectroscopy. Among the tested compounds, eleganolone (0.1–1 µM; 24 h) reverted the neurotoxicity induced by 6-OHDA in about 20%. The neuroprotective effects were mediated by mitochondrial protection, reduction of oxidative stress, inflammation and apoptosis, and inhibition of NF-kB pathway. The results suggest that eleganolone may provide advantages in the treatment of neurodegenerative conditions and, therefore, should be considered for future preclinical studies.

Keywords: Apoptosis; Diterpenes; Marine Natural Products; NF-κB pathway; Neurodegenerative diseases; Oxidative stress.

Reference: Joana Silva, Celso Alves, Susete Pinteus, Patricia Susano, Marco Simões, Miguel Guedes, Alice Martins, Stephanie Rehfeldt, Helena Gaspar, Márcia Goetter, Amparo Alfonso and Rui Pedrosa. (xxx) Disclosing the potential of Eleganolone for Parkinson'sDisease therapeutics: Neuroprotective and anti-inflammatory activities (xxx). *Pharmacology Research*, xxx. ISSN: 1043 – 6618.

Doctoral student contributions:

The doctoral student, Joana Silva, has been involved in the development of all experimental activities carried out in this work, namely: isolation processes, accomplishment of biological assays (cytotoxicity, antioxidant, neuroprotective and anti-inflammatory evaluation) as well as the characterization of intracellular signalling pathways to depict the mechanisms of action underlying the observed activities. Furthermore, the doctoral student was responsible for the treatment of results obtained along the experimental activities, and also for the manuscript draft. However, during the experimental work development, the doctoral student had the support of several researchers, which contribution is described in the following Table:

Table 5.1. Description of the contributions of the co-authors.

Co-authors	Co-author contributions
Celso Alves	Participated in the activities related to neuroprotective assays and characterization of mechanisms of action;
Susete Pinteus	Participated in the activities related to cytotoxic assays;
Patricia Susano	Participated in the activities related to anti-inflammatory assays and characterization of mechanisms of action;
Marco Simões	Participated in the activities related to studied of NF-κB pathway through WB technique;
Miguel Guedes	Participated in the activities related to studied of NF-κB pathway through WB technique;
Alice Martins	Participated in the activities related to the isolation and chemical characterization of compounds;
Stephanie Rehfeldt	Participated in the activities related to anti-inflammatory assays and characterization of mechanisms of action;
Helena Gaspar	Participated in the activities related to the isolation and chemical characterization of compounds;
Márcia Goetter	Participated in the conception and coordination of the study;
Amparo Alfonso	Participated in the conception and coordination of the study;
Rui Pedrosa	Participated in the conception and coordination of the study;

All authors read and approved the final manuscript and are accountable for the integrity of this manuscript.

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CHAPTER 6

NEUROPROTECTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF LOLIOLIDE

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6. NEUROPROTECTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF LOLIOLIDE

In **chapter 6**, the data obtained in this doctoral thesis concerning the neuroprotective and anti-inflammatory potential of isolated compounds from *Codium tomentosum* seaweed are reported. These data resulted in **1 publication**:

1st – publication: Loliolide, a new therapeutic option for neurological diseases? *In vitro* Neuroprotective and anti-inflammatory activities of a monoterpenoid lactone isolated from *Codium tomentosum* – **Published**

Journal: International Journal Molecular Science **Impact Factor:** 4.556

CiteScore 2019: 5.3 **CiteScoretracker 2020:** 5.9

SJR: 1.317 **Quartile:** Q1

Area: Biochemistry (157/381)

Chapter 6 focuses on the isolation and chemical characterization of compounds from *Codium tomentosum* seaweed by applying different chromatographic techniques, such as preparative columns chromatography and semi-preparative HPLC. Structural elucidation was performed by NMR spectroscopy. The antioxidant potential of loliolide was evaluated by different biochemical methods, such as DPPH and FRAP. The neuroprotective activity was evaluated using the 6-OHDA-induced neurotoxicity model in the SH-SY5Y cell line. The anti-inflammatory activity was evaluated using the LPS-induced inflammation model in the RAW 264.7 cell line. The signalling pathawys mechanisms related to the neuroprotective and the anti-inflammatory effects were also evaluated.

All results are available in the following publications:

6.1. Loliolide, a new therapeutic option for neurological diseases? *In vitro* Neuroprotective and anti-inflammatory activities of a monoterpenoid lactone isolated from *Codium tomentosum*

6.1. Loliolide, a new therapeutic option for neurological diseases? *In vitro* Neuroprotective and anti-inflammatory activities of a monoterpenoid lactone isolated from *Codium tomentosum*

Joana Silva [1,2,*], Celso Alves[1] , Susete Pinteus[1], Patricia Susano[1], Marco Simões[1], Miguel Guedes[1], Alice Martins[1] , Stephanie Rehfeldt[3], Helena Gaspar[1,4], Márcia Goetter[3], Amparo Alfonso[2] and Rui Pedrosa [1,*]

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Abstract: Parkinsons Disease (PD) is the second most common neurodegenerative disease worldwide, and is characterized by a progressive degeneration of dopaminergic neurons. Without an effective treatment, it is crucial to find new therapeutic options to fight the neurodegenerative process, which may arise from marine resources. Accordingly, the goal of the present work was to evaluate the ability of the monoterpenoid lactone Loliolide, isolated from the green seaweed *Codium tomentosum*, to prevent neurological cell death mediated by the neurotoxin 6-hydroxydopamine (6-OHDA) on SH-SY5Y cells and their anti-inflammatory effects in RAW 264.7 macrophages. Loliolide was obtained from the diethyl ether extract, purified through column chromatography and identified by NMR spectroscopy. The neuroprotective effects were evaluated by the MTT method. Cells´ exposure to 6-OHDA in the presence of Loliolide led to an increase of cells´ viability in 40%, and this effect was mediated by mitochondrial protection, reduction of oxidative stress condition and apoptosis, and inhibition of the NF-κB pathway. Additionally, Loliolide also suppressed nitric oxide production and inhibited the production of TNF-α and IL-6 pro-inflammatory cytokines. The results suggest that Loliolide can inspire the development of new neuroprotective therapeutic agents and thus, more detailed studies should be considered to validate its pharmacological potential.

Keywords: antioxidant activity; inflammation; marine natural products; neuroprotection; NF-κB pathway; oxidative stress; Parkinson's Disease; seaweeds

Reference: Joana Silva, Celso Alves, Susete Pinteus, Patricia Susano, Marco Simões, Miguel Guedes, Alice Martins, Stephanie Rehfeldt, Helena Gaspar, Márcia Goetter, Amparo Alfonso and Rui Pedrosa (2021). Loliolide, a new therapeutic option for neurological diseases? *In vitro* Neuroprotective and anti-inflammatory activities of a monoterpenoid lactone isolated from *Codium tomentosum*. *International Journal of Molecular Sciences*, 22, 1888. https://doi.org/10.3390/ijms22041888

Doctoral student contributions:

The doctoral student, Joana Silva, has been involved in the development of all experimental activities carried out in this work, namely: isolation processes, accomplishment of biological assays (cytotoxicity, antioxidant, neuroprotective and anti-inflammatory evaluation) as well as the characterization of intracellular signalling pathways to depict the mechanisms of action underlying the observed activities. Furthermore, the doctoral student was responsible for the treatment of results obtained along the experimental activities, and also for the manuscript draft. However, during the experimental work development, the doctoral student had the support of several researchers, which contribution is described in the following Table:

Table 6.1. Description of the contributions of the co-authors.

Co-authors	Co-author contributions
Celso Alves	Participated in the activities related to neuroprotective assays and characterization of mechanisms of action;
Alice Martins	Participated in the activities related to the isolation and chemical characterization of compounds;
Patricia Susano	Participated in the activities related to anti-inflammatory assays and characterization of mechanisms of action;
Marco Simões	Participated in the activities related to studied of NF-κB pathway through WB technique;
Miguel Guedes	Participated in the activities related to studied of NF-κB pathway through WB technique;
Susete Pinteus	Participated in the activities related to antioxidants assays;
Stephanie Rehfeldt	Participated in the activities related to anti-inflammatory assays and characterization of mechanisms of action;
Helena Gaspar	Participated in the activities related to the isolation and chemical characterization of compounds;
Márcia Goetter	Participated in the conception and coordination of the study;
Amparo Alfonso	Participated in the conception and coordination of the study;
Rui pedrosa	Participated in the conception and coordination of the study;

All authors read and approved the final manuscript and are accountable for the integrity of this manuscript

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUDING REMARKS

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7. GENERAL DISCUSSION AND CONCLUDING REMARKS

In the near future, the prevalence of dementia impairments will increase as the world population ages [1]. A study performed by the non-profit group "Alzheimer's Disease International" predicts that there will be around 135 million cases worldwide by mid-century, while the World Health Organization (WHO) estimates that by 2040 the neurodegenerative diseases, such as Parkinson's disease, will overtake cancer to become the second leading cause of death after cardiovascular diseases [2].

Over the past few years, knowledge about the pathophysiology of Parkinson's disease (PD) has been gradually increasing. In great part due to the development of cellular and animal models that play a pivotal role in the understanding of the cellular mechanisms associated with neuronal cell death, allowing to identify possible key targets for the development of selective and effective therapies [3,4]. Although the exact pathophysiology mechanism of PD is not fully elucidated, several evidences indicate that oxidative stress and mitochondrial dysfunction play a critical role in its pathogenesis. As oxidative stress triggers several molecular pathways leading to the progressive loss of neuronal structures and functions, a process termed neurodegeneration, it is considered a major factor in the development of PD [5].

The generation of ROS includes biological processes that release ROS as by-products, such as the mitochondrial electron transport chain and nitric oxide synthase, as responses to inflammatory processes, releasing pro-inflammatory citokines [6,7]. However, most of the free radicals present in cells are generated by the mitochondrial oxidative phosphorylation, through the electron leak from the electron transport chain [8]. These radicals, as superoxide radical, are formed at complexes of the electron transport chain leading to the ROS formation through O_2 consumption by mitochondria [8]. Thus, several studies have been demonstrated that ROS production is strongly related with the membrane potential of the mitochondria, resulting in increased rates of ROS generation [9,10].

Another deleterious species that is produced in the mitochondria through the oxidation of L- arginine to L- citrulline is nitric oxide (NO) that can mediate neurotoxicity and causes neuronal cell death. Additionally, iron concentrations found in tissues, such as Fe (II), in combination with hydrogen peroxide, further contribute to the production of free radicals, which are involved in the degenerative process of dopaminergic neurons due to the proxidative

state of the substantia *nigra pars compacta* where these neurons are found [11]. Therefore, an increased oxidative stress condition may be related with the mitochondrial functional impairment, by inducing a decrease in the Complex I chain activity and cellular metabolism, consequently leading to the reduction of the oxidative phosphorylation, loss of the membrane potential of the mitochondria and release of cytochrome *c* into the cytoplasm, thus contributing to caspase activation, and eventually, cell death of dopaminergic neurons to apoptosis [12].

Since the first marine organisms appeared about 3500 million years ago, they had the opportunity to develop different mechanisms to survive in the more unfriendly ambient; these adverse conditions likely favored the development of such a large number of bioactive molecules to counteract environmental stress. In fact, to survive in extreme habitats or against predators, marine species developed secondary metabolic pathways to produce molecules allowing them to adapt to the environment they live. This way, the production of bioactive compounds has an ecological meaning, since they allow the survival and the adaptation of these marine organisms in the marine environment. Defense strategies by marine organisms include the release of harmful toxins, especially by benthonic species, such as seaweeds [13].

Natural products obtained from marine organisms have been shown to be a potential source of novel therapeutic agents. Among these organisms, seaweeds have been reported for their ability to produce relevant bioactive compounds [14], mainly derived from their secondary metabolism as a defense response to environmental variations [15]. These metabolites are known to exhibit a broad range of bioactive properties such as antioxidant [16], antimicrobial [10], anti-inflammatory [17], anti- tumoral [18], and neuroprotective [19]. Thus, these metabolites can inspire the development of new drugs for the therapy of various pathologies, such as cancer, diabetes, and neurodegenerative diseases [15]. Marine compounds have shown potential as possible therapeutic agents that can slow the processes associated with neuronal cell loss in neurodegenerative diseases, having ability to act in distinct biological targets. Oxidative stress, as described before, is believed to be one of the main triggers of neurodegenerative diseases, particularly in PD, and several studies have been carried out to understand the neuroprotective effects of antioxidant molecules [20]. These molecules have been widely discussed by several researchers as possible therapeutic agents against neuronal cell death, as they have the ability to neutralize free radicals.

The antioxidant potential of seaweeds is well documented in the literature, mainly using the DPPH, QTP, ORAC, and FRAP *in vitro* assays, among others. Seaweeds are known to

produce secondary metabolites capable of eliminating free radicals, which bind to metal ion catalysis to inhibit the continuous production of radicals and protect against lipid peroxidation.

In the present study, a bioguided-assay of the antioxidant and neuroprotective activities of *Bifurcaria bifurcata* and *Codium tomentosum* fractions was carried out. In terms of antioxidant capacity, three complementary methods (DPPH, FRAP, ORAC) were performed and the results correlated with the total phenolic content (TPC) of both seaweeds. Regarding the fractions obtained from *B. bifurcata* they have shown significant antioxidant potential, which can also be correlated with their high levels of TPC. On the other hand, none of the *C. tomentosum* fractions showed significant antioxidant potential when compared to *B. bifurcata*, as can be seen in Chapters 3 and 4. These data suggest that the antioxidant activity of *B. bifurcata* fractions can be mediated by molecules such as polyphenols, which have exhibited great antioxidants activities because of their redox properties, acting as reducing agents [21,22], and hydrogen (H^+) donors, contributing to the production of less reactive radicals, and for the removal of metal ions [23,24]. These results are in accordance with those reported by Pinteus *et al.* (2017) [16], who conducted an antioxidant screening of 27 algae collected from the Peniche coast (Portugal), in which brown algae have shown a relevant antioxidant capacity when compared with red and green seaweed species. Chew *et al.* (2008) [25] have studied aqueous:methanol (1:1) extracts of the seaweeds, *Padina antillarum* (brown), *Caulerpa racemosa* (green) and *Kappaphycus alvarezzi* (red) and also observed that brown seaweed presented higher phenolic content and reducing power when compared with the remaining seaweeds. Furthermore, El-Sheekh *et al.* (2020) [26] also studied the antioxidant potential of four seaweeds, namely *Toania atomaria* (brown), *Padina pavonica* (brown), *Jania rubens* (red) and *Corallina elongata* (red) and observed that *T. atomaria* extract exhibited the highest activity. In recent years, the antioxidant potential of seaweeds has been fully documented in the literature [14,16,20], and has been attributed to the presence of a wide variety of chemical structures with high antioxidant capacity such as phenols, flavonoids, phlorotannins and sulfated polysaccharides [27].

Marine-derived extracts have been previously reported by several studies to exhibit neuroprotective effects against 6-OHDA neurotoxicity and, consequently, this model has been widely used to mimic experimental models of PD [14,19,27,28]. The neuroprotective potential, and associated mechanisms of action, of *B. bifurcata* and *C. tomentosum* fractions were evaluated in the *in vitro* model of PD above mentioned. In this study, it was shown that several

fractions from *B. bifurcata* and *C. tomentosum* are able to promote cells' recovery from the toxicity induced by 6-OHDA exposure, as described in Chapters 3 and 4. The treatment of SH-SY5Y cells with 6-OHDA has promoted a reduction (35-40%) of the cellular viability, however, in the presence of seaweeds' fractions there was an increase of SH-SY5Y cells' viability of 20 - 25% (*B. bifurcata*) and 30-50% (*C. tomentosum*). Our results are consistent with other studies in which methanolic extracts of brown seaweeds (*Sargassum muticum*, *Saccorhiza polyschides)* and dichloromethane extracts of green seaweeds (*C. tomentosum*) increased neuronal cells' viability in 35 - 40%, blocking the toxic effects of 6-OHDA [19]. Park *et al.* (2002) [29] also observed a protective effect of serotonin (17.6 $\mu g.mL^{-1}$) in the PC12 cell model when exposed to DA, inducing an increase of cell' viability approximately of 25%. In addition, Kopalli *et al.* (2013) [30] reported that a methyl ester named methylparaben (1.52 $\mu g.mL^{-1}$), found naturally in blueberries, increased SH-SY5Y cells' viability around 20% in the presence of 6-OHDA. More recently, Ferlazzo *et al.* (2020) [28] described the neuroprotective effect of bergamot juice (BJ) (1%) against 6-OHDA-induced neurotoxicity in differentiated SH-SY5Y cells.

The mechanisms involved in the neuroprotective effects exhibited by seaweeds fractions on the SH-SY5Y cells' viability were studied on different hallmarks associated to PD development. For this purpose, different *in vitro* assays were performed (mitochondrial membrane potential, production of ROS, and Caspase-3 activity) on cells treated with the neurotoxin 6-OHDA, in the presence / absence of the seaweed's fractions. Based on the results obtained in the present work, it was observed that *B. bifurcata* and *C. tomentosum* fractions were able to prevent ROS production and mitochondrial dysfunction promoted by 6-OHDA treatment (Table 3). In addition, due to the Caspase-3 enzyme relevance in cell death by apoptosis, its activity was also determined to understand if the neuroprotective effects of seaweeds fractions were mediated by apoptotic pathways. The exposure of SH-SY5Y cells to 6-OHDA increased its activity, and the treatment with *B. bifurcata* fractions and *C. tomentosum* fractions led to a marked decrease in Caspase-3 activity (**Table 7.1**).

Table 7.1. Illustrative representation of the neuroprotective capacity of *Bifurcaria bifurcata* and *Codium tomentosum* fractions.

		Neuroprotective capacity		
Seaweeds	**Fractions**	**ROS**	**MMP**	**Casp-3**
Bifurcaria bifurcata	DF3	□	□	■
	DF4	□	■	■
	DF5	□	■	□
	MC18F1	□	□	□
	MC18F2	■	□	■
	AEF9	■	■	■
	AEF10	■	■	□
	AEF11	■	■	□
Codium tomentosum	MF2	■	■	■
	DF1	■	■	□
	DF2	■	■	■
	DF5	■	■	■

□ Without activity; ■ With activity

Accordingly, fractions that revealed the best neuroprotective potential were subjected to more refined fractionation and purification steps by means of preparative chromatographic techniques (TLC, CC, HPLC) aiming the isolation of the potential bioactive molecules responsible for the observed activities . *B. bifurcata* fractions afforded three major compounds, eleganolone (**1**)**,** eleganonal (**2**) and manitol (**6**) and in minor amounts, the compounds, bibiolone (**3**), 14,15 - dihydroxy-eleganolone (**4**) and fucosterol (**5**). From the methanolic fraction of *C. tomentosum* it was possible to isolate the bioactive compound loliolide (**7**).

In the late 1960s, the introduction of levodopa in PD therapeutics revolutionized its symptomatic treatment. Levodopa is an amino acid capable of crossing the blood-brain barrier reaching the central nervous system (CNS). However, its prolonged use tends to develop over time a series of motor complications. Dopamine agonists and monoamine oxidase (MAO) inhibitors have also been developed and have not been effective in the reduction of motor symptoms when compared to levodopa. Currently, anti-PD therapies have only symptom-relieving effects, and don't have capacity to provide neuroprotective effects, stop or delay the degeneration of dopaminergic neurons. Thus, it becomes necessary to investigate and develop more effective therapeutic agents for PD, with lower side effects than the current drugs. Accordingly, the marine-derived natural compounds can be useful for the development of new

drugs to PD therapeutics. Currently, twenty-nine marine-derived natural compounds showed effectiveness for the treatment of PD and five compounds have already entered in clinical trials, such as omega-3 fatty acids, inosine, pramipexole, CEP-1347 and GM1 ganglioside [31].

In the present work, the neuroprotective and anti-inflammatory activities of compounds isolated from *B. bifurcata* (eleganolone, eleganonal, fucosterol and mannitol) and from *C. tomentosum* (loliolide) were studied. No cytotoxic effects were observed in the treatments performed with mannitol and loliolide compounds on SH-SY5Y cells using a range of concentrations between 1 - 100 μM. On the other hand, eleganolone exhibited a marked cytotoxic effect in a concentration´ range between 10-100 μM. For this reason, in the remaining assays, the bioactivity of eleganolone was tested in the concentration range between 0.05 -1 μM.

Several studies have reported that marine-derived compounds exhibit neuroprotective effects against 6-OHDA neurotoxicity [32–34]. So, in this work the 6-OHDA-induced neurotoxicity model in SH-SY5Y cell line was used, and it was verified that 6-OHDA singly reduced cell' viability by about 50%. Regarding the neuroprotection assay, only eleganolone (0.5-1 μM), manitol (50-100 μM) and loliolide (50-100 μM) exhibited capacity to promote cells' recovery (20 – 30%) from the 6-OHDA - induced neurotoxicity. However, the result observed with the eleganolone treatment seems to be more promising because it was able to protect cells' viability with a concentration significantly lower, when compared with manitol and loliolide. Effectively, the decrease of neurotoxicity associated with 6-OHDA by marine-derived compounds has already been reported in previous scientific works. Chen and collaborators (2012) [35] evaluated the protective effect of the compound 11 – dehydrosinulariolide, derived from soft corals, against 6-OHDA – induced neurotoxicity in the SH-SY5Y cell line. It was able to increase (27% and 34%) the cells' viability as compared to 6-OHDA treatment, when tested at 0.1 μM and 1 μM, respectively. Yurchenko and collaborators (2018) [36] isolated six compounds from marine fungi *Penicillium* sp., *Aspergillus* sp. and *Aspergillus flocculosus*. The compound 6-hydroxy-*N*-acetyl-β-oxotryptamine was capable of protecting the Neuro2a cells' viability against the damage of 6-OHDA when tested at 10 μM and 50 μM, increasing the cells' viability in 23% and 55%, respectively.

The death of dopaminergic neurons occurs in the central nervous system (CNS) where the neurotransmitter dopamine is synthesized and released into the substancia *nigra* [37].

Currently, it is known that 70% of those dopaminergic neurons in CNS die along PD development, however, the symptoms are not noticed due to compensatory mechanisms of the striatum body [38]. Although the mechanisms responsible for inducing cell death in PD are not completely clarified, it is known that the dopamine metabolism, oxidative stress, mitochondrial dysfunction and neuroinflammation are involved in the death of dopaminergic neurons in CNS [37,39].

Brain is a highly metabolically active organ that relies on oxidative phosphorylation for maintaining energy. One of the first ROS production sites is in the mitochondria, once molecular oxygen can capture electrons from Complex I and III, disturbing electron transport chain leading to DAergic neurons death in SNpc, if not managed with an efficient antioxidant machinery [40]. In addition, as a result of mitochondrial respiratory chain, superoxide anion ($\cdot O_2^-$) is also produced, being a highly reactive sub-product, with capacity to injure components of the electron transport chain and other cellular constituents. Superoxide dismutase (SOD) is often regarded as the first defense line against ROS upswing, being the principal detoxifying enzyme preventing mitochondria damage, playing a main role in preventing neurodegeneration of PD by converting $\cdot O_2^-$ to the less reactive hydrogen peroxide (H_2O_2), which, in turn, generates the most reactive radical, the hydroxyl radical ($HO^{\bullet}$). Although essential to life, playing an indispensable part in cellular cell homeostasis and physiology, supporting signaling events and adaptive cell responses such as hormone synthesis, calcium homeostasis, and cytoskeletal remodeling, among others, these oxidative molecules have to be balanced, otherwise they will promote significant damage in proteins, DNA, causing lipid peroxidation, cell death and disease [41]. In healthy conditions, the human body has a highly effective antioxidative machinery to maintain reactive species at basal levels. However, several biological factors such as age, genetic inheritance and diseases, or external physical aggressors, such as cigarette smoke, pollution, radiation, unhealthy diet, may promote the overproduction of reactive species.

The brain has a comparatively weak endogenous antioxidant defense system relative to other tissues and is susceptible to imbalance in redox homeostasis. Catalase enzyme has an important role in the protection of cells in the detoxification of the generated H_2O_2, for instance during dopamine (DA) metabolism, or even in the oxidative phosphorylation during electron transport chain [42,43]. An inhibition of Catalase activity can result in increased cytotoxicity and ROS production, leading to neurological dysfunctions as in Parkinson's disease [44].

Based on the results obtained on Chapter 3, it was was found that mannitol (50 -100 µM; 6 h) protected from an increased ROS production and Caspase-3 activity promoted by 6-OHDA. However, not showed capacity of decrease mitochondrial dysfunction. On the other hand, based on the results obtained on Chapters 5 and 6, it was found that eleganolone (0.1-10 µM; 6 h) and loliolide (50 -100 µM; 6 h) decreased in about 47 – 100%, and 21 – 23%, respectively, the ROS production induced by 6-OHDA. Furthermore, eleganolone (1 µM; 6 h) and loliolide (100 µM; 6 h) have capacity to stimulate catalase when compared with 6-OHDA. These results are in accordance with those obtained by Alvarino *et al.* (2017) [45] who verified a protective effect of the alkaloid sarain 1, isolated from the sponge *Haliclona* sp., against the damage induced by H_2O_2 on SH-SY5Y cells, decreasing ROS production. However, the authors did not observe changes in Catalase activity. Yurchenko *et al.* (2018) [36] also reported that a melatonin analogue, the 6-hydroxy-*N*-acetyl-β-oxotryptamine (10 µM), isolated from the fungus *Penicillum* sp., protected Neuro2a cells reducing ROS production (23%), when pre-treated 1 h before adding 6-OHDA.

Oxidative stress has been intimately linked to mitochondrial dysfunction. Mitochondria are a vitally organelle involved in the energy metabolism. It generates over 90% of our cellular energy in the form of ATP through oxidative phosphorylation, being involved in various other processes, including the regulation of calcium homeostasis, and stress response [46]. As a consequence of its role, mitochondria are also responsible for more than 90% of cellular ROS production [6], being completely dependent of an efficient antioxidant machinery to prevent oxidative stress condition. In addition to mitochondrial ROS, other factors that may contribute to increased oxidative damage include iron accumulation and an increase in lipid peroxidation burden [41,42], while accumulation of hydroxyl and superoxide radicals due to a decline in glutathione content have also been reported in the brain of PD patients [47].

In fact, mitochondrial dysfunctions play a central role in the neurodegeneration, especially in PD leading to disruption of the respiratory chain and, consequently, to an increase of ROS production, a decrease of mitochondrial Complex I enzyme activity, cytochrome-c release, ATP depletion, and Caspase-3 activation [6,48]. Based on the results presented in Chapter 5, eleganolone prevented mitochondrial dysfunction (0.1 µM; 60%, 0.1 µM; 90% and 1 µM; 60%), increased ATP levels (0.1 µM; 12% and 0.5 µM; 10%) and decreased Caspase -3 activity (0.1 µM; 150%, 0.5 µM; 100% and 1 µM; 230%). On the other hand, as described in Chapter 6, it was verified that loliolide prevented mitochondrial dysfunction (50 µM; 60% and

100 µM; 180%) and decreased Caspase - 3 activity (50 µM; 80% and 100 µM; 100%). However, this compound did not influence the ATP levels. Chen *et al.*(2012) [33] investigated the protection of 11-dehydrosinulariolide, a compound isolated from soft corals, against 6-OHDA-induced apoptosis in SH-SY5Y and verified that the pre-treatment performed with this compound significantly inhibited 6-OHDA - induced Caspase - 3 activation. Nataraj and co-workers (2017) [49] investigated the protective effect of asiatic acid (AA), a triterpene extracted from *Centella asiatica*, in SH-SY5Y cells exposed to rotenone (an inhibitor of mitochondrial Complex I). The exposure of SH-SY5Y cells to rotenone induced a marked overproduction of ROS, mitochondrial dysfunction and apoptosis that were reversed with AA pre-treatment. In addition, Singh and co-workers (2020) [50] also studied the neuroprotective effect of chlorogenic acid (CGA) in a MPTP - induced PD mouse model. The results indicated that CGA treatment improved the mitochondrial complexes I, IV, and V activities as well as the superoxide dismutase and mitochondrial glutathione levels in the midbrain of MPTP-induced mice. CGA also inhibited the activation of proapoptotic proteins, including Bax and Caspase-3, while increased the expression of antiapoptotic protein Bcl-2, preventing the MPTP-mediated apoptotic cascade. These results are in agreement with the results here presented.

Evidences from *post-mortem* studies suggest an involvement of oxidative stress in the degeneration of dopaminergic neurons in PD that have recently been shown to die by apoptosis, but the relationship between oxidative stress and apoptosis has not yet been elucidated [51]. However, in last years, several evidences show that the activation of the transcription factor NF-kB is associated with oxidative stress-induced apoptosis and some researchers verified that this transcription factor can play a role in PD when translocated from the cytoplasm to the nucleus [61]. Under normal conditions, NF-κB is bound to an inhibitor protein, the Ikβα, which sequesters NF-κB in the cytosol. The activation of NF-κB involves the degradation of Ikβα protein, followed by its translocation to nucleus, binding to DNA sequences, inferring damage [50]. Previous works have evidenced that neuronal cell death in PD can be related with the activation of NF-κB transcription factor [47]. In primary cultures of rat mesencephalon was observed that NF-κB translocation is preceded by a transient production of free radicals leading to apoptosis [47]. In the results presented in Chapters 5 and 6, it was possible to observe that the 6-OHDA treatment induced a clearly increase of NF-κB protein levels in the nucleus. On the other hand, the treatment accomplished with loliolide (100 µM) and eleganolone (0.5 µM-1 µM) inhibited the NF-kB translocation from the cytoplasm to nucleus may contribute to

prevent the DNA damage and apoptosis promoted by the exposition to 6-OHDA. Our results are in agreement with Alvarino *et al.* [37] who reported that gracilin A marine - derivates downregulated the NF-kB factor translocation, prompted by H_2O_2 in microglia cells. Chen *et al.* (2012) also observed that 6-OHDA-induced translocation of NF-kB factor to the nucleus that was inhibited by 11- dehydrosinulariolide treatment on SH-SY5Y cells.

Several evidences have suggested that oxidative stress can also lead to the development of neuroinflammation in PD [40], inducing inflammatory responses in microglia [37,39]. Chronic inflammation is characteristic of neurodegenerative diseases such as PD, in which cytotoxic levels of NO and pro-inflammatory cytokines initiate neuronal cell death pathways. The macrophages are known to cross the leaky blood-brain barrier in PD to interact with microglia and stimulate the secretion of inflammatory cytokines causing brain damage via neuroinflammation. Chronic activation of microglia secretes many factors such as pro-inflammatory enzymes (iNOS and COX), pro-inflammatory cytokine (TNF-α, IFN - γ, IL-6 and IL-β), prostaglandins (PGE2), ROS and reactive nitrogen species (RNS), intensifying the occurrence of oxidative stress condition, triggering apoptotic cascades and inflammatory processes in the neurons [52]. As a result, the proximity of microglia with macrophages have attracted increasing attention in relation to the onset and PD progression. In this study, macrophages were exposed to LPS to induce inflammation [53] in the presence of loliolide and eleganolone. Based on the results presented in Chapters 5 and 6 it is possible to observe that both compounds prevented inflammation condition by decreasing NO production and reducing TNF-α and IL-6 levels. Our results are in accordance with Nalli *et al.* (2016) [54] who proved the anti-inflammatory potential of the crude extract and isolated cyclic dipeptide metabolites from marine bacteria *Streptomyces sp*. on key inflammatory mediators like tumor necrosis factor-α and interleukin-6. The authors verified that five of those cyclic peptides cyclo(L-4-OH-Pro-L-Leu), cyclo(L-lle-D-Pro), cyclo(D-trans-4-OH-Pro-D-phe), cyclo(L-Leu-L-Pro) and cyclo(D-Pro-L-Phe) showed good inhibition of both cytokines in lipopolysaccharide-stimulated macrophages. On the other way, also revealed that cyclo(L-Leu-L-Pro) was a specific inhibitor for tumor necrosis factor-α which efficiently inhibited tumor necrosis factor-α release in a dose-dependent manner and decreased lipopolysaccharide induced tumor necrosis factor-α production in human peripheral blood mononuclear cells in both *in vitro* and *in vivo* experiments.

In addition, the interleukin -10 (IL-10) cytokine, a potent anti-inflammatory cytokine, plays a vital role in immunologic system, acting in acute and chronic inflammation. Both up or downregulation of IL-10 cytokine will result in serious immunologic disorders, including neurodegenerative diseases. Studies carried out with the brain tissues of LPS-induced PD mouse model verified that IL-10 decreased the number of activated microglia mediating a protective effect regarding the loss of dopaminergic neurons [55]. Our results showed that LPS treatment did not promote effects on IL-10 expression, neither the exposure to both compounds. Thus, it is possible that it would be necessary to prolong cells' exposure to both, inducer (LPS) and the protective agents (eleganolone and loliolide), to detect this protein production.

In fact, many efforts have been made to identify new anti-inflammatory molecules from natural sources. The terrestrial organisms have been commonly used in traditional medicine to treat inflammatory diseases and have often been ascribed diterpenoid compounds to have anti-inflammatory effects [56]. However, currently, marine organisms also revealed to be an exceptional source of new molecules with therapeutic potential including diterpenoid secondary metabolites, which exhibit notable anti-inflammatory properties. According to some studies, the anti-inflammatory activity of diterpenoids isolated from marine organisms is due to the inhibition of the NF-κB signaling pathway at different levels, once, it plays a crucial role in regulating the inflammatory responses and in the development of various human pathological conditions. Moreover, some marine diterpenoids also have been shown to be inhibitors of prostaglandins and leukotrienes secretion and in some cases, found to be selective inhibitors of 5-lipoxygenase and COX enzymes. These evidences demonstrate that marine diterpenoids can inhibit different pathways involved in inflammation, supporting their potential for anti-inflammatory drugs development. However, little is known about the molecular mechanisms involved in the anti-inflammatory characteristics of marine diterpenoids. Thus, further studies are necessary to better understand their mechanisms of action.

Into this framework, the diterpene eleganolone may be a future drug with anti-inflammatory properties in the treatment of pathologies associated with inflammatory processes as PD. However, further assays are needed to support this potential. Nervertheless, the results presented in this thesis suggest that *B. bifurcata* and *C. tomentosum* seaweeds, may be a source of unique molecules with therapeutic potential. Therefore, the isolation of more molecules from these seaweeds should be considered, since in this work it was patent that seaweeds have not been fully explored as far as potential source of new molecules for PD treatment. In addition,

the eleganolone and loliolide compounds from *B. bifurcata* and *C. tomentosum* isolated in this work have shown to possess capacity to protect neuronal cells from the damaging effects mediated by 6-OHDA, neutralizing its cytotoxicity, preventing mitochondrial dysfunction, apoptosis and inhibiting the NF-kB translocation (Figure 7.1). Additionally, the compounds also exhibited anti-inflammatory effects, decreasing TNF-α and IL-6 levels, thus blocking the inflammatory cascade (Figure 7.1). However, it is very important to keep in mind that good potency, selectivity, and bioavailability of compounds may be not sufficient to be efficient drugs for PD treatment. The compounds need to be able to cross the blood brain barrier (BBB) and attain a certain concentration and duration in the brain to evoke the pharmacodynamic effect necessary to produce the desired therapeutic benefits. Effectively, one of the main bottlenecks associated with the development of new therapeutics for PD is the difficulty of many drugs to reach the brain, due to this highly selective semi-permeant barrier that protects brain and the spinal cord from many foreign substances. Therefore, it is important the development of deeper investigation studies in more complex *in vitro* models, such as co-cultures, to understand the ability of eleganolone and loliolide to cross the BBB, and the possibility of their incorporation into smart delivery systems such as nanoparticles for brain drug delivery, tissue engineering, and new biomaterials.

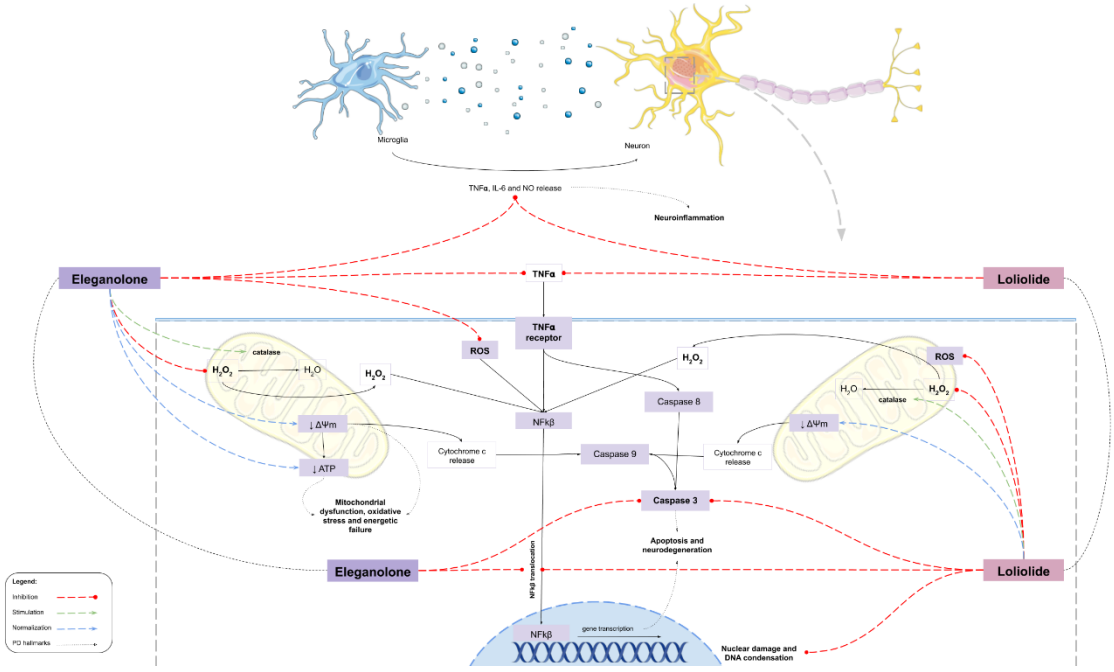


Figure 7.1. Hypothesised mechanisms of action of eleganolone and loliolide in 6-OHDA-induced cell death and in LPS-induced inflammation.

In conclusion, the current lack of studies on seaweed-derived neuroprotective agents evidences the relevance of *B. bifurcata* and *C. tomentosum* seaweeds as a source of new compounds with potential neuroprotective properties, opening new research lines in the future. Among the studied compounds, the linear diterpene eleganolone revealed a significant neuroprotector capacity, acting in several PD hallmarks, reducing oxidative stress, preventing mitochondrial dysfunction, and blocking inflammatory pathways, thus preventing neuronal cell death. Therefore, this work highlights eleganolone as a promising compound for PD therapeutics and should be considered for further detailed studies in more complex *in vitro* and *in vivo* models to validate its therapeutical application in the treatment of PD and other neurodegenerative disorders.

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APPENDIX

Publication: Neuroproctetive effect of seaweeds against 6-hydroxidopamine-induced cell death on an in vitro human neuroblastoma model - **Published**

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This publication focuses on the extraction of several seaweeds by applying different extraction solvents. The bioactive potential of the crude extracts was evaluated through a series of previously optimized *in vitro* assays. The neuroprotective activity was evaluated using the 6-OHDA-induced neurotoxicity model in the SH-SY5Y cell line. PD hallmarks, including oxidative stress and mitochondrial dysfunction, were also evaluated. This publication was a starting point for the work developed along this thesis.

Neuroproctetive effect of seaweeds against 6-hydroxidopamine-induced cell death on an in vitro human neuroblastoma model

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Abstract: Background: Parkinson's disease (PD) is a progressive neurodegenerative disorder of the central nervous system. Although the causes of PD pathogenesis remain incomplete, some evidences has suggested that oxidative stress is an important mediator in its pathogenesis. The aim of this study was to evaluate the protective effects of seaweeds with high antioxidant activity on 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in the human neuroblastoma cell line SH-SY5Y, as well as the associated intracellular signaling pathways. Methods: Cell viability studies were assessed by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium (MTT) bromide assay and the intracellular signaling pathways analyzed were: hydrogen peroxide (H_2O_2) production, changes in the mitochondrial membrane potential and Caspase-3 activity. Results: Exposure of SH-SY5Y cells to 6-OHDA (10–1000 μM) reduced cell's viability in a concentration and timedependent manner. The data suggest that the cell death induced by 6-OHDA was mediated by an increase of H_2O_2 production, the depolarization of mitochondrial membrane potential and the increase of Caspase-3 activity. Extracts from *S. polyshides, P. pavonica, S. muticum, C. tomentosum* and *U. compressa* revealed to efficiently protect cell's viability in the presence of 6-OHDA (100 μM; 24 h). These effects appear to be associated with the reduction of H2O2 cell's production, the protection of mitochondrial membrane's potential and the reduction of Caspase-3 activity. Conclusions: These results suggest that seaweeds can be a promising source of new compounds with neuroprotective potential. Keywords: Parkinson's disease, Substantia nigra, Mitochondrial membrane potential, Caspase – 3 activity, Oxidative stress, Apoptosis, Marine natural bioactive compounds.

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Doctoral student contributions:

In this publication the work was developed entirely by me (doctoral student), having carried out all the main experiences, such as extraction of seaweeds, biological assays (cytotoxicity and neuroprotective) and signalling pathways mechanisms. The treatment of results and article writing were performed by me. However, throughout the work, I had the support of several researchers who helped me in some phases of this work, being their contributions described below:

Table 1.1 Description of the contributions of the co-authors.

Co-authors	Co-author contributions
Celso Alves	Participated in the activities related to cytotoxic and neuroprotective assay;
Susete Pinteus	Participated in the activities related to cytotoxic and neuroprotective assay;
Susana Mendes	Participated in the activities related to date analysis;
Rui Pedrosa	Participated in the conception and coordination of the study;

All authors read and approved the final manuscript and are accountable for the integrity of this manuscript.