Águeda Laura dos Santos Silvestre

Searching for biocompounds in algae and seagrasses with potential use in the treatment of Alzheimer's disease

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Faculdade de Ciências e Tecnologia
Centro de Ciências do Mar – Grupo MarBiotech

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Mestrado **I**ntegrado em **E**ngenharia **B**iológica

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Águeda Laura dos Santos Silvestre

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Custódio L, Ferreira C, Pereira H, **Silvestre L**, Duarte C, Barreira L, Rauter AP, Alberício F and Varela J. (2012). The marine halophytes *Carpobrotus edulis* L. and *Arthrocnemum macrostachyum* L. are a potential source of nutritionally important PUFAs and biocompounds with antioxidant, metal chelating and anticholinesterase inhibitory activities. *Botanica Marina*, 55, 281–288.

Custódio L, **Silvestre L**, Rocha MI, Rodrigues MJ, Vizetto-Duarte C, Barreira L and Varela J. (2016). Methanol extracts from *Cystoseira tamariscifolia* and *Cystoseira nodicaulis* are able to inhibit cholinesterases and protect a human dopaminergic cell line from hydrogen peroxide induced cytotoxicity. *Pharmaceutical Biology*, 5, 1–10.

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Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChI	Acetylthiocholine iodide
AD	Alzheimer's disease
APP	Amyloid precursor protein
BChE	Butyrylcholinesterase
BChI	Butyrithiocoline iodide
CNS	Central nervous system
ChE	Cholinesterase
DFO	Desferrioxamine
DTNB	5, 5-Dithio-bis (2-nitrobenzoic) acid
FDA	US Food and Drug Administration
OS	Oxidative stress
PD	Parkinson's disease
PV	Pirocatechol violet
ROS	Reactive oxygen species
SD	Standard deview

Abstract

The number of older people at risk of developing dementia is growing rapidly worldwide, and Alzheimer's disease (AD) represents the most common cause of dementia in the elderly. The principal characteristics of AD include the presence of amyloid plaques, neurofibrillary tangles, brain atrophy in specific brain areas and loss of the neurotransmitter acetylcholine (ACh), which is hydrolysed by the cholinesterases (ChE) acetylcholinesterase (AChE) and secondly by butyrilcholinesterase (BChE).
Pharmacological treatments currently used to alleviate AD symptoms include ChE inhibitors, but they exhibit bioavailability problems and side effects like hepatotoxicity and gastrointestinal disorders. Thus, there is a high interest in finding better ChE inhibitors from natural sources.

Due to the high oxygen consumption and lipid content, the central nervous system (CNS) is more sensitive to oxidative stress compared to other parts of our body. Thus, special interest has been assigned in nutritional antioxidants and metal chelation therapy as viable neuroprotective approaches for neurodegenerative disorders.

Marine organisms are recognized as rich sources of novel biologically active compounds. However, its application in the treatment of neurological disorders is still scarcely explored. In this context, the main goal of this study was to evaluate the AChE and BChE inhibitory activity of methanol extracts made from different species of macro- and microalgae, seagrasses and halophytes, as well as evaluate their chelating activity on iron (Fe^{2+}) and copper (Cu^{2+}) ions.

The most active species against both enzymes were the brown macroalgae *Cystoseira compressa*, *C. nodicaulis* and *C. tamariscifolia* and the halophytes *Carpobrotus edulis* and *Frankenia laevis*.

The chelating activity was higher for Cu^{2+} than for Fe^{2+} in the majority of the species tested, being the most active the red macroalgae *Plocamium cartilageneum*. These species are thus promising candidates for more detailed *in vitro* and *in vivo* studies aiming their use as sources of innovative products with neuroprotective aplications.

Keywords: Alzheimer's disease; metal chelation; acetylcholinesterase; butyrylcholinesterase; marine natural resources; neuroprotection.

Resumo

O número de pessoas idosas em risco de desenvolver demência cresce rapidamente em todo o mundo e o Alzheimer representa a causa mais comum desta forma de distúrbio. Esta é uma doença que se caracteriza pela presença de microplacas senis, emaranhados neurofibrilares, atrofia do cérebro em áreas específicas e perda do neurotransmissor acetilcolina (ACh), que é hidrolisado principalmente pela acetilcolinesterase (AChE) e, numa segunda instância pela butirilcolinesterase (BChE).
Os tratamentos farmacológicos mais usados actualmente, para o alívio dos sintomas do Alzheimer, incluem inibidores de AChE e BChE, mas devido a problemas de disponibilidade biológica e efeitos colaterais como hepatotoxicidade e desordens gastrointestinais dos medicamentos comummente utilizados existe um grande interesse em encontrar inibidores destas enzimas a partir de recursos naturais.

Devido ao consumo de oxigénio e ao alto teor de lípidos, o sistema nervoso central (SNC) é mais propício a sofrer stress oxidativo em comparação com outras partes do corpo. Assim, enquanto potenciais métodos de neuroproteção, especial interesse tem sido atribuído a antioxidantes nutricionais e terapia de quelação de metais de transição, como o ferro e o cobre, formando complexos inactivos e evitando a formação dos prejudiciais radicais livres.

Os organismos marinhos têm provado, devido à produção de metabolitos secundários, ser fontes riquíssimas de novos compostos biologicamente activos e sem efeitos colaterais. No entanto, a sua aplicação no tratamento de distúrbios neurológicos ainda é pouco explorada e, portanto, o principal propósito deste estudo foi testar a capacidade inibidora da AChE e BChE de extratos metanólicos feitos a partir de diferentes algas, ervas marinhas e plantas halófitas disponíveis na costa sul do Algarve (Portugal), bem como avaliar o seu poder quelante em iões ferro (Fe^{2+}) e cobre (Cu^{2+}).

As espécies mais activas na inibição de ambas as enzimas foram as macroalgas castanhas *Cystoseira compressa*, *C. nodicaulis* e *C. tamariscifolia* e as halófitas *Carpobrotus edulis* e *Frankenia laevis*. A actividade quelante foi maior para o Cu^{2+} do que para o Fe^{2+} na maioria dos casos, sendo a espécie mais activa a macroalga vermelha, *Plocamium cartilageneum*. Estas espécies são, portanto, promissoras candidatas a novos e mais detalhados estudos *in vitro* e *in vivo*, visando o seu uso como fonte de inovadores produtos com aplicações neuroprotetoras.

Palavras-chave: Alzheimer; actividade quelante; acetilcolinesterase; butirilcolinesterase; recursos naturais marinhos; neuroproteção.

1. INTRODUCTION

This section begins with a definition of Alzheimer's disease and its characteristics, followed by information on cholinergic theory and chelating activity. Is also contains some information about the marine natural resources used in this study, such as main components and uses. Finally, the purpose and motivation for conducting this thesis is presented.

1.1. Alzheimer's disease (AD)

Dementia (in Latin: irrationality) is a group of symptoms that may accompany neurological disorders or conditions, appears usually in the elderly and affects important daily living skills such as memory, thinking, comprehension, calculation and language (Holden & Kelly, 2002). It affects approximately 47 million people worldwide, the maiority of them in developing countries and it is estimated that this number could increase to 131 million by 2050 (Alzheimer's Disease International, 2015). The commonest causes of dementia are Alzheimer's disease (AD), Parkinson disease (PD), Dementia with Lewy Bodies and Myasthenia gravis (Holden & Kelly, 2002).

AD was first identified in 1906 by Alois Alzheimer, a German psychiatrist and neuropathologist, but only in the last 40 years research has revealed more about its causes and possible treatments (Zarotsky *et al.*, 2003). However, despite the research in recent years, its exact cause(s) still remains to be clarified (Zarotsky *et al.*, 2003). Nowadays, AD is considered the fourth leading cause of death in developed nations (after heart disease, cancer and strokes) and is characterized by the presence of amyloid plaques, neurofibrillary tangles, neuronal and synaptic loss and brain atrophy in specific brain areas (Natarajan *et al.*, 2009).

1.1.1. Symptoms

The difficulty to process and remember new information is perhaps the most common symptom in patients with AD, due to the fact this is a disease begins affecting areas of the brain responsible for forming new memories. As the disease progresses from mild to moderate and severe, the damage increases and other cognitive and functional capacities are affected, such as: memory loss that disrupts daily life; difficulties in planning or solving daily problems; difficulty in the completion of familiar tasks at home, work or at leisure; confusion with time or place; trouble understanding visual images and spatial relationships; misplacing things and losing the ability to

retrace steps; decreased or poor judgment; withdrawal from work or social activities; changes in mood and personality; aphasia (problems with words in speaking or writing); apraxia (loss of the ability to execute or carry out learned purposeful movements) and agnosia (failure to recognize objects).

In very advanced stages of the disease, people need more care in their day living, becoming increasingly dependent and losing the ability to communicate and recognize relatives. Because of its fragility, the patient becomes increasingly vulnerable to infections, including pneumonia and this can cause ultimately, death.

1.1.2. Causes of AD

Although the exact cause of AD is still unknown, it is believed that this disease develops due to a number of factors, including complex alterations in chemical and electrical processes that take place within the brain.

A normal healthy brain contains billions of neurons, constantly receiveing information from each other in the form of electrical charges travelling down the axon to the end of the neuron – neurotransmitters, which move across microscopic gaps, or synapses, between neurons (Rodgers, 2008). They bind to receptor sites on the dendrites of the next neuron (Fig. 1.1) and AD is the responsible for the disruption of the neuronal network, causing the destruction of memory and thinking skills over time (Rodgers, 2008).

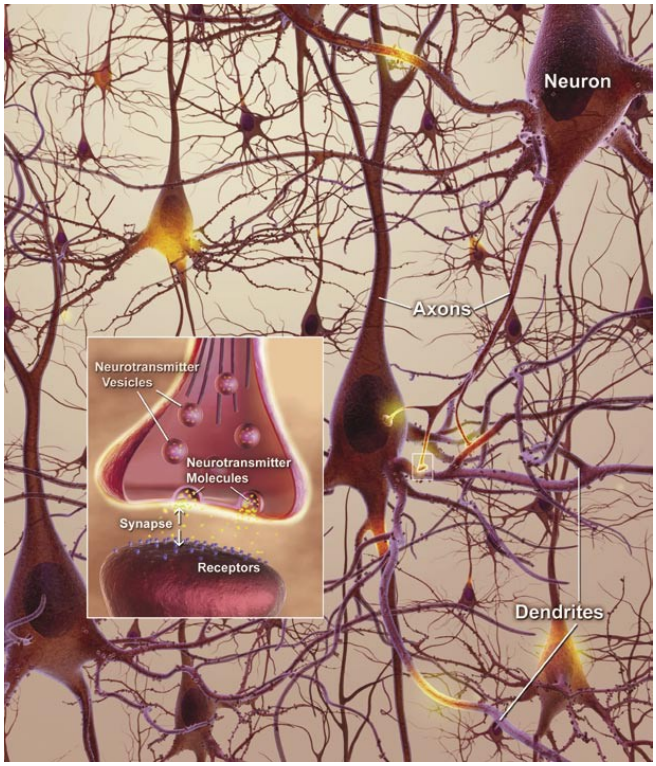

Figure 1.1 Neuronal network in a healthy brain (Rodgers, 2008).

AD is associated with inflammatory processes (Stuchbury and Munch, 2005). Abnormal structures called **β-amyloid plaques** (outside the neurons) and **neurofibrillary tangles** (inside the neurons) are classic biological hallmarks of the disease and can induce inflammation (Ferreira *et al.*, 2006):

- Plaques form with the extracellular β-amyloid peptides accumulation as well as dystrophic neurites, reactive astrocytes, phagocytic cells and protein fragments derived from degenerating cells or liberated from neurons (Jalbert *et al.*, 2008).
  β-amyloid are fragments, result of amyloid precursor protein (APP) cleavage by β-secretase, α-secretase and γ-secretase (Brewer, 2007). These fragments increase in size and become insoluble and consequently toxic, contributing to cell death (Fig. 1.2) (Brewer, 2007).

- Neurofibrillary tangles are made when *tau* protein separates from microtubules, which are used in a normal brain to stabilize critical structures to the cell's internal

nutrients transport system, causing neurotransmitter deficits and neuronal cell death (Jalbert *et al.*, 2008). *Tau* strands aggregate inside the neurons, forming tangles and, therefore, disable the transport system and destroy the cell (Fig. 1.3) (Brewer, 2007).

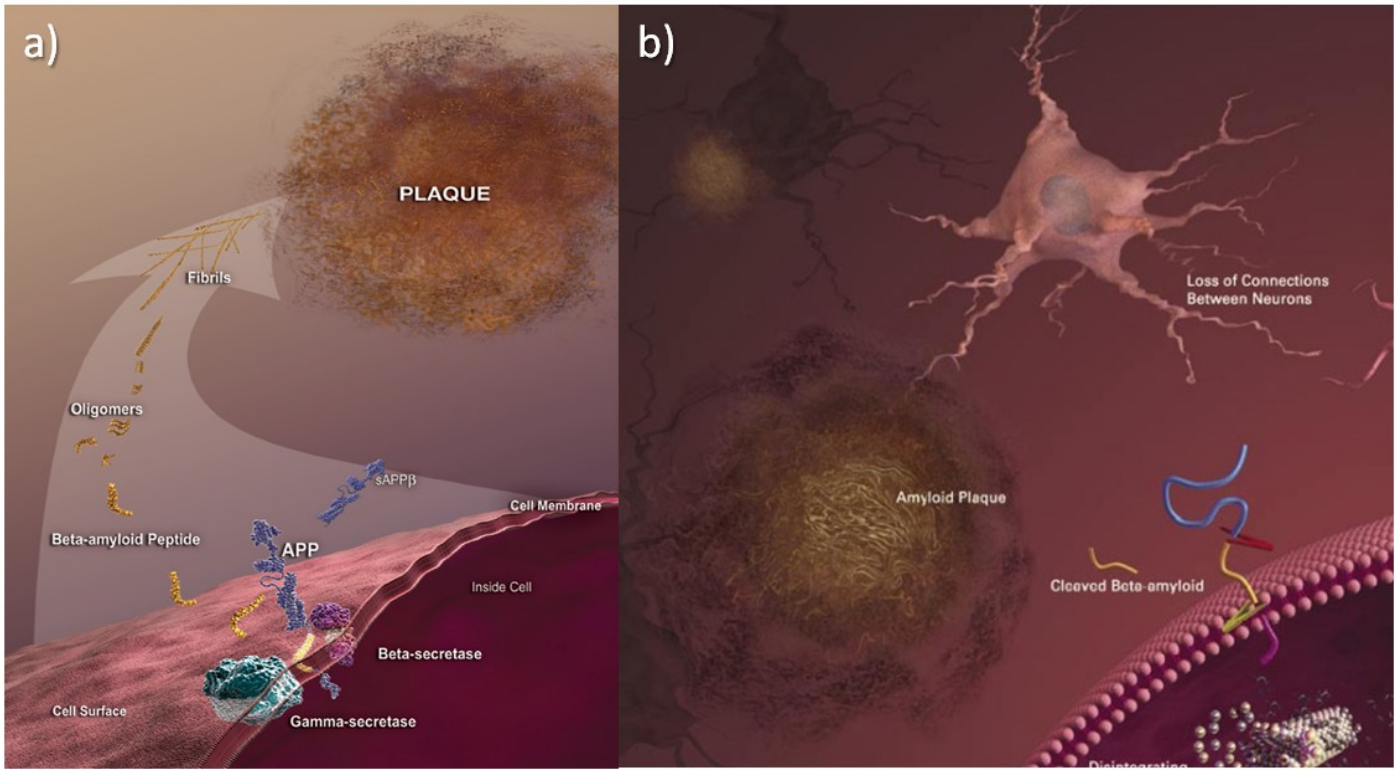

Figure 1.2 Formation of β-amyloid plaques (a, b) (Rodgers, 2008).

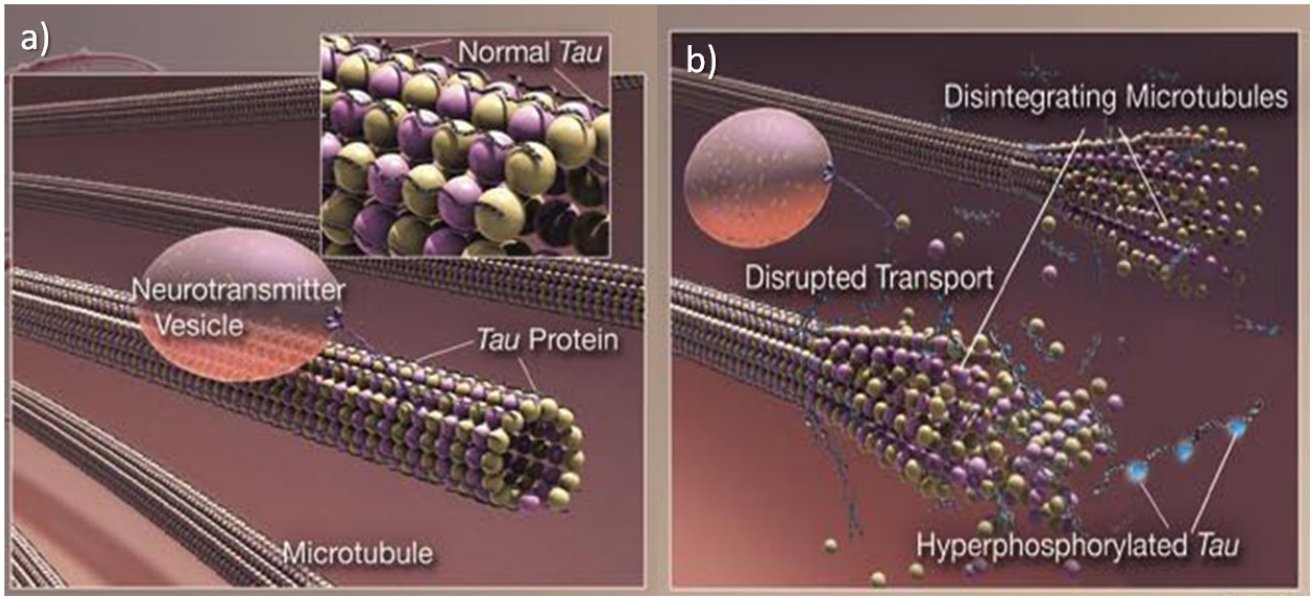

Figure 1.3 General aspect of a healthy neuron (a) and a diseased neuron, exhibiting the formation of *tau* tangles (b) (Rodgers, 2008).

Although many events that happen in the brain of patients with AD are well known, there are still many unclear factors such as what other changes are taking place in the aging brain and its cells and what influence do other diseases, genetics, and lifestyle factors have on the risk of developing AD as the brain and body age (Rodgers, 2008).

1.1.3. Risk Factors for AD

1.1.3.1. Advanced age

People younger than 65 years can develop AD, but the risk increases after this age (Geldmacher, 2010).

1.1.3.2. Family history

Individuals who do not have a first-degree relative with AD are less likely to develop it than those who have and the risk becomes higher when the individual has more than one first-degree relative with AD (Huang *et al.*, 2004).

1.1.3.3. Apolipoprotein E-ε4 (APOE-ε4)

A genetic factor in late-onset Alzheimer's disease is APOE-ε4, which is one of three common forms (ε2, ε3 and ε4) of the APOE gene, responsible for providing the blueprint for a protein that carries cholesterol in the bloodstream (Jalbert *et al.*, 2008). Everyone inherits one form of the APOE gene from each parent, but those who inherit the form APOE-ε4 have increased risk of developing AD in an earlier age (Jalbert *et al.*, 2008). The risk can also increase if the individual inherits two APOE-ε4 genes, but this does not guarantee the development of AD (Jalbert *et al.*, 2008).

On the other hand, APOE-ε4 which has an arginine at position 112 rather than a cysteine, while the other apolipoprotein alleles have cysteine at this position (Reynolds, 1997), may be involved in copper binding and to the diminished antioxidant effect of the E-4 allele (Brewer, 2007).

1.1.3.4. Cardiovascular Disease Risk Factors

The cardiovascular diseases that offer a major risk in developing AD are associated with high cholesterol (especially in midlife), Type 2 diabetes, high blood pressure (especially in midlife), physical inactivity, smoking and obesity (Geldmacher, 2010). Thus, remaining mentally and physically active and consuming a diet low in saturated fats and rich in vegetables, may support heart and brain health (Geldmacher, 2010).

1.1.4. The Cholinergic theory

The cholinergic system is composed by a set of cells that produce and/or are stimulated by the neurotransmitter acetylcholine (ACh) and control the central nervous system (CNS) functions (Gibbs, 2010). ACh is released to travel across the synaptic cleft and the two types of receptors, muscarinic and nicotinic (post-synaptic terminal), respond to ACh, facilitating intracellular communication, memory processing and higher cognitive functions (Fig. 1.4) (Gibbs, 2010). Acetylcholinesterase (AChE) is

the main enzyme that hydrolyzes ACh into a choline and an acetyl groups (Ferreira *et al.*, 2006).

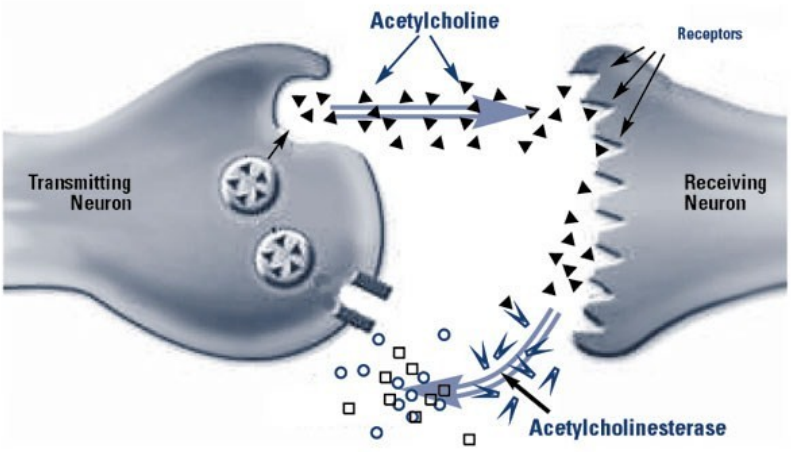

Figure 1.4 Actuation of acetylcholinesterase in a neurotransmission signaled by acetylcholine – After signalling, acetylcholine is released from receptors and broken down by AChE to be recycled in a continuous process. Source: www.vrp.com/brain-health.

The basal forebrain is the brain area where declines in the level of ACh in AD patients can be found and the projection to the hippocampus and neocortex lead to impairments in memory and cognitive functions (Zarotsky *et al.*, 2003; Natarajan *et al.*, 2009).

Acetyltransferase is needed for the synthesis of acetylcholine, and its activity can be reduced up to 90%, as a result of the loss of cholinergic neurons (Zarotsky *et al.*, 2003).

Treatment strategies have therefore focused on replacing the level of ACh, enhancing cholinergic activity in the affected regions of the brain, or inhibiting AChE action, avoiding its degradation (Zarotsky *et al.*, 2003; Natarajan *et al.*, 2009). However this current treatment does not halt the progression of AD, but contribute to modest improvements in memory, thinking and reasoning skills (Zarotsky *et al.*, 2003; Natarajan *et al.*, 2009).

The first organophosphate AChE inhibitor was synthesized in the 1850s and in the 1930s synthetic cholinesterase inhibitors, such as neostigmine, began to be used to treat autonomic nervous system manifestations (Taylor, 1998).

AChE inhibitors not only increase the level of ACh but also prevent the formation of β-amyloid plaques, activating secretase, which acts on amyloid precursor protein thereby preventing neuronal death due to inflammation in AD (Natarajan *et al.*, 2009).

The mammalian brain contains two cholinesterases, AChE and butyrylcholinesterase (BChE) (Kuhl *et al.*, 2006). Butylcholines are not physiological substrates in the brain, and, although their function remains unclear, it has been found to increase progressively in patients with AD. In the human brain, BChE is found in neurons and glial cells, as well as in plaques and neurofibrillary tangles in patients with AD (Zarotsky *et al.*, 2003). So, it is believed that after AChE, BChE plays an important role in the inhibition of ACh (Orhan *et al.*, 2004). Until now, there are no drugs that inhibit BChE as strongly as AChE (Orhan *et al.*, 2004).

1.1.5. Oxidative stress and metal accumulation

Oxidative stress (OS) and the hypothesis that brain metal dysregulation, resulting in reactive oxygen species (ROS) generation from H_2O_2 and inflammatory processes, plays a pivotal role in different clinical disorders, such as neurodegeneration, diabetes, hypothyroidism, liver failure, atherosclerosis, ischemia reperfusion injury, cancer and cardiovascular diseases (e.g. stroke or thalassemia) (Conforti *et al.*, 2009; Weinreb *et al.*, 2009). OS triggers a cascade of events leading to apoptotic/necrotic cell death in neurodegenerative disorders, such as AD, PD, Huntington's disease and amyotrophic lateral sclerosis (Zecca *et al.*, 2004; Tirosh *et al.*, 2007; Ebrahimzadeh *et al.*, 2008). Moreover, due to the high oxygen consumption and

lipid content, the CNS is more sensitive to oxidative stress compared to other parts of our body (Pangestuti & Kim, 2011).

Free radicals, which are generated in mitochondria (Fig. 1.5) can help cells to fight infections, but can also damage the neuron's cell membrane or DNA, because they are very reactive and easily react with other molecules (Rodgers, 2008).

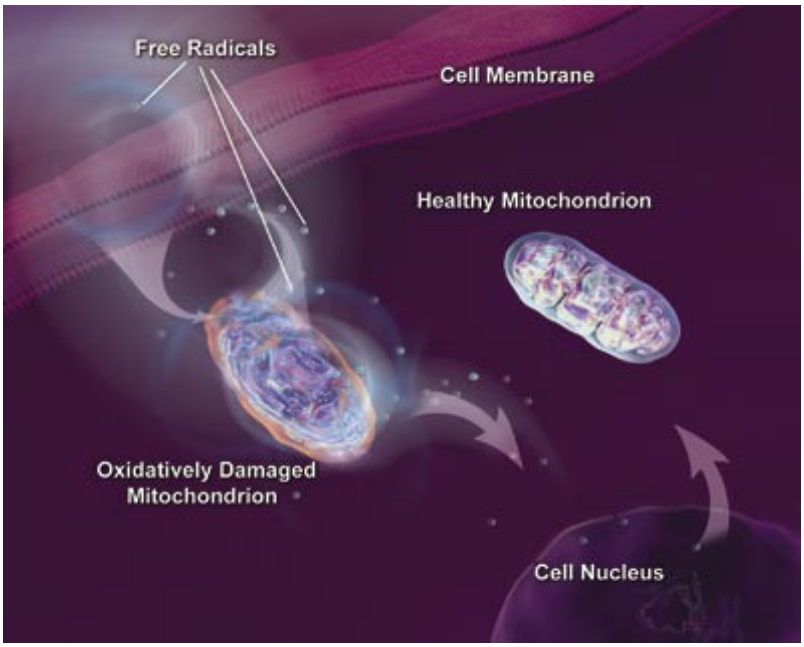

Figure 1.5 Oxidative Stress and *mitochondria* – The arrows indicate the movement of free radicals, which can spread easily from damaged mitochondria to other parts of the cell (Rodgers, 2008).

1.1.5.1. Iron and AD

Iron is involved in several processes (El & Karakaya, 2004; Zecca *et al.*, 2004), such as:

- transport, storage and activation of oxygen (as a central element of the heme molecule, which is a critical part of haemoglobin);
- respiration;
- activity of several enzymes, including cytochromes, which acts in electron transport or tyrosine hydroxylase, which is required for dopamine synthesis;
- synthesis of steroid hormones and bile acids;
- detoxification of external substances in the liver and signal controlling in some neurotransmitters, like dopamine and serotonin systems in the brain.

However, when iron homeostasis is not well regulated it can degrade lipids, proteins and cells as for example astrocytes, microglia or neurons, due to unwanted oxidative reactions (Rival *et al.*, 2001; Zecca *et al.*, 2004; El & Karakaya, 2004). The excess iron, derived from the breakdown of transfused red blood cells, is deposited as hemosiderin and ferritin in the liver, spleen, endocrine organs and myocardium (Ebrahimzadeh *et al.*, 2008).

Consequently, there is increasing evidence that iron accumulation in the brain with age can cause, as mentioned above, a vast range of disorders to the CNS (Zecca *et al.*, 2004).

There are two classes of iron-related neurodegenerative disorders (Zecca *et al.*, 2004):

- Those resulting from iron accumulation in specific brain regions;
- Those resulting from deficiency in iron metabolism and/or homeostasis.

In these disorders are usually involved protein modification, misfolding and aggregation, causing the formation of the intracellular inclusion bodies that are the postmortem characteristics of many neurodegenerative diseases, like AD and PD (Zecca *et al.*, 2004).

Iron might also have a direct impact on plaque formation, due to its action as a modulator of α-secretase in APP cleavage (Huang *et al.* 2000; Rodgers *et al.*, 2002; Kawahara, 2003; Zecca *et al.*, 2004), although some authors argue that by binding iron, β-amyloid might, in fact, protect the surrounding neurons from OS (Zecca *et al.*, 2004).

Iron is also involved in the formation of ROS. ROS are formed when iron (Fe^{2+}) reacts with H_2O_2 to form $^{\bullet}OH$ (hydroxyl radicals), which are very unstable and reactive, *via* the Fenton reaction (*Eq. 1 and 2*; Koschnick & Haller, 2006), initiating the processes of OS and the inflammatory cascade, that result in the production of cytotoxic cytokines in the microglia and surrounding neurons and activation of transcription factors (Rival *et al.*, 2001; El & Karakaya, 2004; Weinreb *et al.*, 2009):

$$O_2^- + Fe^{3+} = O_2 + Fe^{2+} \quad \text{(Equation 1)}$$

$$H_2O_2 + Fe^{2+} = {}^{\bullet}OH + OH^- + Fe^{3+} \quad \text{(Equation 2)}$$

1.1.5.2. Copper and AD

Copper is an essential component of several enzymes and proteins and is critical for numerous reactions vital for life, as for example, antioxidant defense, neuropeptide synthesis and immune function (Brewer *et al.*, 2006; Brewer, 2007). The deficiency on this element leads to anemia and bone marrow suppression, followed by a neurologic syndrome called a myelopathy (Hedera *et al.*, 2003).

However, like iron, copper also participates in the generation of ROS through Fenton chemistry and can produce oxidative damage in much the same manner (Brewer *et al.*, 2007).

At this time the evidences are conflicting whether too much copper is involved in the pathogenesis of AD as well as others neurodegeneratives diseases. Some authors claim that copper is directly related with the onset of neurological diseases (Sayre *et al.*, 2000; Cherny *et al.*, 2001; Nakano *et al.*, 2004; Angeletti *et al.*, 2005; Nelson & Alkon, 2005; Soragni *et al.*, 2008; Kong *et al.*, 2008). However, some authors such as Phinney *et al.* (2003) and Bayer *et al.* (2003) reports *in vivo* studies where amplification of a copper transporter improved brain copper and reduced β-amyloid formation, increasing longevity.

1.1.6. Treatment

The pharmacological treatments currently used to alleviate AD symptoms include antioxidant therapy, the use of ChE inhibitors, nicotinic and muscarinic agonists, estrogen, nerve growth factor (NGF), low molecular lipophilic compounds that can activate neurotrophic factor signaling pathway, nonsteroidal antiinflammatory drugs such as ibuprofen and COX-2 inhibitors, drugs that interfere with β-amilose formation and deposition, and also drugs that attenuate toxicity induced by β-amilose (Park & Kim, 2002).

The changes in the brain of AD patients may begin near 10 years before patients experience symptoms such as memory loss, and this is considered as the ideal period in which the future drugs should be administered (Jalbert *et al.*, 2008).

1.1.6.1. ChE inhibitors

Scientists argue that the inhibition of AChE and BChE represents an effective therapy for AD management (Grossberg, 2003; Darreh-Shori & Soininen, 2010).

However, and despite the high demand for ChE inhibitors for AD and other neurological disorders, only synthetic AChE inhibitors, such as tacrine and donepezil, and the natural products rivastigmine and most recently galanthamine have been approved by FDA (Zarotsky *et al.*, 2003). The latter have been also approved in Europe by the European Registration Bureau and is commercially available as Reminyl® (Fig. 1.6c) (Zarotsky *et al.*, 2003).

Studies have also revealed the relevance of BChE due to its presence in some cholinergic neurons in which AChE is absent, as well as its capability to induce neurotoxicity of some plaques (Greig et al, 2005; Oboh *et al.*, 2015). But only rivastigmine can inhibit both AChE and BChE (Colovic *et al.*, 2013; Pohanka 2014).

The above mentioned drugs exhibit some side effects, as for example, hepatotoxicity, gastrointestinal disorder, anxiety, nervousness, drowsiness, mouth dryness or tiredness and also bioavailability problems, making necessary to find alternative ChE inhibitors from natural sources (Pangestuti and Kim 2011). As examples, *Ginkgo biloba* (Ginkgoaceae) and *Huperzia serrata* (Pteridophyta) have been extensively investigated as natural therapeutic agents for AD patients (Park & Kim, 2002; Conforti *et al.*, 2009).

Galanthamine hydrobromide is a tertiary alkaloid (Fig. 1.6b) that was originally isolated from the *Galanthus worownii* (snowdrop plant) (Fig. 1.6a) (Willis *et al.*, 2009) and is now synthesized for use in the treatment of mild to moderate AD (Zarotsky *et al.*, 2003; Butler, 2005).

Prior to its use in patients with AD, galanthamine was available in Eastern Europe as a curare-reversal agent in anesthesia and as a treatment for neurologic conditions, such as myasthenia gravis (Zarotsky *et al.*, 2003). The interest in this compound as a potential treatment for AD was generated on the basis of its ability to

reach the brain, penetrating the blood barrier and affect cholinergic transmission (Zarotsky *et al.*, 2003).

Figure 1.6 a) General aspect of *Galanthus worownii*
(Source: http://art-nature-garden-passion-bensimon.blogspot.com/);
b) chemical structure of galanthamine (4aS,6R,8aS)-4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro[3a,3,2ef][2]benzazepin-6-ol hydrobromide
(Source: http://dailymed.nlm.nih.gov/dailymed/);
c) Reminyl®, galanthamine Alzheimer's drug
(Source: http://www.sciencephoto.com/media/412296/enlarge).

1.1.6.2. Metal chelation as a neuroprotective strategy

Chelation of the metal ions is considered the main strategy to avoid ROS generation (Ebrahimzadeh *et al.*, 2008).

Antioxidant and other supportive therapies can scavenge ROS and can also attenuate inflammation pathways, protecting red blood cells against oxidant damage (Ferreira *et al.*, 2006; Ebrahimzadeh *et al.* 2008).

Iron chelators are used to form soluble and stable complexes with iron, which are then excreted in the feces and/or urine (Ebrahimzadeh *et al.*, 2008). This iron chelation may result in the improvement of life quality and overall survival (Ebrahimzadeh *et al.*, 2008).

Tirosh *et al.* (2007) reported that the antibiotic iron chelator *Clioquinol* and the continued intramuscular administration of the drug DFO (desferrioxamine) could prevent neurotoxicity in mice and slow the clinical progression of AD. However, *Clioquinol* is highly toxic and DFO is poorly passed through the blood–brain barrier (Tirosh *et al.*, 2007).

In this sense, special interest has been assigned in nutritional antioxidants and metal chelation agents as viable neuroprotective alternative approaches for neurodegenerative disorders (Tirosh *et al.*, 2007; Weinreb *et al.*, 2009). These may have compounds that can chelate metal ions, such as iron and copper to form inactive complexes and prevent the generation of potentially damaging free radicals (Weinreb *et al.*, 2009).

1.2. Marine Natural Resources

The oceans contain about 90% of the world's living biomass, which represents approximately half of the total global biodiversity (Pangestuti & Kim, 2013). This wide diversity of organisms is recognized as an important reservoir of potent and innovative molecules responsible for helping them to survive in the hostile environment characterized by a competition for space, maintenance of unfouled surfaces, deterrence of predation and the ability to successfully reproduce and exhibiting strong pharmacological potential (Salvador *et al.*, 2007).

Among marine organisms, marine algae found attached to rocks in the intertidal zone and washed up on the beach in giant underwater forests (Fig. 1.7) have been identified as an under-exploited resource for bioactive molecules (Natarajan *et al.*, 2009; Pangestuti & Kim, 2011).

Figure 1.7 Kelp forest at Catalina Island, California, USA
(Source: http://www.uwphotographyguide.com/giant-kelp-forests).

Marine algae has been used in traditional medicine in China (Folmer *et al.*, 2010) and as a subsidiary food (Natarajan *et al.*, 2009) for more than 2000 years. Marine algae has also been widely used in Ancient Egyptian and Ayurvedic medicine (Folmer *et al.*, 2010). Specifically, Hamed *et al.*, (2015) postulates that the low incidence of neurodegenerative diseases in East Asia can be related to their high fish

and marine algae consumption. However, there is not enough data currently from clinical trials (Cole *et al.*, 2009).

In Western medicine, the first record of the medicinal use of algae dates back to the 1960's in Italy, in the treatment of breast cancer (Folmer *et al.*, 2010).

In contrast to seagrasses, that have a true root system (Fig. 1.8b), seeds and fruit and veins that carry molecules around the plant, algae have holdfasts and spores (Fig. 1.8a) (Coles *et al.*, 2004).

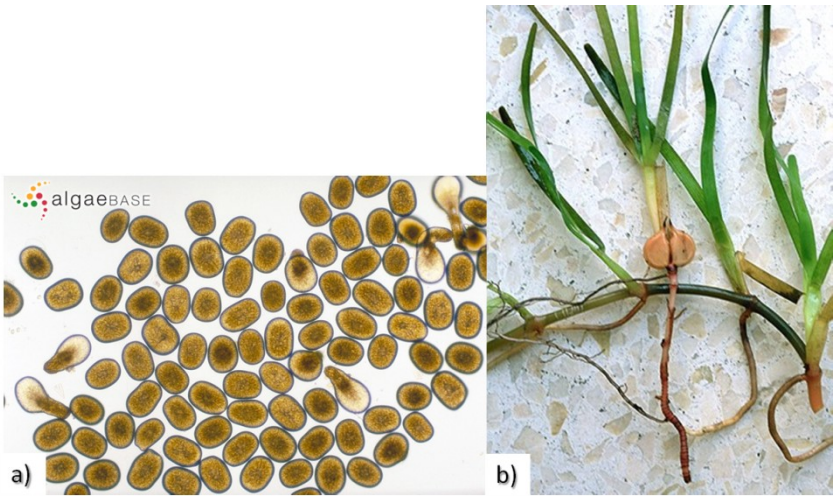

Figure 1.8 Spores from brown macroalgae *Haplospora globosa* (a) (Source: www.algaebase.org); root system of the seagrass *Cymodocea nodosa* (b) (Source: www.terra.es).

Besides their use as antifouling, stabilizers, gelling agents or emulsifiers in food industries, a wide number of seaweeds species exhibit important biomedical applications, such as the treatment of tuberculosis, arthritis, cold, influenza, worm infestations, as a cholesterol lowering drug, ovarian cysts, breast lumps, lymph node swellings and lymphomas (Natarajan *et al.*, 2009), antibacterial, antifungal, antiviral and/or antitumor (Moreau *et al.*, 2006; Kong *et al.*, 2008; Folmer *et al.*, 2010; Vo & Kim, 2010), anticoagulant (Athukorala *et al.*, 2007), antioxidant (Rupérez *et al.*, 2002; El & Karakaya, 2004; Lim *et al.*, 2006; Ganesan *et al.*, 2007), anti-allergic (Li *et al.*, 2008), anti-inflammatory (Kim *et al.*, 2009) or anti-obesity (Maeda *et al.*, 2007; Tsukui *et al.*,

2007; Kong *et al.*, 2010). Apart from all these capabilities, algae can have also interest as neuroprotectants (Natarajan *et al.*, 2009).

1.2.1. Macroalgae

Macroalgae can be classified into three classes based on their pigmentation, namely brown, red and green algae, which are referred to as Ochrophyta, Rhodophyta, and Chlorophyta, respectively (Khan *et al.*, 2010).

Brown macroalgae can contain polysaccharides and diterpenoids (Moreau *et al.*, 2006). These diterpenoids display antitumor effects and may include cyclic diterpenes from *Dictyotaceae* species (Gedaraa *et al.*, 2003) or meroditerpenes from *Cystoseira usneoides* (Moreau *et al.*, 2006; Zubia *et al.*, 2009; Taskin *et al.*, 2010) and *Sargassum tortile* (Moreau *et al.*, 2006). In addition, a linear diterpene, 12-(R)-hydroxygeranylgeraniol, isolated from *Bifurcaria bifurcata* (Culioli *et al.*, 2004) has been reported for its cytotoxicity against cultured human tumor cell lines (Moreau *et al.*, 2006).

Macroalgae polysaccharides has been widely used in the food industry and in medicine (Zvyagintseva *et al.*, 1999) and since the 1940s, its production has attained commercial significance through their application as thickening and gelling agents for several industrial applications (Burtin *et al.*, 2003).

The main polysaccharides of brown macroalgae are fucoidans, laminarans, and alginic acids. In particular, fucoidans and laminarans contents vary from 20 to 50% of defatted alga dry weight (Zvyagintseva *et al.*, 1999).

Fucoidans are nontoxic polyelectrolytes and possess various pharmacological activities, as for example, antioxidant, antibacterial, antiviral, antitumor, immunosuppressive, antipeptic, antilipemic, antigemostatic and anticoagulant (Zvyagintseva *et al.*, 1999). On the other hand, alginic acids can be used for heavy metal binding and as immunostimulators (Zvyagintseva *et al.*, 1999; Chandini *et al.*, 2007; Ye *et al.*, 2008; Koz *et al.*, 2009; Zubia *et al.*, 2009; Chiheb *et al.*, 2009).

Macroalgae and its biocompounds can, thus, exhibit numerous remarkable properties on biological systems, namely antioxidant (Sathya *et al.* 2013), anti-inflammatory (Sugiura *et al.*, 2013), anti-allergic (Sugiura *et al.*, 2009), antimicrobial (Eom *et al.*, 2012), anticancer (Lee *et al.*, 2012), antidiabetic (Lee & Jeon, 2013) and neuroprotective activities (Barbosa *et al.*, 2014).

1.2.2. Microalgae

Microalgae are constituted by a vast array of novel compounds, such as: nutrients (including proteins, vitamins, minerals, fatty acids); carotenoid pigments, such as xanthophylls and carotenes; as well as phenolic acids and tocopherols which are known to exhibit antioxidant properties (Cha *et al.*, 2008; Raposo *et al.*, 2013). Thus, because of the presence of several primary and secondary metabolites in algal cells, microalgal biotechnology has received much interest, with its application in the energy, food, pharmaceutical and cosmetic industries (Olasehinde *et al.*, 2017).

Recently, microalgal biotechnology has proved that it is now possible to produce some carotenoids commercially through aquaculture (Cha *et al.*, 2008). These include, for instance, β-carotene from *Dunaliella*, astaxanthin from *Haematococcus*, and lutein from *Chlorophycean* strains (Cha *et al.*, 2008). Carotenoids are highly bioactive and are reported as potent free radical quenchers, singlet oxygen scavengers, and lipid antioxidants, thereby acting as photoprotectants under conditions of excessive light (Cha *et al.*, 2008). Some carotenoids such as β-carotene and lycopene may reduce the risk of cardiovascular diseases and certain cancers, whereas lutein and zeaxanthin may reduce the risk of eye disorders (Cha *et al.*, 2008). Other carotenoid, such as xanthophylls, extracted from *Chlorella ellipsoidea*, might be also useful as functional ingredients in the prevention of human cancers, since studies have shown that these species have antiproliferative effects, including induction of apoptosis *in vitro* cellular models (Cha *et al.*, 2008; Gardeva *et al.*, 2009). Astaxanthin, another carotenoid, produced by the microalga *Haematococcus pluvialis*, has several applications in nutraceuticals, cosmetics, food and feed industries and exhibits many

essential biological functions, including antioxidant activity and protection against lipid-membrane peroxidation of essential polyunsaturated fatty acids and proteins, DNA damage, and ultraviolet light effects (Cerón *et al.*, 2007). Studies have also shown that they may act through other mechanisms such as gap junction communication, cell growth regulation and modulation of gene expression (Cerón *et al.*, 2007; Cha *et al.*, 2008).

Cosmetics and nutraceuticals industries may also benefit from the use of microalgae biomass as a low cost renewable source of phytosterols and metal chelators compounds, due to its high unsaponifiable content (Gangadhar *et al.*, 2016).

There is also a growing interest in polyunsaturated fatty acids (PUFAs), due to their involvement in human health (Alonso *et al.*, 1998; Barreira *et al.*, 2015). Microalgae are potential sources of these long-chain PUFAs, especially for Eicosapentaenoic acid (EPA, 5,8,11,14,17-cis-eicosapentaenoic acid), which have beneficial effects in the prevention and treatment of certain medical conditions including coronary heart disease, blood platelet aggregation and several carcinomas (Belarbi *et al.*, 2000).

Oher reports have also revealed the anti-inflammatory (Guzman *et al.*, 2001), hypocholesterolemic (Dvir *et al.*, 2015) and antiviral (Huleihel *et al.*, 2002) activities of microalgal-derived extracts and compounds.

Another developing area in the use of microalgae is biodiesel production (Chisti, 2007; Damiani *et al.*, 2010; Pereira *et al.*, 2013a, 2013b) (Fig. 1.9), since they have a fast growth rate and high photosynthesis efficiency, allowing them to be industrially cultivated (Lu *et al.*, 2009). However, since rapid-growing cells contain less oil, biodiesel production from microalgae is not economically feasible yet (Lu *et al.*, 2009). To overcome these biological and technical challenges, Lu *et al.* (2009) did an approach to biodiesel production by a heterotrophic fermentation process with *C. protothecoides*, which produces maximum amounts of algal biomass rich in oil, mainly composed by more than 90% of fatty acids (Lu *et al.*, 2009).

Pereira et al. (2015), for instance, not only proved that microalgae can be a rich source of fatty acids, but also showed that microalgae contain molecules with relevant

bioactivities, including antioxidant, inhibition of BChE and tyrosines, cytotoxic and antileishmanial activities.

Figure 1.9 Biodiesel production by microalgae – closed system bioreactor. Source: http://www.global-greenhouse-warming.com/biodiesel-from-algae.html).

1.2.3. Seagrasses

Seagrasses are a group of flowering plants (angiosperms) with roots, leaves and rhizomes (Coles *et al.*, 2004). They are the only flowering plants that can live underwater and are less primitive than algae, whereby there are only about 60 species of seagrasses around the world (Coles *et al.*, 2004).

Seagrasses occur in protected bays and lagoons and also in deeper waters and the depth at them occurs is limited by water clarity, since most species require high levels of light (Coles *et al.*, 2004).

Although less exploited than algae, seagrasses also contain new commercially valuable phytochemicals (Achamlale *et al.*, 2009). Some of them are used as human

food or as raw material for the production of compounds with nutritional interest in Russia (Achamlale *et al.*, 2009). For example, Achamlale *et al.* (2009) showed that a bioactive pectin from the genus *Zostera*, zosterin, can decrease the toxicity of antitumour drugs and eliminate heavy metals from human organisms.

Others seagrasses such as *Cymodocea nodosa*, used in this work, has an important ecological role in the marine ecosystem, despite that, knowledge of its chemical content is limited (Kontiza *et al.*, 2008).

It has also been described other properties of seagrasses, such as antidepressant activity in humans, due especially to the biologically active compounds, the cyclitols (Kumar *et al.*, 2008; Nuissier *et al.*, 2008) and antiviral, including HIV-1, antioxidant, anti-inflammatory, anticarcinogenic, anti-allergenic and antithrombotic, due to the presence of rosmarinic acid (Achamlale *et al.*, 2009; Custódio *et al.*, 2016).

According to Kumar *et al.* (2008) the use of the roots of the seagrass *Enhalus acoroides* as a remedy against stings of different kinds of rays and scorpion is very popular in India; *Cymodocea spp.* is used as a tranquillizer for babies, as soothing help during pregnancy and against cough and malaria; *Halophila spp.* is a strong medicine against malaria and skin diseases and found to be very effective in early stages of leprosy.

1.2.4. Halophytes

Although halophytes represent a small fraction of the overall plant population (aprox. 2%), they display important roles in the environment, such as desalinization and prevention of soil erosion, loss of biodiversity and bioproductivity (Gago *et al.*, 2011). These plants can survive in different environments, such as salt marshes and estuaries, cliffs and dunes near the ocean, and some are adapted for near-desert environments where water supplies may be limited and highly saline (Gago *et al.*, 2011).

Halophytes are considered as good sources of food, fibre and bioenergy (Gago *et al.*, 2011). Some halophytes, such as *Salicornia ssp.*, *Aster tripolium*, *Atriplex ssp.* or *Inula crithmoides* are consumed in Europe as fresh or cooked gourmet foods, for example, in salads as a substitute of salt (Gago *et al.*, 2011; Ventura *et al.*, 2011). They have a high nutritional content, which includes proteins, carbohydrates, fiber, calcium, potassium, magnesium, iron, manganese, copper, vitamin C and β-carotene (Gago *et al.*, 2011).

It has been reported some important therapeutic applications of different halophyte species, as for example, *Salicornia spp.*, including immunomodulation, antioxidant and antitumor proporties (Chung *et al.*, 2005).

Recently, Medini *et al.* (2015) suggested the strong potential of the halophyte *Limonium densiflorum* as a source of phenolic compounds, marked as having great potencial in the food and pharmaceutical industry. In turn, *Arthrocnemum macrostachyum* is rich in phenolics and flavonoids and is also a potential source of antioxidants (Custódio *et al.*, 2012 (a); Rodrigues *et al.*, 2014). These secondary metabolites play different roles in the physiology and cellular mechanisms of plants, including pigmentation as well as resistance to pests, predators and oxidative stress (Barreira *et al.*, 2017). The stress conditions to which the halophytes are exposed (high salinities and UV radiation), usually trigger to a production of ROS and hence often have antioxidant capacity, attributed to their phenolic compounds (Barreira *et al.*, 2017; Ksouri *et al.*, 2008). This is the case of *H. italicum* subsp. *picardii* flowers, with similar or even higher antioxidant potential than the commercial green and herbal red teas, which has also showed moderate anti-diabetic potential and low toxicity in *in vitro* models (Pereira *et al.*, 2017a). In another study, Pereira *et al.* (2017b) also tested the leaves and the flowers infusions of the halophyte species *Crithmum maritimum* L. and observed its high antioxidant potencial.

Regarding to ChE inhibitors, Rodrigues *et al.* (2017) has identified a bioactive compound from *Juncus acutus*, junconol, and proved its capacity to inhibit the enzyme AChE on neuronal and glial cells *in vitro*.

1.3. Objective

The number of older people at risk of developing dementia is growing rapidly worldwide, and AD represents the most common cause of dementia in the elderly (Olasehinde, 2017).

The search for novel anticholinesterases compounds from natural resources as therapeutics agents for AD and other CNS disorders is based on the need for agents targeted to brain areas affected, with reduced toxicity and side-effects.

Marine natural products are considered as important sources of novel biologically active compounds, but its application in the treatment of neurological disorders is still rather unexplored. In this context, the objective of this study was to screen for the AChE and BChE inhibitory activity of some commonly available macro- and microalgae, seagrasses and halophytes species of the southern coast of Algarve (Portugal), as well as evaluate their chelating activity on iron and copper ions.

2. MATERIALS AND METHODS

In this chapter it is described the processes used in the evaluation of the algae, seagrasses and halophytes, as potential new sources of bioactive compounds with neuroprotective activity.

2.1. Plant material

The list of the species included in this work is presented in Table 2.1.

Macroalgae (Fig. 2.1) and seagrasses (Fig. 2.2) samples were collected on the Algarve coast in July-November 2009. Species identification was made by Dr. Aschwin Engelen (Centre of Marine Sciences, University of Algarve, Portugal). Samples were washed in seawater, kept cold until arrival to the laboratory, washed with tap water, freeze dried, ground with a coffee grinder and stored at -20°C.

Microalgae (Fig. 2.3) samples were provided by NECTON S.A. as a solid dark green frozen paste and were stored at -20°C.

The halophytes samples were collected in the Ria Formosa lagoon (Fig. 2.4), in July-August 2010, which is an area that extends 60 km along the southern coast of Portugal (Algarve), covering approximately 18,400 hectares (Gago *et al.*, 2011). Aerial parts were washed with tap water, dried at 40°C for 48h, ground with a coffee grinder and stored at room temperature. The taxonomical classification was performed by the botanist Dr. Manuel J. Pinto (National Museum of Natural History, University of Lisbon, Botanical Garden, Portugal).

Table 2.1. List of the species included in this work.

Macroalgae		
Brown	**Red**	**Green**
Cladostephus spongiosus	*Asparagopsis armata*	*Chaetomorpha sp.*
Cystoseira compressa	*Cladophora albida*	*Codium fragile*
C. humilis	*Jania sp.*	*Codium sp.*
C. nodicalis	*Peyssonnelia sp.*	*Enteromorpha sp.*
C. tamariscifolia	*Plocamium cartilagineum*	*Ulva sp.*
C. usneoides	*Pterocladiella capillacea*	
Dictyota dichotoma		
D. spiralis		
Halopteris scoparia		
Sargassum vulgare		
Taonia atomaria		

Microalgae	Seagrasses	Halophytes
Botryococcus braunii	*Cymodocea nodosa*	*Arthrocnemum macrostachyum*
Chlorella minutissima	*Zostera noltei*	*Carpobrotus edulis*
Isochrysis galbana T-ISO		*Frankenia hevipes*
Neochloris oleoabundans		*Mesebriantemum cristalinum*
Scenedesmus sp.		*Salicornia ramosissima*
		Salsola vermiculata
		Sarcocornia fruticosa
		Spartina maritima

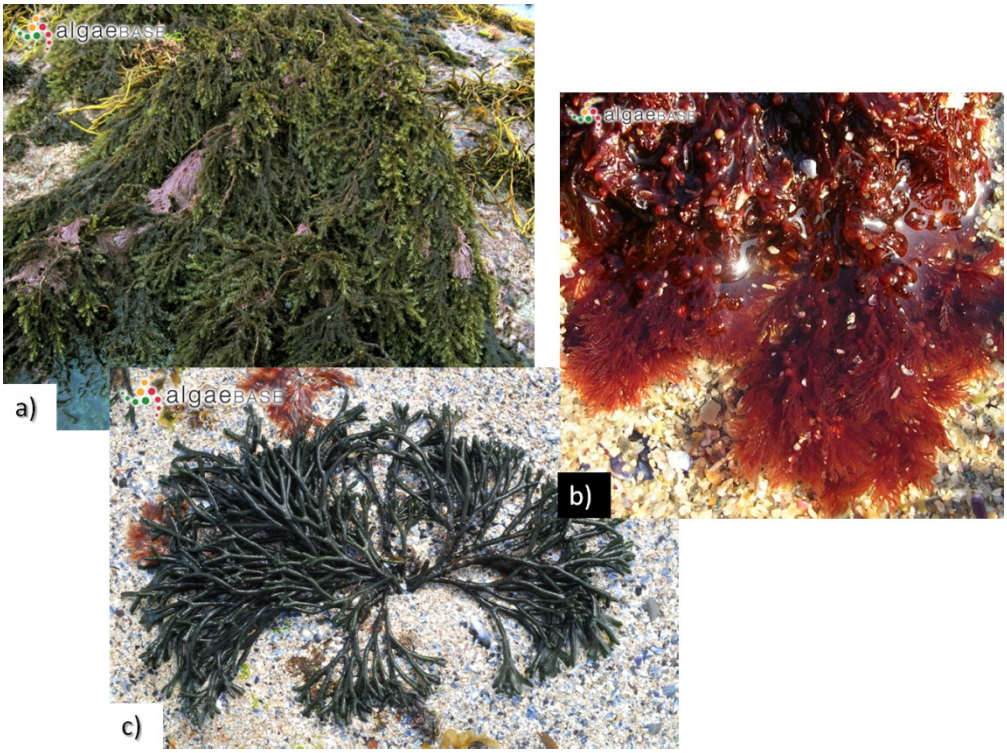

Figure 2.1 General aspects of some macroalgae species included in this work. *Cystoseira tamariscifolia* (a) (brown), *Plocamium cartilagineum* (b) (red) and *Codium fragile* (c) (green). Source: www.algaebase.org

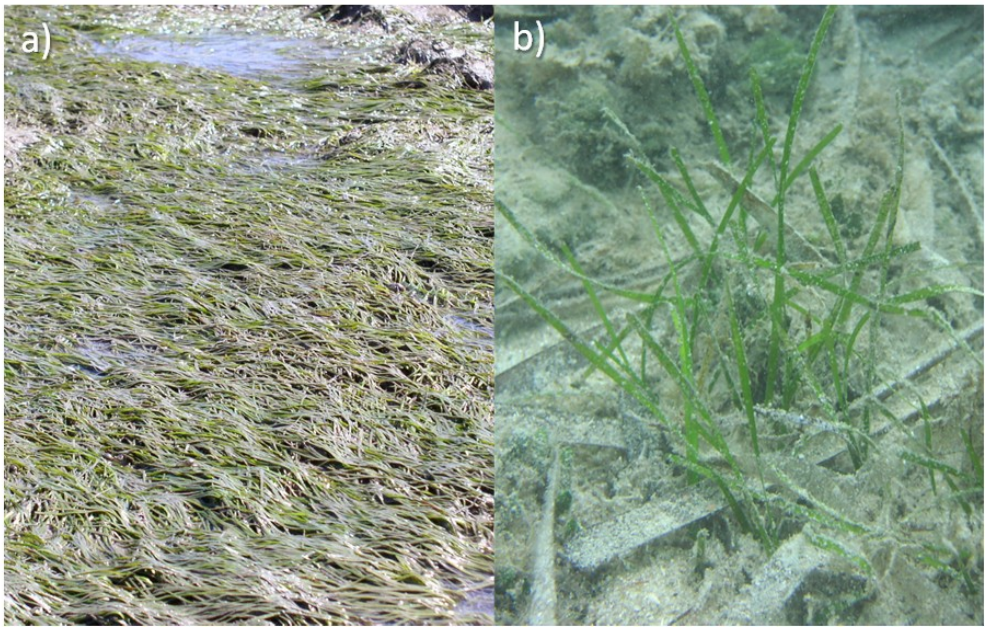

Figure 2.2 General aspect of the seagrasses included in this work. *Zostera noltei* (a) and *Cymodocea nodosa* (b) (Source: Borum *et al.*, 2004).

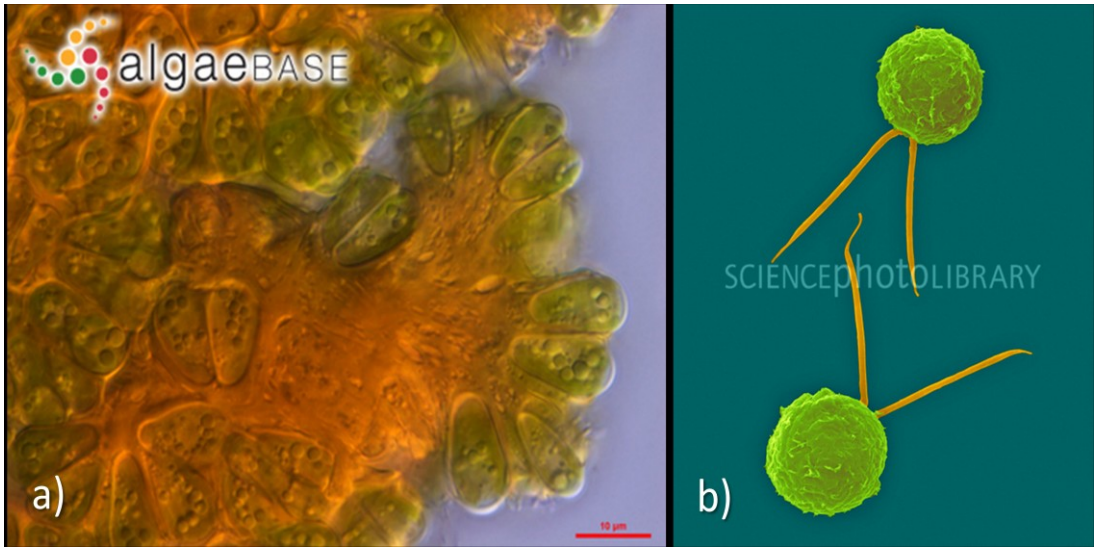

Figure 2.3 General aspects of some microalgae species included in this work. *Botryococcus braunii* (a) (Source: www.algaebase.org) and *Isochrysis galbana* (b) (Source: www.sciencephoto.com)

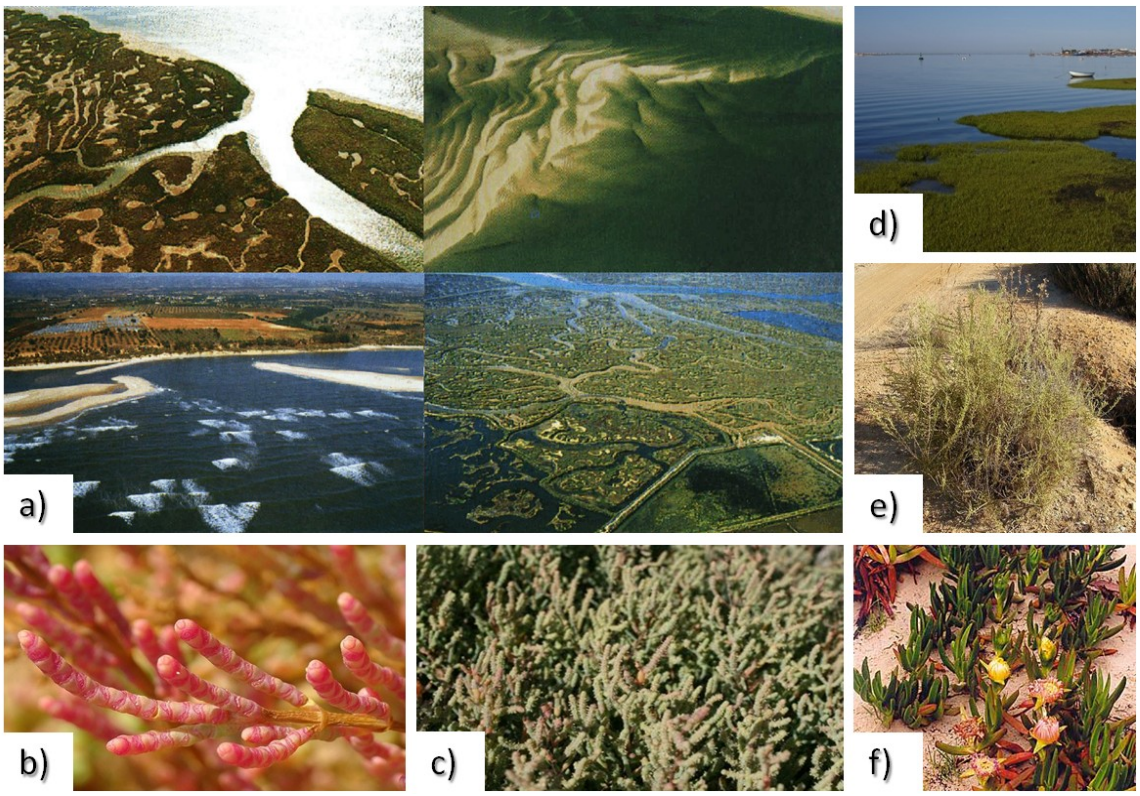

Figure 2.4 Ria Formosa lagoon, Algarve (a) (Source: http://algarvecom.blogspot.com) and some species of the halophytes species included in this work: *Salicornia ramossisima* (b) (Source: http://www.flickr.com), *Sarcocornia fruticosa* (c) (Source: http://plantas-e-pessoas.blogspot.com), *Spartina maritima* (d) (Source: http://portal.icnb.pt), *Salsola vermiculata* (e) (Source: http://www.flickr.com) and *Carpobrotus edulis* (f) (Source: http://o-blog-verde.blogs.sapo.pt).

2.2. Extraction

Aliquots (1 g) of milled samples were mixed with 20 mL of methanol and homogenized in an Ultra Turrax T25 (IKA Labortechnik Basic) in order to disrupt cells (1900 rpm in two cycles of 1 minute each). Then, the volume was made up to 40 mL with methanol, and samples were extracted for 16h at room temperature (RT, approximately 20^{o}C) with stirring.

The extracts were then centrifuged (3000 rpm, 15 min, 20°C, BECKMAN COULTER ALLEGRA 6R CENTRIFUGE), the upper layer carefully removed, filtered with Whatmann nº 4 filters and dried in a vacuum evaporator (temperature below 50°C, ROTAVAPOR R-114). Dried extracts were weight, resuspended in methanol at the concentration of 10mg/mL and stored at -20°C. Each extraction was repeated 3 times.

2.3. Metal chelating activity

The Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex according to Custódio *et al.* (2012, b).

Ferrozine can quantitatively form complexes with Fe^{2+}. However, in the presence of chelating agents, the complex formation is disrupted with the result that the purple colour of the complex is decreased. Measurement of colour reduction, therefore, allows for the estimation of the chelating activity of the coexisting chelator (Ebrahimzadeh *et al.* 2008).

In 96-well microplates, 30 µL of the extracts were added to 200 µL of distilled water and then immediately mixed with 30 µL of $FeCl_2$ (0.1 mg/ml water).

After 30 minutes, 12.5 µL of ferrozine solution (40 mM in water) was added.

Samples were then incubated at RT for 10 min. and the absorbance was measured in a microplate reader (BioTek Synergy 4) at 562 nm.

The Cu^{2+}-chelating activity was determined using pirocatechol violet (PV – indicator for metal titration) according to Saiga *et al.* (2003) and Megías *et al*. (2009).

In 96-well microplates, 30 µL of the extracts were mixed with 200 µL of Na acetate buffer (50 mM, pH 6), 100 µL of $CuSO_45H_2O$ (5×10^{-5} g/mL) and 6 µL of PV (4 mM in Na acetate buffer).

The complex of PV with $CuSO_4$ is blue and the colour changes to yellow when PV dissociates from Cu^{2+} ion in the presence of chelating agents. The absorbance was measured in a microplate reader (BioTek Synergy 4) at 632 nm.

Due to the color of the extracts, it was necessary to use a colour control, which absorbance was subtracted to the absorbance of the samples, thus only the values related to the complex ferrozine-Fe^{2+} or to the complex PV-$CuSO_4$, in iron and copper chelating activity, respectively. In the first case, the sample was added to 242.5 µL of distilled water, which volume is the same comprised by the distilled water, $FeCl_2$ and ferrozine. In the second case, the sample was added to 306 µL of buffer, which volume is the same comprised by buffer, $CuSO_45H_2O$ and PV.

The extracts were evaluated at the concentrations of 1, 5 and 10 mg/mL and results were expressed as percentage of chelating activity relative to a negative control containing methanol in place of the sample. A solution of the synthetic metal chelator ethylenediaminetetraacetic acid (EDTA) at the concentration of 1 mg/mL was used as positive control.

The percentage of chelating activity of Fe^{2+} and Cu^{2+} was determined by using the next formula:

$$\text{Fe}^{2+} \text{ or } \text{Cu}^{2+} \text{ chelating activity } (\%) = \left(\frac{\text{A}_{\text{control}} - \text{A}_{\text{sample}}}{\text{A}_{\text{control}}}\right) \times 100$$

Where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the sample, which was obtained by subtracting the absorbance of the colour control.

2.4. AChE and BChE inhibitory activity

The AChE and BChE inhibitory activities were assessed by the Ellman's colorimetric assay (Ellman *et al.*, 1961), according to previously described methods (Orhan *et al.* 2006, 2009; Custódio *et al.*, 2012 (b)).

Briefly, 140 µL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µL of the extracts at the concentrations of 1, 5 and 10 mg/mL and 20 µL of AChE or BChE (0.28 U/mL) solution were mixed in 96-well microplates and incubated at RT for 15 min. Then, 10 µL of the substract acetylthiocholine iodide (AChI) or butyrithiocoline iodide (BChI) (4 mg/mL) were added to initiate the reaction, together with 20 µL of a solution of the dye 5,5-Dithio-bis (2-nitrobenzoic) acid (DTNB) at the concentration of 1.2 mg/mL.

The hydrolysis of AChI and BChI was monitored by the formation of the yellow 5-thio-2-nitrobenzoato anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm using 96-well microplate reader (BioTek Synergy 4).

Results were expressed as percentage of inhibitory activity relative to a negative control containing methanol in place of the sample. Galanthamine was used as reference at the concentration of 1 mg/mL.

The percentage of inhibition of AChE and BChE was determined by using the formula:

$$\text{AChE or BChE inhibitory activity } (\%) = \left(\frac{\text{A}_{\text{control}} - \text{A}_{\text{sample}}}{\text{A}_{\text{control}}}\right) \times 100$$

Where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the samples obtained by subtracting the absorbance of the colour control.

2.5. Statistical analysis

All the experiments were carried at least in triplicate and the results were expressed as mean ± standard deview (SD).

One-way analysis of variance (ANOVA) was used to compare the mean values of each method, using STATISTICA for Windows (release 7, STATISTICA INC).

A significant difference between the means of parameters was determined by using Kruskal-Wallis multiple comparison tests. A p-value of less than 0.05 was considered significant.

3. RESULTS

This chapter is divided in two sections, one for the analysis of chelating activity on iron and copper and other for the analysis of inhibitory activity on AChE and BChE.

3.1. Iron and copper chelating activity

3.2. ChE inhibitory activity

3.1. Iron and copper chelating activity

In this work we applied the classification suggested by Vinutha *et al.* (2007) for the AChE inhibitory activity to the metal chelating activity, to make easier the interpretation of the results. In this sense, metal chelating activity was classified as: potent (>50% activity), moderate (30–50% activity), low (<30% activity) or nil (<5% activity).

3.1.1. Fe^{2+} chelating activity

In the used method, the higher the chelation of ions by the sample, the smaller the number of ions available for reaction with ferrozine, so the reaction does not get the purple colour characteristic of the complex Fe^{2+}-ferrozine. Thus, the lower the absorbance of the reaction mixture, the higher the Fe^{2+}-chelating ability (Fig. 3.1).

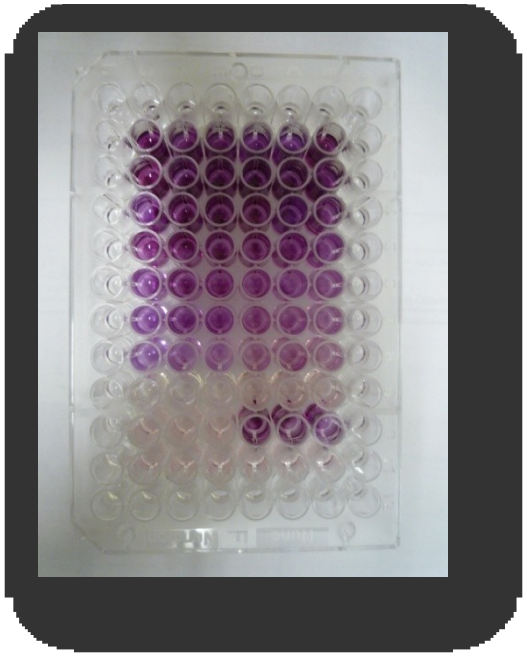

Figure 3.1 Example of 96-well microplates with an iron chelating assay employing methanol extracts with low Fe^{2+} chelating activity. The concentrations of the extracts are increasing from top to bottom. The darker the reaction, the lower the chelating activity of the tested extract.

3.1.1.1. Macroalgae

The chelating activity varied between groups of macroalgae and significant differences were found between them (Fig. 3.2).

The best results were obtained in the group of red macroalgae, followed by the brown macroalgae and for last, the green macroalgae, where no relevant activities were detected (Fig. 3.2).

Regarding brown macroalgae, the species *D. dichotoma* displayed the highest iron chelating potential, with a value of 52.7% at 10 mg/mL (Fig. 3.2). The species *P. cartilagineum* (red) also had a potent chelating activity (83.4% at 10 mg/mL), while for the group of green macroalgae, the maximum value was obtained with the species *Enteromorpha sp* (30.5%; Fig. 3.2) at the highest concentration tested.

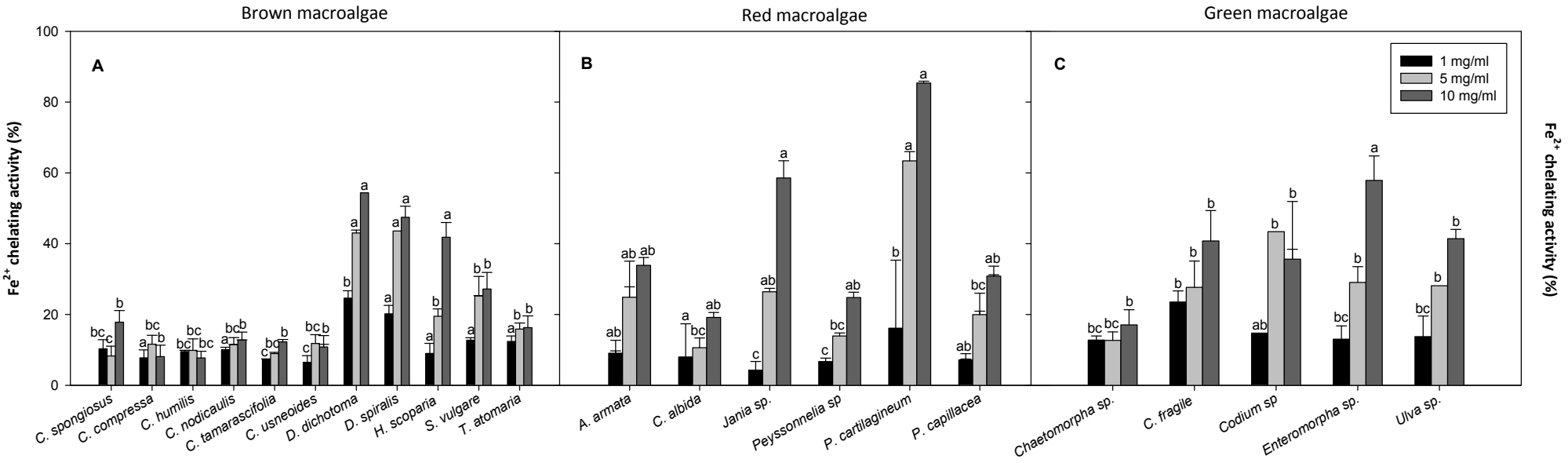

Figure 3.2 Fe^{2+} chelating activity (%) of methanol extracts of brown (A), red (B) and green (C) macroalgae species. For the same group (brown, red or green), bars labelled with different letters are significantly different at $p<0.05$ (Kruskal-Wallis multiple comparison test).

3.1.1.2. Microalgae

The species *I. galbana* was the most active followed by *B. braunii* with maximum values of 64.4% and 59.7% at the concentration of 10 mg/mL, respectively (Fig. 3.3).

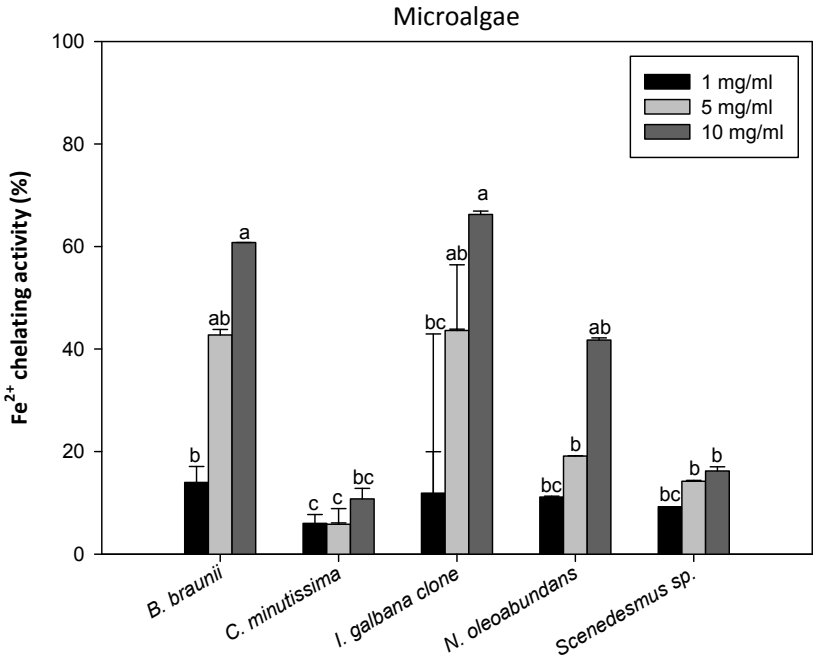

Figure 3.3 Fe^{2+} chelating activity (%) of methanol extracts of microalgae species. Bars labelled with different letters are significantly different at $p<0.05$ (Kruskal-Wallis multiple comparison test).

3.1.1.3. Seagrasses

None of the seagrasses tested had relevant capacity to chelate iron (Fig. 3.4).

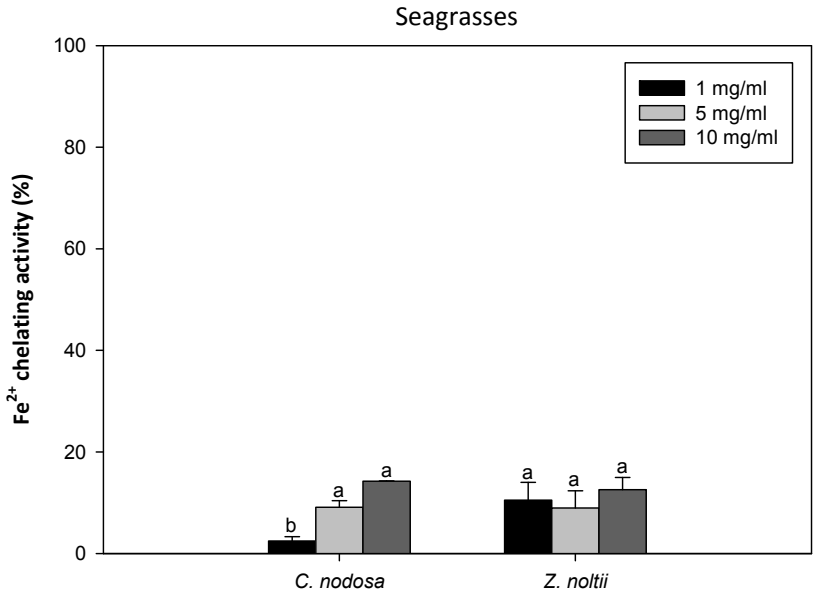

Figure 3.4 Fe^{2+} chelating activity (%) of methanol extracts of *C. nodosa* and *Z. noltei* species. Bars labelled with different letters are significantly different at $p<0.05$ (Kruskal-Wallis multiple comparison test).

3.1.1.4. Halophytes

Two species had moderate capacity to chelate iron at 10 mg/mL, namely *M. cristalinum* (35.6%) and *S. ramosissima* (38%) (Fig. 3.5).

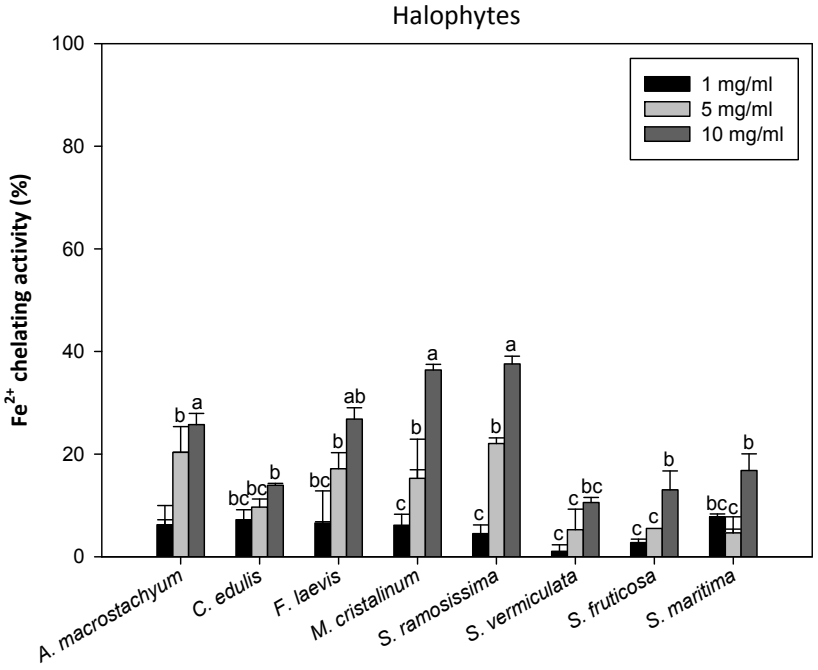

Figure 3.5 Fe^{2+} chelating activity (%) of methanol extracts of halophytes species. Bars labelled with different letters are significantly different at $p<0.05$ (Kruskal-Wallis multiple comparison test).

3.1.2. Cu^{2+} chelating activity

In this assay, it is observed a decrease in the characteristic blue colour of the complex Cu^{2+}-PV to a yellowish colour with the increase of the chelating activity of the tested sample (Fig. 3.6).

In general, the extracts had the capacity to chelate Cu^{2+}, and this activity was concentration dependent, increasing with increasing concentrations of the extracts (Fig. 3.6).

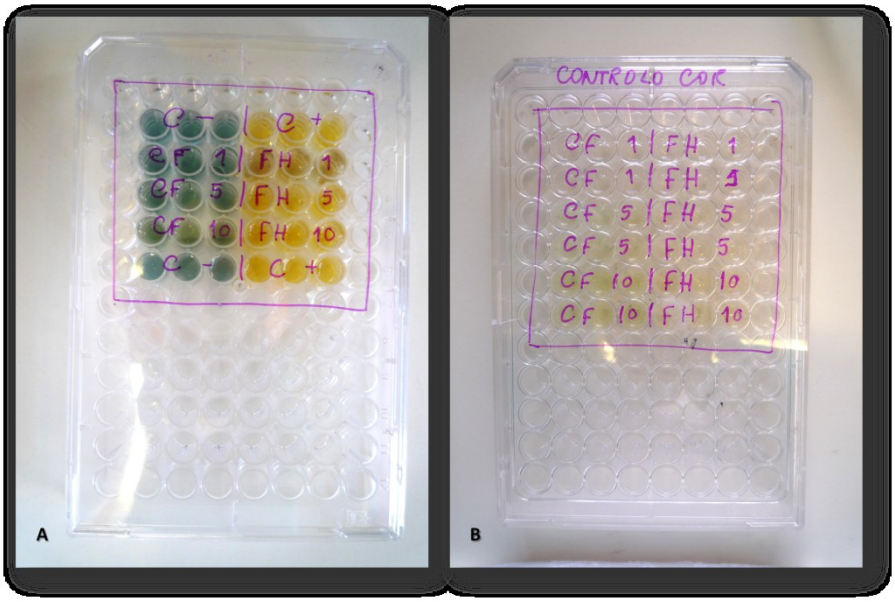

Figure 3.6 General aspect of a copper chelation assay made in 96-well microplates. Plate A) negative control (C-); positive control (C+); methanol extracts of the green macroalgae *C. fragile* at the concentrations of 1 (CF1), 5 (CF5) and 10 (CF10) mg/mL; methanol extracts of the halophyte *F. laevis* at the concentrations of 1 (FH1), 5 (FH5) and 10 (FH10) mg/mL. Plate B) colour controls made to the assays using the species in plate A.

3.1.2.1. Macroalgae

The copper chelating activity was significantly higher in brown and red macroalgae extracts (Fig. 3.7).

Regarding brown algae, the best result was obtained with the species *H. scoparia* (64.0%) at the concentration of 10 mg/mL (Fig. 3.7). Three of the red macroalgae tested had potent activities, namely *C. albida* (62.7%), *P. cartilagineum* (63.9%) and *P. capillacea* (62.4%) at the concentration of 10 mg/mL (Fig. 3.7). The green species *Enteromorpha sp.* exhibited potent ability to chelate copper ions (56.1%), at the concentration of 10 mg/mL (Fig. 3.7).

Of all the macroalgae tested, the best preventing the formation of the complex Cu^{2+}-PV was the brown algae *H. scoparia* and the red algae *P. cartilagineum and P. capillacea*, with similar activities.

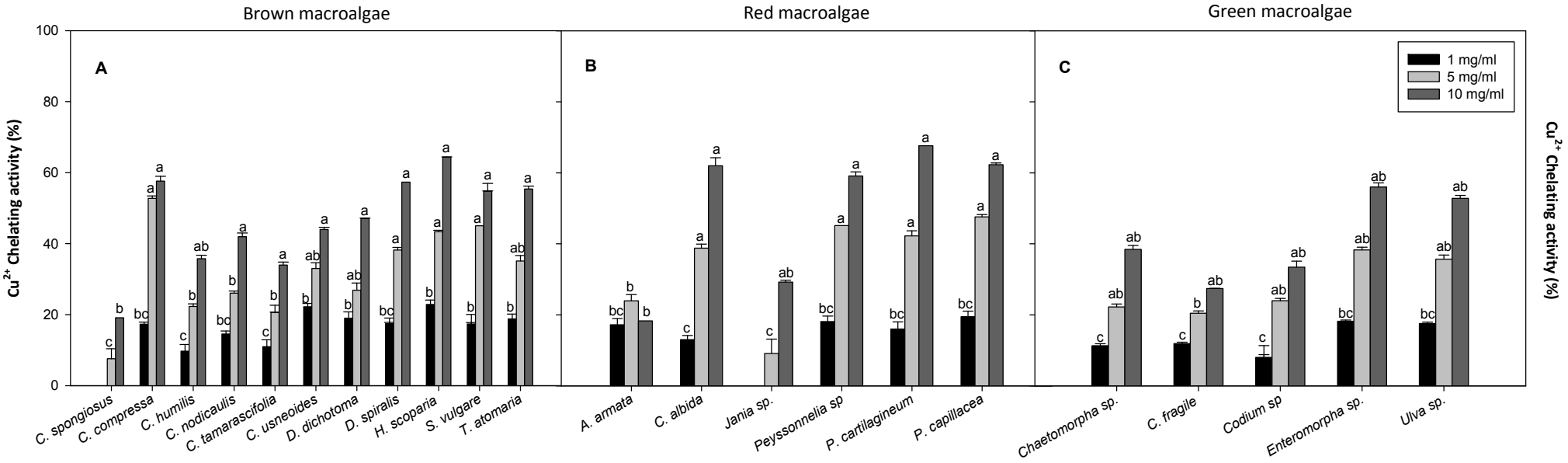

Figure 3.7 Cu^{2+} chelating activity (%) of methanol extracts of brown (A), red (B) and green (C) macroalgae species. For the same group (brown, red or green), bars labelled with different letters are significantly different at $p<0.05$ (Kruskal-Wallis multiple comparison test).

3.1.2.2. Microalgae

Four of the five species tested had more than 40% of Cu^{2+} chelating capacity even at a concentration of 5 mg/mL and in the case of *I. galbana* and *N. Oleoabundans,* a potent activity was observed at the higher concentration tested, 69,7% and 67,5%, respectively (Fig. 3.8).

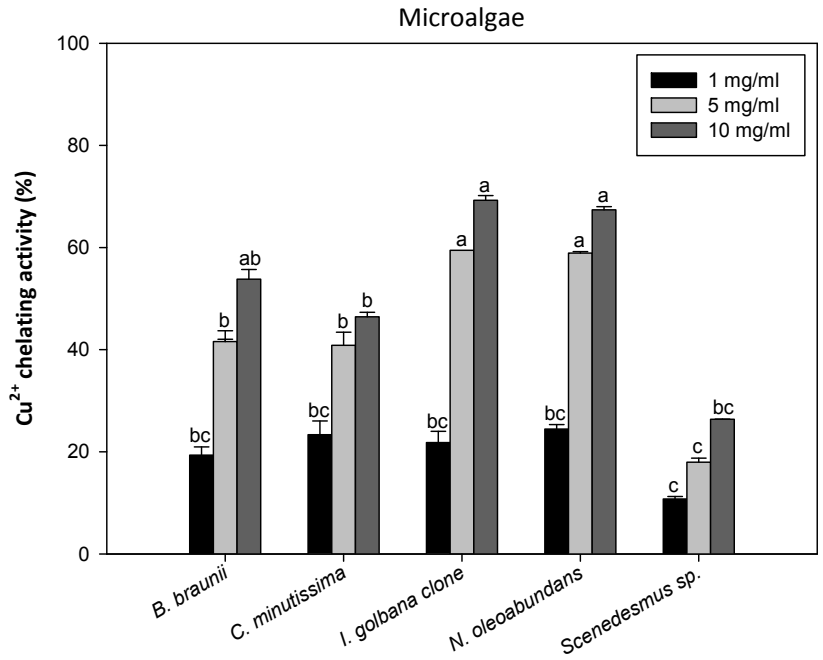

Figure 3.8 Cu^{2+} chelating activity (%) of methanol extracts of microalgae species. Bars labelled with different letters are significantly different at $p < 0.05$ (Kruskal-Wallis multiple comparison test).

3.1.2.3. Seagrasses

The species *Z. noltei* and *C. nodosa* exhibited potent and moderate capacity to chelate Cu^{2+} ions, with values of 54.8% and 49.3%, respectively, for the highest concentration tested (Fig. 3.9).

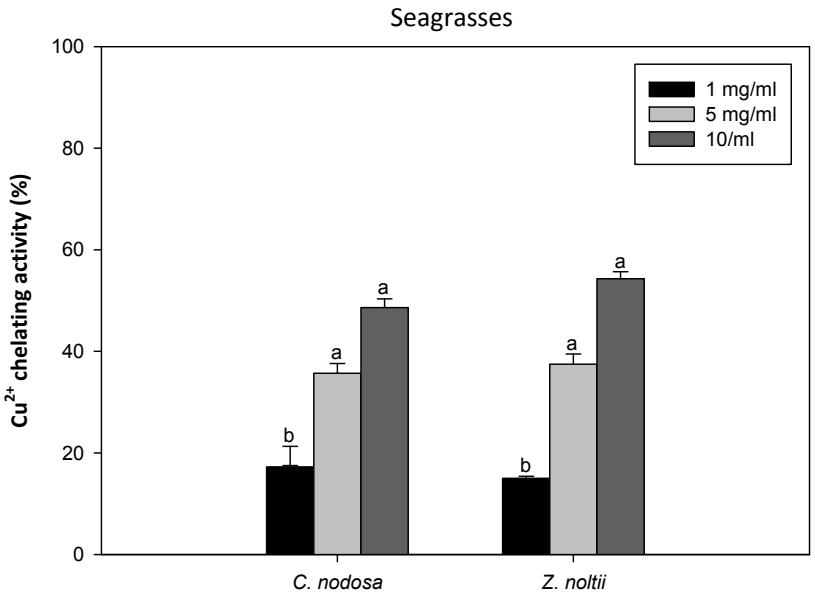

Figure 3.9 Cu^{2+} chelating activity (%) of methanol extracts of *C. nodosa* and *Z. noltei* species. Bars labelled with different letters are significantly different at $p < 0.05$ (Kruskal-Wallis multiple comparison test).

3.1.2.4. Halophytes

Halophytes species displayed the highest values of Cu^{2+} chelation. In fact, 75% of the species had a potent capacity to chelate Cu^{2+} and the best result was obtained with *F. laevis* with a potent activity even at the lowest concentration tested (1 mg/mL: 73.9%) (Fig. 3.10).

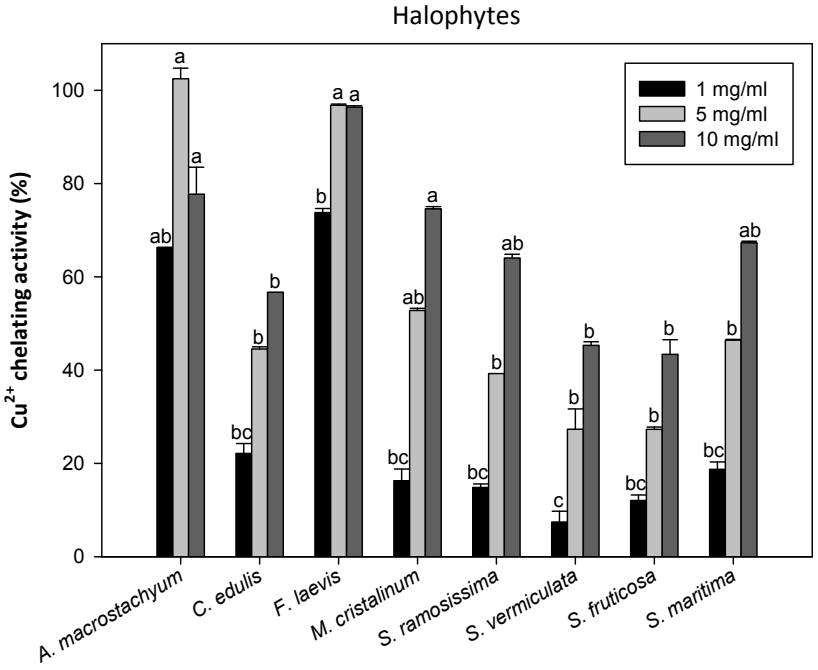

Figure 3.10 Cu^{2+} chelating activity (%) of methanol extracts of halophytes species. Bars labelled with different letters are significantly different at $p < 0.05$ (Kruskal-Wallis multiple comparison test).

Compared with iron, the chelating activity was higher for copper in the majority of the cases.

3.2. ChE Inhibitory activity

The inhibitory activity (%) of AChE and BChE was also classified according to Vinutha *et al.* (2007) as potent (>50%), moderate (30-50%), low (<30%) or nil (<5%).

3.2.1. AChE inhibitory activity

The AChE inhibitory activity of the different species is summarized in Fig. 3.11 for macroalgae, Table 3.1 for microalgae, Table 3.2 for seagrasses and Fig. 3.12 for halophytes.

In general, the group with most potent capacity to inhibit AChE was the group of brown macroalgae.

3.2.1.1. Macroalgae

The AChE inhibitory activity of the extracts varied significantly among the three types of macroalgae tested, being highest in the group of brown macroalgae and lowest in the green macroalgae group (Fig. 3.11).

The brown species *C. compressa* and *C. tamariscifolia* had the highest AChE inhibition, with high values at the lower concentration tested (1 mg/mL), 71.6% and 70.8%, respectively (Fig. 3.11).

In the rodophyta group, only the specie *A. armata* displayed a potent inhibition (58.6%) at the concentration of 10 mg/mL (Fig. 3.11). Generally, all the chlorophyta species displayed nil or low AChE inhibitory activity (Fig. 3.11).

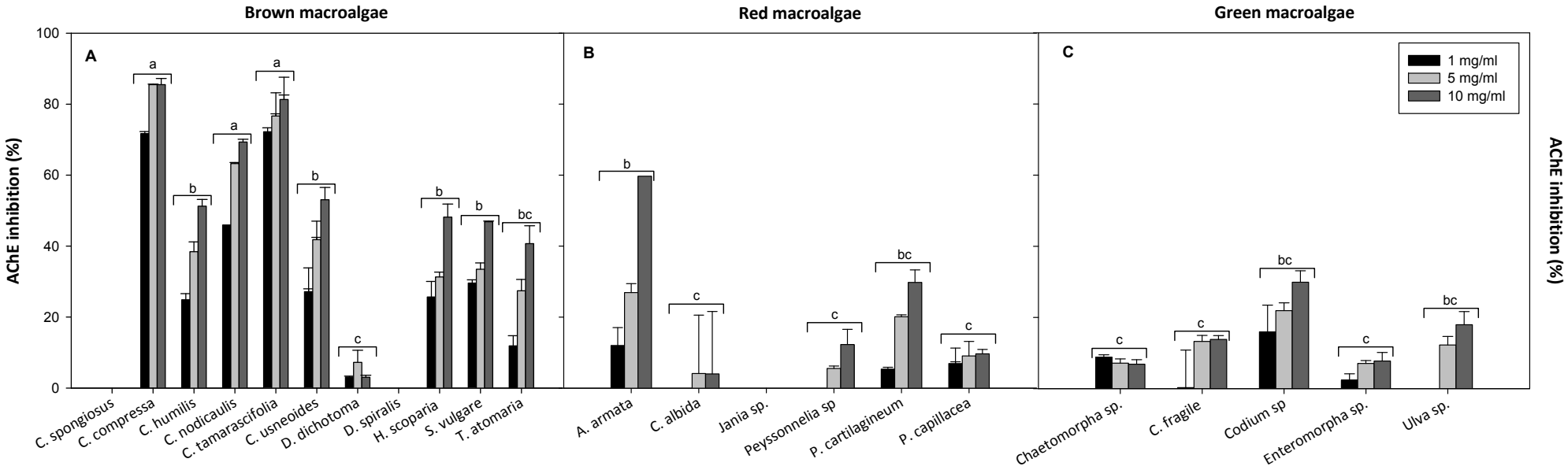

Figure 3.11 AChE inhibitory activity (%) of methanol extracts of brown (A), red (B) and green (C) macroalgae species. Bars groups labelled with different letters are significantly different at $p<0.05$ (Kruskal-Wallis multiple comparison tests).

3.2.1.2. Microalgae

All the species of microalgae tested showed nil or low inhibitory activity against AChE (Table 3.1).

Table 3.1. AChE inhibitory activity (%) of methanol extracts of microalgae species.

	AChE INHIBITORY ACTIVITY (%)		
Concentration / Microalgae	**1 mg/mL**	**5 mg/mL**	**10 mg/mL**
B. braunii	9.7 ± 1.8[a]	6.0 ± 1.1[a]	n.a.
C. minutissima	n.a.	n.a.	n.a.
I. galbana T-ISO	13.0 ± 2.4[a]	16.0 ± 2.7[a]	16.2 ± 3.4[a]
N. oleoabundans	n.a.	n.a.	n.a.
Scenedesmus sp.	n.a.	n.a.	n.a.
Galanthamine[a]	90.5 ± 0.6		

[a] Positive control (1 mg/mL). Data presented as mean ± SD of at least 3 independent experiments.
n.a. – No activity. In the same row values labelled with different letters are significantly different at $p < 0.05$ (Kruskal-Wallis multiple comparison test).

3.2.1.3. Seagrasses

The species *Z. noltei* had no inhibitory effect on AChE, while *C. nodosa* exhibited a moderate activity at the concentration of 10 mg/mL (Table 3.2).

Table 3.2. AChE inhibitory activity (%) of methanol extracts of seagrasses species.

	AChE INHIBITORY ACTIVITY (%)		
Concentration / Seagrasses	**1 mg/mL**	**5 mg/mL**	**10 mg/mL**
C. nodosa	13.0 ± 1.95[c]	29.3 ± 2.60[b]	36.7 ± 1.18[a]
Z. noltei	n.a.	n.a.	n.a.
Galanthamine[a]	90.5 ± 0.6		

[a]Positive control (1 mg/mL). Data presented as mean ± SD of at least 3 independent experiments.
n.a. – No activity. In the same row values labelled with different letters are significantly different at $p < 0.05$ (Kruskal-Wallis multiple comparison test).

3.2.1.4. Halophytes

The most bioactive species were *C. edulis* and *F. laevis* with potent inhibitory activity at the lowest concentration tested, 56.4% and 78.3%, respectively (Fig. 3.12).

With the exception of *A. macrostachyum*, which showed a moderate activity at the concentrations of 5 and 10 mg/mL (Fig. 3.12), all the other species had low or nil activities at those concentrations (Fig. 3.12).

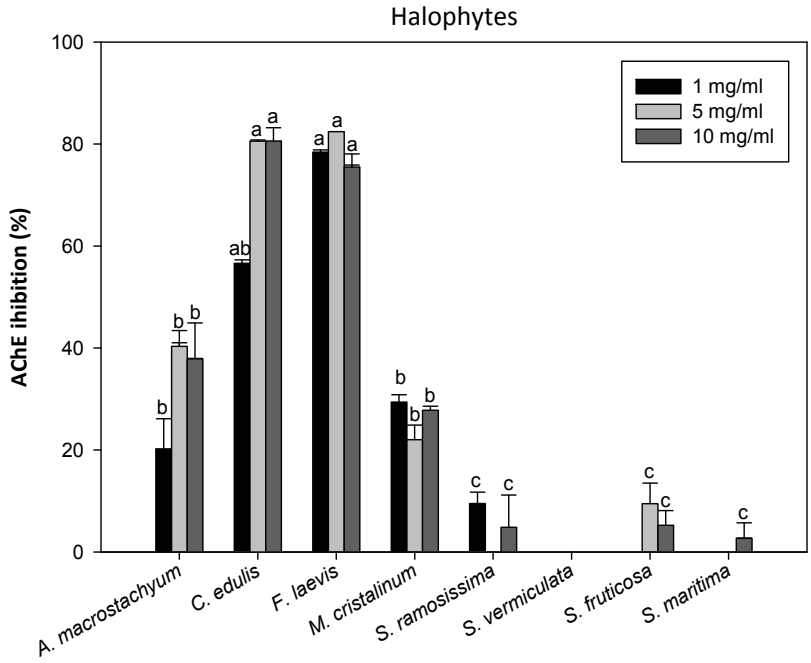

Figure 3.12 AChE inhibitory activity (%) of methanol extracts of halophytes species. Bars labelled with different letters are significantly different at $p < 0.05$ (Kruskal-Wallis multiple comparison test).

3.2.2. BChE inhibitory activity

In order to further explore the ChE inhibitory properties of the active extracts, those with potent activity (>50%) against AChE were tested on BChE and the results are summarized on Fig. 3.13.

The highest inhibitory activity against BChE was achieved with the application of the extracts from the *Cystoseira* genus, in particular the species *C. nodicaulis* (92.2%) and *C. tamariscifolia* (100%) and also with the halophytes species *C. edulis* (76.8%) and *F. laevis* (93.2%; Fig. 3.13), all applied at the concentration of 10 mg/mL.

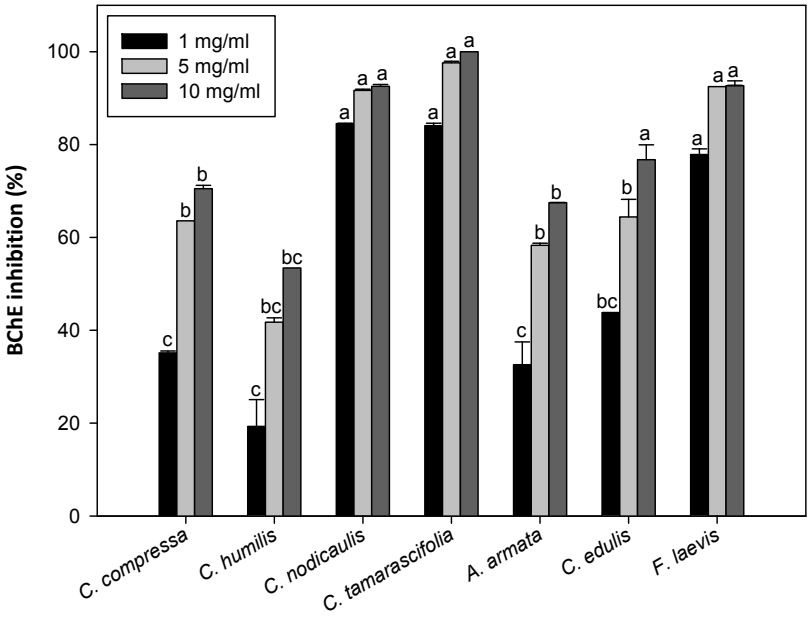

Figure 3.13 BChE inhibitory activity (%) of methanol extracts of 4 brown macroalgae, 1 red macroalgae and 2 halophytes. Bars labelled with different letters are significantly different at $p < 0.05$ (Kruskal-Wallis multiple comparison test).

4. DISCUSSION

Here the results are discussed and compared with the literature.

4.1. Iron and copper chelating activity

4.2. AChE and BChE Inhibitory activity

4.1. Iron and copper chelating activity

One of the factors that can cause neurodegenerative diseases such as AD, PD and Huntington's disease can be the total iron and copper concentration increasing in some brain regions (Brewer, 2010).

Iron might have a direct impact on plaque formation, due to its action as a modulator in α-secretase to cleave APP (Huang *et al.* 2000; Rodgers *et al.*, 2002; Kawahara, 2003; Zecca *et al.*, 2004). In turn, *tau* protein, a component of neurofibrillary tangles, binds copper, required for its aggregation (Soragni *et al.*, 2008).

Moreover, these transition metals generate free radicals from peroxides *via* Fenton reactions and, therefore, the reduction of their levels can offer protection against oxidative damage (Rival *et al.*, 2001).

In this work, the chelating activity was higher for copper than in iron in the majority of the cases and the most promising was the species *P. cartilagineum* (Fig. 3.2).

There are some studies reporting the iron chelating properties of different macroalgae species. The methanolic extracts of the green macroalgae, *Ulva rigida*, and the brown macroalgae, *Fucus spiralis*, collected from Marocco coastal area, displayed interesting iron chelating capacity (Chernane *et al.*, 2014).

Extracts using chloroform, methanol, petroleum ether and ethyl acetate from the red macroalga *Asparagopsis taxiformis*, collected in the south coastal of India, have also displayed relevant values of Fe^{2+}-chelating activity (Neethu *et al.*, 2017).

In a study by Alencar *et al.* (2016) a moderate Fe^{2+}-chelating activity was observed in ethanol and hexane extracts of the red macroalgae *Pterocladiella capillacea*.

The green macroalgae *Ulva faciata* and the red *Gracilaria corticata* can also highly chelate ferrous ions, especially extracts using dichloromethane and ethylacetate (Taheri, 2016).

There are very few reports of research on microalgae capacity to chelate metals. Custódio *et al.* (2012b) observed that hexane extracts of microalgae species namely *Tetraselmis chuii*, *Nannochloropsis oculata*, *Chlorella minutissima* and *Rhodomonas* could chelate Cu^{2+} ions. In another study, water extract from *N. oculata* also showed a relevant Cu^{2+}- chelating activity (Custódio *et al.*, 2014).

More recently, Trabelsi *et al.* (2016) tested the microalgae *Graesiella sp.* and find its elevated potencial on Fe^{2+}-chelating activity.

A few publications were found about the metal chelating capacity of seagrasses and halophytes. El & Karakaya (2004), who tested some greens used as traditional dishes in Mediterranean diet, including the halophyte *Salicornia europaea* (methanolic extracts) observed that this species had the capacity to chelate iron. Besides, methanolic extract of the halophyte *Limonium algarvense*, also collected in the south of Portugal, showed a relevant copper chelating activity (Rodrigues *et al.*, 2015).

All together the results suggests that the species *P. cartilagineum* and *I. galbana* could be sources of natural products and / or compounds with metal chelation potential, and thus, with interest in the management of oxidative stress-linked neurological diseases.

4.2. AChE and BChE Inhibitory activity

There has been some research on the biological effect of plants traditionally used either in infusions or in traditional remedies as AChE inhibitors *in vitro* and also as memory enhancers *in vivo* (Tildesley *et al.*, 2003). These studies are carried out in order to find new molecules or a group of molecules that can be used in the AD therapy without the toxicity of the synthesized chemical compounds (Ferreira *et al.*, 2006).

Marine resources, particularly macroalgae, are a promising source of biocompounds with a wide range of bioactivities, including neuroprotective effects (Barbosa *et al.* 2014). Despite this, some authors, as for example, Grosso *et al.* (2014), believe that few of these sources will be successfully marketed, since its consumption could jeopardize the marine ecosystem's sustainability (Barbosa *et al.* 2014). In order to overcome this problem, it is crucial to implement new and greener strategies like efficient cultivation techniques (Reddy *et al.*, 2008).

From the results obtained in this work, it is clear that, regarding macroalgae, the extracts of the brown algae *C. compressa*, *C. nodicaulis* and *C. tamariscifolia* are capable to inhibit both AChE and BChE *in vitro*. Natural extracts with dual anti-ChE activity may be appropriate to patients at a moderate stage of AD, where the level of AChE use has not yet significantly declined but where the possibility that BChE could hydrolyse ACh exists (Mesulam et al., 2002). In adition, the use of compounds with dual ChE inhibition are reported to increase the efficacy of the treatment and expand their uses to other disorders from the CNS, as for example Down syndrome and also to patients suffering from traumatic brain injuries (Giacobini, 2004).

In a previous report, Andrade *et al*. (2013) evaluated the AChE and BCHE inhibitory properties of ethanol extracts of different brown, red and green algae. Contrary to our results those authors obtained a strong AChE inhibition in extracts from *C. usneoides* and no activity in those of *C. tamariscifolia* and *A. armata*. Still in that work, a moderate activity against BChE was observed after application of samples

from *C. tamariscifolia*. The differences between our funding and those from other authors are most probably due to the different collection sites and / or exctration methods.

The high ChE inhibitory capacity of *C. tamariscifolia*, *C. compressa* and *C. nodicaulis*, can be related with the presence of phenolic compounds in these species (Custódio *et al.*, 2016). Phenolic compounds are one of the most therapeutically useful molecules due to their capacity to prevent oxidative stress-mediated disorders, including neurodegenerative disorders (Rodríguez-Morató *et al.*, 2015). In algae phenolic compounds are related with the defence of the thallus against for example UV-induced damage and herbivory (Abdala-Díaz *et al.*, 2006). *Cystoseira tamariscifolia*, one of the most active species, was previously reported to contain high levels of hydroxycinnamic acids, which were reported as neuroprotective agents in *in vivo* studies in rats and mice (Cheng *et al.*, 2008; Tsai *et al.*, 2011).

There are other reports on the inhibition of AChE and BChE by different extracts of brown macroalgae, such as ethanolic extracts of the species *Ecklonia stolonifera* (Yoon *et al.*, 2008) and chloroform extracts of the *Sargassum sagamianum* (Choi *et al.*, 2007; Natarajan *et al.*, 2009) and from methanolic extracts of the green *Ulva reticulata* (Suganthy *et al.*, 2010). The last species, *U. reticulata*, exhibited a higher inhibitory activity towards AChE and BChE than the anti-AChEs in current clinical use, making this seaweed an interesting potential source of compounds for AD management (Choi *et al.*, 2007; Natarajan *et al.*, 2009).

The following macroalgae were also found to be capable to inhibit AChE: *Ishige okamurae*, *Dictyota humifusa* and *Padina gymnospora* (brown) and *Hypnea valentiae* and *Gracilaria edulis* (red) (Stirk *et al.*, 2007; Yoon *et al.*, 2009; Suganthy *et al.*, 2010). Moreover, *H. valentiae* (red), *Enteromorpha intestinalis* (green) and *Dictyota dichotoma* (brown) displayed a strong BChE inhibitory activity (Suganthy *et al.*, 2010).

Kannan *et al.* (2013) have proved that extracts using methanol, hexane, dichloromethane, ethyl acetate and butanol of the brown macroalga *Ecklonia maxima*,

from the coast of South Africa, display great AChE inhibitory activity, probably because of the phlorotannins, compounds with a large molecular size and a big number of hydroxyl groups, which are able to modulate the interaction with AChE and consequent inhibition of the enzyme.

In this work no relevant ChE inhibory capacity was detected on extracts from microalgae. There are few reports on the AChE inhibitory potential of microalgae species. Contrary to our results in a previous work (Custódio *et al.*, 2012 (b)), it was observed that an hexane extract from *Chlorella minutissima* had high capacity to inhibit AChE, while ether and water extracts from *Scenedesmus* spp and *I. galbana*, and ether and hexane extracts from *B. braunii* were able to inhibit AChE (Custódio et al., 2014, 2015).

These differences maybe ascribed to the different extracts tested, that allows for the extraction of compounds with different inhibitory capacity against cholinesterases (Custódio et al., 2012 (b), 2014, 2015).

Moreover, it is also known that the composition of bioactive compounds of a natural extract obtained from algae varies significantly with the conditions to which the organisms are subjected such as algae size, age, tissue type, salinity, season, nutrient levels, light intensity and water temperature (Lopes *et al.*, 2012).

The seagrasses included in this work showed no capacity to inhibit AChE or BChE. Similar results were obtained with methanol crude extracts and fractions of *Z. noltei* (Custódio *et al.*, 2016).

As for the halophyte species*, C. edulis* and *F. laevis* had the highest dual inhition against both enzymes. Similar results were previously reported for methanol extracts from *C. edulis* (Custódio *et al.*, 2012 (a); Rocha *et al.*, 2017), and the activity can be related with its high phenolic content (Rocha *et al.*, 2017).

Carpobrotus edulis, usually called sour fig, is a medicinal and edible species native to the coast of South Africa. Sour fig has different traditional uses, as for

example, for the treatment of diarrhoea, tuberculosis, diabetes mellitus, to treat gum infections and burn wounds (Van Wyk, 2008; Van Wyk *et al.*, 1997; Van Der Watt and Pretorius, 2001; Thring and Weitz, 2006; Martins *et al.*, 2011; Ksouri *et al.*, 2012; Omoruyi *et al.*, 2012). This species was introduced in the southern and western Europe, including Portugal, for landscaping and to stabilize coastal sand dunes. Nevertheless, it became invasive mainly due to its high successful reproduction and dispersal capacity.

Sour fig is edible and has a nutritional profile adequate for human consumption (Rocha *et al.*, 2017). Moreover, it has a high antioxidant potential and exhibits multiple *in vitro* neuroprotective features (Rocha *et al.*, 2017) which suggests that it is a potential source of natural products able to improve cognitive functions.

5. CONCLUSION AND FUTURE PERSPECTIVES

Presentation of the conclusions of this work, according to the most significant results obtained and future perspectives.

This study provides data regarding the *in vitro* metal chelating and cholinesterase inhibitory activities of several species of algae, includind macro- and micro-, seagrasses and halophytes species.

The objectives of this thesis have been successfully achieved, since several species displayed relevant activities and thus, were identified as interesting potential sources of innovative products and /or molecules able to improve cognitive functions and, thus, for the management of neurodegenerative diseases.

Specifically, the extracts of the brown macroalgae species *C. compressa*, *C. nodicaulis* and *C. tamariscifolia* and from the halophytes *C. edulis* and *F. laevis* displayed both AChE and BChE inhibitory activities and may be a potential source of novel products useful in alleviating the symptoms associated with AD and other neurogenerative ailments. Also the red algae *P. cartilagineum* and the microalgae *I. galbana* may contain strong metal-chelating compounds. Thus, these species are promising candidates for more detailed *in vitro* and *in vivo* studies.

As for future perspectives, further studies would be needed in order to further explore their therapeutic potential, as for example, through the isolation and identification of the bioactive compounds present in the extracts; elucidation of the mechanisms of actions and by performing *in vivo* studies using mammalian models of neurodegeneration.

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