Universidade do Minho

Escola de Ciências

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Neuroprotective Effect of macroalgae and its phytochemicals

outubro de 2024

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UMinho | 2024

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Neuroprotective Effect of macroalgae and its phytochemicals

Master's Dissertation

Master in Molecular Genetics

Work developed under supervision of:

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outubro de 2024

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Agradecimentos

Um agradecimento especial ao professor Rui Pedro Soares Oliveira, pela sua orientação ao longo deste ano. A sua persistência e exigência para comigo foram cruciais para o meu desenvolvimento académico. São poucos os professores que nos marcam o percurso escolar, mas tenho muito orgulho em dizer que fui uma aluna desde a licenciatura até a este momento de um professor tão admirável.

Agradeço também aos meus colegas de laboratório, Ana Teixeira, Jorge, Nuno, João e Vera por todos os momentos de ajuda, espírito de entreajuda e conversas de apoio quando as coisas não correm como planeado.

Às minhas amigas e colegas de curso, que me acompanharam desde o inicio torcendo pelo meu sucesso académico e dando me apoio sempre que precisei. A vossa companhia, persistência e palavras foram muito especiais.

Às minhas irmãs, Inês Costa, Inês Morgado, Dara Machado, Carolina Gonçalves e Catarina Macedo que foram todo o meu suporte e apoio na minha vida, especialmente nesta etapa académica. Graças a vocês sei o que é excelência académica e tenho um exemplo a seguir. Obrigada.

À minha família, à minha mãe e ao meu pai, por darem tudo que tinham e podiam para que não me faltasse nada para eu poder estudar. Agradeço à tia Fia, tia Nuziat e à tia Natalia, por serem exemplos de mulheres fortes e torceram por este momento comigo, ajudando-me todos os dias ao longo deste ano. Ao Tiago, por ter estado presente em todos os momentos cruciais deste ano dando me um apoio incondicional, para que não me faltasse força, apoio e uma mão para segurar.

A todos que de alguma forma apoiaram-me no meu percurso académico, muito obrigada.

Statement of integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information of falsification of results along the process leading to its elaboration.

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Abstract

Neurodegenerative diseases, defined by the progressive loss of cells of the central and peripheral nervous system, represent a substantial and escalating global health concern. The development of novel therapeutic strategies is of vital importance, as the current treatments are primarily focused on symptom management and often have adverse side effects. Natural products, particularly those derived from plants, algae, fungi and bacteria, have long been recognised as a valuable source of medicinal compounds, with ongoing research indicating their potential for combating a range of diseases. In this context, algae have emerged as a promising natural resource, rich in diverse bioactive compounds such as fucoidans, flavonoids, fucoxanthin, among others which have been identified as having significant potential for neuroprotection.

This study aimed to investigate the neuroprotective potential of a variety of macroalgae extracts. The initial step involved the evaluation of macroalgae extracts obtained from the company SeaExpert for their antioxidant effect using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, Ferric Reducing Antioxidant Power (FRAP) assay, and Iron Chelating Activity (ICA) assay. Of these, *Cystoseira humilis* (CH), *Pterocladiella capillacea* (PC), *Sargassum vulgare* (SV) and *Zonaria tournefortii* (ZT) extracts demonstrated considerable antioxidant activity, thereby justifying further investigation in a *Saccharomyces cerevisiae* model. The resistance of yeast cells to oxidative stress was increased by extracts derived from PP, SV and ZT, which also attenuated intracellular oxidation caused by H_2O_2. Furthermore, these extracts demonstrated the capacity to reduce α-synuclein aggregation in a yeast strain that expressed human α-synuclein for the purpose of studying protein aggregation in relation to Parkinson's disease (PD) with PP, SV and ZT also exhibiting the ability to decrease huntingtin aggregation in a strain that expressed huntingtin, which is associated with Huntington's disease.

The findings of this research identify algae as potential sources of antioxidant and protein antiaggregating compounds, with promising applications in the treatment of neurodegenerative diseases.

Keywords: Neurodegenerative diseases, *Saccharomyces cerevisiae, Padina pavonica, Sargassum vulgare, Zonaria tournefortii*

Resumo

As doenças neurodegenerativas, definidas pela perda progressiva de células do sistema nervoso central e periférico, representam uma preocupação substancial e crescente para a saúde global. O desenvolvimento de novas estratégias terapêuticas é de vital importância, pois os tratamentos atuais são focados principalmente na gestão dos sintomas e muitas vezes têm efeitos colaterais adversos. Os produtos naturais, especialmente aqueles derivados de plantas, algas, fungos e bactérias, há muito são reconhecidos como uma fonte valiosa de compostos medicinais, com pesquisas contínuas indicando o seu potencial no combate a uma variedade de doenças. Nesse contexto, as algas emergiram como um recurso natural promissor, rico em diversos compostos bioativos, como fucoidanos, flavonóides e fucoxantina, entre outros, que foram identificados como tendo um potencial significativo para a neuroproteção.

O presente estudo teve como objetivo investigar o potencial neuroprotetor de uma variedade de estratos de macroalgas. O primeiro passo envolveu a avaliação de extratos de macroalgas obtidos da empresa SeaExpert quanto ao seu efeito antioxidante, utilizando o ensaio 2,2-difenil-1-picrilhidrazil (DPPH), ensaio de Poder Antioxidante de Redução Férrica (FRAP) e o ensaio de Atividade Quelante de Ferro (ICA). Dentre esses extratos, os de *Cystoseira humilis* (CH), *Pterocladiella capilácea* (PC), *Sargassum vulgare* (SV) e *Zonaria tournefortii* (ZT) demonstraram considerável atividade antioxidante, justificando uma investigação mais aprofundada num modelo de *Saccharomyces cerevisiae*. A resistência das células de levedura ao stress oxidativo foi aumentada pelos extratos derivados de PP, SV e ZT, que também atenuaram a oxidação intracelular causada por H_2O_2. Além disso, estes extratos mostraram atividade antiagregante de α-sinucleína numa estirpe de levedura que expressa a α-sinucleína humana para estudos de agregação proteica da doença de Parkinson (PD), com PP, SV e ZT também exibindo a capacidade de diminuir a agregação de huntingtina numa estirpe que expressa a huntingtina, associada à doença de Huntington.

Os resultados desta pesquisa identificam as algas como potenciais fontes de compostos antioxidantes e antiagregantes de proteínas, com aplicações promissoras no tratamento de doenças neurodegenerativas.

Palavras-chave: Doenças neurodegenerativas, *Saccharomyces cerevisiae, Padina pavonica, Sargassum vulgare, Zonaria tournefortii*

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List of Abbreviations and Acronyms

AChE - acetylcholinesterase

AD – Alzheimer's disease

APP – Amyloid precursor protein

Aβ – Amyloid β

BuChE – butyrylcholinesterase

CNS – Central nervous system

DCF – 2',7'-dichlorofluorescein

dH2O – dionized water

DMSO - Dimethylsulfoxide

DPPH – 2,2-Diphenyl-1-picrylhydrazyl

EtOH- ethanol

FRAP - Ferric Reducing Antioxidant Power

H2DCFDA - fluorochrome 2',7'-dichlrofluorescein diacetate

HD - Huntington's disease

HTT – Huntingtin

ICA – Iron Chelating Activity

MPTP - pyridine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

OD - optical density

PBS – Phosphate buffer saline

PD – Parkinson's disease

PI - Propidium iodide

PolyQ - Polyglutamine

PP- *Padina pavonica*

Rpm – rotations per minute

SPs - sulfated polysaccharides

SV- *Sargassum vulgare*

TPTZ - 2,4,6-tris (2-pyridyl)-s-triazine

TYRO - tyrosinase

UPR - Unfolded protein response

ZT- *Zonaria tournefortti*

αSYN – α-synuclein

1. Introduction

1.1. Neurodegenerative Diseases

The achievements in public and individual health have resulted in a significant increase in human lifespan. Life expectancy has increased steadily in recent decades, which is undoubtedly a remarkable achievement in the 21st century (Prince et al., 2015). The extended lifespan has resulted in a larger population of older individuals, which has led to an increase in the prevalence of age-related diseases, particularly neurodegenerative disorders (Duncan, 2011). The prevalence of neurodegenerative diseases leading to dementia is calculated by the European dementia meta-analysis (EURDEM), the overall prevalence of neurodegenerative disease leading to dementia is 1.6% for males and 1% for females in the 65-69 age group. This percentage rises to 11% for males and 12.6% for females in the 85-89 age group (Lobo et al.,2000).

Neurodegenerative diseases are a wide range of neurological disorders that affect the lives of millions of people worldwide and involve the gradual loss of neurons in either the central or peripheral nervous system (Wilson et al., 2023). Neurodegenerative diseases are characterized by the collapse of the structure and function of neural networks and the loss of neurons due to theirs terminally differentiated nature, which makes it difficult for them to efficiently renew themselves. As a result, the core communication circuitry breaks down, leading to impaired memory, cognition, behaviour, sensory and motor function (Wilson et al., 2023).

Research over decades has identified genetic factors and biochemical pathways involved in neurodegenerative diseases (Wilson et al., 2023). This has resulted in the identification of eight hallmarks of neurodegenerative diseases, shown in Figure 1.

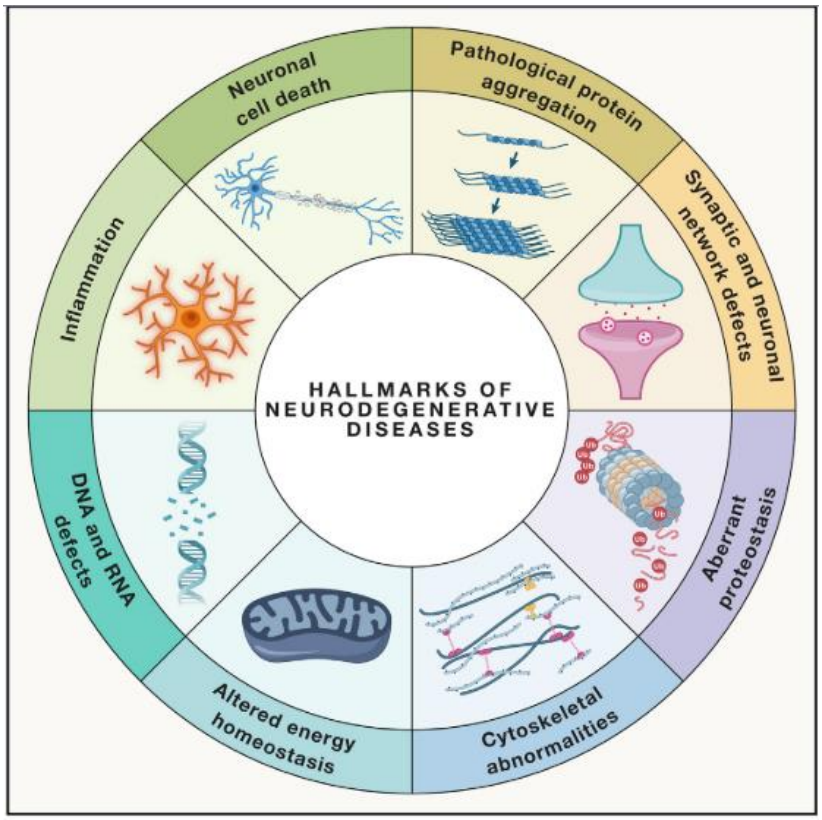


Figure 1. Hallmarks of neurodegenerative diseases. Many genetic factors and biochemical pathways underlying many neurodegenerative diseases have been identified. This resulted in the identification of eight hallmarks of neurodegenerative diseases: pathological protein aggregation, synaptic and neuronal network dysfunction, aberrant proteostasis, cytoskeletal abnormalities, altered energy homeostasis, DNA and RNA defects, inflammation, and neuronal cell death. Adapted from Wilson et al., 2023.

Neurodegenerative diseases often have multiple contributing factors. Some common risk factors associated include age, education, gender, genetics, lifestyle, medical conditions and environmental factors, listed in Table 1. Understanding these complex interplays is crucial for developing effective treatments and preventive strategies.

Table 1. List of risk factors associated with Alzheimer's disease (AD). Based on (Henderson, 1988)

Types of risk	Risk factors
Demographic	Age
	Education
	Gender
	Race
	Social class
Genetics	Amyloid precursor protein (APP)
	Presenilin 1 and 2 (PSEN1 and 2)
	Apolipoprotein E (APOE)
	Clusterin gene (CLU)
	Estrogen receptor gene (ESR)
	Transferrin gene (Tf)
Lifestyle	Alcohol
	Lack of exercise
	Malnutrition
	Poor diet
	Smoking
Medical	Cancer
	Cardiovascular disease
	Immune system dysfunction
	Obesity
	Stroke
	Traumatic brain injury
Environmental	Air pollution
	Organic solvents
	Vitamin deficiency
	Geographic location

1.1.1. Alzheimer's Disease

Alzheimer's disease stands as the predominant cause of dementia, rapidly emerging as one of the deadliest and most burdening diseases of this century (Scheltens et al., 2021). In 2018, Alzheimer's Disease International, the international federation of Alzheimer and dementia in official relations with the World Health Organization, predicted that around 50 million people worldwide had dementia (Prince et al., 2015). They expect this number to triple by 2050, with most affected people residing in low to middle-income countries. Recent data suggests that dementia cases in Europe will double by 2050 (Scheltens et al., 2021).

The two main pathological hallmarks of AD are amyloid plaques and neurofibrillary tangles (Ballard et al., 2011). Specific modifications occur in amyloid β (Aβ) processing, such as the cleavage of amyloid precursor protein (APP) into Aβ peptides. The specific accumulation of neurotoxic Aβ derived from the post-translational proteolysis of APP in the central nervous system appears to represent a major pathological step in the progression of AD (Selkoe, 2001). AD has been thought to occur due to the accumulation of aggregated neurotoxic Aβ appearing in specific brain regions (hippocampus and cerebral cortex), triggering an inflammatory response, neuronal cell death and gradual cognitive decline (Ling et al., 2003).

There are two pathways of APP enzymatic processing, amyloidogenic and non-amyloidogenic. In the amyloidogenic pathway, the amino-terminus of APP is cleaved by β-secretase, whereas the APP C-terminus is cleaved by γ-secretase. β-secretase cleaves APP between residues Met671 and Asp672, giving rise to a soluble peptide of APP and the C99 fragment. The γ-secretase cleavage site is located in the vicinity of residue 712 of the C-terminus. γ-secretase is capable of cleaving the C-terminus region in Val711 or Ile713, resulting in the production of the short peptide Aβ (Aβ40) or the long peptide (Aβ42). In addition to the Aβ, a C-terminal peptide of approximately 50 residues of APP is also released, which is also known as the APP intracellular domain (AICD; Figure 2; Hernández-Zimbrón & Rivas-Arancibia, 2014). Conversely, the non-amyloidogenic pathway does not produce the Aβ peptide. APP can be processed by α-secretase (TACE), which cleaves the APP fragments between Lys687 and Leu688, thus producing a long soluble domain. Similarly, the C-terminal fragment containing P3 and C83 domain if formed by α-secretase at residue 711 or 713 to release the P3 fragment (Figure 2; Hernández-Zimbrón & Rivas-Arancibia, 2014).

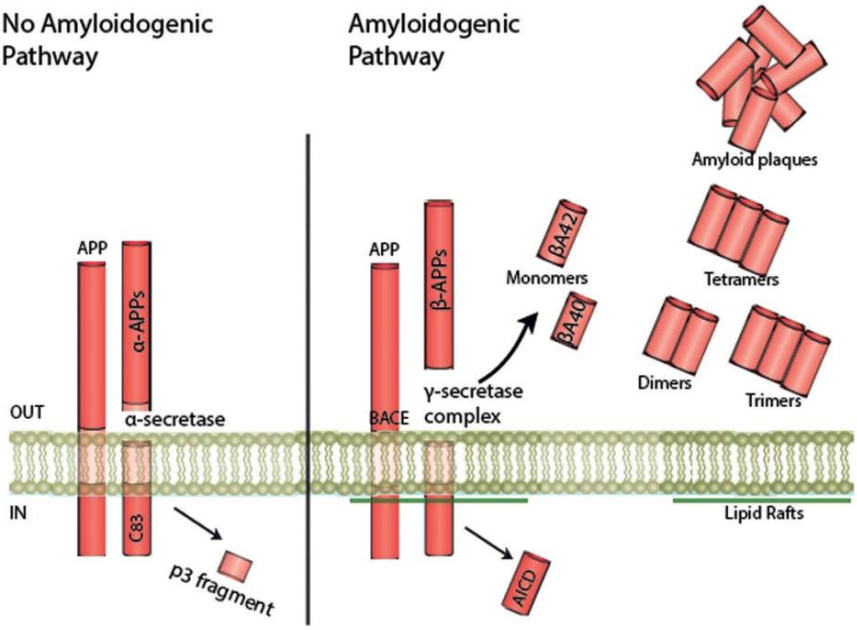


Figure 2 Amyloidogenic and non-amyloidogenic pathways of APP. In non-amyloidogenic pathway (left side) APP is cleaved by α-secretase, producing a soluble fragment known as α-APPs. This cleavage occurs within the Aβ region, preventing the formation of Aβ peptide. The remaining membrane fragment, C83, is further cleaved by γ-secretase, releasing the APP Intracellular Domain (AICD) into the cytoplasm and producing the P3 fragment, which is non-toxic. Amyloidogenic Pathway (right side): APP is first cleaved by β-site-cleaving enzyme (BACE1), generating a soluble fragment called β-APPs. The remaining membrane-bound fragment is then cleaved by γ-secretase, producing Aβ peptide, including βA40 and βA42. These peptides aggregate to form monomers, dimers and eventually amyloid plaques, which are associated with AD. Adapted from Hernández-Zimbrón & Rivas-Arancibia, 2014.

Tau is a microtubule-associated protein that is the main constituent of neurofibrillary tangles (Ballard et al., 2011). Under normal conditions, Tau is a soluble protein responsible for stabilizing microtubules within the neuron. However, when insoluble aggregates form due to tau hyperphosphorylation, the neurons suffer with the appearance of neurofibrillary tangles, disrupting the synaptic function(Ballard et al., 2011).

1.1.2. Parkinson's Disease

Parkinson's disease (PD) represents a rapidly increasing neurodegenerative condition. In most populations, 3-5% of PD cases can be attributed to genetic causes linked to known PD genes (Bloem et al., 2021). The cause of PD is likely to be multifactorial (Chade et al., 2006). Genetic susceptibility and combinations of risk and protective factors are likely to contribute to the

development of the disease. The discovery of a cluster of parkinsonism produced by the neurotoxic pyridine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the 1980s enhanced the study of nongenetic causes of PD. MPTP is a prodrug to the neurotoxin MPP+, which causes the development of permanent symptoms of PD by the death of dopaminergic neurons in the substantia nigra of the brain. This finding emphasized the potential for environmental toxins to be involved in the aetiology of PD, shifting some research focus towards understanding how non-genetic factors, such as exposure towards environmental toxins, might contribute to the disease. Since that time, epidemiological investigations have suggested risk factors, though the results do not establish causality (Chade et al., 2006).

Significant evidence associates α- synuclein (αSYN), a small and highly conserved presynaptic protein which function remains unknown, to PD. The synuclein family consist of three members: α-, β- and γ-synuclein. αSYN has been identified as the main component of Lewy bodies, the characteristic protein deposits that are the hallmarks of PD (Breydo et al., 2012). PD can be induced as the result of three missense mutations in the αSYN gene, corresponding to Ala30Pro, Glu46Lys and Ala53Thr substitutions in αSYN. PD can also be a result of the overexpression of the wild-type αSYN protein due to gene triplication (A. B. Singleton et al., 2003, A. Singleton et al., 2004).

Synucleinopathies are a group of disorders characterized by the progressive aggregation of insoluble fibrillary αSYN in neurons and glia, which is associated with neurodegeneration. This underlies a wide spectrum of clinical syndromes, including movement disorders. The disorders include Parkinson, dementia and autonomic dysfunction (multiple system atrophy). From a pathogenic perspective, these disorders arise from metabolic disturbances in αSYN, such as increased synthesis and oligomer formation due to insufficient degradation (Jellinger, 2010).

1.1.3. Huntington's Disease

George Huntington, a young physician, was the first to publish a description of the disorder that would later be named after him in 1872 (Finkbeiner, 2015). Huntington's disease (HD) is an inherited autosomal dominant disorder that causes neurological, cognitive and psychiatric symptoms (Anderson & Marder, 2001).

A number of motor symptoms may be observed in individuals with HD, including chorea, which typically manifests in the hands, feet and face during the early stages of the disease. Dystonia is also a common symptom, although less prevalent in the late stages of the disease. Additionally, speech and swallowing impairment are frequently observed, as are irregularities in eye movement, which contribute to a slower reaction time and difficulty driving (Anderson & Marder, 2001).

The mutation responsible for HD results in a repeated sequence of cytosine, adenine and guanine (CAG) in exon 1, located in 4p16.3, the huntingtin gene, encoding a chain of polyglutamine (polyQ) residues in the huntingtin protein. Individuals with less than 35 CAG repeats do not develop the disease, while those with more than 35 CAG repeats are at high risk of developing HD (Bates, 2003). When the number of CAG exceeds 35-45, the phenotypes are usually observed (Bauer & Nukina, 2009).

The accumulation of polyQ within neurons, both intranuclearly and cytoplasmicly, has been identified as a potential cause of a toxic gain of function. Aggregates are commonly observed in areas of the brain in patients affected by this condition. These inclusions have been associated with a loss of neuronal viability (Bauer & Nukina, 2009).

1.2. Therapeutic Strategies

Despite years of research, there are still no approved therapies that effectively slow down or prevent the progression of AD, PD and HD. The existing treatments only alleviate symptoms and are not able to address the root cause (Chopade et al., 2023, Eje et al., 2023). The current approved therapies for AD are based on acetylcholinesterase (AChE) inhibitors, including Razadyne, Aricept and Exelon. There are also NMDA receptors antagonists, such as Namenda, used in the treatment of AD, shown in Figure 3.

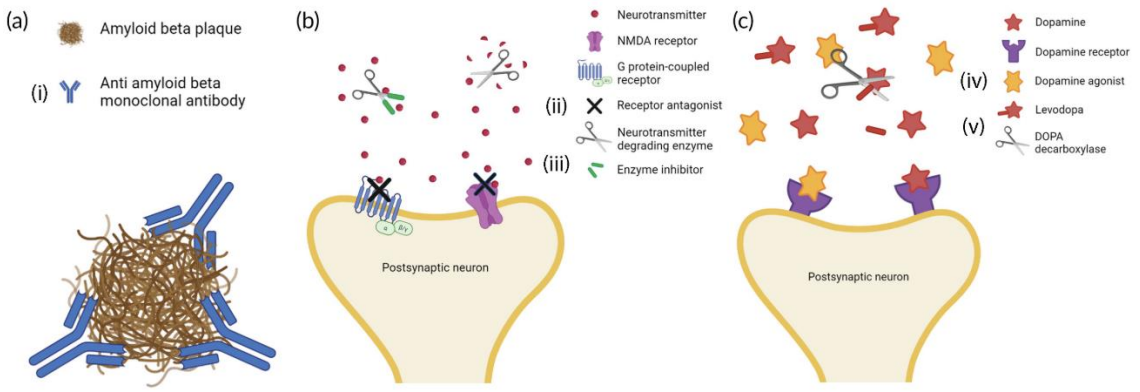


Figure 3 Therapy mechanisms of drugs in Alzheimer's disease (AD) and Parkinson's disease (PD). Pannels a, b and c show mechanisms specific to AD and PD. Adapted from Chopade et al., 2023.

In the case of PD, the most commonly used therapies are based on dopamine agonists, including Mirapex and Requip. Additionally, there are monamine oxidase inhibitors such as Zelapar and Azilect utilized in PD. Their efficacy is based on the modulation of neurotransmitters within the CNS (Chopade et al., 2023). Symptomatic therapies for HD are also available and are employed with some regularity in the treatment of chorea and psychiatric aspects of the condition. However, the efficacy of these treatments can vary considerably between individual patients, and a substantial minority of patients experience adverse reactions (Barker & Mason, 2019;Eje et al., 2023).

1.3. Protein Misfolding

Proteins are fundamental to life processes. Alterations in protein structure are reflected in their function. Misfolded proteins and their aggregates present a significant risk to the cell. Cells have a diverse but integrated network of protection mechanisms, such as molecular chaperones and protein degradation factors in order to regulate and contain problems caused by protein misfolding (Ajmal, 2023).

Heat shock proteins (Hsp) are responsible for housekeeping functions. As molecular chaperones, they facilitate the correct folding of polypeptides by binding with nascent proteins via their C terminal domain. Many Hsp are also upregulated by stress, with inducible Hsp preventing protein denaturation and incorrect polypeptide aggregation. It has been proposed that Hsp may prevent protein unfolding or aggregation and enhance cell survival (Trivedi, 2007).

Intracellular protein aggregation is a hallmark of various neurodegenerative disorders. Mutations cause abnormal misfolded protein processing that overtakes the cell's quality-control mechanisms resulting in the deposition of protein aggregates in the cytosol, extracellular space and/or nucleus (Forman et al., 2004).

1.4. Oxidative Stress

Oxidative stress is a condition in which there is an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defence system. It can arise due to excessive ROS production, inadequate antioxidant defences or both (Patel et al., 2018). This imbalance leads to the accumulation of ROS, which can cause damage to cellular components such as lipids, proteins, and DNA. In neurons, this can lead to neurodegenerative diseases such as AD and PD (Olasehinde et al., 2017).

Several factors can contribute to oxidative stress, including exposure to environmental toxins, radiation, infections, and metabolic disorders. Under normal conditions, the body has a well-developed antioxidant system that neutralizes ROS and prevents oxidative damage (Moradas-Ferreira et al., 1996). This defence system includes enzymes such as superoxide dismutase, catalase and glutathione peroxidase, as well as non-enzymatic molecules such as vitamin C, vitamin E, and glutathione (Moradas-Ferreira et al., 1996). However, when ROS levels exceed the antioxidant capacity of the body, oxidative stress occurs. The resulting damage can lead to alterations in cellular signalling pathways, energy metabolism, and ultimately cell death. In neurons, oxidative stress can contribute to the accumulation of misfolded proteins and the formation of aggregates, which are characteristic features of neurodegenerative diseases. Also, ROS can cause oxidative DNA damage, which may lead to inflammation, accelerated ageing, and increased susceptibility to cancer and neurodegenerative diseases (Thanan et al., 2014).

Neurons in the central nervous system (CNS) consume high amounts of oxygen and have a longer lifespan, leading to chronic exposure to elevated levels of ROS. The accumulation of genome damage, including oxidized bases and single-strand breaks resulting from ROS exposure, as well as their deficient or diminished repair within the brain, have been suggested as contributing factors in the development of various neurological disorders, such as AD and PD (Hegde et al., 2011).

It is thought that free radicals produced during oxidative stress may have a pathological significance in AD, PD and HD (Cross et al., 1987). Several lines of evidence suggest that oxidative stress is an early occurrence in AD, happening before cytopathology, and therefore may have a significant role in the disease's pathogenesis (Zhu et al., 2004).

Protein structure and function are susceptible to alteration by ROS produced either by cell metabolism or by external oxidants. While catalases, superoxide dismutases and peroxidases facilitate the maintenance of non-toxic levels of ROS, amino acid side chain modification occurs as a consequence (Herrero et al., 2008). Therefore, understanding the mechanisms of oxidative stress and developing strategies to mitigate its effects is crucial in the prevention and treatment of neurodegenerative diseases.

1.5. Biological activity of extracts

The potential of natural sources for drug discovery has been recognized for some time. Many of the medications currently in use in healthcare have been discovered from natural sources, such as plants and algae (Yuan et al., 2016). Natural products, evolved over millions of years, have unique chemical diversity, resulting in diverse biological activities and drug-like properties, some of which have proven effective in treating diseases (Yuan et al., 2016). However, there remains a need to identify new drugs with high potency and pharmacological effects, as further therapeutics targets are identified through medical research.

Alzheimer's disease is characterized by the accumulation and aggregation Aβ peptides in the brain (Lin et al., 2020). Recent studies have shown that the sulfated polysaccharide ulvan extracted from the macroalga *Ulva lactuca* has potent antioxidant properties (Yang et al., 2021). These properties make ulvan a promising candidate for the development of antioxidants and the treatment of related diseases (Liu et al., 2021). By inhibiting Aβ aggregation, ulvan may be able to reduce the accumulation of Aβ peptides in neuronal cells, which could slow the progression of Alzheimer's disease.

Beside this, recent research has shown the potential of *Sargassum muticum*, a brown macroalga, as a source of functional ingredients against oxidative stress-related conditions. The findings suggest that *S. muticum* contains compounds with the ability to decrease cell's oxidative stress levels by blocking the production of H_2O_2 (Pinteus et al., 2017). These characteristics

demonstrate the potential of *S. muticum* as an excellent source of functional ingredients against oxidative stress conditions. The Sargassaceae family presents interesting studies reporting various biological activities such as neuroprotective effects. For instance, *Sargassum vulgare* has been found to exhibit neuroprotective effects in cultured neuronal cells under induced oxidative stress (Pinteus et al., 2017). Also, a recent study found that fucoidans extracted from *Fucus vesiculosus* have neuroprotective properties against the damaging effects of Aβ and H_2O_2 in cells (Alghazwi et al., 2019). These fucoidans were shown to decrease the aggregation of Aβ, which reduced the cytotoxicity caused by Aβ and H_2O_2 In addition, they reduced Aβ-induced cell death and increased the growth of neurites, which are important for maintaining healthy brain function (Mohd Sairazi & Sirajudeen, 2020).

1.6. Macroalgae and Neuroprotective Compounds

Macroalgae, more commonly known as seaweeds, are large, photosynthetic organisms that inhabit either marine of freshwater environments respectively. In contrast to microalgae, which are unicellular and microscopically small, macroalgae are multicellular and observable with the naked eye. They are classified into three main phyla: The phyla Chlorophyta (green algae), Rhodophyta (red algae), and Phaeophyceae (brown algae) are distinguished by their unique pigmentation, cellular structure, and ecological roles (Sudhakar et al., 2018).

Marine algae are acknowledged as a rich source of structurally diverse biologically active compounds with significant potential for use in pharmaceutical and biomedical applications. It has been demonstrated that compounds derived from marine algae display a range of allergic, anti-cancer, anti-inflammatory and anti-obesity. Additionally, scientific studies have shed light on the neuroprotective properties of marine algae.

Recent research has indicated that seaweed, which is a rich source of bioactive compounds, may have the potential to mitigate and combat neurodegenerative diseases These bioactive constituents have demonstrated promising properties in the prevention and mitigation of neurodegeneration. Their mechanisms of action involve intricate pathways that contribute to neuronal survival, reduction of oxidative stress, inhibition of neuroinflammation, and modulation of protein aggregation processes (Pereira & Valado, 2023).

Bioactive compounds derived from algae can be classified into several chemical groups, each exhibiting distinct therapeutic properties. Among pigments, phycobiliproteins and carotenoids, such as phycocyanin and fucoxanthin, have been shown to possess potent antioxidant and neuroprotective activities (Chini Zittelli et al., 2023). Polysaccharides, including alginates and fucoidans, have demonstrated the capacity to influence the immune system, reduce inflammatory processes and protet against the damaging effects of neurotoxin exposure (Fitton, 2011;Liu & Sun, 2020;Dimitrova-Shumkovska et al., 2020). Another important group of compounds with neuroprotective properties is polyphenols, which include flavonoids, catechins and phenolic acids(Mandel & Youdim, 2004). These have been demonstrated to reduce oxidative stress , which is associated with neurodegenerative diseases. Furthermore, omega-3 fatty acids (EPA andDHA) present in algae have been demonstrated to possess anti-inflammatory and neuroprotective properties(Y. Liu et al., 2022). Certain algae produce alkaloids that exhibit antimicrobial and neuroprotective properties (Mandel & Youdim, 2004; Alghazeer et al., 2013). Terpenoids, including those of the diterpenoids class, have been demonstrated to exhibit antioxidant and anti-inflammatory properties(Graßmann, 2005; Wang et al., 2019). In addition, strerols such as fucosterol are acknowledged for their antioxidant properties and role in regulating cholesterol (Abdul et al., 2016). Each of these groups contributes to the therapeutic potential of algae, particularly in the context of chronic and neurodegenerative diseases.

Flavonoids have been proven to be used as therapeutic molecules for HD (Schulte & Littleton, 2011). It has also been reported that the polyphenolic extract demonstrated to reduce the formation of amyloid deposits and to significantly attenuate cognitive deterioration in a mouse model of AD via its antioxidant activity (Rezai-Zadeh et al., 2005,Rezai-Zadeh et al., 2008) and the promotion of APP degradation into non-amyloidogenic peptides (Jeon et al., 2003; Fernandez et al., 2010). An antioxidant polysaccharide, porphyrin, derived from the red algae *Pyropia haitanensis*, has been introduced as a protective agent against neurotoxicity induced by Aβ in a mouse model of AD. Porphyran has been demonstrated to possess neuroprotective properties, which may assist in the alleviation of the adverse effects of Aβ (Zhang et al., 2020).

1.6.1. Padina pavonica

Padina pavonica, commonly known as peacock's tail, is a remarkable species of brown algae in the family Dictyotaceae. This marine alga is characterised by its unique fan-shaped

calcified fronds. It is found primarily in warm temperate and tropical waters, including the Mediterranean Sea and the Atlantic Ocean. As a vital component of coastal marine ecosystems, *P. pavonica* serves both an ecological and a bio-indicator role, contributing to habitat complexity and reflecting environmental health. Furthermore, the alga's complex chemical composition, which includes bioactive compounds such as polysaccharides, sterols, and fatty acids, highlights its potential for use in pharmaceutical and cosmetic applications. A qualitative and quantitative estimation of terpenes and sterols from the brown algae *Padina* revealed the presence of 19 terpenes and 5 sterols. The ash content of *Padina* was found to be 30-48%, while the carbohydrate content ranged from 25-39%. The total dietary fibre constituted 27-39% on a dry basis, with the protein content at 5-7% and lipid content at 1,6-1,8%. Additionally, various *Padina* species have been found to contain fatty acids with 14-22 carbon atoms. (Wahbeh, 1997,Kamenarska et al., 2002, El Shoubaky & Salem, 2014). These compounds have been shown to possess a range of biological activities, including anti-inflammatory, antimicrobial, and skin-firming properties, making *P. pavonica* a promising candidate for future exploitation.

The antioxidant activity of *P. pavonica* is of significant importance in the prevention of damage caused by oxidative stress in neurodegenerative diseases. The literature has demonstrated that *P. pavonica* (acetate extract) exhibits DPPH and ABTS radical scavenging activity above 50% (Olasehinde et al., 2019). Additionally, *P. pavonica* has been shown to possess neuroprotective properties, as evidenced by its ability to enhance the viability of SH-SY5Y cells originated from a neuroblastoma, and to inhibit caspase-3 activity, thereby reducing the neurotoxic effects induced by 6-OHDA, a neurotoxin commonly used in research to induce oxidative stress and to model neurodegenerative conditions, particularly PD (Silva et al., 2018).

1.6.2. Sargassum vulgare

The species *Sargassum vulgare*, belonging to the genus *Sargassum*, has attracted scientific interest due to the diverse range of bioactive compounds it contains. Of these, the sulphated polysaccharides (SPs) are of particular interest. SPs have been shown to confer significant health benefits, including antioxidant activity, which enables the scavenging of free radicals and the protection of cells from oxidative stress and damage (Chouh et al., 2022). Furthermore, SPs have been demonstrated to possess anti-inflammatory properties, which are of particular importance in the management of chronic inflammatory conditions (Chouh et al., 2022).

Additionally, they have been shown to exhibit anticoagulant effects, which can reduce the risk of thrombosis and promote cardiovascular health (Dore et al., 2013).

Another group of bioactive compounds present in *S. vulgare* is polyphenols. These compounds have demonstrated potential for the inhibition of cancer cell growth and the induction of apoptosis, making them a promising class of agents for cancer therapy (Abhishek & Dilip, 2021). Moreover, they exhibit anti-inflammatory properties as well. Of particular interest are fucoidans, a type of SPs found in the cell walls of brown seaweed such as *S. vulgare*. It has been demonstrated that fucoidans possess antiviral properties (Sun et al., 2020).

Studies have shown that *S. vulgare*, a brown algae found along the Mediterranean coast, inhibits enzymes associated with neurological diseases such as acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and tyrosinase (TYRO, Custódio et al., 2016). These findings suggest that compounds in *S. vulgare* may have potential therapeutic applications in conditions such as AD and other neurodegenerative disorders.

1.6.3. Zonaria tournefortii

The majority of the *Zonaria* species are distributed worldwide and are found in a variety of habitats. A total of 18 species have been identified, with *Zonaria diesingiana*, *Zonaria farlowii*, *Zonaria tournefortii*, and *Zonaria turneriana* representing the extensively studied (Cardoso et al., 2022). The biological traits of *Zonaria* spp. Indicate that these seaweeds are highly adaptable. They are capable of modulating nutrient uptake in accordance with prevailing abiotic and biotic conditions. The biochemical composition of *Zonaria* spp. has been subject of study, with the identification of potential bioactive compounds, including phenols, sterols, fatty acids and other lipids and lipophilic substances (Fonseca et al., 2021). Additionally, some species have been found to possess relevant bioactivities, including antioxidant, antimicrobial, and anticancer properties (Cardoso et al., 2022).

In the organism *Z. tournefortii*, researchers have identified the compound chromone, which has demonstrated potential for therapeutic use through the inhibition of GSK3β enzyme activity (Ahmed et al., 2020) This inhibition is of considerable significance, as GSK3β is an enzyme linked to a number of diseases, including neurodegenerative disorders and diabetes (Lei et al., 2011).

This suggests that that chromone from *Z. tournefortii* could prove to be a valuable compound for the development of treatments for these conditions.

2. Objectives

Neurodegenerative diseases are characterized by the progressive loss of neurons and their functions, which ultimately results in significant physical and psychological impairment for affected individuals and their families. As the global population continues to age, driven by advances in medical care that increase life expectancy, the incidence of these disease continues to rise. This highlights the urgent necessity for the development of efficacious treatments. The current treatments are primarily symptomatic rather than offering a cure or prevention, thereby emphasising the critical need for new therapeutic strategies.

In this context, the main goal of this study is to identify natural marine sources of new molecules with pharmacological activity that could potentially be used to prevent or cure neurodegenerative diseases. Algae, a diverse group of photosynthetic organisms, have demonstrated the potential to produce bioactive compounds with therapeutic properties.

To achieve this goal, we will assess the antioxidant activity of the macroalgae and identify neuroprotective activity as well. For this purpose, the biological model *Saccharomyces cerevisiae* and respective mutant strains will be used to assess anti-aggregation activity of the extracts, and the ability of the algae extracts to promote the expression of chaperones, which are involved in the protection against stress.

The results of this study will provide insight into the potential of algae extracts as therapeutic agents for neurodegenerative diseases. Identification of algae extracts or compounds with anti-aggregation activity would represent a significant step forward in the development of effective treatments for these neurodegenerative diseases.

3. Materials & Methods

3.1. Plant material and preparation of extracts

The algae were collected by the company SeaExpert in the Atlantic Ocean near Faial Island, Azores (Table 2). All the plant material tested in this study was collected by manual harvesting or with cutting tools and supplied in a dehydrated form.

Table 2: Algae species used in this study and type of drying.

Algae species and abbreviation	Type of drying
Asparagopsis armata (AA)	Dark
Asparagopsis taxiformis (AT)	Dark
Cystoseira humilis (CH)	Sun
Gongolaria abis-marina (GAM)	Dark
Halopteris scoparia (HS)	Dark
Padina pavonica (PP)	Dark
Pterocladiella capillacea (PC)	Dark
Sargassum vulgare (SV)	Sun
Zonaria tournefortii (ZT)	Sun

In order to prepare the algae extract, 4.8 g of the dried alga were incubated with 80 mL 100% ethanol (etOH) during 24 h at 50 °C 150 rotations per minute (rpm). The supernatant was collected and 40 mL of 100% etOH were added for a second extraction during 60 min at 50 °C 150 rpm. The supernatants were mixed, and this process was repeated twice. Each supernatant collected was centrifuged at 5000 *g* for 8 min and filtered afterwards. The algae solution that was obtained was evaporated in a rotary evaporator (BUCHI Rotavapor R-200) at 40 °C. The dry extracts were then dissolved in 10 mL deionized H_2O (dH_2O) and frozen at -80 °C. The sample was lyophilized and the obtained powder material was stored at room temperature until use and stock solutions were prepared at 50 mg/mL using DMSO as solvent.

3.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

In order to evaluate the antioxidant characteristics of the algae extracts, a DPPH radical scavenging assay was conducted. This assay quantifies the capacity of antioxidants to neutralise free radicals. The unpaired electron of the nitrogen atom in DPPH is reduced when it accepts a hydrogen atom from antioxidants, thereby converting into hydrazine. Consequently, the solution's colour shifts from violet to yellow, indicating the degree of antioxidant activity (Kedare & Singh, 2011).

The DPPH stock solution was prepared with 19,7 mg in 50 mL 100% etOH. This solution was diluted to an optical density at 500 nm (OD_{500}) of 1.0. A range of concentrations from the extract was tested, from 1 mg/mL to 0.03125 mg/mL. In a 96 well plate, each well was added 10 µL extract and 140 µL DPPH stock solution. The absorbance at 515 nm was read after 60 min incubation in the dark. The blank of the samples consisted of 140 µL 100% etOH and 10 µL of each extract. The control was 140 µL DPPH and 10 µL 100% etOH. The radical scavenging activity of test samples, expressed as percentage inhibition of DPPH, was calculated according to the formula: % inhibition = $[(A_B - A_A)/A_B] \times 100$, where A_B and A_A are the absorbance values of the control and of the test sample, respectively. To assess the IC_{50} of the extracts in the DPPH assay, linear regression analysis was performed for all extracts.

3.3. Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay is a relatively simple, rapid, and cost-effective direct method of measuring the combined antioxidant activity of reductive antioxidants in a test sample. The assay employs the reduction of ferric ions (Fe^{3+}) t ferrous ions (Fe^{2+}) as the signal or indicator reaction, which is accompanied by a colour change.

In this assay, a range of concentrations from the extract was tested, from 1 mg/mL to 0.03125 mg/mL. In a 96 well plate each well was added 20 µL extract, 15 µL dH_2O and 150 µL of working solution. To summarize, the FRAP reagent was freshly prepared by combining 2.5 mL of a 10 mM 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3$ and 25 mL 300 mM acetate buffer at pH 3.6. The solution was then warmed to 37 °C prior to use. The absorbance of the reaction mixture at 593 nm was then measured spectrophotometrically after incubation for 30 min in the dark. The blank consisted of 20 µL of each extract and 165 µL dH_2O.

3.4. Iron Chelating Activity (ICA) assay

The Iron Chelating Activity (ICA) assay represents a fundamental analytical technique employed to assess the capacity of diverse compounds to bind and sequester iron ions, thereby preventing their involvement in oxidative processes that are potentially damaging to biological systems. The presence of iron, particularly in its free ionic forms (Fe^{2+} and Fe^{3+}), has the potential to catalyse the formation of ROS through Fenton and Haber-Weiss reactions. This can result in the development of and subsequent cellular damage.

The ICA assay entails incubating the extract with a known concentration of iron ions, (Fe^{2+}), followed by the addition of a chromogenic agent, ferrozine, which forms a coloured complex with the remaining unbound iron. The decrease of colour intensity, measured spectrophotometrically, is indicative of the amount of iron chelated by the test compound. Iron chelating activity was determined by mixing 50 µL of sample with 50 µL of 0.12 mM $FeSO_4$ and 50 µL of 0.6 mM ferrozine and incubation for 15 min in the dark. As mentioned above, a range of concentrations from the extract was tested from 1 mg/mL to 0.03125 mg/mL in a 96 well plate. The blank consisted of 50 µL of sample, 50 µL $FeSO_4$ and 50 µL of dH_2O. The absorbance at 562 nm was measured at 15 min after ferrozine addition (Cian et al., 2016).

3.5. Strains, media and growth conditions

The strains of *S. cerevisiae* utilized in this study are presented in Table 3. The cultures of strains BY4741, SNCA WT, SNCA 153T and *ssa2* were preserved on YPDA medium plates, the composition of which is listed in the supplementary material. The strains HTT 25Q and HTT 103Q were maintained in minimal medium, listed in supplementary material as well. Yeast cultures were incubated at 30 °C for 48 h and stored at 4 °C in YPDA plates with refreshment occurring every two weeks. With the purpose of conducting assays, a pre-inoculum of the strains BY4741, SNCA WT and SNCA A53T was prepared collecting a single colony from the stock culture plate and suspending it in YPD, as detailed in supplementary material. Pre-inoculum of strains HTT 25Q and HTT 103Q, was prepared in SCG (supplemented with amino acids) medium and incubated for 30 °C, 200 rpm. Growth was monitored by optical density at 600 nm (OD_{600}). The transformed strains SNCA WT, SNCA A53T, HTT 25Q and HTT 103Q were washed twice with PBS 1x at 25 °C and

centrifuged at 5000 g. Subsequently, the cells were suspended again, but in SCG medium with galactose, listed in supplementary material.

Table 3: *S. cerevisiae* strains used in this study.

Yeast and Mutant Strains	Name	Genotype	Origin	Institution
Wild type	BY4741	*MATa his3D1 leu2D0 met15D0 ura3D0*	-	-
Transformed from W303-1A	SNCA WT	W303-1A [*pRS306 GALSNCAWT::GFP*]	Paula Ludovico and Belém Marques	University of Minho
	SNCA A53T	W303-1A [*pRS306 GALSNCAA53T::GFP*]		
	HTT 25Q	W3031-A [*pRS425 GALHTT25Q::GFP*]	Robert Manson	University of Leicester
	HTT 103Q	W303-1A [*pRS425 GALHTT103Q::GFP*]		
Transformed from BY4741				
	ssb1	BY4741 *SSB1-GFP*	Gunter Kramer	University of Heidelberg

3.6. Flow Cytometry for Intracellular Oxidation

An overnight culture of *S. cerevisiae* strain BY4741 was diluted to OD_{600}=0.1 and cultured until OD_{600}=0.4 in YPD medium. The cells were washed as above mentioned with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) and diluted to $0D_{600}$=0.002 with the same buffer. Five hundred microliters were taken as a control for autofluorescence and stored at 4 °C until analysis. Propidium iodide (PI) and the fluorochrome 2',7'-dichlrofluorescein diacetate (H2DCFDA) were added to the final concentration of 3 ug/mL and 50 µM, respectively, and the cells were incubated for 1 h in the dark at 200 rpm and 30 °C. The cell suspension was divided into 500 µL aliquots and treated with algae extracts at 250, 500 or 1000 µg/mL and 5 mM H_2O_2. Positive and negative controls were treated with 5 mM H_2O_2 and DMSO (solvent of extracts in the same amount as the highest concentration of extract used in treatments), respectively. The cells were incubated for 20 min in the dark and ~20,000 events were analysed on an Epics XL flow cytometer Beckman Coulter. Controls of H2DCFDA and PI were also utilised to account for any overlap between the emission of H2DCFDA. The percentage of non-oxidized and viable cells was quantified through the CytExpert 2.5 software.

3.7. Assessment of anti-aggregation activity

The strains SNCA WT, SNCA A53T, HTT 25Q and HTT 103Q were incubated overnight in YPD medium at 30 °C 200 rpm. Growth was monitored by spectrophotometry at 600 nm. Cells were diluted to OD_{600}=0.4 and washed twice with PBS, at 20 °C and 5000 *g*. The cell culture was resuspended in SCG medium and divided into aliquots that were incubated with 1 mg/mL of PP, SV or ZT. The control cells were treated with DMSO. All strains were incubated at 30 °C and a rotation speed of 200 rpm during 6 h for strains SNCA WT and SNCA A53T and 18 h for strains HTT 25Q and HTT 103Q. All samples were analysed using Leica DM5000B fluorescence microscope. At least 200 cells per experiment condition were counted for the assessment of anti-aggregation activity. The control group and the corresponding experimental conditions were subjected to consistent settings for exposure, gain, and intensity.

The anti-aggregation activity was evaluated through the calculation of the percentage of cells without inclusions in the strains treated with extracts in comparison with their corresponding controls. The percentage of cells without inclusions was determined by dividing the number of cells lacking inclusions by the total number of cells, and subsequently multiplying by 100.

3.8. Expression of Chaperones

Pre inocula of strain *ssb1* were prepared in YPD medium and incubated overnight at 30 °C 200 rpm. Cells were diluted to OD_{600}= 0.1 and incubated under the sane conditions until OD_{600}=0.4. Expression of chaperones was analysed in *ssb1* strain treated with 1 mg/mL PP, SV or ZT. In this strain, GFP is replacing the gene that encodes Ssb1 involved in unfolded protein response (UPR). All of the conditions were submitted to 42 °C and observed in fluorescence microscope at time 0, 15 and 30 min to analyse the levels of expression. Prior to imaging, cells were centrifuged 2700 *g* for 1 min, washed and resuspended in PBS.

3.9. Statistical Analysis

All statistical analysis were performed using GraphPad Prism 8.0.1 software. To assess the IC_{50} of the extracts in the DPPH assay, linear regression analysis was performed for all extracts. Furthermore, to compare the viability of *S. cerevisiae* cells in the presence of the extract and H_2O_2, the percentage of cells without aggregates in *S. cerevisiae* and for flow cytometry assays, one-way ANOVA test was used. The results were expressed as mean ± standard error (SEM) from at least three independent assays. A statistically significant difference was defined as $p \leq 0.05$ and is indicated in the figures with * ($p \leq 0.05$), **($p < 0.01$), ***($p < 0.001$), ****($p < 0.0001$).

4. Results and Discussion

4.1. *Asparagopsis armata* and *Asparagopsis taxiformis* algae extracts showed scavenging against DPPH

The antioxidant capacity of the samples was initially evaluated using the DPPH assay, prior to their utilisation in experiments involving biological models. The initial evaluation was conducted due to the established association between oxidative stress and the development and progression of numerous neurodegenerative diseases. A colorimetric analysis can be employed to determine whether a compound exhibits antiradical activity and has the potential to provide neuroprotection. From the extracts tested, AA and AT exhibited IC_{50} values that can be considered as representing considerable activity (Figure 4).

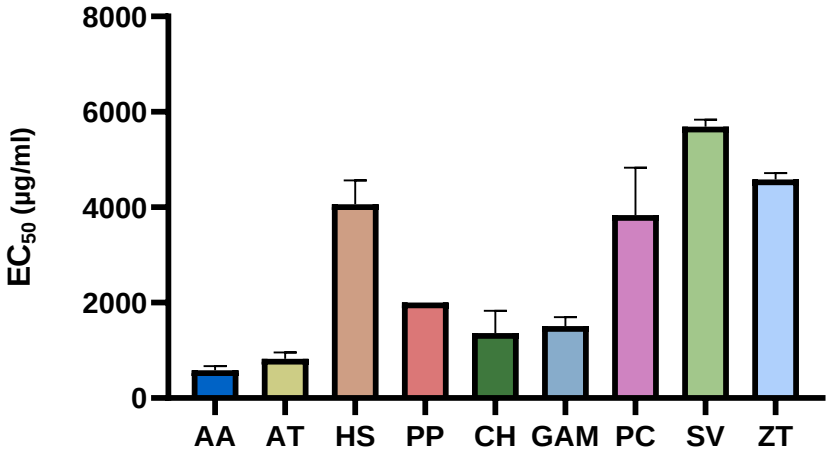


Figure 4 The scavenging DPPH activity represented as EC_{50} of macroalgae extracts *Asparagopsis armata* (AA*), Asparagopsis taxiformis* (AT), *Halopteris scoparia* (HS), *Padina pavonica* (PP), *Cystoseira humillis* (CH), *Gongolaria abis-marina* (GAM), *Pterocladiella capilacea* (PC), *Sargassum vulgare* (SV) and *Zonaria tournefortii* (ZT) with antioxidant potential, after 60 min of DPPH reaction. Results are presented as mean ± SEM of three independent replicates.

The results indicate that the reported EC_{50} values are influenced by a number of factors, including the type of solvent used, the location of harvesting and the prevailing environmental conditions (Stengel et al., 2011). Additionally, the stage of growth and the time of harvest also have an impact on the observed values. The selection of solvent has a significant impact on the extraction of secondary metabolites from algae. The polarities of different solvents vary which allows them to extract different classes of compounds. For instance, polar solvents such as methanol or ethanol

are more efficacious at extracting hydrophilic antioxidants, including phenolic compounds, whereas non-polar solvents like hexane are more effective for lipophilic compounds, including carotenoids. The location of harvesting has a considerable impact on the concentration and composition of secondary metabolites. Environmental factors, including temperature, light exposure, salinity and nutrient availability, can influence the metabolic pathways (Stengel et al., 2011). Stress conditions such as high salinity or nutrient deprivation, can induce the production of secondary metabolites as a defensive response, leading to elevated antioxidant activity (Stengel et al., 2011).

The results of the antioxidant activity assays conducted using the DPPH method demonstrated notable variability among the various extracts, which can be attributed to factors such as the solvent utilized and the environmental conditions prevailing during the harvesting process. In comparing the results of this study with those reported in the literature, it was found that the *Asparagopsis armata* (AA) extract exhibited higher antioxidant activity, with a lower EC_{50} value than that reported by Lee et al (2020). The EC_{50} was determined to be 261.44 µg/mL, which is lower to the value of 580.93 µg/mL obtained in our study. This discrepancy is presumably attributable to the utilisation of a more polar solvent in our extract, which is more efficacious in extracting hydrophilic compounds (Stengel et al., 2011). For *Asparagopsis taxiformis* (AT), our findings exhibited markedly elevated activity in comparison to those documented by (Ktari et al., 2022), potentially due to the employment of a more suitable solvent for extracting particular antioxidants. Conversely, extracts such as *Halopteris scoparia* (HS) and *Padina pavonica* (PP) exhibited lower antioxidant activity in the present study than that reported in the literature (Hadjkacem et al., 2023;(Kerzabi-Kanoun et al., 2021). Such discrepancies may be attributed to fluctuations in environmental circumstances during the harvesting process or discrepancies in the extraction methodology. The brown seaweed HS reported in Hadjkacem et al. was harvested from Tunisia in February and the solvent used was methanol, while our was harvested in Azores and the solvent used was ethanol. On the other hand, PP reported in Kerzarbi-Kanoun et al. was collected in the Northwest Coast of Algeria during May and the solvent used was methanol. With regard to *Cystoseira humillis* (CH), the reduced antioxidant activity observed in comparison to the findings of Belattmania et al (2016) may be attributed to differences in the growth stage or time of year when the samples were harvested. It is established that these factors exert an influence on the concentration of secondary metabolites (Stengel et al., 2011). Furthermore, the extracts of *Gongolaria abis marina* (GAM) and *Pterocladiella capillacea* (PC) exhibited higher EC_{50} values documented in other research studies (Rosa et al., 2023;Amorim et al., 2020). It is postulated that

these discrepancies can be explained by the variations in solvents used, as previous studies indicate that solvent polarity directly affects the efficiency of bioactive compound extraction. The antioxidant activity of *Sargassum vulgare* (SV) and *Zonaria tournefortii* (ZT) was previously found to be considerably lower, (Arguelles et al., 2019; Hamiche et al., 2018) a result that aligns with previous studies indicating that harvest season and environmental conditions are of significant influence on the antioxidant activity of these extracts (Stengel et al., 2011). Variables such as solvent type, environmental conditions and harvest location are of importance when evaluating the antioxidant activity of algae extracts, as they can significantly influence the observed differences.

4.2. FRAP assay reveals strong antioxidant activity in *Cystoseira humillis* and *Pterocladiella capilacea* algae extracts

The ferrous reducing antioxidant power (FRAP) was also performed prior to experiments in biological models. The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, coloured form in the presence of antioxidants. As depicted in Figure 5, overall, CH and PC displayed higher values of antioxidant activities when compared with the other extracts, AA, AT, GAM, HS, PP, SV and ZT.

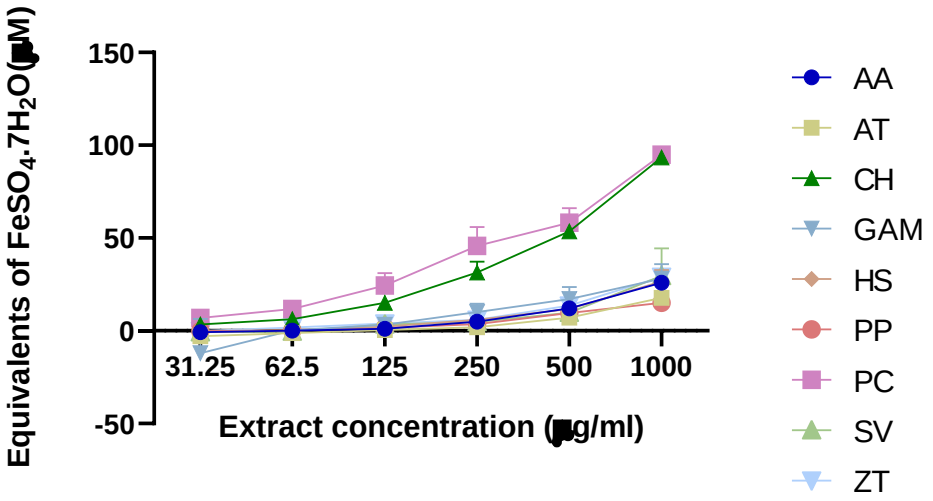


Figure 5 The antioxidant activity represented as Equivalents of $FeSO_4.7H_20$ (µM) of macroalgae extracts *Asparagopsis armata* (AA), *Asparagopsis taxiformis* (AT), *Halopteris scoparia* (HS), *Padina pavonica* (PP), *Cystoseira humillis* (CH), *Gongolaria abis-marina* (GAM), *Pterocladiella capilacea* (PC), *Sargassum vulgare* (SV) and *Zonaria tournefortii* (ZT) with higher antioxidant potential, after 30 min of FRAP reaction. Results are presented as mean ± SEM of three independent replicates.

When comparing with the literature, we found reported antioxidant activity, although the measurement units differ from our study. For example, Amorim et al., (2020) reported an EC_{50} value of 5.57 mg/mL for PC, while in our study the antioxidant potential was 94.97 equivalents of $FeSO_4.7H_2O$ (µM). Similarly, CH showed high antioxidant activity in aqueous extract of shade-dried samples, obtaining a value of 151.0 ± 18.4 Fe^{2+} Eq/g dried weight (Julião et al., 2021). In this present study, the obtained value was 93.34 equivalents of $FeSO_4.7H_2O$ (µM). In the case of the remaining macroalgae species examined in this study, no comparable values could be identified in the existing literature. Despite this, both PC and CH exhibited a consistently high antioxidant potential, thereby supporting the hypothesis that these macroalgae contain considerable quantities of antioxidant compounds. Given the intricate composition of algae extracts, which encompass a multitude of antioxidant components, it is inherently challenging to quantify each antioxidant individually. Nevertheless, the correlation between the FRAP values obtained in this study and those documented in the literature suggest a positive relationship, thereby further corroborating the elevated antioxidant activity of these extracts.

4.3. *Pterocladiella capillacea, Sargassum vulgare*, and *Zonaria tournefortii* extracts achieve low EC_{50} in ICA assay

The iron chelation capacity of compounds like algal extracts can be determined through the use of the ICA assay. Regulation of the activity of free iron itself is also very important, as a surplus of this metal has been described to catalyse formation of ROS via the Fenton reaction. This process can result in oxidative damage in biological systems. The extracts might interfere with this because of their antioxidant properties, in part due to their capacity of chelate iron. This assay works using the addition of ferrous ions Fe^{2+} to the extracts, the chelating agents in the extract bind these ions which makes them less available to complex with other reagents. This is usually determined using a chromogenic reagent, ferrozine, to form a coloured complex with Fe^{2+}. Decrease in colour intensity indicates iron-chelating ability of extract which may demonstrate antioxidant properties. Consequently, the use of chelating agents can prevent this by binding to free iron, thereby reducing its availability for the production of ROS.

Results shown in Figure 6 demonstrate that among all of the extracts tested, SV and ZT extracts were able to achieve low EC_{50} in this assay, which translates to high antioxidant activity. For purposes of comparison, a low EC_{50} value is indicative of high antioxidant activity, with EDTA

typically employed as a standard reference value. The extract PC showed a value of EC_{50} -74.4 µg/mL. This negative value is likely indicative of interference of non-specific interactions with the assay, such as turbidity, rather than the true iron chelating activity. The SV extract exhibited the second-highest antioxidant activity, with an EC_{50} value of 215.9 µg/mL while the ZT extract demonstrated a low EC_{50} value of 418.4 µg/mL. These values indicate a notable chelating potential in comparison to the EDTA reference. As long as we know, this is the first study on the chelating activity of these extracts. Furthermore, as far as we know, no previous reports in the literature have addressed the antioxidant properties of SV, ZT or PC in ICA assay. Therefore, our findings provide novel insights into the potential of these macroalgae as antioxidant agents.

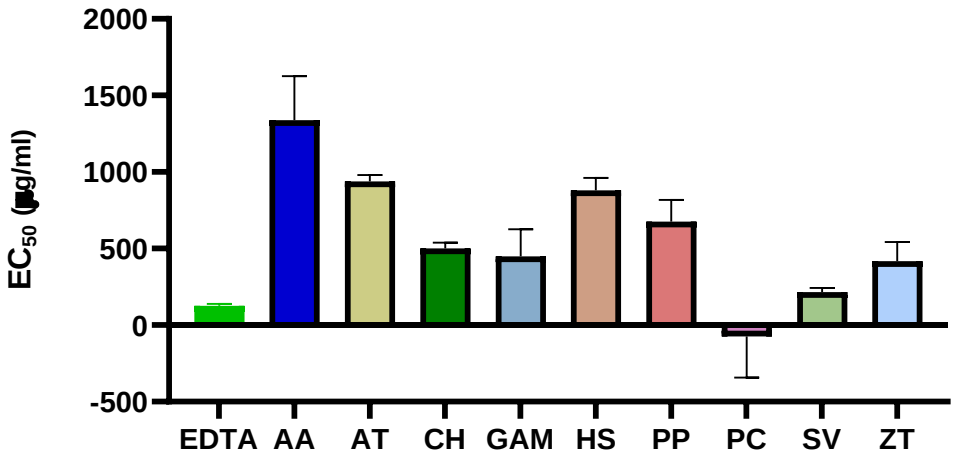


Figure 6 The ICA activity represented as EC_{50} of macroalgae extracts *Asparagopsis armata* (AA), *Asparagopsis taxiformis* (AT), *Halopteris scoparia* (HS), *Padina pavonica* (PP), *Cystoseira humillis* (CH), *Gongolaria abis-marina* (GAM), *Pterocladiella capilacea* (PC), *Sargassum vulgare* (SV) and *Zonaria tournefortii* (ZT) with higher antioxidant potential, after 15 min of reaction. Results are presented as Mean ± SEM of three independent replicates.

4.4. Extracts from *Padina pavonica, Sargassum vulgare* and *Zonaria tournefortii* decreased intracellular oxidation caused by H_2O_2

The *in vitro* assays, namely DPPH, FRAP and ICA, revealed that the extracts of CH, PC, SV and ZT exhibited the most favourable outcomes. Moreover, a review of the existing literature revealed evidence supporting antioxidant activity of these extracts. This provided the basis for proceeding with *in vivo* assays to further evaluate the antioxidant potential of CH, PC, SV and ZT.

The antioxidant protection provided by the algal extracts CH, PC, SV and ZT was investigated by evaluating the *S. cerevisiae* strain BY4741 after exposure to H_2O_2, an oxidative stress inducer. These experiments were carried out as complementary assays in the screening of the extracts to select the samples with a higher antioxidant activity.

PI allows to differentiate between viable and non-viable cells. Once inside the cell, H2DCFDA is subjected to enzymatic deacetylation by esterases, resulting in the formation of the non-fluorescent compound H2DCF, which remains trapped within the cell. Upon oxidation, H2DCF is converted into the fluorescent compound 2',7'-dichlorofluorescein (DCF), which enables the measurement of cellular oxidation levels. It is anticipated that the fluorescence increase, detected at 530 nm, is directly proportional to the concentration of ROS (Oliveira et al., 2020). Since DCF emits green fluorescence in the presence of H_2O_2 independently of whether the cells are alive or dead, PI was also employed to assess cell viability, ensuring that the stress applied was not excessive enough to kill the cells. This allowed us to analyse only the viable cells, which are the ones susceptible to protection by the extract.

The CH extract demonstrated antioxidant activity at the intracellular level at concentrations of 250, 500 and 1000 µg/mL (Figure 7A; $p<0.0001$). At the concentration of 1000 µg/mL, the extract exhibited great observable activity (Figure 7B). This was evidenced by a population of approximately 10 % of cells H2DCFDA +.The PP extract demonstrated considerable antioxidant activity at concentrations of 250, 500 and 1000 µg/mL, with greater cell population exhibiting positive signal to H2DCFDA (Figure 7D; $p<0.0001$). This finding is consistent with the observations illustrated by the blue line in Figure 7C, where a deviation of the line peaks is observed. With regard to the SV extract, it demonstrated considerable antioxidant activity across the entire range of concentrations tested (Figures E and F). These results indicate that CH, PP, SV and ZT extracts are able to prevent oxidative stress in viable cells of BY4741 *S. cerevisiae* strain. Nevertheless, the ZT extract was capable of maintaining a population of living and protected oxidative stress ($p<0.001$).

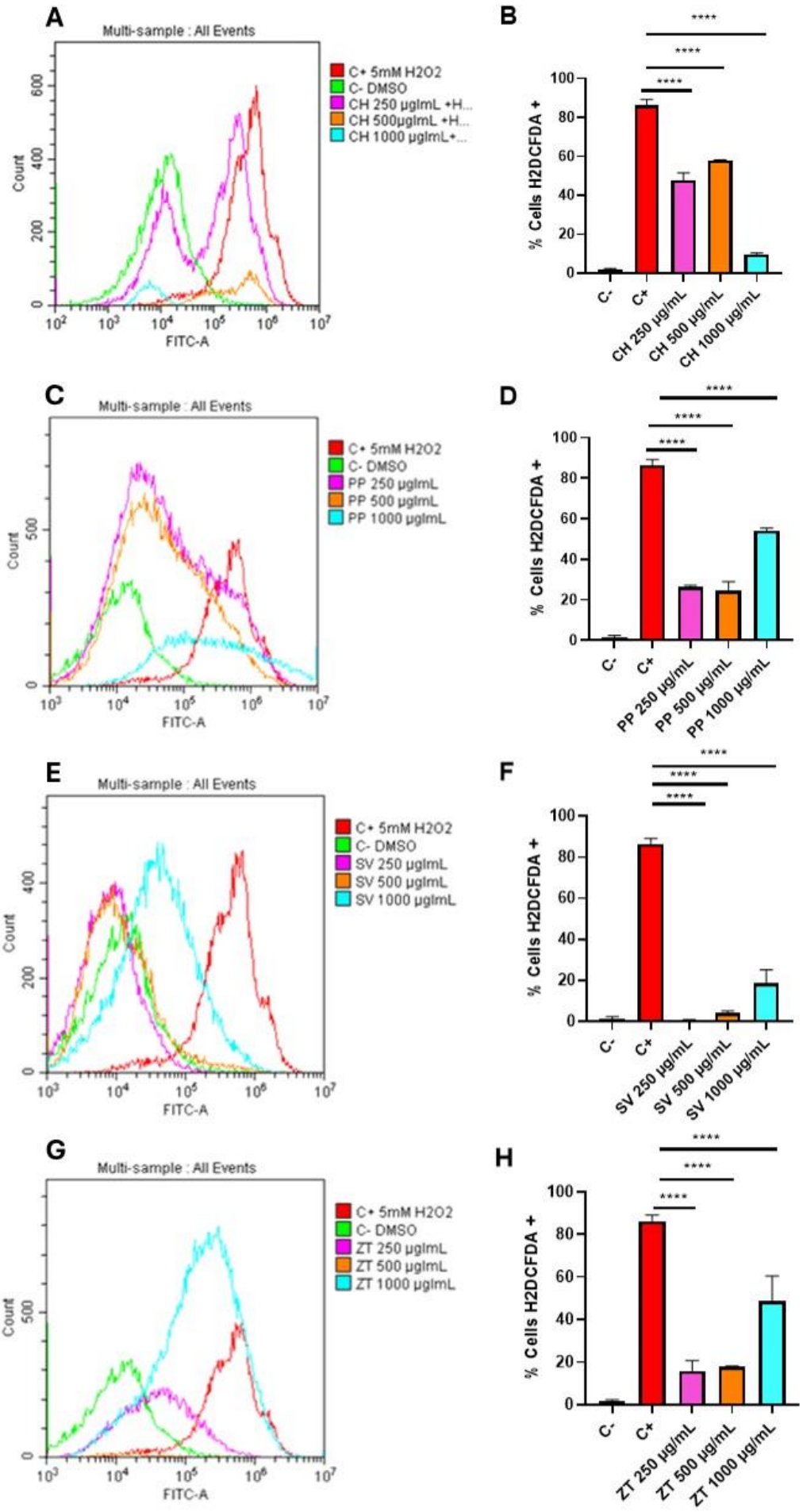

A
Multi-sample : All Events
C+ 5mM H2O2
C- DMSO
CH 250 µg/mL +H...
CH 500µg/mL +H...
CH 1000 µg/mL+...
Count
FITC-A
B
% Cells H2DCFDA +
C-
C+
CH 250 µg/mL
CH 500 µg/mL
CH 1000 µg/mL
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C
Multi-sample : All Events
C+ 5mM H2O2
C- DMSO
PP 250 µg/mL
PP 500 µg/mL
PP 1000 µg/mL
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PP 250 µg/mL
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C- DMSO
SV 250 µg/mL
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C- DMSO
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ZT 500 µg/mL
ZT 1000 µg/mL
Count
FITC-A
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% Cells H2DCFDA +
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C+
ZT 250 µg/mL
ZT 500 µg/mL
ZT 1000 µg/mL
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Figure 7 *Cystoseira humillis* (CH; A and B), *Padina pavonica* (PP; C and D), *Sargassum vulgare* (SV; E and F) and *Zonaria tournefortii* (ZT; Gand H) extracts lowers intracellular oxidation induced by H_2O_2 in *Saccharomyces cerevisiae* strain BY4741. Cells from an exponentially growing culture were treated with 250, 500 or 1000 μg/mL of each extract and 5 mM H_2O_2 (pink line, orange line and blue lines, respectively; A, C, E and G), incubated for 20 min at 30 °C, 200 rpm, labelled with 2',7'-dichlorofluorescein diacetate (50 μM; H2DCFDA) and propidium iodide (3 μg/ml) in the dark, at 30 °C for 1h and the fluorescence was measured by flow cytometry. Negative controls were prepared with cells treated only with DMSO (C- DMSO, green lines; A, C, E and G) and positive controls were prepared with cells treated only with 5 mM H_2O_2 (C+ 5 mM H_2O_2, red lines; A, C, E and G). All histograms (A, C, E and G) were made with data from PI- negative cells and bar charts were prepared with data from H2DCFDA+ negative cells. Results shown in A, C, E and G are representative of at least three independent experiments. Results represented in B, D, F, and H are presented as mean ±SEM. (Standard Error of the Mean), and statistical analysis was performed using a one-way ANOVA, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

The results of this *in vivo* assay are in accordance with those obtained in the DPPH, ICA and FRAP assays. The extracts have been observed to demonstrate the capacity to prevent oxidative stress in through a range of mechanisms. In the DPPH assay, AA and AT extracts demonstrated the capacity to act as radical scavengers, neutralising free radicals that could otherwise cause cellular damage. In the FRAP assay, PC and CH extracts demonstrated their reducing power by donating electrons to reduce ferric FE^{3+} to ferrous Fe^{2+}, reflecting their antioxidant capacity. Finally, in the ICA assay, the SV and ZT extracts exhibited metal-chelating activity, which prevents the formation of ROS by limiting the availability of metal ions that catalyse oxidative reactions. Collectively, these mechanisms contribute to the extracts ability to mitigate oxidative stress, protection against cellular damage.

A study conducted by Fujiwara et al. (2007), demonstrated that *Uncaria rhynchophylla* contains various alkaloids, such as corynoxeine and rhynchophylline, which are believed to have neuroprotective properties, including antioxidant activity that may help reduce oxidative stress in neurons. By utilizing flow cytometry to quantify the antioxidant activity, researchers could provide a clearer understanding of how *U. rhynchophylla* not only inhibits amyloid aggregation but also mitigates oxidative stress, potentially offering a dual therapeutic approach for AD.

4.5. *Padina pavonica, Sargassum vulgare* and *Zonaria tournefortii* treatment increased the number of cells without α-synuclein inclusions

A literature search was conducted to identify algae with neuroprotective activity among those tested in antioxidant assays. Following a review of the relevant literature, the decision was taken to proceed with the extracts of PP, SV and ZT. It has been reported that PP has neuroprotective potential and bioactive metabolites, with pharmacological insights and therapeutic advances (Hannan et al., 2020). Additionally, it has been demonstrated to possess neuroprotective potential in a human cellular stress model (Silva et al., 2020). SV has been shown to possess antioxidant activity in rats, as well as positive results from phytochemical and pharmacological screening (Shreadah et al., 2018; Abdel-Alim et al., 2023). While there is no direct evidence linking ZT to neuroprotective activity, studies have reported its health and metabolic benefits (Cardoso et al., 2022).

To assess the antiaggregant capacity of PP, SV and ZT in proteins associated with PD and HD, genetically modified yeast strains were used. These yeast strains were designed to produce human alpha-synuclein or huntingtin proteins fused to GFP, allowing to observe aggregates of these proteins under the fluorescence microscope as bright green spots. The GAL promoter, which is activated when galactose is the sole carbon and energy source, transcriptionally regulates the expression of synuclein and huntingtin fused to GFP. Therefore, in order to induce the expression of synuclein of huntingtin proteins fused to GFP, we cultured the cells in a medium in which the carbon and energy source was galactose. This allowed us to test whether the extracts tested were able to prevent the formation of synuclein aggregates in the cells. The strain SNCA A53T is a genetically engineered strain carrying the SNCA gene with this specific mutation. The mutation A53T refers to the replacement of alanine by threonine at residue 53 in α-synuclein. The A53T mutations is specifically associated with familial forms of PD and known to promote intracerebral deposition of cytotoxic α-synuclein-oligomers.

The culture treated with DMSO, the solvent of extracts, is shown in Figure 8A, together with the cultures treated using PP (Figure 8B), SV (Figure 8C) and ZT (Figure 8D) for 6 h after induction of the SNCA A53T strain in a medium containing galactose as the carbon and energy source. Subsequently, the percentage of cells without protein inclusions was determined in accordance with the methodology outlined by (Popova et al., 2021). The images demonstrate that the fluorescence intensity and the number of cells without fluorescent inclusions were significantly

greater in the treatments with PP, SV and ZT (Figures 8B, 8C and 8D) compared to the DMSO control (Figure 8A). The differences between the treatments were statistically significant, with p-values indicating these effects ($p<0.0001$). The results of the quantitative analysis revealed notable discrepancies between the SNCA A53T strain treated with the PP, SV and ZT extracts and the DMSO control group (Figure 8E). It can be concluded that the addition of 1 mg/mL of PP, SV or ZT extracts resulted in a notable reduction in the number of cells without protein aggregates present in the SNCA A53T strain. These findings suggest that these compounds may have the capacity to effectively minimise α-synuclein aggregation.

A simultaneous cell viability assay was conducted to assess the toxicity of the extracts to the cells. The results demonstrated that the viability of the cells treated with the extracts was comparable to that of the control cells, indicating that the extracts were not toxic and that the reduction in α-synuclein aggregation was not attributable to cell toxicity (Figure 8F).

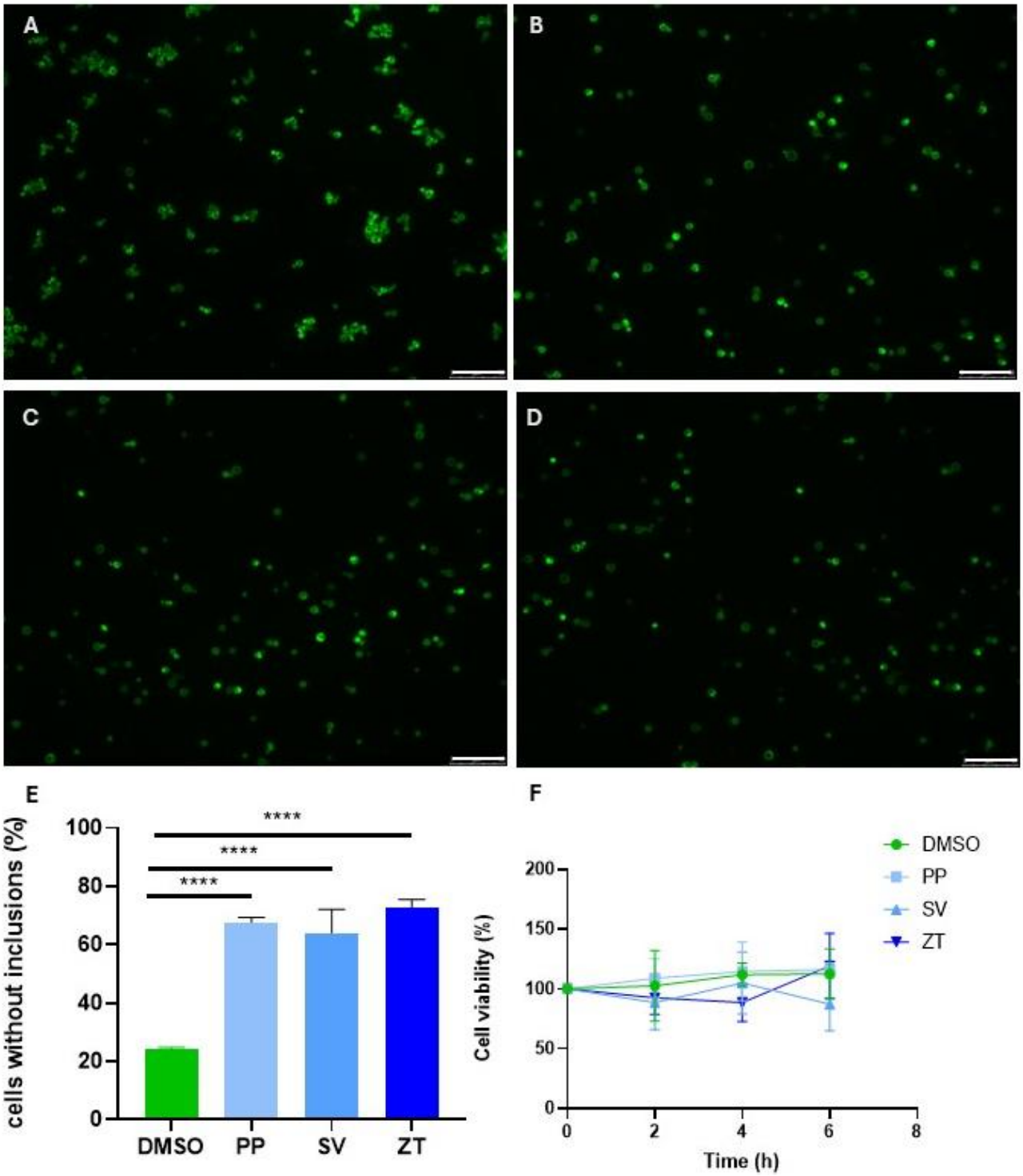


Figure 8 Treatment with *Padina pavonica* (PP; B*), Sargassum vulgare* (SV; C) and *Zonaria tournefortii* (ZT; D) at 1 mg/mL reduces α-synuclein A53T aggregation in *Saccharomyces cerevisiae* SNCA A53T strain. Cells were incubated in SCG medium to induce α-synuclein A53T fused to GFP expression in the presence of DMSO control (A). All the extracts PP (B), SV (C) and ZT (D) at 1 mg/mL, after 6 h of incubation, were observed in fluorescence microscopy for the presence of fluorescent green dots. Cells without intracellular fluorescent spots were counted and the percentage was calculated and plotted (E). Scale bar represents 25 µm. Viability along time was assessed as percentage of colony-forming units assuming o min as 100% viability (F). Results are present as Mean ± SEM of three independent replicates and statistical analysis was performed by one-way ANOVA.

The strain SNCA WT expresses the wild type form of the SNCA gene, encoding normal, non-mutated α-synuclein. This strain was used to test whether the extract inhibits the aggregation of wild type α-synuclein. We also tested a mutant form, A53T, which is associated with disease manifestation. The objective of this study is to examine the aggregation behaviour of the wild type α-synuclein in response to the extracts, with a subsequent comparison to the mutant form, which is linked to more severe neurodegenerative effects. The same experiment was done with strain SNCA WT, expressing αSynWT-GFP, the expected results were obtained in the control treatment with DMSO (Figure 9A). Significant differences were found in the PP (Figure 9B), SV (Figure 9C) and ZT (Figure 9D) extracts ($p<0.001$). Both strains SNCA A53T and SNCA WT showed similar percentage of cells without protein inclusions (Figure 8E and 9E). Significant differences were observed when the extracts PP, SV and ZT were used ($p<0.001$). As in the case of the SNCA A53T strain, all extracts regarding SNCA WT strain showed a viability assessment profile similar to that of the control. This suggests that these extracts are not toxic to these cells. These results indicate that α-synuclein aggregates are indeed reduced by these compounds and that this reduction is not caused by cellular toxicity or loss in viability (Figure 9F).

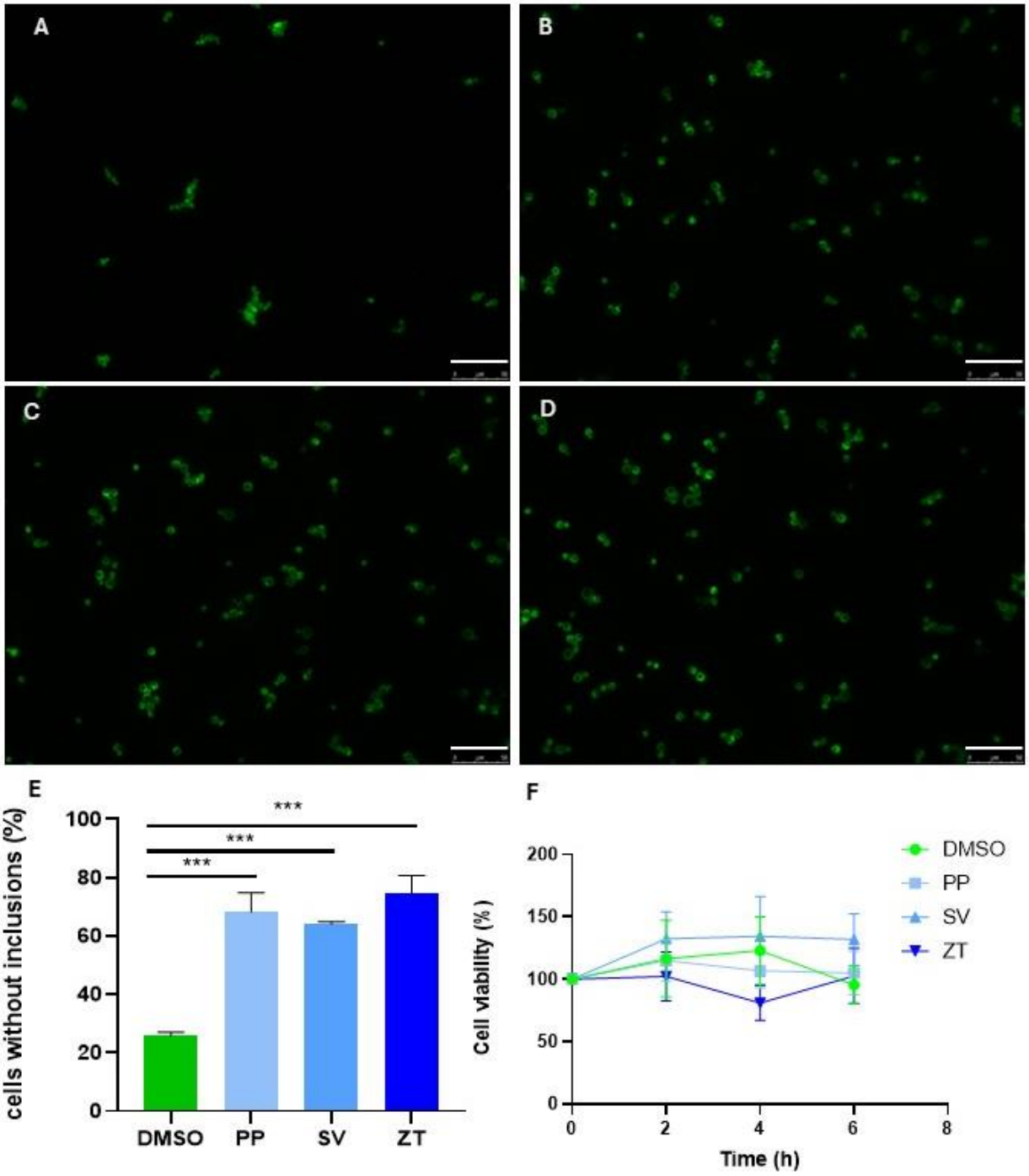


Figure 9 Treatment with *Padina pavonica* (PP; B), *Sargassum vulgare* (SV; C) and *Zonaria tournefortii* (ZT; D) at 1 mg/mL reduces αsyn-GFP aggregation in *Saccharomyces cerevisiae* SNCA WT strain. Cells were incubated in SCG medium to induce αsynuclein fused to GFP expression in the presence of DMSO control (A). All the extracts PP (B), SV (C) and ZT (D) at 1 mg/mL, after 6 h of incubation, were observed in fluorescence microscopy for the presence of fluorescent green dots. Cells without intracellular fluorescent spots were counted and the percentage was calculated and plotted (E). Scale bar represents 25 µm. Viability along time was assessed as percentage of colony-forming units assuming o min as 100% viability (F). Results are present as Mean ± SEM of three independent replicates and statistical analysis was performed by one-way ANOVA.

Previous research conducted by Popova et al. (2021) revealed a comparable prevalence of α-synuclein aggregates in the control treatment. This result is in accordance with our findings. The results can be explained by the induction of Hsp70 activity. Heat shock proteins (HSPs) are a family of molecular chaperones that are induced by stress and are responsible for protecting the cells against protein misfolding (Putcha et al., 2010). They detect exposed hydrophobic areas on the surface of misfolded proteins and help to refold or degrade them. The role of Hsp70 and Hsp90 in regulating α-synuclein protein aggregates in PD has been elucidated through the investigation of strategies designed to act against protein aggregation, as evidenced by several studies in this field. In the case of neurodegenerative diseases, molecular chaperones Hsp70 and Hsp90 are able to interact with α-synuclein as well modulate its folding, stabilization or degradation (Daturpalli et al., 2013). For instance, studies have shown that using geldanamycin pretreatment in neuroglioma cells resulted in approximately a 50% reduction in α-synuclein aggregation, underscoring the significance of Hsp70 in early intervention and protein folding regulation in PD (Mclean et al., 2004). The accumulation of α-synuclein resulting from aggregation is a notable characteristic in PD. Hsp90 prevents α-synuclein degradation through stabilization and Hsp70 promotes its breakdown in a ubiquitin-proteasome-dependent manner. A study suggests that inhibition of Hsp90 or activation of Hsp70 may mediate the attenuation of α-synuclein toxicity. This balance is important for the protein quality control and thus could open a new therapeutical for PD by targeting these pathways (Pratt et al., 2014).

4.6. *Padina pavonica, Sargassum vulgare* and *Zonaria tournefortii* treatment increased the number of cells without huntingtin inclusions

The genetic construction of HTT fused with GFP was also regulated by the GAL promoter, similar to the strains SNCA A53T and SNCA WT mutations, with the exception of the induction and incubation time. The HTT 25Q strain is characterised by the presence of a polyglutamine (polyQ) tract comprising 25 consecutive glutamine (Q) residues in the huntingtin protein. This strain is distinguished by a relatively normal and stable polyQ length in the human huntingtin protein, consequently making it a commonly utilised control or even reference strain in studies aimed at elucidating the aetiology of HD. The HTT103Q strain, by contrast, features a huntingtin protein with an extended polyQ tract of 103 consecutive glutamine residues. This strain has been genetically

modified to replicate the specific mutation responsible for HD in humans, where an aberrant expansion of the polyQ tract ultimately gives rise to the disease's hallmark pathological consequences.

In these assays, we induced for 16 h the expression of HTT103Q-GFP (Figure 10) and HTT25Q-GFP in the *S. cerevisiae* HTT 103Q and HTT 25Q strains, respectively. After the administration of DMSO serving as the control (Figure 10A), 1 mg/mL PP (Figure 10B), 1 mg/mL SV (Figure 10C) and 1 mg/mL SV (Figure 10D), the cells were submitted to a 16 h incubation period. After this time, the cells were observed in a fluorescence microscope and images were captured, in order to calculate the percentage of cells without inclusions.

The HTT 25Q strain is responsible for expressing the HTT25Q-GFP protein, associated with healthy patients in HD. Again, significant differences were observed in the control group, treated with DMSO (Figure 11A), in comparison with PP (Figure 11B), SV (Figure 11C), and ZT (Figure 11D) extracts, at the concentration of 1 mg/mL (p<0.0001). In fact, the presence of these extracts in cells expressing HTT25Q-GFP prevents the formation of protein aggregates significantly (Figure 11E). A viability assay was also conducted, and the results shown in Figure 11F, indicate that PP, SV and ZT extracts are not toxic to the HTT 25Q strain. The results of our analysis revealed notable distinctions in the HTT 103Q strain when exposed to PP, SV and ZT extracts, in comparison to the control group (Figure 10E; p<0.0001). In fact, all the cells treated with extracts PP, SV and ZT showed a higher percentage of cells ranging from 60% to 70% of cells without protein inclusions, leading to antiaggregating properties (Figure 10E). This indicates that PP, SV and ZT extracts have potential acting as antiaggregant agents in HD. Simultaneously, a viability assay was conducted in order to evaluate the toxicity of PP, SV and ZT extracts on the cells. The results show that after 16 h of incubation, the cells treated with the PP, SV and ZT extracts are similar to the control group (DMSO), shown in Figure 10F. This is a strong indicator that our extracts are not toxic to the Saccharomyces cerevisiae HTT 103Q strain.

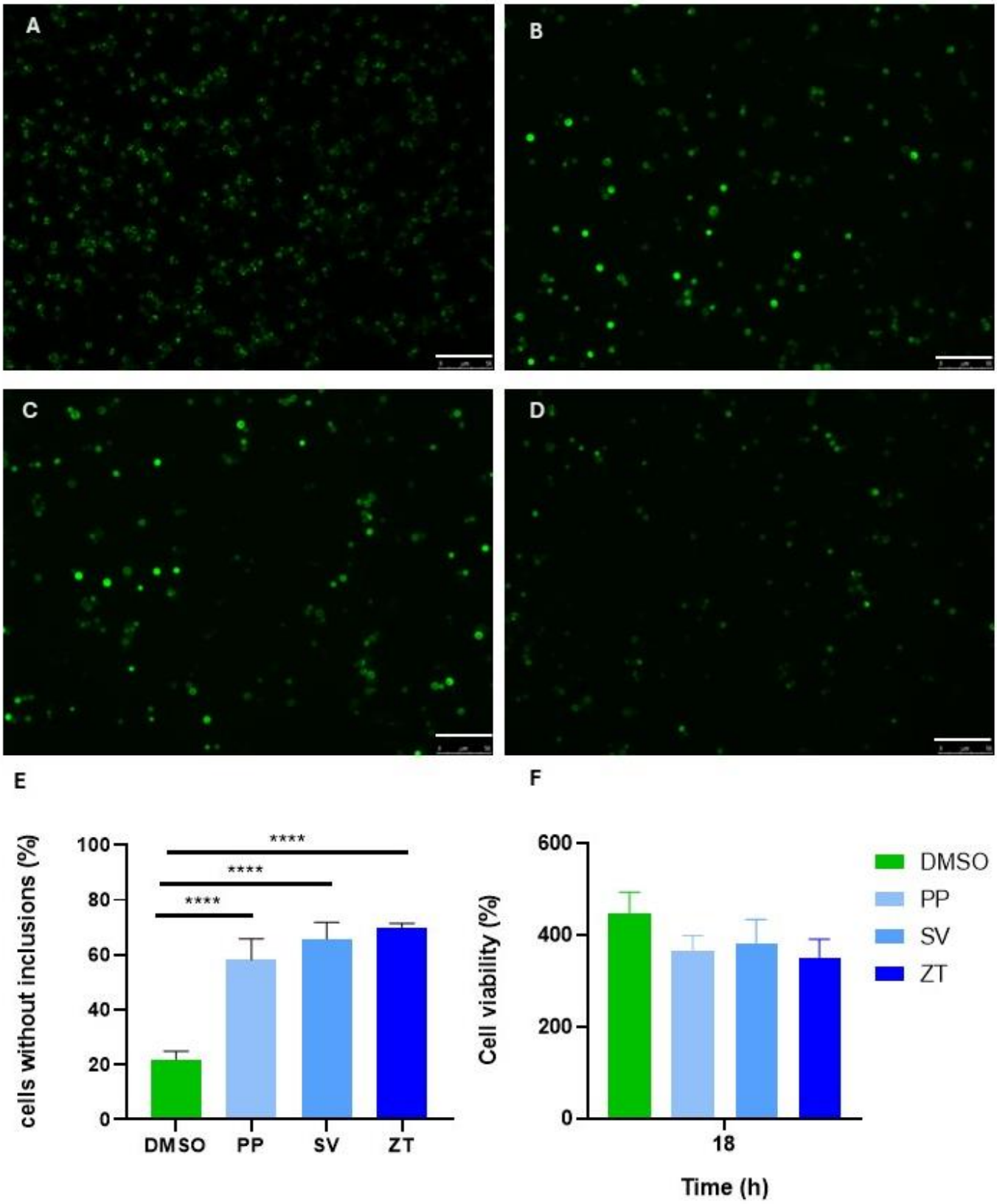


Figure 10 Treatment with *Padina pavonica* (PP; B), *Sargassum vulgare* (SV; C) and *Zonaria tournefortii* (ZT; D) at 1 mg/mL reduces HTT103Q-GFP aggregation in *Saccharomyces cerevisiae* HTT 103Q strain. Cells were incubated in SCG medium to induce HTT103Q fused to GFP expression in the presence of DMSO control (A). All the extracts PP (B), SV (C) and ZT (D) at 1 mg/mL, after 6 h of incubation, were observed in fluorescence microscopy for the presence of fluorescent green dots. Cells without intracellular fluorescent spots were counted and the percentage was calculated and plotted (E). Scale bar represents 25 µm. Viability along time was assessed as percentage of colony-forming units assuming o min as 100% viability (F). Results are present as Mean ± SEM of three independent replicates and statistical analysis was performed by one-way ANOVA.

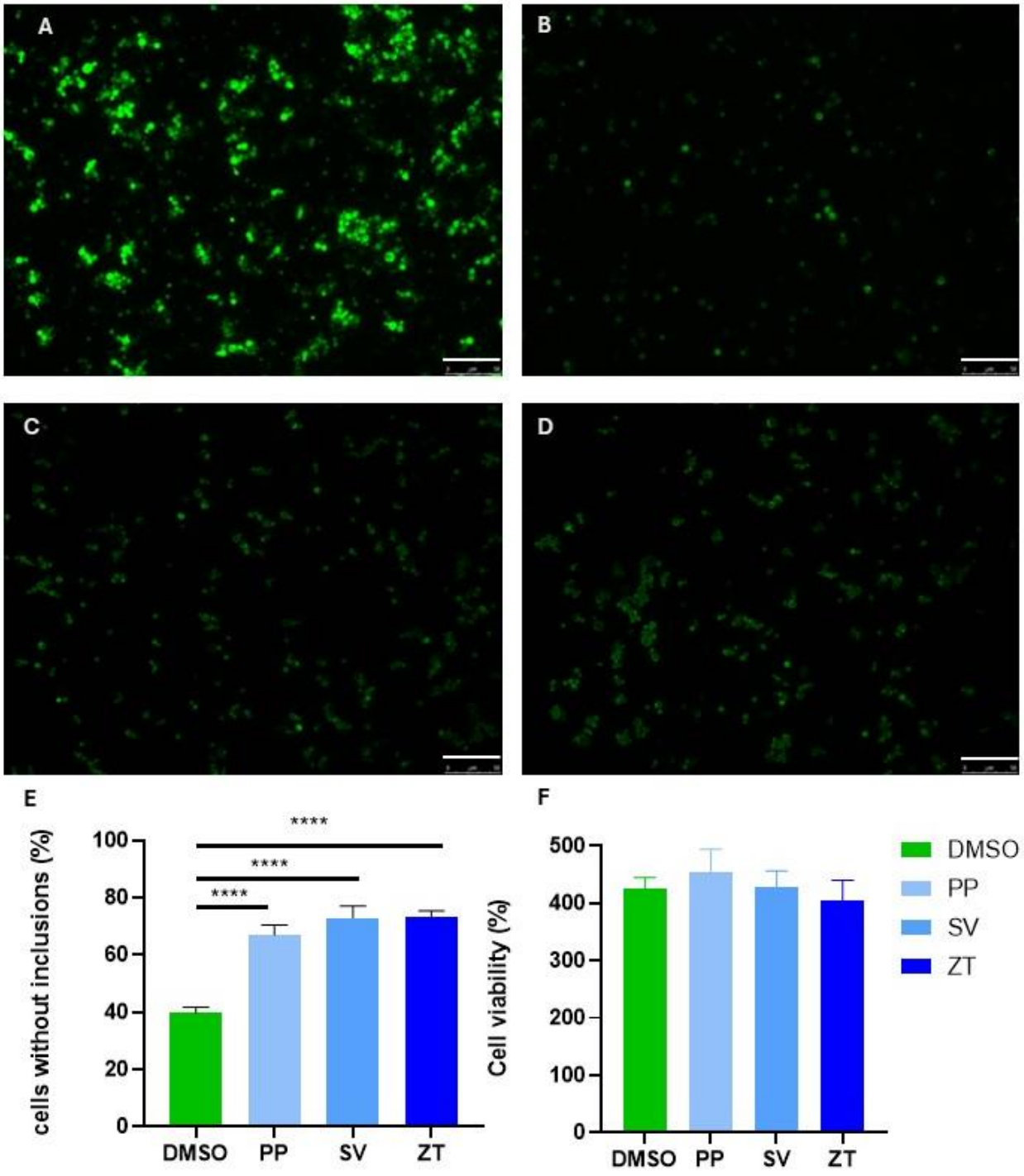


Figure 11 Treatment with *Padina pavonica* (PP; B), *Sargassum vulgare* (SV; C) and *Zonaria tournefortii* (ZT; D) at 1 mg/mL reduces HTT25Q-GFP aggregation in *Saccharomyces cerevisiae* HTT 25Q strain. Cells were incubated in SCG medium to induce HTT25Q fused to GFP expression in the presence of DMSO control (A). All the extracts PP (B), SV (C) and ZT (D) at 1 mg/mL, after 6 h of incubation, were observed in fluorescence microscopy for the presence of fluorescent green dots. Cells without intracellular fluorescent spots were counted and the percentage was calculated and plotted (E). Scale bar represents 25 µm. Viability along time was assessed as percentage of colony-forming

units assuming o min as 100% viability (F). Results are present as Mean ± SEM of three independent replicates and statistical analysis was performed by one-way ANOVA.

These yeast models are valuable tools for the investigation of the underlying processes that drive HD, particularly in elucidating the mechanisms by which mutant huntingtin protein causes cell dysfunction. The expression of these HTT variants in yeast enables researchers to observe the effects of protein aggregation, toxicity, and the cellular pathways involved in HD progression (Mason & Giorgini, 2011). These yeast models provide a more straightforward system for examining these processes, thus enabling scientists to conduct high-throughput screens in order to identify potential therapeutic targets and compounds (Hofer et al., 2018). The importance of these strains crucial to elucidate the mechanisms underlying mutant HTT- induced cytotoxicity. This includes the identification of the autophagy and proteasome systems as key players in the pathway of protein aggregate clearance. Furthermore, this highlights the importance of autophagy and chaperone-mediated pathways in maintaining protein homeostasis and mitigating the toxic effects of mutant HTT (Hofer et al., 2018). In fact, findings show that chaperones, particularly Hsp104, are able to operate with the objective of reducing both the formation of aggregates and cell death (Carmichael et al., 2000). *In vivo* studies realized in rat models show that the presence of Hsp104 and Hsp27 alter the distribution and size of HTT inclusions, increasing the number of smaller, non-nuclear inclusions while reducing larger nuclear aggregates (Perrin et al., 2007). These results demonstrate that Hsp104 and Hsp27 have protective effects in mammalian models of HD, suggesting their potential as therapeutic targets for treatment (Perrin et al., 2007).

4.7. Activation of chaperone expression with *Padina pavonica, Sargassum vulgare,* and *Zonaria tournefortii* treatment

To investigate the mechanisms responsible for the neuroprotective effects of PP, SV and ZT extracts, we used the mutant strain of *S. cerevisiae ssb1*, in which GFP was used to replace the coding sequence of *SSB1,* involved in the unfolded protein response (UPR), under the control of the native promoter SSB1. This reporter system allowed us to evaluate the extent to which the expression of this gene is induced at the transcriptional level. The expression of chaperones is low under normal conditions, so if the mechanism of action of the extracts PP, SV and ZT involves activating chaperones, we will be able to see GFP overexpressed, with fluorescence proportional to its expression.

In particular, *SSB1* gene plays a pivotal role in the folding and quality control of proteins, particularly during the translation phase, responsible for encoding Ssb1, a member of the Hsp70 family (Chernoff et al., 1999). It interacts with nascent polypeptides emerging from the ribosome, thereby facilitating the correct folding of these newly synthesized proteins. Ssb1 plays an essential role in maintaining proteostasis, preventing protein aggregation and assisting in the correct folding of proteins within the cell (Ohba, 1997).

An assay was conducted to explore the possibility of PP, SV and ZT exerting their anti-aggregant activity through the overexpression of the *SSB1* gene. As control, cells were exposed to DMSO (Figure 12A), and treatment with PP (Figure 12B), SV (Figure 12C) and ZT (Figure 12D) at 1mg/mL for 30 min. The analysis by fluorescence microscopy did not show any differences in fluorescence intensity (Figure 12).

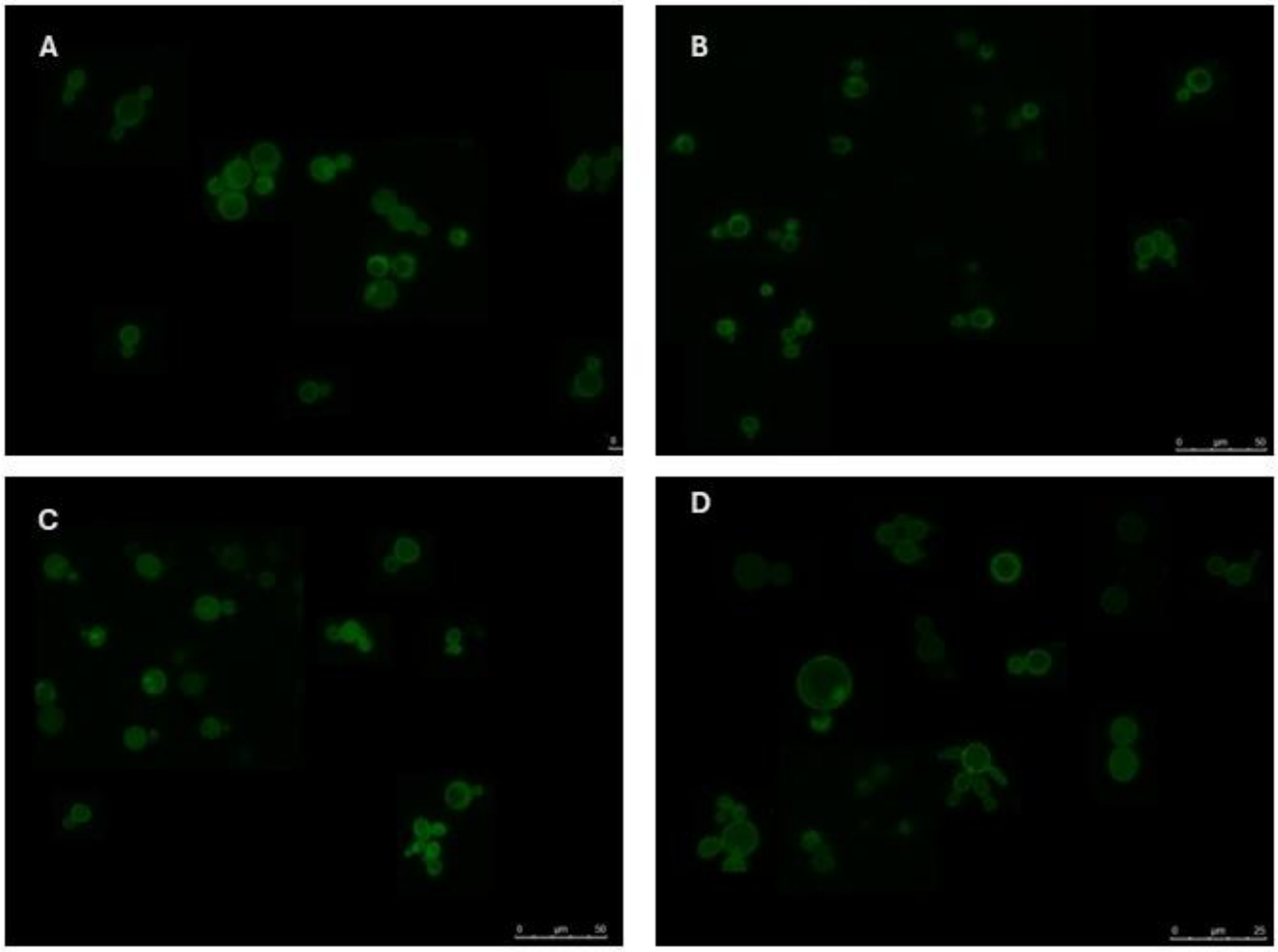


Figure 12 Influence of extracts from *Padina pavonica* (PP), *Sargassum vulgare (*SV) *and Zonaria tournefortii* (ZT) on the activation of the SSB1 promoter (A-D) assessed with the reporter gene GFP. GFP fluorescence images were acquired in *Saccharomyces cerevisiae* treated with DMSO (A), 1 mg/mL PP (B), 1 mg/mL SV (C) and 1 mg/mL ZT (D) for 30 min. The results are representative of three independent replicates.

In yeast cells, Ssb1 plays a pivotal role in protein folding and quality control during translation, the process by which cells synthesize proteins (Nelson et al., 1992). The results indicate that the extracts do not affect the expression of Ssb1. However, other chaperones, such as Ssa2 and Hsp104, may still be involved in the observed anti-aggregation effects. Ssa2, a member of the Hsp70 family, plays an important role in protein folding, preventing the formation of aggregates, and facilitating the refolding of misfolded proteins. Hsp104, on the other hand, plays a pivotal role in disaggregating and refolding large protein aggregates, working in conjunction with other chaperones such as Hsp70. Given that these chaperones also contribute to proteostasis, it cannot be excluded that the extracts induce the expression of activity of Ssa2, Hsp104, or other related chaperones. Further investigation is required to ascertain the veracity of the hypothesis.

Another potential mechanism by which the extracts may exert their anti-aggregation effects is through the activation of cellular protein degradation systems, such as the proteasome. The proteasome is important for maintaining proteostasis by facilitating the degradation of misfolded, damaged or aggregated proteins that could accumulate and disrupt cellular function. With regard to neurodegenerative disorders, there is compelling evidence that proteasomal dysfunction is linked to the pathological accumulation of protein aggregates (McKinnon & Tabrizi, 2014). By enhancing proteasome function, the extracts could promote the clearance of these toxic aggregates, thereby preventing further cellular damage (Davidson & Pickering, 2023). In addition to the proteasome, other degradation pathways such as autophagy, may also be involved in the clearance of protein aggregates (Kiriyama & Nochi, 2015). Consequently, the observed protective effects of the extracts may involve not only the inhibition of protein aggregation but also the activation of pathways responsible for the degradation of already formed aggregates.

5. Conclusion and Future Perspectives

The present study highlights the potential of macroalgae to develop an innovative treatment strategy against neurodegenerative diseases. Neurodegenerative diseases are progressive deteriorating disorders of the structure and function of the nervous system (NS), including moderate-to-severe impairment in cognition & activity of daily living; examples include somatic dysfunction & unfamiliarity with PD, AD or HD. Although there have been multiple research efforts, to date the current therapeutic strategies are symptomatic rather than targeting underlying causes. The discovery of natural compounds, especially from marine resources offers a promising alternative. Marine organisms exhibit a remarkable biological diversity and produce a diverse range of secondary metabolites which are often shaped by the extreme environments in which they live. These distinctive conditions can result in the synthesis of highly active compounds in terms of protection against stress, which can have neuroprotective properties, making algae extracts a promising candidate for the treatment and management of disease progression. Nevertheless, very little exploration has been done, which make these compounds promising for their neuroprotective actions, indicating a need for further research into their neuroprotective actions.

The present study reports that macroalgae-derived extracts suggest neuroprotective effect by assessing the antioxidant and anti-aggregant activity in *S. cerevisiae*. Of particular interest is the antiradical action attributed to these extracts, this activity is implicated in protecting cells from oxidative stress, a major factor contributing to the onset of neurodegenerative disease. The yeast model of PD expressing human α-synuclein, incubated with algae extracts displayed anti-aggregation activity which reduced protein misfolding, a feature common to ND. This anti-aggregation effect could have therapeutic implications, as it suggests that effective compounds may not only prevent the onset of such diseases by protecting against protein misfolding, but also potentially reverse disease progression by disrupting existing aggregates. By their means it was noted that protein aggregation - long suspected to be one of the molecular mechanisms that leads to neurodegeneration in diseases like PD and HD - is inhibited.

Although these outcomes are encouraging, they represent a limitation to the present investigation because a very detailed chemical assessment of the macroalgae extracts has not been performed. While the nature of the bioactive components responsible for PP, SV and ZT extract-induced neuroprotection is not yet identified, this is necessary to link these extracts usage and the curative effect observed in brain injury with the corresponding functions. However, many

studies have already been carried out regarding various bioactive compounds present in brown macroalgae such as fucoxanthin, polyunsaturated fatty acids (PUFAs), phlorotannins etc., and their important biological effects (Belattmania et al., 2016; Abdul et al., 2016; Chini Zittelli et al., 2023).

Further studies should aim at the thorough chemical characterization of the PP, SV and ZT extracts in order to identify which is/are molecule(s) that likely contribute to promote neuroprotection. Furthermore, after being isolated and purified, these compounds could be tested in mammalian models of neurodegeneration. Furthermore, bioavailability optimization strategies for these compounds should be explored. As an example, bioactive compounds could be employed for microencapsulation techniques, which protect this compound from degradation and ensure a superior absorption rate in the body(Munin and Edwards-Lévy, 2011; Vieira, Pastrana and Fuciños, 2020). This can open new horizons to develop dietary supplements or functional foods in the prevention or treatment of neurodegenerative diseases. Considering the rising demand for natural, dietary-based methods to improve health conditions such as chronic diseases, the exploitation of these bioactive compounds from macroalgae in nutraceutical applications seems interesting.

In addition to the Alzheimer's facet of this study, there are bigger questions here about how we can salvage marine resources in a way that is sustainable and ensures their long-term survival. Seaweeds, or macroalgae, are considered as rich in bioactive compounds, providing beneficial effects on nutritional values (high dietary fibre content, minerals, proteins and PUFAs; low saturated fats). In addition to its scenario in the industrial field, introducing seaweed into diet has been shown to provide a lot of health advantages (Lomartire et al., 2021). There are a few epidemiological studies suggesting that populations with high contact with seaweed inside their diets specifically within Southeast Asia have decreased incidences of neurodegenerative diseases (Jorm and Jolley, 1998). Though the full nature of this relationship is not yet fully known, it highlights seaweed as a promising new product in both dietary and therapeutic applications.

Our study adds to the ever-increasing body of research supporting the beneficial effects of seaweed while providing insight into novel ways they might be employed as a treatment for neurodegenerative conditions. The ocean is still a largely untapped source of bioactive compounds with therapeutic potential, and this study shows that macroalgae could prove to be an interesting niche as contributors of novel bioactive agents. With the increasing worldwide prevalence of neurodegenerative diseases, new, natural therapeutic agents are needed. These findings open the

door to more studies into the therapeutic use of marine derived molecules and stress the importance of sustainable exploration of oceanic resources.

To conclude, this study highlights that macroalgae extracts are an effective and sustainable avenue in the development of novel therapeutics specific for neurodegenerative disorders, specifically PP, SV and ZT. However, it offers neuroprotection in yeast model by lowering the oxidative stress and preventing the protein aggregation. With further investigations on the plant extract, we estimate that future emerging phenolic compounds from plants used medicinally may serve as auxiliary therapeutics to these agents. The sustainable use of marine resources, such as macroalgae, may therefore provide a pathway to the development of lasting and truly disease-modifying therapies for neurodegenerative diseases, still an unmet burden in medicine today.

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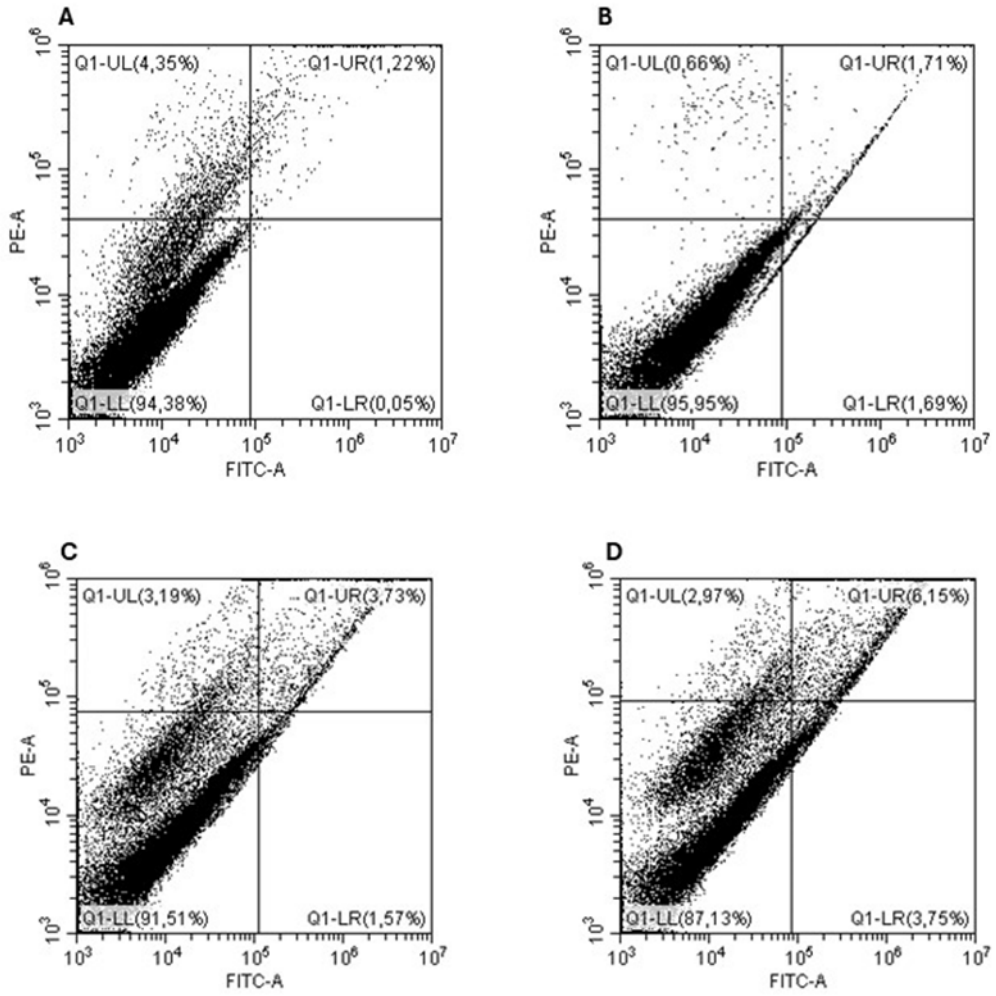
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7. Supplementary Material

Supplementary Table 1: Media and Solutions composition

Media or Buffer	Composition
YPD	1% Yeast extract, 2% dextrose, 2% peptone
YPDA	1% Yeast extract, 2% dextrose, 2% peptone, 2% agar
SCG (supplemented with aminoacids) used for SNCA strain	0.67% YNB without aminoacids, 2% dextrose, 2% agar, 80 mg/L adenine, 80 mg/L histidine and 80 mg/L leucine
SCG (supplemented with aminoacids) for HTT strain	0.67% YNB without aminoacids, 2% dextrose, 1,4 g/L dropout, 80 mg/L histidine, 80 mg/L tryptophan and 80 mg/L uracil
SGCD for HTT strain	0.67% YNB without aminoacids, 2% dextrose, 2% agar, 1,4 g/L drop-out, 80 mg/L histidine, 80 mg/L tryptophan and 80 mg/L uracil
PBS	NaCl: 137 mM; KCl: 2.7 mM; Na2HPO4: 10 mM; KH2PO4: 1.8 Mm

Anexo 1. Cystoseira humillis (CH; A), Padina pavonica (PP; B), Sargassum vulgare (SV;



C) and Zonaria tournefortii (ZT; D) extract did not affect cell viability in Sccharomyces cerevisiae strain BY4741. Cells were treated with with 1000 µg/mL of each extract and 5 mM H_2o_2 for 20 min at 30 °C, 200 rpm, labelled with 2',7'-dichlrofluorescein diacetate (50 µM; H2DCFDA) and propidium iodide (3 µg/mL) in the dark, for 1 h and the fluorescence was measured by flow cytometry. Percentage of negative events for PI in CH treatment was 94,38% (A); in PP treatment was 95,95% (B); in SV treatment was 91,51% (C) and in the ZT treatment was 83,13% (D)