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Salt marsh plants as source of bioactive compounds

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Declaração de autoria de trabalho

Declaro ser o autor 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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Abbreviations

AA	Arachidonic acid
ACh	Acethylcholine
AChE	Acethylcholinesterase
AD	Alzheimer's disease
APP	Amyloid precursor protein
Aβ	Amyloid-β peptide
BChE	Butyrylcholinesterase
BHA	Butylated hydroxytoluene
BHT	Butylated hydroxytoluene
ChE	Cholinesterase
CNS	Central nervous system
DCH	Docosohexanoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DOPA	Dihydroxyphenylalanine
DOPA-quinone	Dihydroxyphenylalanine
DPPH	1,1-diphenyl-2picrylhydrazyl
DTNB	5,5-dithio-bis(2-nitrobenzoic) acid
EDTA	Ethylenediamine tetraacetic acid
IL-1β	Interleukin-1β
LB	Lewis bodys
LH	Unsaturated lipids
LOO˙	Peroxyl radical
LOOH	Lipid hydroperoxides
LPS	Lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
ND	Neurodegenerative diseases
NFTs	Neurofibrillary tangles

NO	Nitric oxide
PBS	Phosphate buffer
PD	Parkinson's disease
PHFs	Paired helical filaments
PUFAs	Polyunsaturated fatty acids
PV	Pyrocatechol violet
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SNpc	Substantia nigra pars compact
SOD	Superoxide dismutase
TH	Tyrosine hydroxylase
TYRO	Tyrosinase

Abstract

Neurdegenerative diseases are characterized by the progressive decline of neuronal functions. Oxidative stress is considered the principal initiator of this problem, and contributes to the appearance of neurodegenerative diseases. In this study it was investigated the potential source of biocompounds with neuroprotective features of halophytes species. An initial screening of methanol extract of 26 species was made, using the 1,1-diphenyl-2picrylhydrazyl (DPPH) radical scavenging assay. The species *Carpobrotus edulis* was selected for the next assays, and used to prepare hexane, dichloromethane, chloroform, ethyl acetate and methanol extracts. The antioxidant activity was evaluated through different and complementary methods, namely the DPPH radical scavenging activity, iron and copper chelating activity, iron reducing power and nitric oxide (NO) scavenging activity, and for the phytochemical analysis, including total flavonoid content (TFC), total condensed tannin content (TCTC) and total phenolic content (TPC). These extracts were also tested for inhibition against enzymes related to neurodegenerative disorders, namely acetylcholinesterase (AChE), butirylcholinesterase (BChE) and tyrosinase (TYRO).

The *in vitro* anti-inflammatory activity of the extracts was determined through the evaluation of the inhibition of NO production in lipopolysaccharide (LPS)-stimulated N9 microglial cells. The neuroprotective activity was tested through the H_2O_2- induced injury in SH-SY5Y cell line, and the cellular viability measured by the MTT method. The methanol extract had the highest activity on the DPPH assay, and also the highest copper chelating activity and NO-scavenging activity. The hexane extract had the highest iron chelating activity, while the ethyl acetate extract had the highest iron reducing power activity. The methanol extract had the highest levels of total flavonoid content (TFC) and total phenolic content (TPC), while the dichloromethane extract had the highest total condensed tannin content (TCTC). All the extracts tested had a protective effect against H_2O_2- induced injury in SH-SY5Y cells, especially the methanol extract, which also had the highest anti-inflammatory activity in N9 microglial cells.

Keywords: Halophytes, *C. edulis*, plant extracts, antioxidant activity, anti-inflammatory activity, enzyme inhibition, nitric oxide, phenolic compounds.

Resumo

As doenças neurodegenerativas são caracterizadas pela progressiva degeneração do sistema nervoso. A perda contínua e específica de células neuronais pode conduzir a uma situação de doenças, como a doença de Alzheimer (DA) ou a doença de Parkinson (DP). O stress oxidativo é considerado uma das maiores e mais frequentes causas ou processo iniciador deste tipo de doenças. O envelhecimento conduz a um variado número de problemas para a saúde, incluindo o aumento de espécies reativas de oxigénio (ERO) que podem provocar danos celulares. A DA, a principal causa de demência na população, é caracterizada por distúrbios de comportamento, perda de memória e a diminuição da atividade cognitiva. Na DP os movimentos involuntários, associados a tremores, perda de força muscular e rigidez muscular são sintomas característicos desta doença. Em ambos os casos, a acumulação de neurotoxinas, de algumas proteínas e ERO conduzem a estados de inflamação crónica, perda de capacidade sináptica, levando assim à morte neuronal e ao agravamento dos sintomas. Diversos estudos têm provado a eficácia de compostos antioxidantes na redução da formação de ERO e de fortalecimento dos mecanismos de defesa dos organismos. Torna-se assim fundamental a pesquisa de produtos naturais que possuam propriedades antioxidantes e anti-inflamatórias. As plantas, devido às condições em que habitam, principalmente as halófitas, desenvolveram sistemas antioxidantes eficientes para proteção dos seus sistemas. As halófitas são capazes de sobreviver a ambientes com elevado teor de salinidade. Deste modo, podem desenvolver stress oxidativo, que é regulado por diversos mecanismos que facilitam a retenção e aquisição de água, proteção de cloroplastos, e manutenção da homeostase iónica. Essencialmente, estas ações compreendem a síntese de osmólitos (pequenos glícidos, polióis, aminoácidos e metilaminas), proteínas específicas (ex. enzimas antioxidantes, proteínas transportadoras de iões) e moléculas antioxidantes (ex. compostos fenólicos, carotenóides e vitaminas). Isto pode explicar a utilização de algumas espécies de halófitas em países orientais, na medicina tradicional e na alimentação. Para, além disso, sabe-se que as moléculas presentes em plantas, vulgarmente designadas por fitoquímicos, possuem propriedades antioxidantes e parecem ter uma atividade anti-inflamatória, eventualmente prevenindo o desenvolvimento destas doenças, através da inativação de algumas enzimas, prevenindo o stress oxidativo ou

ainda evitando a inflamação neuronal. O uso de compostos provenientes de halófitas para a prevenção de doenças neurodegenerativas ainda está pouco estudado. Neste estudo fez-se um screening inicial com 26 espécies de halófitas, fazendo um estudo da atividade antioxidante através do radical 1,1-difenil-2picrylhydrazyl (DPPH). A espécie *Carpobrotus edulis* foi selecionada para os seguintes ensaios. Foi efetuada uma extração sequencial com os solventes n-hexano, diclorometano, clorofórmio, acetato de etilo e metanol. Assim este estudo teve como objetivo estudar a atividade antioxidante, anti-inflamatória e neuroprotectora de extratos desta espécie. Através dos métodos de redução de radicais livres DPPH, da atividade quelante do cobre e do ferro, da atividade redutora do ferro e potencial de eliminação do óxido nitrico (NO) foi medida a capacidade antioxidante destes extratos. Os teores totais de compostos fenólicos, flavonóides e taninos foram igualmente determinados. Foi também testada a capacidade inibitória destes extratos contra algumas enzimas específicas, como a acetilcolinesterase (AChE), a butirilcolinesterase (BChE) e a tirosinase. Foram determinadas ainda a atividade anti-inflamatória e neuroprotectora em células de neuroblastoma (SH-SY5Y) e células de microglia (linha celular N9). Para o estudo da atividade anti-inflamatória foi utilizado o ensaio de Griess, que é um método simples e rápido para avaliar indiretamente a produção de óxido nitríco em meio aquoso, através da medição de nitritos, que é um produto final estável do óxido nitríco, na presença de oxigénio. Este método permite determinar o potencial inibitório dos extratos sobre a produção de óxido nitríco, na linha celular de células da microglia N9 estimulada com lipopolissacarídeo (LPS). A atividade neuroprotectora foi avaliada através do método de MTT, na linha celular SH-SY5Y estimuladas com peróxido de hidrogénio (H_2O_2), previamente tratadas com os extratos, procedendo-se posteriormente à medição da viabilidade celular. Todos os extratos mostraram possuir atividade antioxidante contra o radical DPPH, principalmente o extrato metanólico com uma atividade anti radical de 96.1%, para a concentração de 1 mg/mL. O extrato metanólico permitiu obter ainda a maior atividade quelante do cobre (48.27% a 10 mg/mL), e de no radical NO (41.4% a 10 mg/mL), enquanto na atividade quelante do ferro o extrato de hexano foi o mais ativo (81.32% a 10 mg/mL). A atividade redutora do ferro mais elevada foi obtida com o extrato de acetato de etilo (76.63% a 10 mg/mL). O extrato de metanol apresentou a maior concentração de flavonóides e fenólicos totais, enquanto o extrato de diclorometano

apresentou o teor mais elevado de taninos totais. A maior capacidade inibitória da AChE foi obtida com a aplicação do extrato de metanol (43.2% a 10 mg/mL), enquanto o de acetato de etilo foi o mais eficaz a inibir a BChE (60.4% a 1 mg/mL). Os extratos não apresentaram citotoxicidade nas linhas celulares utilizadas nos ensaios de neuroprotecção. Os extratos apresentaram capacidade para proteger a linha celular SH-SY5Y contra o stress oxidativo induzido pela aplicação de água oxigenada, traduzido numa redução da viabilidade para cerca de 50%, e o melhor resultado foi obtido com o extrato de metanol (143.5% de viabilidade celular a 50 μg/mL). A aplicação do extrato de metanol permitiu ainda uma redução significativa da produção de NO por células de microglia. Em conclusão, a espécie halófita *C. edulis* mostrou ser uma potencial fonte de compostos interessantes para o possível tratamento ou prevenção das doenças neurodegenerativas, em particular o extrato metanólico uma vez que foi o que permitiu obter melhores resultados.

Palavras-chave: *Carpobrotus edulis*, halófitas, Alzheimer, Parkinson, atividade antioxidante, compostos fenólicos, inibição enzimática, atividade anti-inflamatória, atividade neuroprotectora,

1. Introduction

1.1 Bioactive compounds

Bioactive compounds (BioA) can be characterized as crucial and non-crucial constituents that occur in small amounts in plant and animal products, and can influence cellular and physiological activities, contributing to a better health when included in the diet (Kris-Etherton *et al*., 2002, 2004; Biesalski *et al*., 2009). Some examples of BioA are flavonoids, isoflavones, phytoestrogens and monotrepenes, which can be found in many vegetables (Kris-Etherthon *et al*., 2002). These compounds display several biological activities, namely antioxidant, anti-inflammatory and anti-tumoral (Kris-Etherton *et al*., 2004; Ye *et al*., 2013). Apart from this activities, the definition of BioA implies that it displays comproved health benefits (Kris-Etherton *et al*., 2004; Biesalski *et al*., 2009), and can been taken through the diet or by medication.

Higher variety in fruit and vegetable intake has been associated with a lower risk of several chronic diseases and have been widely used to prevent and treat some disorders (Koppula *et al*., 2012; Ye *et al*., 2013). BioA have become popular due to have none or limited side effects, in contrast with synthetic drugs (Koppula *et al*., 2012). It is increased the search for BioA with a view to discover new antioxidant potential therapeutic compounds to attenuate or treat neurodegenerative diseases (Koppula *et al*., 2012).

1.2. Neurodegenerative diseases

Neurodegenerative diseases (ND) are often hereditary and sporadic conditions which are characterized by progressive nervous system dysfunction, induced by deposits of abnormal proteins and other factors, which leads to a neuronal degeneration in particular areas of the brain (Ross *et al*., 2004). Alzheimer's disease (AD) and Parkinson's disease (PD) are examples of ND, and are characterized by the continued loss of specific neuronal cells in association with protein aggregation (Barnham *et al*., 2004; Ross *et al*., 2004). The oxidative stress is closely linked to these diseases, and is probably the responsible for the dysfunction of intracellular components, like proteins or neuronal cell death, which contributes to the development of these diseases (Barnham *et al*., 2004).

The increasing age in population is deeply linked to the increase in the prevalence of several forms of ND (Forman *et al*., 2004). It is expected that by 2025, 75% of the world's population over 60 years of age living in developing countries will suffer of ND (Forman *et al*., 2004). The search for effective treatments or preventive interventions for ND is extremely important because in the near future the emotional, societal and financial burden of these aging-related disorders will be very high (Forman *et al*., 2004).

1.2.1 Alzheimer's disease

Alzheimer's disease (AD) is one of the main causes of dementia in aged population, affecting more than 35 million people worldwide (Prince *et al*., 2012). It was first identified in 1907 by Alois Alzheimer, in a 51-year-old woman, which exhibited personality changes, progressively worsening memory loss, time disorientation and language disturbances, despite the relatively normal neurological function (Nixon 2002; Forman *et al*., 2004). After autopsy, her brain revealed evidences of cerebral atrophy (Nixon 2002). There are many factors that contribute to AD, such as hypertension, oxidative stress, inflammation and hypercholesterolemia (Gamba *et al*., 2011). There are two forms of AD, the sporadic form, which affects 90% of the patients, and the familiar predisposition, that includes the remaining 10% (Nixon 2002). However, the two forms of the disease show the same neuropathology features (Nixon 2002). AD is normally recognized by characteristic symptoms namely behaviour disturbances, memory loss and decreasing of cognitive activity (Chitranshi *et al*., 2012), induced by the decrease of the forebrain cholinergic neurons, the most affected in this disease (García-Ayllón *et al*., 2011). With the advances of neuroimaging techniques, it is now possible to detect and measure the metabolism, atrophy and inflammation in brain, and the presence of plaques and neurofibrillary tangles (NFTs), as well the brain activity (Perrin *et al*., 2009). The research and development of biomarkers in AD is crucial to measure the structural and functional changes in the brain (Perrin *et al*., 2009). Biomarkers like brain volume, atrophy, level of glucose metabolism in the brain, regional activity changes, amyloid-plaque and NFTs formation, inflammation and oxidative stress are crucial on the preclinical phase of AD to make a diagnosis and prognosis, even before irreversible neurological damage occurs (Perrin *et al*., 2009; Alzheimer's & Dementia, 2011).

In Europe there are 7.3 million people with AD (Alzheimer-europe.org, 2014). In Portugal it is estimated that there are more than 180 000 cases, and due to the increase of elderly population it is estimated that the number of AD cases will increase (Alzheimer-europe.org, 2014). Every year, 1.4 million Europeans develop dementia meaning that every 24 seconds a new case is diagnosed. It is expected that by 2050 the number of the cases increases to 16.2 million (Forman *et al*., 2004; Alzheimer-europe.org, 2014).

1.2.1.1. Neuropathology of AD

AD is characterized by extracellular deposits of amyloid-β peptide (Aβ), which form senile plaques and formation of intracellular neurofibrillary tangles (NFTs) composed of paired helical filaments (PHFs) of hyperphosphorylated protein tau (Resende *et al*., 2008; Fig. 1.1). There are several studies showing that the accumulation of amyloid plaques and NFTs plays an important role in neurodegeneration during and before the clinical phase of the disease (Nixon 2002). These structures are present in brain areas such as those involved in learning and memory, like the cortex and hippocampus (Nixon 2002; Resende *et al*., 2008). Such areas typically exhibit synaptic and neuronal loss, with cholinergic and glutamatergic neurons being the most affected (Resende *et al*., 2008). AD is also accompanied by inflammation, and neuronal, axonal and synaptic loss dysfunction (Perrin *et al*., 2009). These features are also found in cognitively normal elderly individuals and need to reach a certain threshold to be noticed by clinical symptoms (Perrin *et al*., 2009).

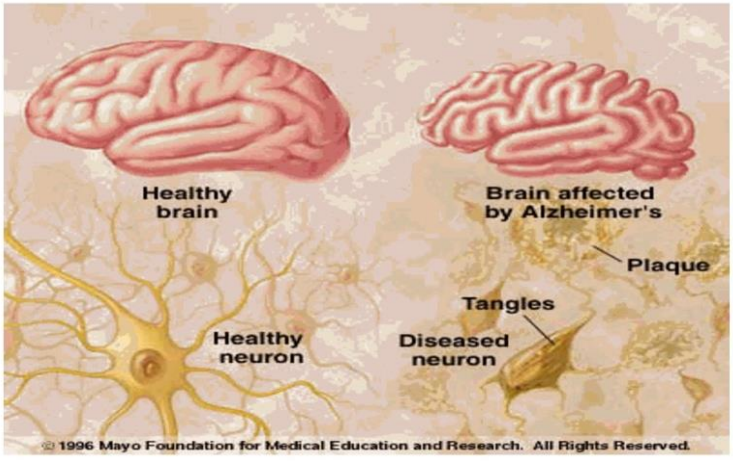

Figure 1.1: Comparison of a healthy brain and a brain affected by AD, showing senile plaques and NFTs tangles leading to cortex atrophy. (Source http://img.docstoccdn.com/thumb/orig/90531693.png).

AD has 4 clinical stages, described as "very mild/mild cognitive impairment" (MCI), "mild", "moderate" and "severe" (Fig. 1.2). In the beginning of the "very mild" stage, the disease is correlated not with plaques or NFTs, but with synaptic and neuronal loss, supporting the idea that plaques and NFTs need to accumulate 10 to 15 years before cognitive decline is noticeable (Perrin *et al*., 2009).

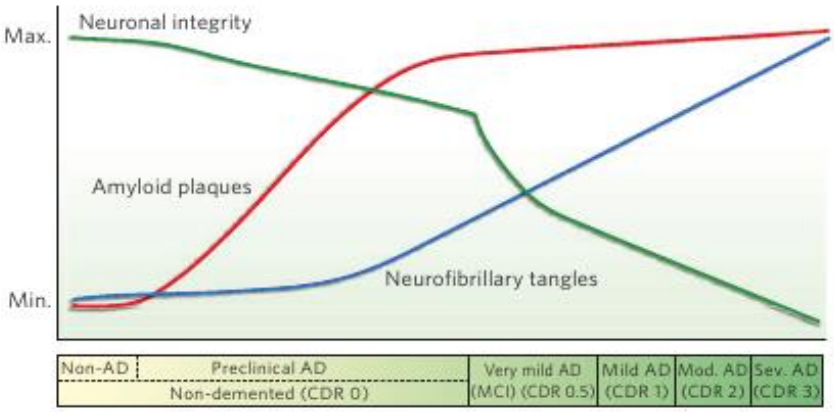

Figure 1.2: Progressive clinical stages of AD, "very mild/mild cognitive impairment" (MCI), "mild", "moderate" and "severe", correspond to clinical dementia rating (CDR) scores of 0.5, 1, 2 and 3, respectively. These stages are associated with abundant amyloid plaques (red line), the gradual accumulation of NFTs (blue line) and synaptic and neuronal loss in certain brain regions (green line). (Source: Perrin *et al.*, 2009).

1.2.1.2 β-amyloid plaques and neurofibrillary tangles

As stated before, the most common hallmarks of AD is the formation of senile plaques and NFTs (Perrin *et al.*, 2009) (Fig. 1.1).

Senile plaques are composed by extracellular aggregates of Aβ. This peptide is formed from a larger amyloid precursor protein (APP), a transmembrane protein, which, in normal cases, is cleaved first by α-secretase and then by γ-secretase (Goedert *et al.*, 2006; LaFerla *et al.*, 2007; Karran *et al.*, 2011). In AD cases the first cleavage was made by β-secretase, which creates a shorter fragment known as β-amyloid (or Aβ) (LaFerla *et al.*, 2007). When fragments of Aβ aggregate they became toxic, interfering with the normal neuronal function (Butterfield *et al.*, 2001; Perrin *et al.*, 2009). With time these aggregates

increase in size and become soluble, originating the senile plaques and thus contributing to cell death (Butterfield *et al.,* 2001; Perrin *et al.,* 2009; Alzheimer's & Dementia, 2011). The deposition of Aβ plaques is responsible for a chronic inflammatory response involving the activation of microglial cells (Nixon 2002; Dumont and Beal, 2011). Those reactions explains the presence of additional cell types in senile plaques, namely microglia and reactive astrocytes (Butterfield *et al.,* 2001; Nixon 2002). Some of them accelerates the fibrillation of Aβ with the consequent formation of toxic products, which leads to a local development of dystrophic neurites (Butterfield *et al.,* 2001; Nixon 2002). The primary damage to neurites initiates local Aβ overproduction, neurite degeneration-regeneration responses, and secondary inflammatory reactions involved in the removal of cellular debris (Nixon 2002). All these events cause more damage to the neurons through the formation of more toxic products and Aβ (Nixon 2002).

1.2.1.3. Cholinesterases

Neurochemical studies show that the brain of AD patients has a disturbance of acethylcholine (ACh) metabolism, which led to the formulation of the "cholinergic hypothesis" (Carvajal and Inestrosa, 2011). This hypothesis postulates that the accumulation of Aβ and tau protein is connected to the loss of cholinergic neurons (Carvajal and Inestrosa, 2011).

Acetylcholinesterase (AChE) is a selective enzyme responsible for the hydrolysis of the cholinergic neurotransmitter ACh (Fig. 1.3; Ciro *et al.,* 2012; Wang *et al.,* 2012). AChE is present in all cholinergic structures, as well as in a subpopulation of non-cholinergic neurons and in glial cells (Ciro *et al.,* 2012; Wang *et al.,* 2012). Butyrylcholinesterase (BChE) also hydrolyses ACh and is present in a subpopulation of cortical and subcortical neurons and in glial cells (Ciro *et al.,* 2012; Wang *et al.,* 2012). It was demonstrated that intense cholinesterase (ChE) activity occurs in senile plaques and neurofibrillar tangles, which influences the aggregation of Aβ (Ciro *et al.,* 2012). AChE inhibitors have been shown to interfere with the production of Aβ, by decreasing the levels of APP, thus reducing the toxicity effects associated with Aβ production and aggregation (Ciro *et al.,* 2012). Moreover, it is known that the expression of BChE increases as plaques go through the process of maturation, from the diffuse type to the pathologic compact variety (Ciro *et*

al., 2012). Thus, it is suggested that the influence of BChE in the pathology of plaques is greater than that of AChE, being essential in AD plaque maturation (Wang *et al.*, 2012; Ciro *et al.*, 2012). BChE shows to be a great contributor to the loss of ACh in AD brain, and is inhibition can lower the levels of Aβ peptide (Wang *et al.*, 2012).

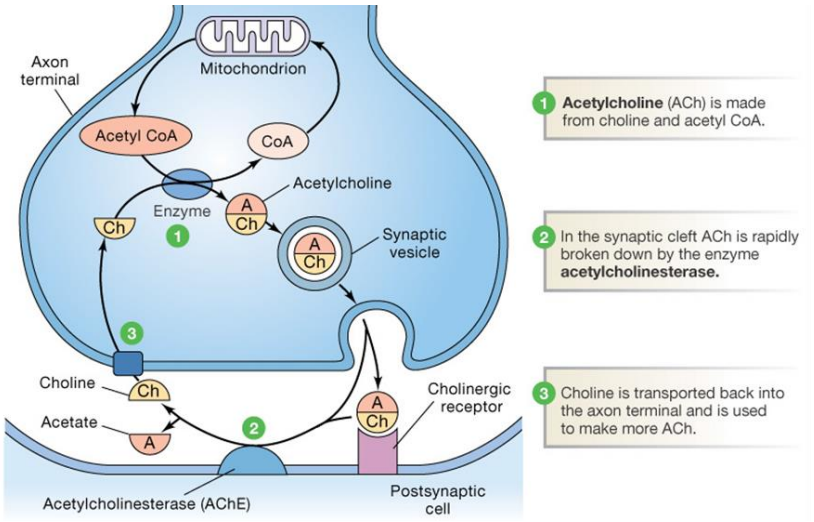

Figure 1.3: Schematic representation of synthesis of ACh. 1 - Acetylcholine is made from choline and acetyl CoA being afterwards incorporated and stored in presynaptic vesicles. Presynaptic vesicles then move or are emptied into the synaptic cleft, and the diffusion of ACh across the synaptic cleft to the postsynaptic ACh receptors occurs through binding of ACh to postsynaptic ACh receptor. 2 - After signalling, acethylcholine is released from receptors and broken down by acethylcholinesterase to be recycled in a continuous process. 3 – Choline is reuptaked into the presynaptic neuron. (Source: http://peaknootropics.com/wp-content/uploads/2013/08/acetylcholine-metabolism.png).

1.2.2. Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative pathology which affects 2-3% of the population above the age of 65 (Fasano *et al.*, 2006) and 5% above 85 years, and is the second most common ND, after AD (Hasegawa *et al.*, 2008; Bartels *et al.*, 2009; Shulman *et al.*, 2011). In Portugal there are between 13000 and 15000 people affected with this disease (Associação Protuguesa de Doentes de Parkinson, 2012).

PD was initially reported by James Parkinson in 1817, and was based on six cases with a disease called then "Shaking Palsy", which symptoms were involuntary tremulous motion, reduced muscular power, higher propensity to bend the trunk forward, and to pass from walking to a running pace, but where the cognitive abilities have not been injured (Zhang *et al.,* 2000; Bartels *et al.,* 2009). The neuropathological hallmark of PD, the Lewy body (LB), was described around 1912 by Friederichy Lewy (Forman *et al.,* 2004, Bartels *et al.,* 2009; Shulman *et al.,* 2011). Around 1919, Konstantin Tretiakoff associated the loss of pigmented cells in the *substantia nigra pars compacta* (SNpc; the deep portion of substantia nigra) with the PD progression (Bartels *et al.,* 2009). In the 1950's Arvid Carlsson found a relationship between the decrease of dopamine and PD (Bartels *et al.,* 2009).

There are two forms of PD: a sporadic form, which affects 95% of all patients, and familial form accounting for about 5% of all cases (Hald and Lotharius, 2005). The familial form is associated with mutations in at least five genes, such as SNCA, PARK, PINK1, DJ1, UCHL1 and LRRK2 (Fig. 1.4; Hald and Lotharius, 2005; Tan and Skipper, 2007). The SNCA and PARK1 genes are linked to α-synuclein protein, promoting abnormal protein aggregation like showed in Fig. 1.4 (Tan and Skipper, 2007). Mutations in PARK and UCH-L1 cause a damage in the ability of the cellular machinery to detect and eliminate misfolded proteins (Duaer and Przedbarski., 2003). DJ1 gene have a role in the oxidative stress response, either as a redox sensor protein or as an antioxidant (Tan and Skipper, 2007).

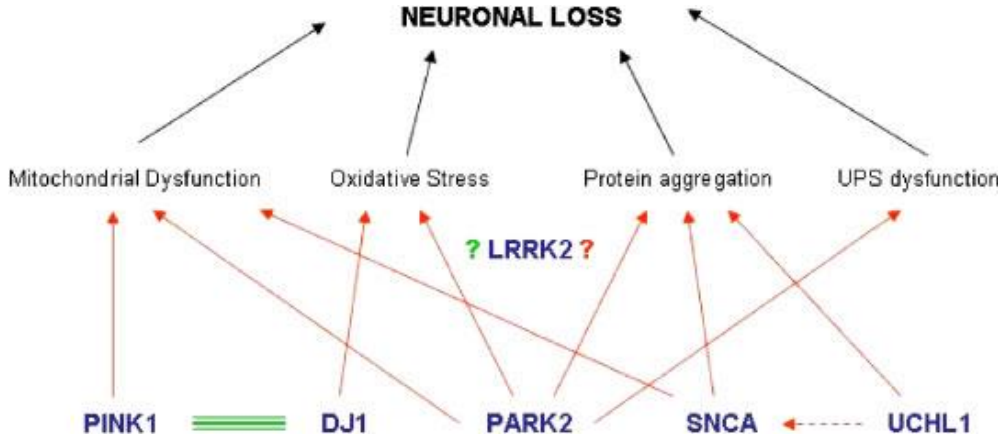

Figure 1.4: Scheme of potential interactions between the encoded proteins of the different PD genes

The aetiology of the sporadic form is still unknown (Zhang *et al.,* 2000; Asanuma *et al.,* 2003; Hald and Lotharius, 2005). Currently the most widely accepted hypothesis says that PD is a result of both genetics and environmental factors, leading to the failure of mitochondria, development of oxidative stress and occurrence of dopaminergic cells death (Hasegawa *et al.,* 2008; Bartels *et al.,* 2009; Nakashima *et al.,* 2012). PD manifests through bradykinesia (extreme slowness of movements and reflexes), rest tremor, muscular rigidity, gait abnormalities and postural instability and in some cases dementia (Zhang *et al.,* 2000; Montiel, 2006; Mosley *et al.,* 2006; Zecca *et al.,* 2008; Hauser and Hastings, 2013).

1.2.2.1. Neuropathology of PD

The cell body of dopaminergic neurons project from the SNpc, which is responsible for the body movements, to the caudate nucleus and striatum, where neurons release dopamine (Zhang *et al.,* 2000; Dauer and Przedbarski, 2003; Fasano *et al.,* 2006; Mosley *et al.,* 2006; Hauser and Hastings, 2013). In dopaminergic neurons, which also contain neuromelanin that confers a characteristic pigmentation to the SNpc area, the production of dopamine is affected and decreased, leading to cell degeneration and death (Fasano *et al.,* 2006; Shulman *et al.,* 2011; Hauser and Hastings, 2013). This fact is shown by the depigmentation of SNpc, as shown in Fig 1.5. (Duaer and Przedbarski, 2003). This dopaminergic cell loss in SNpc results in dysfunctional motor movements (Shulman *et al.,* 2011).

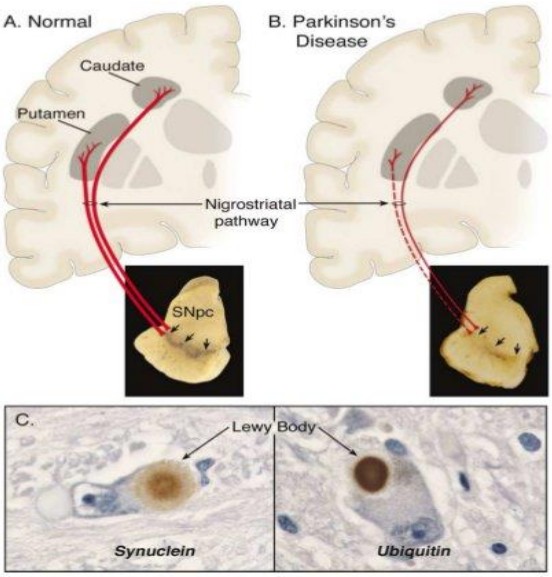

Figure 1.5: Neuropathology of PD. (A) Schematic representation of the normal dopaminergic neurons whose cell bodies are located in the SNpc (black arrows). These neurons project (thick solid red lines, the nigrostriatal pathway) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). (B) Schematic representation showing the degeneration of nigrostriatal pathway in Parkinson's patients there is a marked loss of dopaminergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line). (C) Immunohistochemical labeling of intraneuronal inclusions, termed Lewy bodies, in a SNC dopaminergic neuron showing the differences between a normal neuron and a neuron from a PD patient. (Source: Duaer and Przedbarski, 2003).

The LB is an intraneuronal proteinaceous cytoplasmic inclusions, mostly composed by α-synuclein, a small acidic presynaptic protein, which is involved in vesicular transport (Fig. 1.5C; Fasano *et al.,* 2006; Shulman *et al.*, 2011; Irwin *et al.,* 2013).

Although the causes of PD are not yet fully understood, data obtained from several studies support the theory that mitochondrial dysfunction and oxidative stress are involved in the progression of the pathogenic disease, which is strongly linked to dopaminergic neuronal loss (Mosley *et al.,* 2006; Hasegawa *et al.,* 2010; Hauser and Hastings, 2013). Besides these two factors, others, such as inflammation, excitotoxic mechanisms and toxic actions of nitric oxide (NO) are involved in PD development (Jenner 2003, Hasegawa *et al.,* 2010; Hauser and Hastings, 2013).

The microglia cells are important in surveillance and host defence in brain, aiding in the identification and removal of cells who are programed to death (Jenner 2003; Liu *et al.*, 2003). These cells are particularly sensitive to changes in their environment and turn active in case of injury or infection (Liu *et al.,* 2003; Hald and Lotharius 2005). Several soluble factors are secreted by activated microglia cells, some have neuroprotective function, but the majority are neurotoxic and have proinflammatory activity, such as NO, superoxide anion and fatty acid metabolites (Liu *et al.*, 2003; Hald and Lotharius 2005). Some experiments established a relationship between neuronal degeneration in PD and massive activated microglia, suggesting that activated microglia have a toxic effect in neurons (Liu *et al.*, 2003; Hald and Lotharius 2005).

1.2.2.2. Tyrosinase

Tyrosinase, also known as polyphenol oxidase, is a copper-containing monooxygenase enzyme widely distributed in organisms (Nerya *et al.*, 2003; Chang 2012). This enzyme catalyses the formation of melanin pigment from tyrosine, oxidation of several phenolic compounds, the synthesis of amino acid based antibiotics among others (Senol *et al.*, 2010; Chang 2012). It is implicated in the hydroxylation of tyrosine to DOPAquinone (dihydroxyphenylalanine-quinone), and oxidation of DOPAquinone to DOPA (dihydroxyphenylalanine), through dopamine-quinone via contributing to neuromelanin (NM) formation (Fig. 1.6; Asanuma *et al.*, 2003; Greggio *et al.*, 2005; Chang 2012).

Dopamine is a potent neuromodulator in the central nervous system (CNS) that can be found in neuronal tissues and body fluids, in the form of large organic cations (Maciejewska *et al*., 2011). In fact dopamine is linked to PD, but the symptoms are recognized in AD patients too, showing that disturbance of dopamine metabolism may be closely associated also with AD (Silva and Ming, 2007). Catecholamines, dopamine, epinephrine, norepinephrine and DOPA are catechol-containing neurotransmitters, which are involved in cognitive, behavioural, physical, physiological and psychological functions (Silva and Ming, 2007). The oxidation of these molecules can cause severe alterations in brain activity, leading to neuronal death. Metabolic malfunctions of neurotransmitters are known phenomena in the physiology of AD and PD and have also been suggested to be

related to the neuropathology of these diseases (Silva and Ming, 2007).

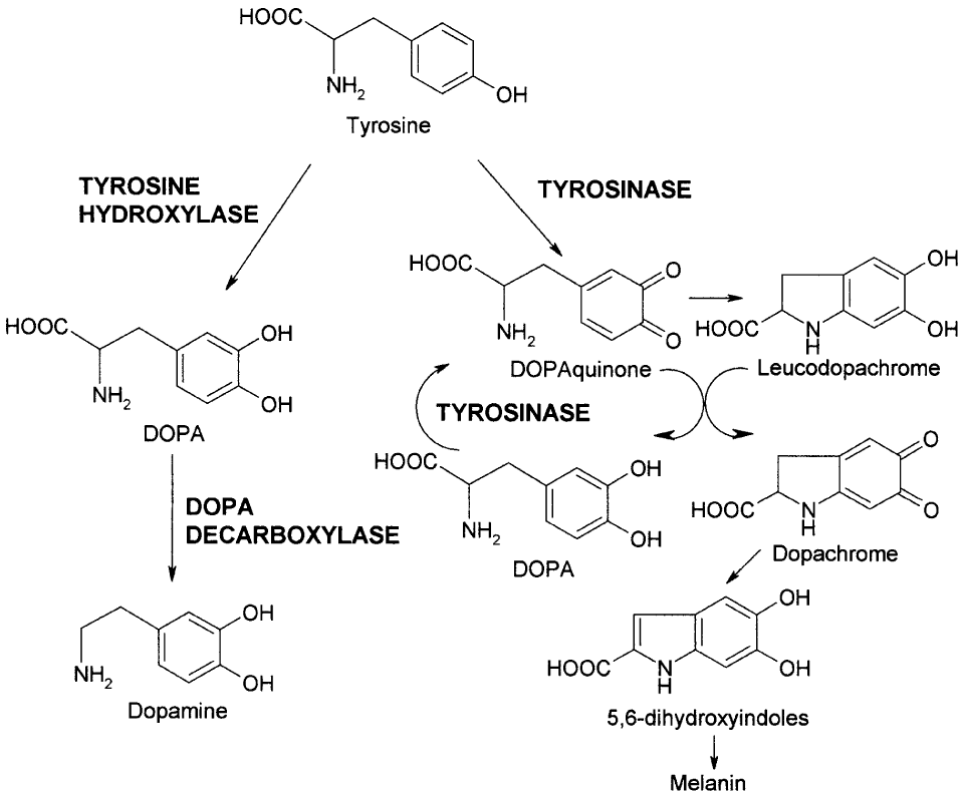

Figure 1.6: Tyrosine metabolism. (Source: Fiore *et al.*, 2004).

Tyrosine is converted into DOPA through tyrosine hydroxylase (TH), being then carboxylated to form dopamine (Fig. 1.6; Xu *et al.*, 1998; Asanuma *et al.*, 2003). Dopamine, in the presence of tyrosinase, covalently modifies TH, which becomes disabled (Greggio *et al.*, 2005). Tyrosinase can oxidise the catechol ring of dopamine turning it into the high reactive species dopamine-quinone, as showed in Fig. 1.7, which is responsible for the toxicity of the neurotransmitter (Greggio *et al.*, 2005). The oxidation of dopamine inactivates dopamine transporters, the glutamate transporter and the mitochondrial respiration (Xu *et al*., 1998; Greggio *et al.*, 2005; Hwang 2013). Dopamine-quinones can react with cysteine residues in the cytosol of neurons, and form protein adducts, which lead to irreversible modification or inactivation of functional proteins (Greggio *et al.*, 2005). These quinones can also react with α-synuclein, stabilizing this toxic fibril intermediate, a dominant cause in PD (Greggio *et al.*, 2005).

Tyrosinase activity may also result in an increase in toxic effects of hydrogen peroxide (H_2O_2), being the peroxide a hallmark of oxidative stress and also an inhibitor of dopamine transporter (Gregiio *et al.,* 2005), increasing the oxidative stress in neurons.

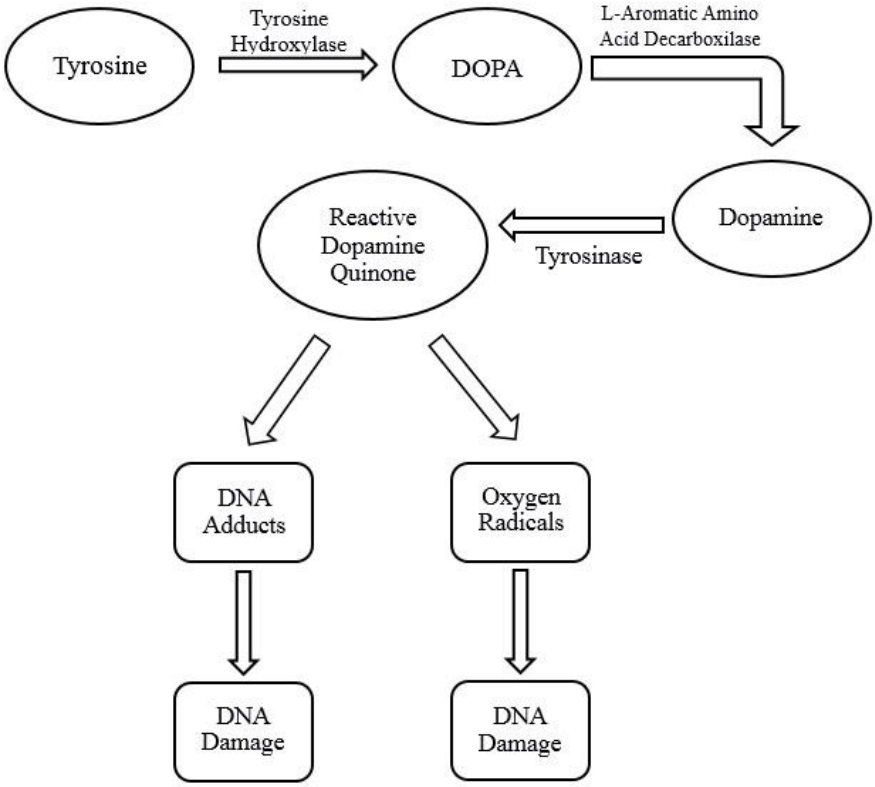

Figure 1.7: Potential pathway for the synthesis of dopamine quinone (DA-quinone) and consequent damages. The DA-quinone is highly reactive and can change DNA or enhanced the formation of reactive oxygen radicals (superoxide, hydroxyl and peroxide). (Source: Stokes *et al.,* 1996).

Neuromelanin is a dark insoluble polymer produced from dopamine oxidation that provide the dark pigmentation to the *substantia nigra* (Hwang 2013). Tyrosinase can accelerate the induction of catecholamine quinone derivates, due to its oxidase activity leading to dopamine neurotoxicity and neurodegeneration (Khan *et al*., 2007). Extraneuronal neuromelanin deposition has been observed in the brains of PD patients (Hwang 2013). When this neuromelanin is added to microglia cultures levels of NO increase, causing a strong microglia activation and consequently loss of dopaminergic neurons (Hwang 2013).

Since these discoveries, the research of tyrosinase inhibitors as a target for the treatment of PD has become increasingly important. The current treatment relies on the administration of L-dopa, to increase dopamine levels in the brain, though a long-therm therapy may cause adverse reactions (Asanuma *et al.,* 2003).

1.3. Oxidative stress, antioxidants and ND

Oxidative stress has been regarded as a crucial factor in neurodegeneration (Friedman *et al.,* 2011). Reactive oxygen species (ROS) and reactive nitrogen species (RNS), include radical anion superoxide ($O_2^{-\bullet}$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), and nitric oxide radical ($NO^{\bullet}$). ROS and RNS are normal by-products of cellular metabolism, but react strongly with multiple molecules, leading to neurodegeneration (Behl and Moosmann, 2002; Barnham *et al*, 2004; Friedman *et al*, 2011; Hwang 2013; Taylor *et al*, 2013). Through different pathways, such as direct interaction with redox-active metals and oxygen species via reactions, like Fenton and Haber-Weiss reaction, the radical $^{\bullet}OH$ has produced being the most reactive one of all ROS (Taylor *et al*., 2013).

The brain is characterized by a high oxygen consumption rate and lipid content, and by a relative shortage of antioxidant enzymes, compared with other tissues, making the CNS especially vulnerable to oxidative stress (Markesbery, 1996; Friedman *et al.,* 2011). The chemical origin of ROS is the reaction between molecular oxygen with redox-active metals iron (Fe^{3+}) and copper (Cu^{2+}) (Barnham *et al.,* 2004). To sustain many functions, the brain needs an elevated concentration of metal iron, but has a low capacity to manage the oxidative stress and low regeneration ability (Barnham *et al.,* 2004).

Due to high content of polyunsaturated fatty acids (PUFA), like arachidonic (AA), and docosohexanoic acid (DCH), brain are more sensitive to oxidation, being an important mechanism of neurodegeneration (Butterfield and Lauderback, 2002; Floyd and Hensley, 2002). AA and DCH are sensitive to free radical attack and PUFA decreased with the increase of lipid peroxidation (Butterfield and Lauderback, 2002). Lipid peroxidation is a process where molecular oxygen is integrated into unsaturated lipids (LH) and form lipid hydroperoxides (LOOH), like showed in Fig. 1.8 (Minotti and Aust 1992).

$I^{\bullet} + LH \rightarrow IH + L^{\bullet}$ initiation

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ O_2 addition

$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ propagation

Figure 1.8: Reactions involved in lipid peroxidation. (Source: Minotti and Aust 1992).

These reactions are promoted by an "initiator" ($I^{\bullet}$), which surpass the dissociation energy of an allylic bond and this cause a hydrogen abstraction and formation of a lipid alkyl radical ($L^{\bullet}$; Minotti and Aust 1992). This alkyl radical can add oxygen to form lipid peroxyl radicals ($LOO^{\bullet}$) which can liberate LOOH *via* hydrogen abstraction from a neighbour alkyl radical (Minotti and Aust 1992). It is necessary external oxidants, like $^{\bullet}OH$, to initiate lipid peroxidation, but once started the reaction this will propagate and form new LOOH (Minotti and Aust 1992; Valko *et al*., 2006). Transition metals, like Fe^{2+}, increase the propagation of lipid peroxidation reaction (Minotti and Aust 1992; Valko *et al*., 2006). Fe^{2+} enters into Fenton and Haber-Weiss reaction, producing $^{\bullet}OH$. The increasing formation of $^{\bullet}OH$ enhances the lipid peroxidation (Fig. 1.8 and 1.9; Minotti and Aust 1992; Behl and Moosmann, 2002; Barnham *et al.*, 2004; Friedman *et al.*, 2011; Hwang 2013; Taylor *et al.*, 2013).

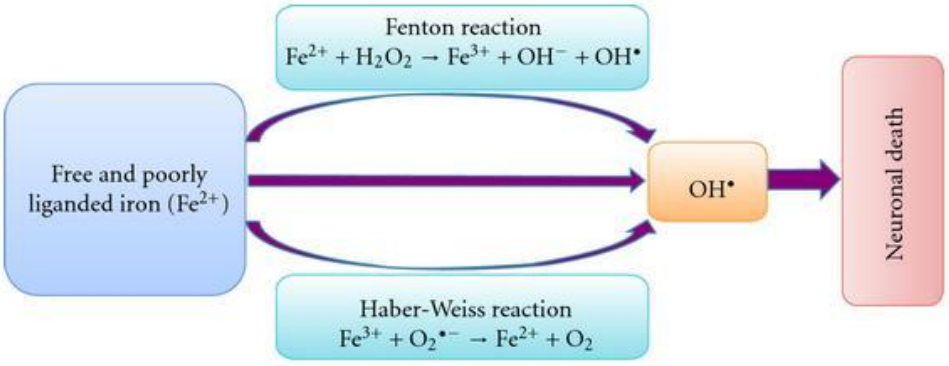

Figure 1.9: Schematic representation of hydroxyl radical production. In the Fenton reaction the Fe^{2+} reacts with H_2O_2 generating a very reactive and damage $OH^{\bullet}$. Ferric iron (Fe^{3+}) react with $O_2^{\bullet-}$ through the Haber-Weiss reaction, leading to the regeneration of Fe^{2+}. The production of highly reactive $OH^{\bullet}$ promote the oxidative stress, lipid peroxidation, mitochondrial dysfunction, increases in intracellular calcium concentration, and lastly neuronal cell death. (Source: http://www.hindawi.com/journals/msi/2011/606807.fig.002.jpg).

It is known that mitochondria, redox-active metals and inflammation (via activated microglia) are potential causes of oxidative stress in the brain (Fig. 1.10 and Fig. 1.11; Dumont and Beal, 2011). Oxidative stress is therefore considered one of the main causes for the development of different ND, including AD and PD (Halliwell 2001; Behl and Moosmann, 2002; Barnham *et al.*, 2004).

In a brain of an AD patient, the content of Fe^{2+} and Cu^{2+} is increased, and can result in the production of free radicals (Jomova *et al.*, 2010). Aβ plaques have been describe as metallic sinks because of the high concentrations of Cu, Fe and zinc (Zn) that have been found in this deposits (Barnham *et al.*, 2004). *In vitro* studies evidenced that Cu^{2+} and Fe^{3+} induced peptide aggregation (Barnham *et al.*, 2004). The homeostasis of Cu, Fe and Zn and their respective proteins, are severely modified in the AD brain (Barnham *et al*., 2004). Cu^{2+} and Fe^{3+} bound to Aβ may induce redox chemical reactions that reduce the oxidation state of both metals leading to the production of H_2O_2, and promoting the Fenton reaction. This, in turn, leads to the generation of extremely toxic $OH^{\bullet}$ radicals inducing an inflammatory response by microglial cells (Barnham *et al.*, 2004; Silva and Ming, 2007; Jomova *et al.*, 2010). Aβ cause lipid peroxidation in brain cell membranes, and leads to 4-hydroxy-2-nonenal (HNE) and acrolein formation (Butterfield and Lauderback, 2002). These reactive alkenals can alter the conformation of membrane proteins and are toxic to neurons (Butterfield and Lauderback, 2002). Protein oxidation can lead to diminished specific protein function which can induce cell death (Butterfield and Lauderback, 2002).

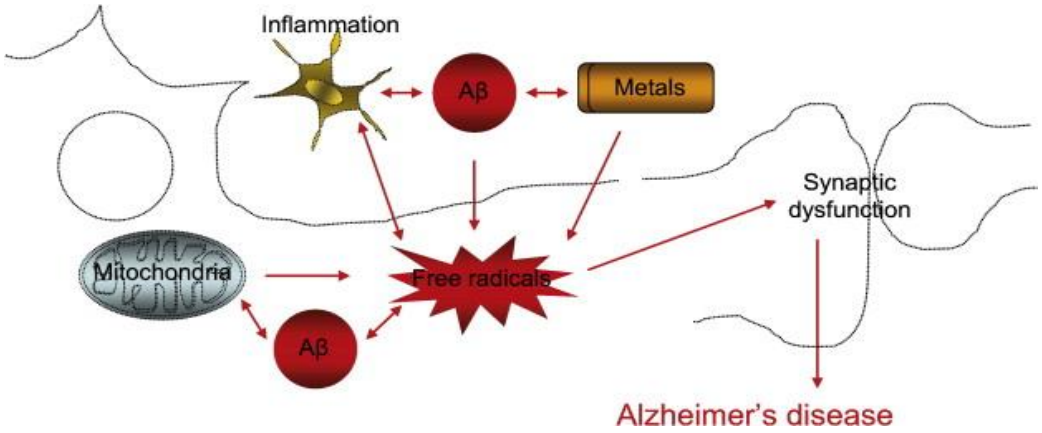

Figure 1.10: Scheme of the generation and role of free radicals in AD. Several key players, such as metals and Aβ, can exacerbate free radical production. Once accumulated inside the cell, free radicals can cause lipid, protein, DNA, and RNA damage and exacerbate AD pathogenesis. (Source: Dumont and Beal, 2011).

Dopaminergic neurons are vulnerable to oxidative stress due to the elevated consumption of oxygen, low levels of antioxidant enzymes and the synthesis and storage of dopamine (Taylor *et al.*, 2013; Hwang 2013). The neurotransmitter dopamine is stable when inside synaptic vesicles, however outside the vesicles, as in a damaged neuron, dopamine is metabolised or auto-oxidised by in an oxygen rich environment producing ROS (Taylor *et al.*, 2013; Hwang 2013). The oxidation of dopamine generates H_2O_2, reactive quinones and $O_2^{-\bullet}$, leading to a highly oxidative stress conditions (Taylor *et al.*, 2013). Neuromelanin, present in dopaminergic neurons, is a potent activator of microglia when released from dying cells, thereby increasing the sensitivity of this neurons to oxidative stress-mediated cell death, and promoting neuroinflammation (Hwang 2013; Taylor *et al.*, 2013).

High levels of iron can also be an important factor in the initiation and promotion of neuronal death, due to interaction with neuromelanin and dopamine (Barnham *et al.*, 2004; Taylor *et al.*, 2013; Hwang 2013). Since the SNpc has increased levels of microglia cells, the activation of this cells caused by injuries, is more pronounced in PD (Taylor *et al.*, 2013). Such proneness to pro-inflammatory conditions has been related to elevated oxidative stress produced through the Fenton reaction (Fig. 1.8) (Hwang 2013; Taylor *et al.*,

2013). Dopamine-quinone has the ability to directly modify some proteins, such as α-synuclein, whose dysfunction and accumulation has been linked to PD, being another cause of oxidative stress (Hwang 2013). α-synuclein is a synaptic vesicle protein involved in neurotransmitter release, vesicle turnover, endoplasmic reticulum trafficking and synaptic plasticity, as shown in Fig. 1.11 (Taylor *et al.,* 2013). The covalently modification of α-synuclein, by dopamine-quinone, leads to conversion into a cytotoxic protofibril form (Hwang 2013). The mutation of the α-synuclein gene can increase its expression leading to formation of oligomers and finally insoluble polymers or fibrils (Barnham *et al.*, 2004; Taylor *et al.,* 2013). Both forms have been proved to be neurotoxic by inhibiting the complex I activity of mitochondria thus inducing leakage of dopamine into the cytosol, which in turn promotes dopamine quinone production and consequently increased ROS production and oxidative stress (Fig. 1.11; Jomova *et al.,* 2010; Choi *et al.,* 2012; Hwang 2013; Taylor *et al.,* 2013). Damages caused to the mitochondrial complex I in the electron transport chain leads to a leak of electrons, increasing ROS generation (Hwang 2013).

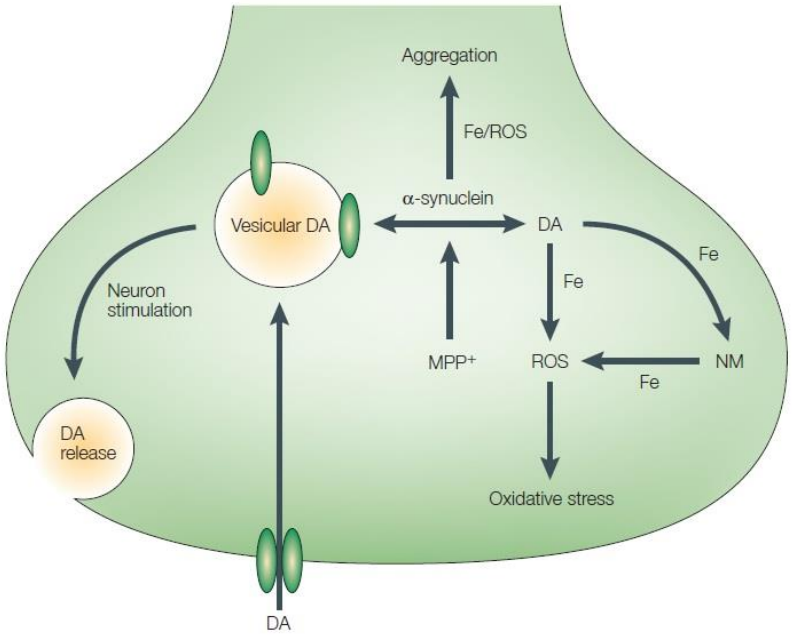

Figure 1.11: Oxidative stress in PD. The rule of DA and α-synuclein in oxidative stress process. The formation of NM leads to oxidative stress. (Source: Barnham *et al.,* 2004).

Oxidative stress occurs when there is an imbalance between ROS production and antioxidant defences leading to oxidation of biomolecules and severe cellular damage (Fig. 1.12; Markesbery, 1996; Halliwell 2001; Orhan, *et al* 2007; Hauser and Hastings, 2013; Hwang 2013), namely DNA modification, enzymes inactivation, and structural proteins destruction (Behl and Moosmann, 2002; Barnham *et al.,* 2004). Oxidative stress is present in most diseases and contributes to tissue injury. In order to cope with the overproduction of ROS, cells are endowed with several defence systems, being the antioxidants among the most important (Khan 2002; Hwang 2013). Antioxidants are compounds, synthetic or natural, that inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions (Velioglu *et al*., 1998; Moreno *et al*., 2006). Natural antioxidants can be phenolic compounds, such as tocopherols, flavonoids and phenolic acids, nitrogen compounds, like alkaloids, chlorophyll derivates, amino acids and amines, and carotenoids as well as ascorbic acid (vitamin C) (Velioglu *et al*., 1998). Phenolic compounds are secondary metabolites present in a large abundance in plant tissues (Blokhina *et al*., 2003). Their antioxidants properties arise from their high reactivity as hydrogen or electron donors, from the ability of the polyphenol-derived radical to stabilize the unpaired electron, and from their ability to chelate transition metals ions, stopping the Fenton's reaction (Blokhina *et al*., 2003). The ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membrane, is another mechanism who confers antioxidant properties to these compounds (Blokhina *et al*., 2003). Synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used, but it is known now their carcinogenic potential, making the research for natural antioxidant drugs extremely important (Velioglu *et al*., 1998; Custódio *et al*., 2011).

Moreover the antioxidants can be defined as enzymatic or non-enzymatic (Khan 2002; Hwang 2013). Non-enzymatic antioxidants, like tocopherol (vitamin E) and ascorbic acid (vitamin C), phenolic compounds and other small molecules, and enzymatic antioxidants, such as catalases and peroxidases, which regulates the intracellular level of H_2O_2, superoxide dismutases (SOD), which catalyses the dismutation of superoxide to H_2O_2, and repair enzymes (Khan 2002; Blokhina *et al*., 2003). All this molecules work together in order to alleviate the ROS production by directly quenching oxygen radicals before they promote damages to cells (Khan 2002; Blokhina *et al*., 2003).

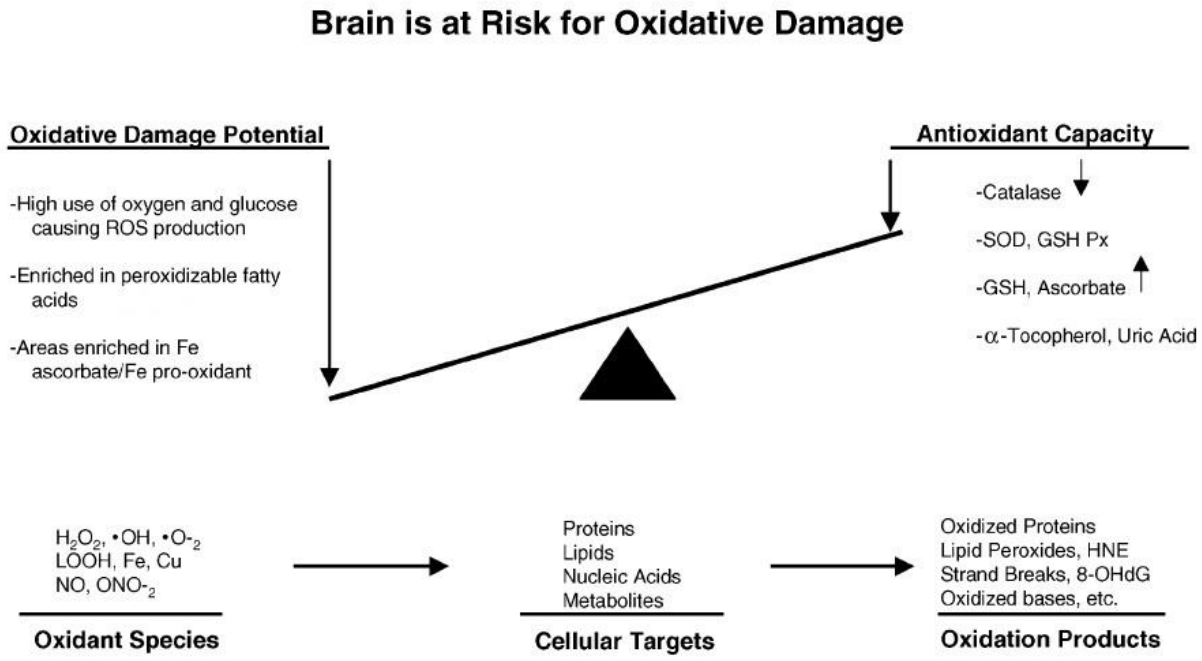

Figure 1.12: Schematic representation of how oxidative damage is exerted on brain. The oxidant species noted are produced and interact with cellular targets producing unique oxidation products and they, in some cases in turn, exert oxidative stress upon the tissue also. (Source: Floyd and Hensley 2002).

1.4. Neuroinflammation: the role of microglia

Microglia cells are the resident phagocytes and innate immune cells of the brain, and play an important role in inflammatory responses being important in homoeostasis, as well as in various pathologies of the CNS (Liu *et al* 2012). Reactive microglia are hallmarks of AD, and these brain cells are likely to contribute to the mechanisms of neuronal damage and cognitive loss associated with that disease (Branden *et al.,* 2012). The activation states in microglia are similar to the macrophages and exhibit a functional plasticity during activation states (Branden *et al.,* 2012). In inactive or quiescent state, these cells have a surveillance function, while in an active state they have two functionally distinct states, M1 and M2 with different functions (Branden *et al.,* 2012). Microbial compounds and pro-inflammatory cytokines induce the classical activation of macrophages, the M1 state in microglia cells (Branden *et al.,* 2012. The M2 state, also known as alternative activation state (Liu *et al* 2012), is responsible for anti-inflammatory activity by blocking the release of pro-inflammatory cytokines, ingesting debris, promoting tissue repair and releasing neurotrophic factors (Branden *et al.,* 2012). The microglia cells respond to exogenous and endogenous stimuli originating a cascade of inflammatory molecules (Liu *et al* 2012). They can be activated in response to lipopolysaccharide (LPS) stimulation, and in consequence of this activation, cells secrete ROS and pro-inflammatory cytokines, such as interleukin-1β (IL-1β), which cause neuronal injuries (Branden *et al.*, 2012). IL-1β is one of the molecules responsible for neuroinflammation, and has a direct effect on neurons causing glial cells and neurons to release multiple inflammatory mediators leading to self-amplifying neuroinflammation (Zhou Wu *et al.*, 2013).

Microglial cells are activated when the protein β-amyloid form oligomers and amyloid deposits, and migrate towards plaques as they are formed (Fig. 1.13; Perrin *et al.*, 2009). The astrocytes become reactive, and numerous inflammatory mediators, signalling molecules, oxidative processes, complement cascades and modulators of protease activities are released (Perrin *et al.,* 2009). Dendrites and axons become dystrophic, due to this transport process malfunctions, leading to altterations in brain metabolism (Perrin *et al.*, 2009). Apart from the formation of NFTs, neurons suffer many other changes: synapses are lost because they are dysfunctional, and neurons die (Perrin *et al.*, 2009).

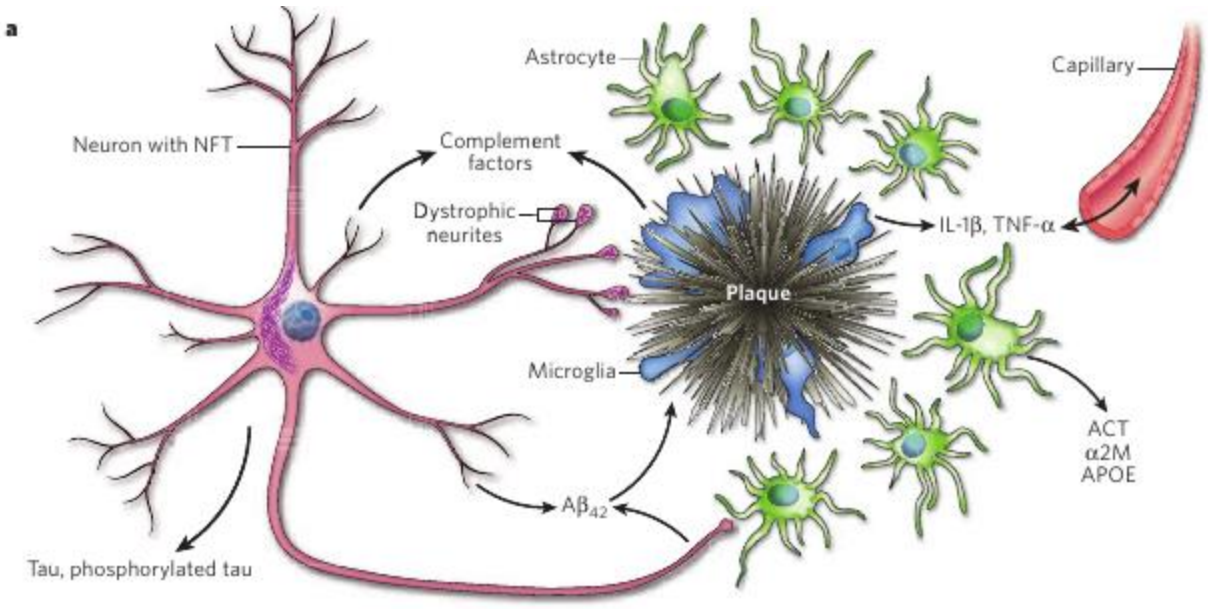

Figure 1.13: Schematic histological representation of biomarkers in relation to Alzheimer's disease pathology. Produced by neurons, $A\beta_{42}$ becomes deposited in plaques, which activate microglia. Microglia release cytokines (for example IL-1β and TNF-α). Microglia and neurons also produce complement factors that can be activated by amyloid-β aggregates, and cause synapse loss. Tau becomes hyperphosphorylated and aggregates into NFTs in neurons and dystrophic neurites around plaques; its mechanism of release from neurons is uncertain. (Source: Perrin *et al.*, 2009).

1.5. Actual treatments for AD and PD

Some studies show that AChE inhibitors have beneficial effects on cognitive, functional, and behavioural symptoms of AD (Scarpini *et al*., 2003). There are four cholinesterase inhibitors approved by the US Food and Drug Administration, the tacrine, donepezil, rivastigmine and galantamine (Scarpini *et al*., 2003). In Europe the availability differs from country to country, and only rivastigmine has been approved centrally by European Agency for the Evaluation of Medical Products (EMEA; Scarpini *et al*., 2003). Tacrine, the first approved drug for AD, is a potent non-selective inhibitor of both AChE and BChE (Franco *et al*., 2005). Although this hepatotoxicity and absence of selectivity have reduced is therapeutic use, the search of tacrine analogues is still of interest (Franco *et al*., 2005). Rivastigmine inhibits both AChE and BChE, while donepezil inhibits only

AChE (Bullock *et al*., 2005). These therapeutic drugs have common adverse events such as nausea, vomiting, diarrhoea, dizziness and weight loss (Hansen *et al*., 2008).

In PD the widely used and effective treatment is with L-DOPA and has been shown to benefit certain cognitive functions, although detrimental effects can also develop following L-DOPA therapy (Cools 2006). L-DOPA impairs some, but improves other complex cognitive abilities (Cools 2006). A long-term treatment with this medicament can lead to a disabling abnormal involuntary movements, also known as L-DOPA induced Dyskinesia (LID), characterized by uncontrolled and repetitive movement in the axis, arms, legs and oro-facial zone (Bravo *et al*., 2014). These facts are a serious limitation to the use of L-DOPA, and become important the search for new way of treatments and new therapeutic targets (Bravo *et al*., 2014).

Because of all these facts presented it becomes even more important the research for natural therapies and also new targets, with the prospect of being more efficient and with fewer side effects.

1.6 Halophytes

Halophytes can live in extreme saline conditions and grow and reproduce in different saline biotypes, such as dunes, rocky coasts, saline depressions, saline inland deserts and in salt marches (Ksouri *et al.*, 2011). In order to survive in these habitats, these plants have developed the ability to resist to toxic ROS, prooduced in response to unfavourable environment conditions: they are endowed with a powerful antioxidant system, including enzymes and secondary metabolites, which can delay the oxidation of molecules through the inhibition of the initiation and propagation of oxidative chain reaction (Ksouri *et al.*, 2011). Due to its unique chemical content, halophytes are a promising source of bioactive compounds, such as vitamins, polyunsaturated fatty acids (PUFAS), and phenolic compounds (Ksouri *et al.*, 2011). The importance of these compounds has been recognized in connection with health promotion, disease risk reduction and decrease in health care costs (Ksouri *et al.*, 2011).

In the last years scientific research has focused in terrestrial and marine natural products with commercial and scientific interests, having found already several thousand

novel molecules with biological activities (Haefner 2003). Studies of bioactivities in halophytes from the Coast of Portugal are however limited, despite the great abundance of this plants in this particularly region (Haefner 2003; Custodio *et al.*, 2012)

1.6.1. *Carpobrotus edulis*

Carpobrotus edulis (Syn. *Mesembryanthemum edule*; sour fig, *Aizoaceae*, Fig. 1.14), is a succulent perennial halophyte spread along the coastal areas of Europe, Africa, Australia and California. In the past it was introduced in Portugal to stabilized and fix coastal sand dunes (Conser and Connor 2009). Nowadays it is considered an invasive species which form impenetrable mats that occupy large areas, preventing the development of native vegetation due to the acidification of the soil that they induce (Conser and Connor 2009).

Sour fig is used in traditional medicine in some areas of South Africa, to treat fungal and bacterial infections and some diseases like tuberculosis, throat infections, sinusitis, dysentery, diarrhoea, infantile eczema, mouth ulcers, toothache and oral and vaginal thrust (Martins *et al* 2011; Ksouri *et al.*, 2011; Custodio *et al.*, 2013). Compounds such as uvaol, β-amyrin, oleanolic acid, catechin, epicatechin and monogalactosyldiacylglycerol have been isolated from *C. edulis*, and their anticancer activity has been suggested (Martins *et al* 2011; Custodio *et al.*, 2013). Moreover phenolic compounds from *C. edulis*, show antioxidant activity, anti-atherosclerotic and anti-inflammatory properties, and protect against congestive heart failure.

Figure 1.14. *Carpobrotus edulis*. (Source: http://www.onlyfoods.net/wpcontent/uploads/2012/09/Carpobrotus-Edulis.jpg).

1.6. Aims of the thesis

There are several studies on the antioxidant, anti-inflammatory and cytotoxicity activities of different plant species. However, the reports on these bioactivities on halophytes are rare. Halophytes are choose to this work because they are potential source of bioactive compounds, whom normally exhibits interesting activities. In this context, the major goal of this work was to study the *in vitro* antioxidant, enzyme inhibition, like Acetylcholinesterase, Butirylcholinesterase and Tyrosinase, neuroprotective, anti-inflammatory activities of different extracts of selected halophyte species common in the southern Portugal (Algarve).

These activities were selected in order to:

- Evaluate the neuroprotective potential activity, through the determination of the antioxidant capacity of halophyte extracts;
- Evaluate the anti-inflammatory activity through nitric oxide inhibitory effect of halophyte extracts;
- Investigate the enzyme inhibitory activity of halophyte extracts.

2. Materials and Methods

2.1 Plant material

In this work 26 halophyte species were used, namely, *Halimione portulacoides, Beta maritima, Carpobrotus edulis, Cotula coronopifolia, Frankenia laevis, Hordeum marinum, Juncus acutus, Limonium algarvense, L. ferulaceum, Limoniastrum monopetalum, Mesembryanthemum crystallinum, Phragmites australis, Plantago coronopus, Polygonum maritimum, Polypogon maritimus, Salicornia ramosissima, Salsola vermiculata, Sarcocornia fruticosa subsp. alpini, S. fruticosa subsp. perennis, Scirpus maritimus, Spartina densiflora, S. maritima, Sporobolus sp., Suaeda vera, Tamarix africana* and *Lampranthus sp*. Biomass was collected in Southern Portugal, namely in Ludo, Praia de Faro and Castro Marim, during the summer of 2010. The aerial parts of the above mentioned species were cleaned from extraneous matter, dried at 40°C for 2-3 days, powdered and stored at -20°C.

2.2 Supplements, chemicals and culture media

Sodium nitrite, sodium acetate, 1,1-diphenyl-2picrylhydrazyl (DPPH), electric eel acethylcolinesterase (AChE) (type-VI-S, EC 3.1.1.7), horse serum butyrylcholinesterase (BChE) (EC 3.1.1.8), galanthamine, acetylthiocholine iodide and butyrylthiocholine chloride, 5,5-dithio-bis(2-nitrobenzoic) acid (DTNB), lipopolysaccharide (LPS) from *Escherichia coli*, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), tyrosinase (EC 1.14.1.8.1, 30 U, mushroom tyrosinase), N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) were purchased from Sigma-Aldrich (Germany). Merck (Germany) supplied Folin-Ciocalteau (F-C) phenol reagent, phosphoric acid and ferrospectral. Lonza (Belgium) provided Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin, L-glutamine and penicillin/streptomycin. Dimethylsulfoxide (DMSO), ethylenediamine tetraacetic acid (EDTA), copper (II) sulphate and additional reagents and solvents were obtained from VWR International (Belgium)

2.3. Preparation of the extracts

For the initial screening, dried biomass was powdered and a single overnight methanol (1:40 w/v) extraction was performed, at room temperature with stirring. The extracts were then filtered with filter paper (Whatman no. 4) and concentrated at 40-50ºC under reduced pressure, and dried under a gentle flow of nitrogen. Dried extracts were weighed, and dissolved in DMSO at a concentration of 10 mg/mL.

C. edulis was subjected to sequential extraction with different polarities solvents in order to obtain different compounds with different polarities in each extract. That way we have an arrangement of different compounds with different activities, and can choose the extract(s) who better fit in our study. Dried sample of *C.edulis*, were extracted in a Soxhlet apparatus for 2 hours with each solvent, in the order: n-hexane, dichloromethane, chloroform, ethyl acetate, and methanol. In each extraction 50 gr of sample and 250 mL of solvent were used. Extracts were filtered with filter paper (Whatman no. 4), and evaporated under reduced pressure. Dried extracts were weighed, dissolved in DMSO to a final concentration of 50 mg/mL and stored at 4ºC.

2.4 Determination of antioxidant activity

2.4.1. Radical scavenging activity (RSA) of DPPH

The DPPH assay was performed according to the method of Brand-Williams *et al*. (1995), as described by Moreno *et al*. (2006). Samples (22 µL) were mixed with 200 µL of methanol DPPH solution (120 µM) in 96-wells microplates and incubated for 30 min. at room temperature (RT), in the dark. Hexane, dichloromethane, chloroform and ethyl acetate extracts were tested at the concentrations of 1, 5 and 10 mg/mL. Methanol extract was tested at 0.010, 0.100 and 1 mg/mL. A colour control was made to exclude any absorbance by the crude extract. This control was made by adding 22 µL of extract plus 200 µL of methanol solvent. The DPPH solution was used as a negative control, while butylated hydroxytoluene (BHT, E320) was used as positive control at the concentration of 1 mg/mL. The absorbance was measured at 517 nm using a Biotek Synergy 4 microplate reader and antioxidant activity (%) was calculated according to the following equations:

% inhibition = (Absorbance of negative control – Absorbance of test sample) x 100 / Absorbance negative control

Where

Absorbance of test sample = Absorbance of test solution – Absorbance of colour control

2.4.2 Iron (ICA) chelating activity

ICA was determined according to the method described by Megías *et al*. (2009). The samples (30 µL) were mixed in 96-well microplates with 250 µL of 100 mM Na acetate buffer (pH 4.9) and 30 µL of an aqueous $FeCl_2$ solution (0.1 mg/mL, w/v). After 30 min., 12.5 µL of 40 mM ferrozine aqueous solution were added. The change in colour was measured at 562 nm in a microplate reader (Biotek Synergy 4), and the results expressed as percentage inhibition, relatively to a negative control solution containing methanol or DMSO in place of the sample. Extracts were tested at the concentrations of 1, 5 and 10 mg/mL. The synthetic metal chelator EDTA was used as a standard at 1 mg/mL.

2.4.3 Copper (CCA) chelating activity

CCA was evaluated using pyrocatechol violet (PV) according to Megías *et al*. (2009). Samples (30 µL) were mixed in 96-well microplates with 200 µL of 50 mM Na acetate buffer (pH 6.0), 6 µL of 4 mM PV dissolved in the buffer and 100 µL of $CuSO_4.5H_2O$ (50 µg/mL, w/v). The change in colour was measured at 632 nm, using a microplate reader (Biotek Synergy 4). Results were expressed as percentage of inhibition relatively to a negative control solution containing methanol or DMSO in place of the sample. Extracts were tested at the concentrations of 1, 5 and 10 mg/mL. The synthetic metal chelator EDTA was used as a standard at 1 mg/mL.

2.4.4. Iron Reducing power

Iron reducing power was determined according to the method described by Megías *et al*. (2009). The samples (50 µL) were mixed in 96-well microplates with 50 µL of distilled water and 50 µL of 1% potassium ferricyanide and incubated at 50ºC for 20 min. Then, 50 µL of 10% trichloroacetic acid and 10 µL of 0.1% ferric chloride were added and incubated for 20 min. at 50ºC. Absorbances were read at 700 nm on a microplate reader (Biotek

Synergy 4). Samples were tested at 1, 5 and 10 mg/mL. BHT (butylated hydroxytoluene) was used as positive control at 1 mg/mL. Results were expressed relatively to the positive control.

% Activity = (100 x absorbance of test sample) / Absorbance positive control

Where

Absorbance of test sample = Absorbance of test solution – Absorbance of colour control

2.4.5. Nitric oxide (NO) - scavenging activity

NO scavenging activity was measured according to Ebrahimzadeh *et al*. (2010), adapted to 96 well plates. Briefly, 50 µL of the extracts were mixed with 50 µL of 10 mM sodium nitroprusside in phosphate buffer (PBS) in 96-well microplates, and incubated for 90 min at RT. Then, 50 µL of Griess reagent (1% of sulphanilamide and 0.1% of naphthylethylenediamine in 2.5% HPO_3) were added and the change colour was recorded at 546 nm using a microplate reader (Biotek Synergy 4). Ascorbic acid was used as positive control at 1 mg/mL. Results were expressed as percentage of activity, relatively to a negative control Containing DMSO in place of the sample.

2.5. Phytochemical analysis

2.5.1 Total flavonoid content (TFC)

TFC was determined by the aluminium chloride ($AlCl_3$) colorimetric method adapted to 96-well microplates (Zou *et al*., 2012). Aliquots (30 µL) of the extracts at 1 mg/mL were mixed with 180 µL of distilled water, and 10 µL of 5% sodium nitrite ($NaNO_2$). After 6 min. of incubation, 20 µL of 10% of $AlCl_3$ (in methanol) were added and the solution was further incubate for another 6 min. Then, 60 µL of 4% sodium hydroxide (NaOH) was added, the mixture further incubated for 15 min, and the absorbance measured at 510 nm in a microplate reader (Biotek Synergy 4). TFC was calculated based on a standard curve of rutin solutions and results expressed as milligram of rutin equivalents per gram of dried extract (mg RE/ g dry weight, DW).

2.5.2. Total condensed tannin content (TCTC)

TCTC of the extracts was evaluated by the 4-dimethylaminocinnamaldehyde (DCAMA) colorimetric method adapted from the vanillin method to 96-well microplates (Zou *et al*., 2012). 10 µL of each extract at 1 mg/mL were mixed with 200 µL of 1% of DCAMA (in methanol), and 100 µL of 37% HCl. After 15 min of incubation the absorbance was read at 640 nm in a microplate reader (Biotek Synergy 4). TCTC was calculated based on a standard curve of catechin solutions and results expressed as milligrams of catechin equivalents per gram of dried extract (mg CE/ g, DW).

2.5.3. Total phenolic content (TPC)

TPC of the extracts was determined by the Folin-Ciocalteau (F-C) colorimetric assay according to Velioglu *et al*. (1998). The experiments were performed in 96-well plates: 5 µL of each extract at 1 mg/mL were mixed with 100 µL of F-C (1:10, v/v in water). After 5 min, 100 µL of sodium carbonate solution (75 g/L) were added, and the mixture incubated at RT for 90 min, in the dark. The absorbance of each sample was measured at 725 nm in a microplate reader (Biotek Synergy 4). Results were calculated based on a standard curve of gallic acid solutions and results expressed as milligrams of gallic acid equivalents per gram of dried extract (mg GAE/ g, DW).

2.6. Enzyme inhibition assays

2.6.1. Acetylcholinesterase (AChE) and butirylcholinesterase (BChE) inhibition

The inhibitory activity of AChE and BChE was determined according to the methods described by Orhan *et al*. (2007). Briefly, 140 µL of 0.1 mM sodium phosphate buffer (pH 8.0) were mixed with 20 µL of the extracts at 1, 5 and 10 mg/mL, and 20 µL of AChE/BChE (0.28 U/mL) in 96-well microplates. After a 15 min incubation period at 37ºC, 10 µL of acetylthiocholine iodide (ATChI) or butyrylthiocholine chloride (BTChI), at 4 mg/mL were added to start the hydrolysis reaction, catalysed by the enzymes. Then, 20 µL of 5,5-dithiobis-(2-nitrobenzoic) acid (DTNB) (1.2 mg/mL) were added to form the yellow 5-thio-2-nitrobenzoate anion as a result of DTNB reaction with the thiocholines, produced as a result of AChE or BChE activity. This anion was detected at 412 nm, in a 96-well microplate reader (Biotek Synergy 4). Galanthamine, an anticholinesterase alkaloid-type

drug isolated from the bulbs of snowdrop (*Galanthus sp.*) was used as a positive control at 1 mg/mL.

2.6.2. Tyrosinase (TYRO) inhibition

TYRO inhibition was determined according to the method described by Nerya *et al*. (2003) with some modifications. Samples were diluted in 50 mM potassium phosphate buffer solution (pH 6.5) to obtain concentrations of 1, 5 and 10 mg/mL. Extracts (70 µL) were mixed in 96-well microplates with 30 µL of tyrosinase (333 Units/mL in buffer), and incubated for 5 min at RT. Then, 10 µL of substrate (2 mM L-Tyrosine) were added, incubated at RT for 30 min, and the optical density read at 492 nm using a microplate reader (Biotek Synergy 4). Results were expressed as % of tyrosinase inhibition relatively to a negative control solution containing potassium phosphate buffer solution in place of the sample. The positive control used was arbutin at 1 mg/mL.

2.7. Neuroprotective activity using cell-based method

2.7.1. Protective effect against H_2O_2-induced injury in neuronal cells

2.7.1.1. Cell culture and viability determination

The human neuroblastoma cell line (SH-SY5Y cells) was maintained in Dulbecco's modified eagle medium (DMEM) with 4500 mg/mL of glucose, 10% heat inactivated fetal bovine seurm (FBS), l-glutamine (2 mM), penicillin (50 U/mL) and streptomicin (50 µg/mL), and maintained in an incubator at 37ºC, in a 5.1% CO_2 humidified atmosphere. To evaluate the effect of the extracts on the viability of SH-SY5Y cells, they were seeded in 96-well plates at a density of 2×10^4 cell/well and incubated for 24 h. Then, 100 µl of the extracts at 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL were added and plates further incubated for 24 h. Cell viability was determined by the MTT assay (Mosmann, 1983). Briefly, 2h prior to the end of the incubation period, 20 µL of MTT (5 mg/mL in PBS) were added to each well and incubated at 37ºC. Then, 150 µL of DMSO were added to each well in order to dissolve the formazan crystals produced and absorbance was measured at 590 nm (Biotek Synergy 4). Samples allowing cell viability higher than 80% were selected and used to assess their protective effect of the extracts against H_2O_2 induced oxidative stress on SH-SY5Y cells.

2.7.1.2. Protective effect against H_2O_2 induced oxidative stress on SH-SY5Y cells

SH-SY5Y cells were seeded at a density of 2×10^4 cells/well in 96-well culture plates, left to adhere overnight, treated with non-toxic concentrations of the extracts in culture medium and incubated for 24 h. Then, H_2O_2 (1 mM) was added for 30 min. and cell viability was determined by the MTT assay (Mosmann, 1983) as described in section 2.7.1.1. A stock solution of H_2O_2 was prepared on phosphate-buffered saline (PBS, pH 7.4) and diluted with DMEM without FBS immediately before use.

2.7.2. Anti-inflammatory activity on LPS-stimulated microglia cells

2.7.2.1. Cell culture and viability determination

The murine microglial cell line N9 was cultured in Dulbecco's modified eagle medium (DMEM) in the same conditions described in section 2.7.1.1 for neuronal cells. To evaluate the effect of the extracts on the viability of N9 cells, extracts were applied at concentrations ranging from 3.125 to 100 μg/mL, incubated for 24 h, and cell viability was determined by the MTT assay (Mosmann, 1983). Samples allowing cell viability higher than 80% were selected to assess for anti-inflammatory activity, through inhibition of NO production by LPS-stimulated microglia cells.

2.7.2.2. Quantification of nitric oxide (NO) production on LPS-stimulated microglia cells

N9 cells were seeded at a density of 1×10^6 cells/well in 24-well plates and incubated for 24h. Then, cells were treated with 200 μL of the extracts at various concentrations (3.125, 6.25, 12.5, 25, 50 and 100 μg/mL), in serum- and phenol-free culture medium, for 24 h, with 200 μL of LPS (10 μg/mL). Control cells were treated with DMSO at the highest concentration used in test wells (0.5%, v/v) with and without LPS. The NO production in the cell culture medium was measured spectrophotometrically using the Griess reaction (Miranda *et al*., 2001). Stock Griess solution was prepared with equal amounts of a solution of 1% (w/v) sulphanilamide + 0.1% of N-(1-Naphthyl)-ethylenediamine dihydrochloride (NED) and 2.5% (v/v) phosphoric acid. After the incubation period with the plant extracts and/or LPS, 100 μL of the culture supernatants were mixed with the 100 μL of Griess reagent in 96-well microplates. After 20 min, of incubation at RT, in the dark, absorbance

was measured at 540 nm on a microplate reader (Biotek Synergy 4). Nitrite concentration was determined using a calibration curve prepared with several concentrations (1.7, 3.1, 6.2, 12.5, 25, 50 and 100 μM) of sodium nitrite as standard. Results were expressed as μM of nitric oxide.

2.8 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM), and all experiments were conducted at least in triplicate. Analysis of variance (ANOVA) using the Duncan's honest significant difference (HSD) test was used to assess differences between means. Samples were considered significantly different when $P < 0.05$. The SPSS statistical package for Windows (release 15.0, SPSS INC) was used.

3. Results

3.1 Determination of antioxidant activity

3.1.1 Radical scavenging activity (RSA) of DPPH•

In this work, methanolic extracts from twenty six halophytes species were screened for RSA on the DPPH radical. The extracts were tested at 1 mg/mL and results are summarized on Table 3.1.

Table 3.1. RSA on DPPH free radical• (%) of methanolic extracts of 26 species of halophytes collected in the Southern Portugal, applied at the concentration of 1 mg/mL.

Species	DPPH 1mg/mL
Halimione portulacoides	6.95 ± 1.46
Beta maritima	n.a
Carpobrotus edulis	95.7 ± 0.2*
Cotula coronopifolia	18.7 ± 0.3
Frankenia laevis	95.2 ± 0.0*
Hordeum marinum	20.9 ± 0.2
Juncus acutus	29.8 ± 0.5
Limonium algarvense	95.7 ± 0.3*
Limonium ferulaceum	91.7 ± 0.3
Limoniastrum monopetalum	63.5 ± 1.0
Mesembryanthemum crystallinum	10.9 ± 0.4
Phragmites australis	39.5 ± 0.9
Plantago coronopus	31.2 ± 0.3
Polygonum maritimum	95.4 ± 0.4*
Polypogon maritimus	46.4 ± 1.0
Salicornia ramosissima	19.9 ± 0.4
Salsola vermiculata	40.1 ± 0.5
Sarcocornia fruticosa subs. alpini	93.1 ± 0.1
Sarcocornia fruticosa subs. perenis	18.3 ± 0.3
Scirpus maritimus	75.8 ± 0.8
Spartina densiflora	8.72 ± 0.64
Spartina maritime	2.45 ± 0.48
Sporobolus sp.	20.9 ± 0.5
Suaeda vera	29.5 ± 0.3
Tamarix africana	95.0 ± 0.1*
Lampranthus sp.	69.8 ± 0.7
BHT[a]	89.1 ± 0.1

Each value corresponds to mean ± SEM (n = 12). [a] Positive control; * significantly higher that the positive control ($p < 0.05$). n.a: no activity.

Several species had high antioxidant activity, and the best results were obtained with *C. edulis* (95.7%), *F. laevis* (95.1%), *L. algarvense* (95.6%), *P. maritimum* (95.3%) and *T. africana* (95.0%), all significantly higher than the pure antioxidant compound BHT used at the same concentration as the samples. Based on these results we selected *C. edulis* for further work, having in mind not only its high RSA, but also the fact that it is an invasive species in several locations, including Portugal (Rodrigues *et al*, 2014). In fact, populations of *C. edulis* are often submitted to physical removal in order to reduce the area occupied by this species; a biotechnological use for the resulting biomass would provide a sustainable use of such an abundant resource (Rodrigues *et al*, 2014).

Dried biomass of *C. edulis* was sequentially extracted in a Soxhlet apparatus using solvents of increasing polarities namely hexane, dichloromethane, chloroform, ethyl acetate and methanol. Those extracts where then tested for RSA activity using the DPPH assay at concentrations ranging from 0.010 mg/mL to 10 mg/mL (Fig. 3.1).

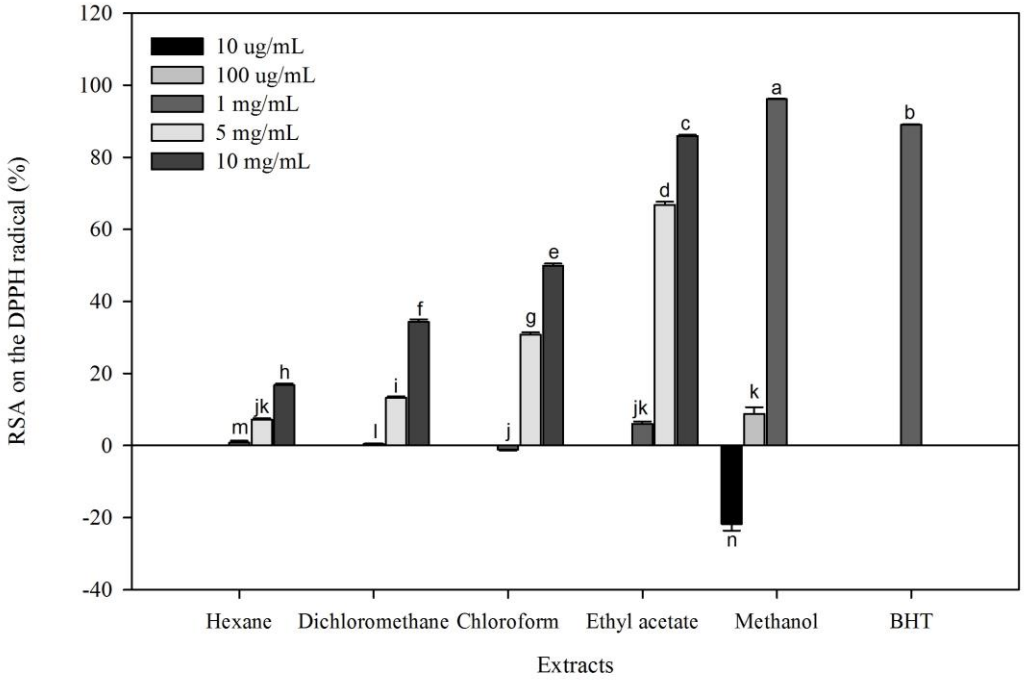

Figure 3.1: RSA on the DPPH radical of the extracts of *C. edulis* and positive control (BHT). Solid bars and errors bars represent the average and standard error values, respectively (n = 12). Results are calculated in respect to the negative control. Significant differences between concentrations, between extracts and the positive control were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-n).

All the extracts showed a dose-dependent RSA against DPPH• and the highest activity was observed with the methanol extract at the concentration of 1 mg/mL, with a RSA value of 96.1%, significantly higher than the one obtained with the positive control used, BHT (89.1%; $p<0.05$; Fig. 3.1).

3.1.2 Iron (ICA) and copper (CCA) chelating activity

The extracts were tested for ICA and CCA at the concentrations of 1, 5 and 10 mg/mL (Table 3.2). All extracts had an increasing ICA with increasing concentrations, and the hexane extract had the highest ICA (81.3 %) at the highest concentration tested (10 mg/mL; Table 3.2).

Table 3.2. Iron and copper chelating activity of extracts of *C. edulis*.

	ICA			**CCA**		
Extract / Compound	**1 mg/mL**	**5 mg/mL**	**10 mg/mL**	**1 mg/mL**	**5 mg/mL**	**10 mg/mL**
Hexane	45.7 ± 2.0^{g}	72.7 ± 0.9^{cd}	81.3 ± 1.8^{b}	n.a	n.a	n.a
Dichloromethane	50.8 ± 2.2^{ef}	68.6 ± 1.5^{d}	76.3 ± 1.3^{c}	3.4 ± 2.2^{gh}	8.1 ± 1.5^{f}	7.1 ± 1.5^{f}
Chloroform	31.4 ± 1.7^{h}	46.3 ± 1.6^{fg}	51.6 ± 1.4^{e}	n.a	n.a	0.5 ± 0.8^{h}
Ethyl Acetate	n.a	n.a	n.a	3.1 ± 0.8^{gh}	12.9 ± 0.9^{e}	25.9 ± 1.1^{d}
Methanol	12.7 ± 1.9^{i}	32.7 ± 0.8^{h}	45.3 ± 1.3^{g}	5.81 ± 2.1^{fg}	30.8 ± 1.5^{c}	48.3 ± 0.7^{b}
EDTA*	89.7 ± 0.4^{a}			89.2 ± 0.5^{a}		

Each value represents mean ± SEM (n = 12); *positive control at concentration at 1 mg/mL. Significant differences between concentrations, extracts and positive control were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-h). n.a: no activity.

Regarding the CCA, only the ethyl acetate and methanol extracts had an activity dependent of the extract concentration, and the highest result was obtained with the methanol extract at the concentration of 10mg/mL (48.3%, Table 2). None extract showed a higher activity than the obtained with positive control (EDTA).

3.1.3 Iron reducing power

In this assay, the Fe^{3+} to Fe^{2+} reduction power of the extracts was tested at 1, 5 and 10 mg/ml, and results are depicted on Fig. 3.2.

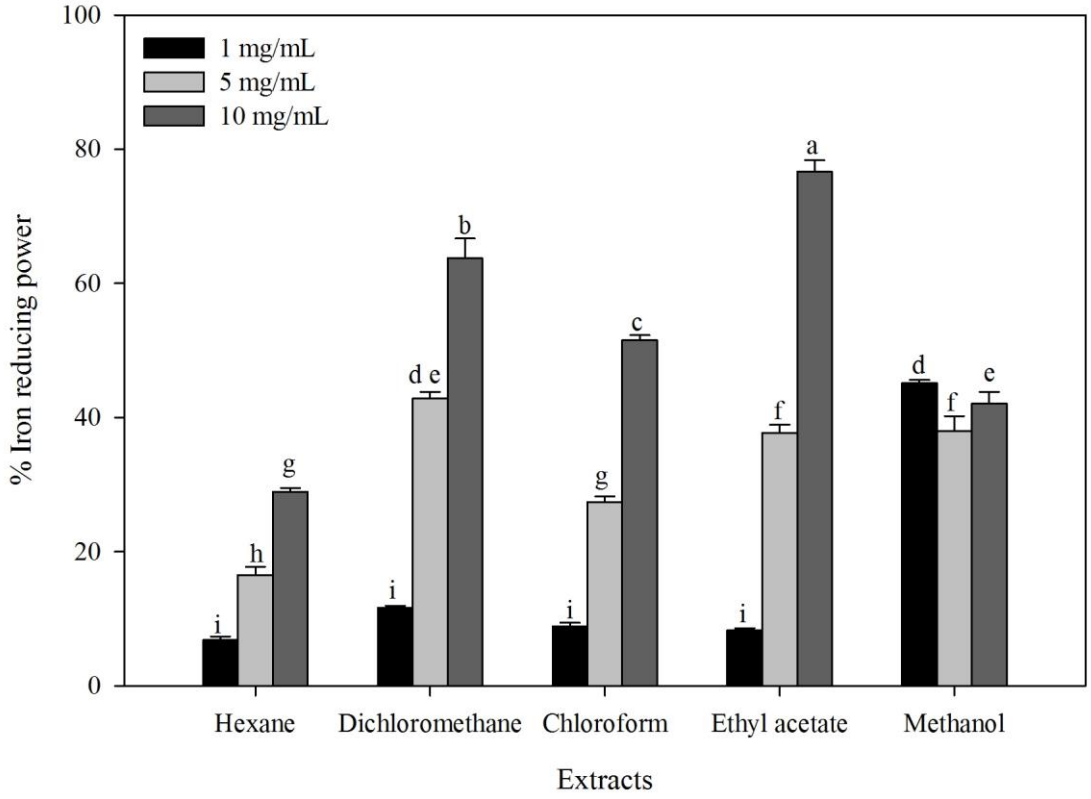

Figure 3.2: Iron reducing activity of extracts of *C.edulis*. Solid bars and error bars represent the average and standard errors values respectively (n = 12). Results are calculated in respect to the BHT, used as a positive control. Significant differences between concentrations and extracts were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-i).

The hexane, dichloromethane, chloroform and ethyl acetate extracts showed an increasing reducing power activity with increasing concentrations, and the highest result was obtained with the ethyl acetate extract at the concentration of 10mg/mL (76.6 %).

3.1.4. Nitric oxide (NO)-scavenging activity

The NO scavenging capacity of the extracts was determined at the concentrations of 1, 5 and 10 mg/mL, and results are summarized on Fig. 3.3.

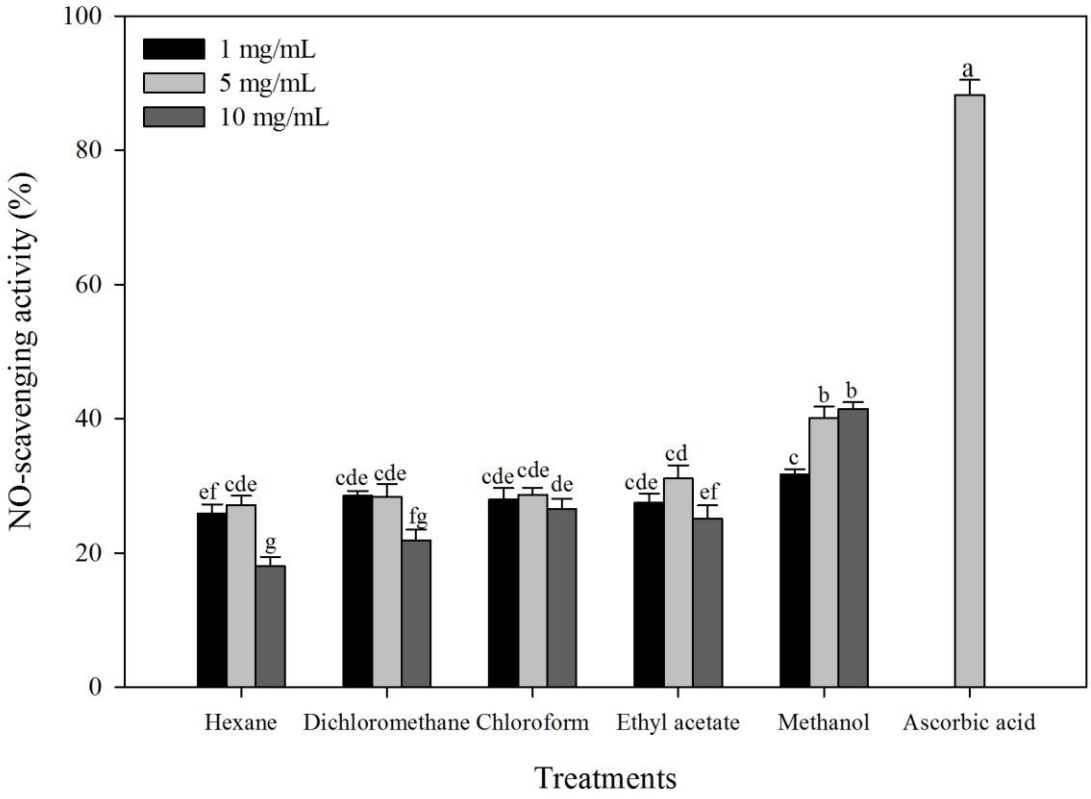

Figure 3.3. NO scavenging activity of extracts of *C.edulis*. Each value represents mean ± SEM (n = 12). Significant differences between extracts and concentrations were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-b). Ascorbic acid was used as a positive control at the concentration of 1 mg/mL.

Only the methanol extract had a dose-response effect, with an increase in the % of NO-scavenging activity with increasing concentrations of the extract, and the highest activities were obtained with the concentrations of 5 and 10 mg/mL, (40.1% and 41.5%, respectively. The hexane and the dichloromethane extracts had a decreasing NO scavenging activity from the concentrations of 1 and 5 mg/mL to the highest concentration tested (10mg/mL), while ethyl acetate had a decrease from the concentration 5 to 10mg/mL.

3.2. Total phenolics (TPC), flavonoids (TFC) and condensed tannins content (TCTC)

The methanol extracts had the highest TPC (378.8 mg GAE/g, DW), followed by the ethyl acetate (260.5 mg GAE/g, DW) and chloroform (219.6 mg GAE/g, DW; Table 3.3).

Table 3.3. Total phenolics (TPC, mg GAE/g DW), flavonoids (TFC, mg RE/g DW) and condensed tannins contents (TCTC, mg CE/g DW) of *C. edulis* extracts.

Extract	TPC (mg GAE/g, DW)	TFC (mg RE/g, DW)	TCTC (mg CE/g, DW)
Hexane	196 ± 3^{c}	299 ± 5^{d}	95.4 ± 6.4^{a}
Dichloromethane	200 ± 7^{c}	369 ± 8^{b}	102 ± 10^{a}
Chloroform	219 ± 4^{bc}	319 ± 5^{c}	94.6 ± 6^{a}
Ethyl acetate	260 ± 4^{b}	383 ± 6^{b}	100 ± 5^{a}
Methanol	378 ± 4^{a}	587 ± 6^{a}	58.0 ± 2.7^{b}

Results are expressed as mean ± SEM (n = 12) and are calculated in respect to the negative control. Significant differences between extracts were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-d).

The methanol extracts had the highest level of flavonoids (587 mg RE/g, DW), followed by the ethyl acetate (383 mg RE/g, DW), dichloromethane extracts (369 mg RE/g, DW), chloroform (318 mg RE/g, DW) and hexane extracts (298 mg RE/g, DW; Table 3.3). Regarding tannins, the highest amounts were observed in the dichloromethane (102 mg CE/ g, DW), ethyl acetate (100 mg CE/ g, DW), hexane (95.3 mg CE/ g, DW) and chloroform extracts (94.6 mg CE/ g, DW), which are statistically similar, followed by the methanol extract (58 mg CE/ g, DW) (Table 3.3).

3.3. Enzyme inhibition assays

3.3.1. Cholinesterase inhibition

The extracts were tested for inhibition of enzymes related with neurodegenerative diseases, namely AChE, BChE and TYRO, and results are depicted on Tables 3.4 (AChE), 3.5 (BChE) and 3.6 (TYRO). The inhibitory activity (%) was classified as potent (>50%), moderate (30-50%), low (<30%) and nil (<5%) (Vinutha *et al*, 2007).

According to this classification the ethyl acetate and the methanol extracts had potent AChE inhibition, even at the lowest concentration tested (1 mg/mL), and the highest results were obtained with the methanol extract, with inhibition values of 75.1% (1 mg/mL), 87.2% (5 mg/mL) and 86.2% (10 mg/mL). Dichloromethane also had potent AChE inhibition at 10 mg/mL (62.9%), while the remaining extracts had moderate activity at the highest concentration tested. There is no significant difference in inhibition of AChE activity among the methanol extract and positive control galantamine (p<0.05).

Table 3.4. AChE inhibitory activity of extracts of *C. edulis*.

Extract	1 mg/mL	5 mg/mL	10 mg/mL	Galanthamine
Hexane	n.a	14.4 ± 4.6^{g}	37.0 ± 5.2^{def}	89.9 ± 0.7^{a}
Dichloromethane	21.4 ± 3.2^{fg}	31.4 ± 3.5^{ef}	62.9 ± 7.5^{bc}	
Chloroform	44.4 ± 2.3^{de}	49.4 ± 7.9^{cd}	36.8 ± 7.0^{def}	
Ethyl acetate	50.4 ± 7.7^{cd}	80.8 ± 2.8^{a}	75.6 ± 1.9^{ab}	
Methanol	75.1 ± 0.7^{ab}	87.2 ± 1.1^{a}	86.2 ± 0.7^{a}	

Each value represents mean ± SEM of at least 6 replicates. Significant differences between concentrations of the same extract were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-g). Galanthamine was used as a positive control, at 1 mg/mL. n.a: no activity.

The hexane extract had a potent BChE inhibition at all tested concentrations. The chloroform extract showed a potent BChE inhibition at 1 and 5 mg/mL, and a moderate BChE inhibition at the highest concentration tested (10 mg/mL). The dichloromethane, ethyl acetate and methanol extracts at the lowest concentration tested (1 mg/mL) show a potent BChE inhibition, and the remaining extracts and concentration has a moderate BChE inhibition, except for the ethyl acetate at concentration at 10 mg/mL, which had a low BChE activity.

Table 3.5. BChE inhibitory activity of extracts of *C. edulis*.

Extract	**1 mg/mL**	**5 mg/mL**	**10 mg/mL**	**Galanthamine**
Hexane	59.4 ± 1.04^{b}	57.8 ± 1.21^{bc}	58.6 ± 3.29^{b}	81.3 ± 1.3^{a}
Dichloromethane	56.4 ± 1.21^{bc}	43.6 ± 1.69^{de}	35.6 ± 1.85^{f}	
Chloroform	58.7 ± 1.09^{b}	55.4 ± 1.93^{bc}	40.2 ± 3.05^{ef}	
Ethyl acetate	60.4 ± 0.86^{b}	39.2 ± 2.96^{ef}	21.2 ± 0.81^{g}	
Methanol	52.1 ± 1.2^{c}	46.2 ± 2.11^{d}	40.5 ± 1.08^{ef}	

Each value represents mean ± SEM of at least 6 replicates. Significant differences between concentrations of the same extract were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-g). Galanthamine was used as a positive control, at 1 mg/mL.

3.3.2. Tyrosinase inhibition

The methanol extract had a moderate TYRO inhibition at all concentrations tested while the ethyl acetate extract have the lowest TYRO inhibition, at all concentrations. The dichloromethane extract had a moderate TYRO inhibition at concentration at 5 mg/mL (32.0% ± 1.2) and at 10 mg/mL (36.5% ± 2.5).

Table 3.6. Tyrosinase inhibitory activity of extracts of *C.edulis*.

Extract	1 mg/mL	5 mg/mL	10 mg/mL	Arbutin
Hexane	6.5 ± 1.1^{gh}	14.9 ± 1.0^{f}	36.9 ± 0.6^{c}	78.2 ± 2.1^{a}
Dichloromethane	25.8 ± 1.1^{e}	32.0 ± 1.2^{d}	36.5 ± 2.5^{cd}	
Chloroform	n.d	n.d	n.d	
Ethyl acetate	9.3 ± 0.5^{g}	6.9 ± 0.7^{g}	2.2 ± 1.6^{h}	
Methanol	43.1 ± 1.7^{b}	33.4 ± 0.8^{cd}	42.6 ± 0.9^{b}	

Each value represents mean ± SEM (n = 12). Significant differences between concentrations of the same extract were determined by the Duncan HSD test ($p < 0.05$) and are indicated by different letters (a-h). Arbutin was used as positive control, at the concentration of 1 mg/mL. (n.d – not dertemined).

3.4. Neuroprotective activity using cell-based method

3.4.1. Protective effect against H_2O_2-induced injury in neuronal cells

In this work the protective effect of *C.edulis* extracts against cell injury caused by H_2O_2 treatment was evaluated on the human neuroblastoma SH-SY5Y cell line. In order to find the non-toxic concentrations samples were first applied at different concentrations (3.125, 6.25, 12.5, 25, 50 and 100 μg/mL) on SH-SY5Y cells, and viability was determined by the MTT method. Results were compared with control cells treated with DMSO at the maximum concentration used in the wells (0.5%, (v/v)).

All the extracts significantly increased the viability of SH-SY5Y cells, especially the methanol extract at the concentration of 100 μg/mL (287.7% of cell viability; Fig. 3.4), and thus, all samples were used to determine the protective effect against H_2O_2 – induced injury.

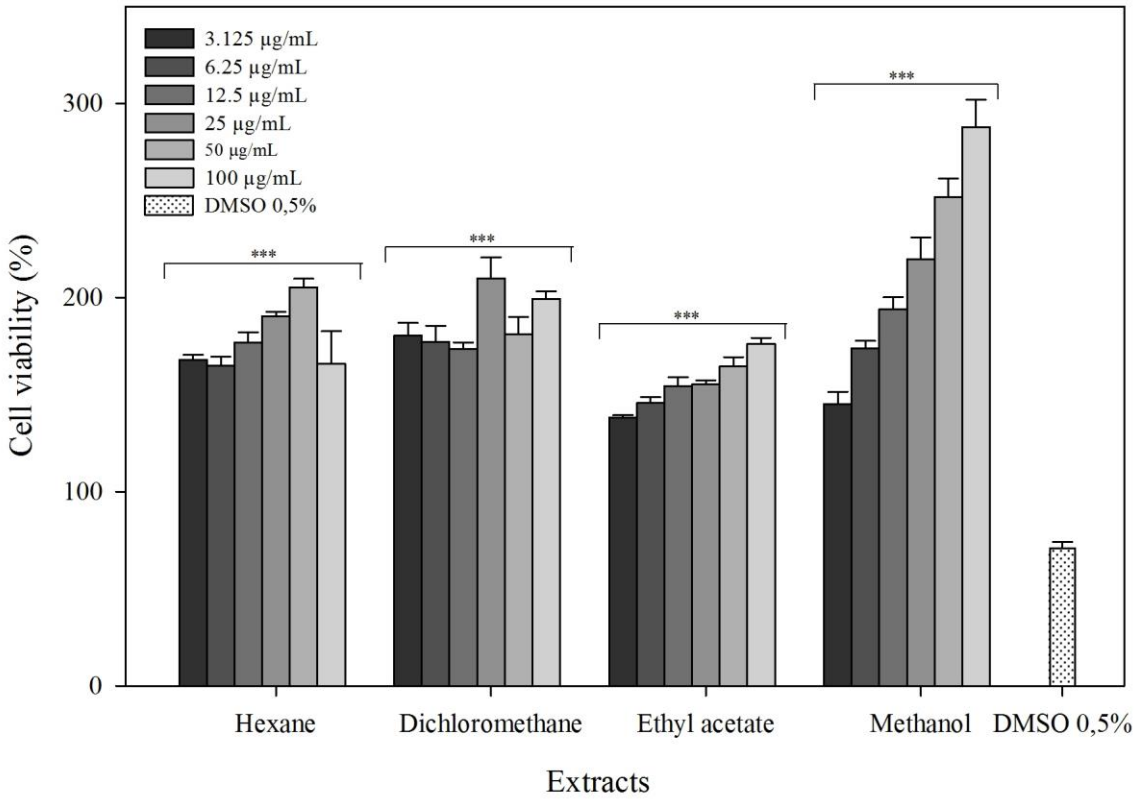

Figure 3.4. Cytotoxic effect of extracts of *C.edulis* in a human neuroblastoma cell line (SH-SY5Y cells). Solid bars and errors bars represent the average and standart error values, respectively (n = 6). *** Indicate a significan increase of cell viability of treated cells compared to control cells containing DMSO (0.5%, v/v; $p < 0.001$.

In order to select the H_2O_2 concentration required to reduce cell viability by 50% cells were treated with different concentrations of H_2O_2 (0.25 – 1 mM) for different periods of incubation (15-60 min.) and cell viability was evaluated by the MTT assay. As can be seen on Fig. 3.5 the treatment of SH-SY5Y with 1 mM of H_2O_2 for 30 min. resulted in a reduction of cell viability to 48.9%. Thus, these conditions were selected and used in the following assays.

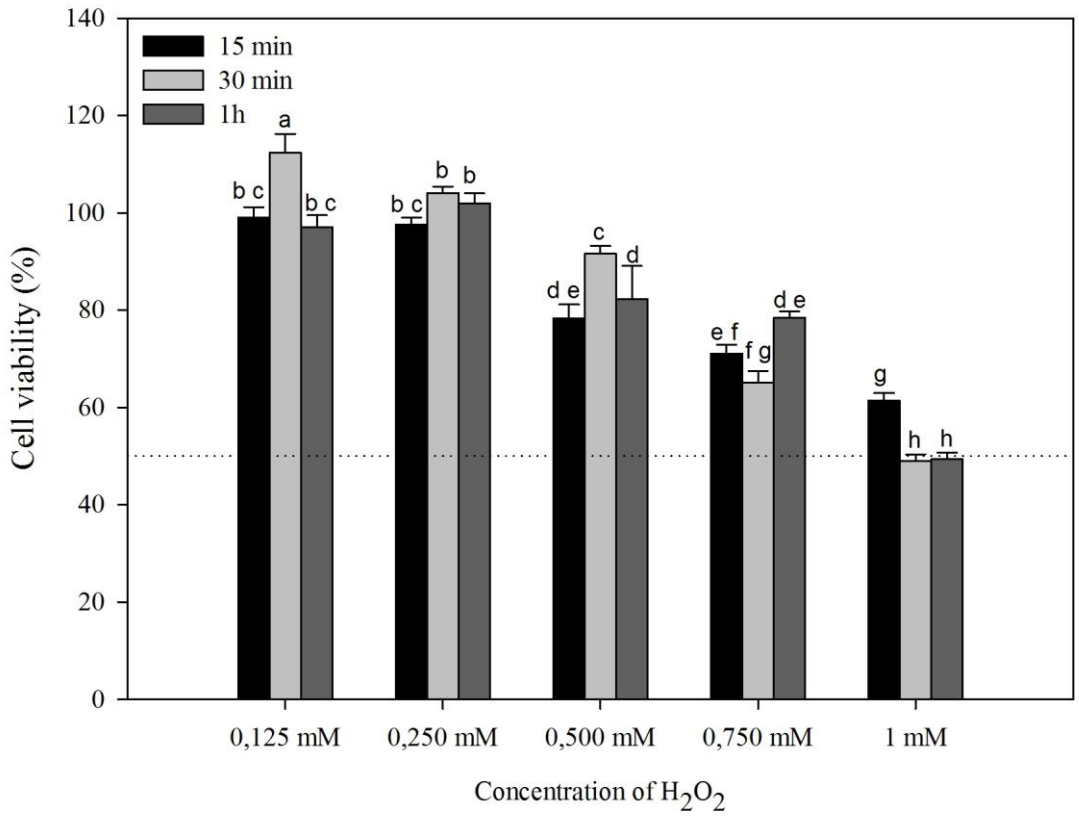

Figure 3.5. Cytotoxic effect of different concentrations of H_2O_2 applied for different time period on SH-SY5Y cells. Solid bars and error bars represent the average and standard error values, respectively (n = 6). The horizontal dashed line represents 50% of cell viability. Significant differences between extracts were determined by the Duncan HSD test (p < 0.05) and are indicated by different letters (a-h).

In order to determine the neuroprotective potential of *C. edulis*, hexane, dichloromethane, ethyl acetate and methanol extracts were applied to SH-SY5Y cells at concentrations ranging from 3.125 to 100 μg/mL for 24h.After extract removal, cells were challenged with H_2O_2 (1 mM for 15 min), and dissolved in culture medium containing DMSO at the highest concentration used (0.5% v/v). Cell viability was measured by the MTT method. As can be seen on Fig. 3.6 the pre-treatment of the cells with the extracts before exposure to H_2O_2 resulted in an increase in cell viability, except for the ethyl acetate extract at the concentration of 25 μg/mL. The dichloromethane and methanol extracts had the highest capacity to protect cells from H_2O_2 injury, with values of cell viability of 105.1% and 143.4%, respectively, at the concentration of 50 μg/mL.

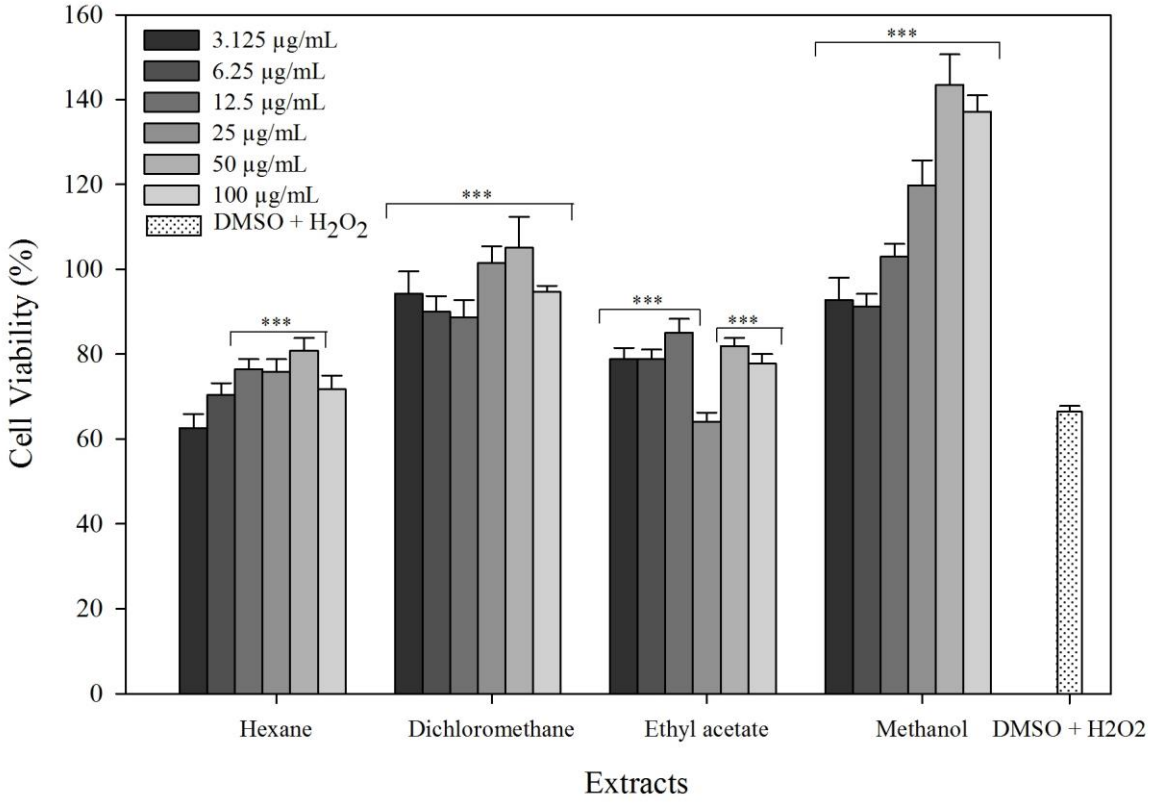

Figure 3.6. Protective effect of the *C. edulis* extracts in SH-SY5Y cells pre-treated with the extracts at the concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL for 24h, and exposed to 1mM H_2O_2 for 15 min. Solid bars and error bars represent the average and standard error values, respectively (n = 6). *** Indicate a significan increase of cell viability of treated cells compared to control cells containing DMSO (0.5%, v/v; $p < 0.001$.

3.4.2. Anti-inflammatory activity on LPS-stimulated microglia cells

In order to evaluate the anti-inflammatory activity of the *C. edulis* extracts on LPS-stimulated microglia cells, the cytotoxic effect of different concentrations of the extracts (3.125, 6.25, 12.5, 25, 50 and 100 µg/mL) was first determined for 24h on N9 cells. Cell viability was determined by the MTT method and results were compared with control cells treated with DMSO (0.5%, v/v).

An increase in cell viability was observed after application of all samples (Fig. 3.7). Thus, and having in mind the results obtained in the previous assays, the methanol extract was selected to be tested for anti-inflammatory activity.

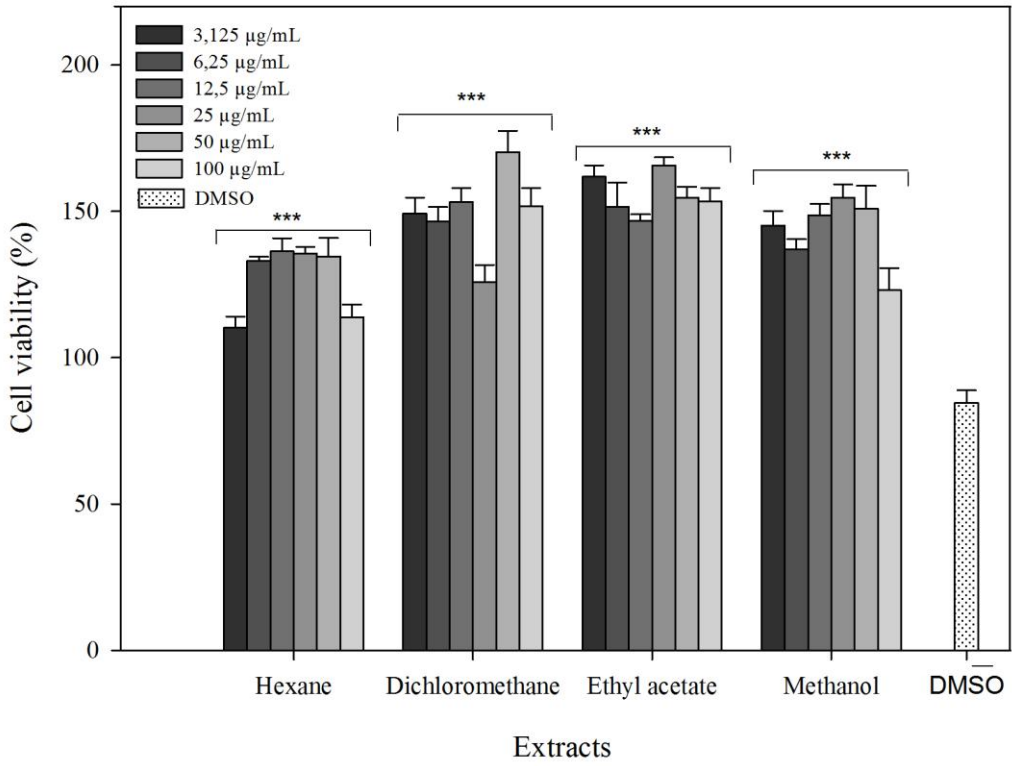

Figure 3.7. Cytotoxic effect of extracts of *C. edulis* in murine microglia cells (N9 cell line). Solid bars and errors bars represent the average and standard error values, respectively (*n* =6). *** Indicate a significan increase of cell viability of treated cells compared to control cells containing DMSO (0.5%, v/v; $p < 0.001$).

For the evaluation of the anti-inflammatory activity, the N9 cells were stimulated with 200 µL of LPS during 24h in the presence or absence of methanol extract, and the level of nitrites in the culture medium was measured by the Griess method. The incubation of N9 cells for 24h in the presence of LPS (10 µg/mL) induced the production of nitrites.

The methanol extract was selected for this assay because it was, in a general way, the extract whit the better results and it is a viable candidate for future works.

The application of the methanol extract at the concentration of 100 µg/mL significantly reduced NO production (1.53 µM), when compared with control cells (6.66 µM), corresponding to a 77% of decrease in NO production (Fig. 3.8).

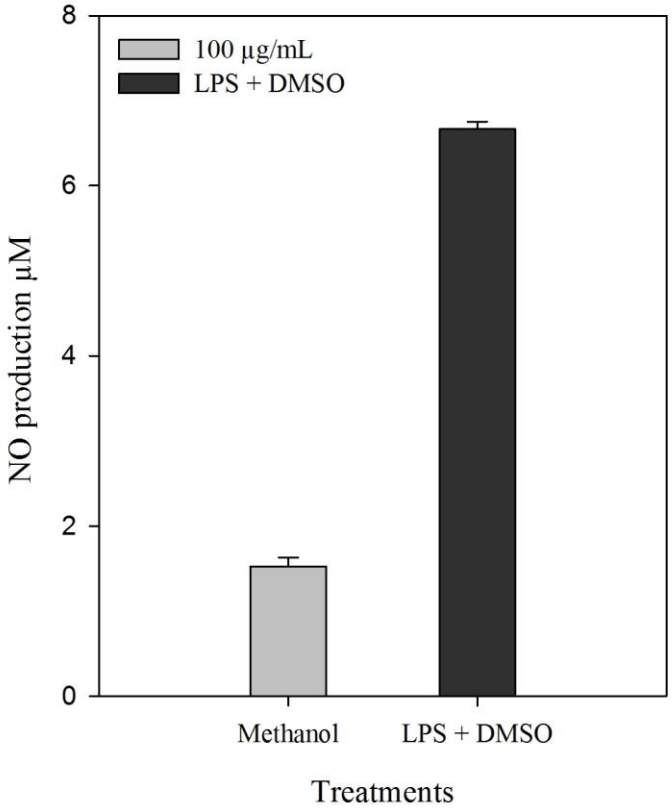

Figure 3.8. NO production in N9 cell line. Cells were treated with 10 µg/mL of LPS alone, or in combination with selected extract concentration. After 24h of incubation the NO concentration was determined using the Griess reagent. Solid bars and errors bars represent the average and standard error values, respectively (*n*=5). Results were expressed as µM of NO production.

4. Discussion

4.1. Antioxidant activities

In this work it was performed a screening of the RSA on the DPPH radical of methanol extracts leaves of 26 halophytes, and it was identified at least 5 species with a high antioxidant activity, namely *C. edulis* (95.7%), *F. laevis* (95.2%), *L. algarvense* (95.7%), *P. maritimum* (94.5%) and *T. africana* (95.0%), all higher than the positive control (89.1%).

The species *C. edulis* was selected for further studies due to its high RSA, and also because it is an invasive plant and very abundant in our region. The high RSA of *C.edulis* are in agreement with previous studies showing that it has a strong antioxidant activity, possible due to is high phenolic content (Falleh *et al*., 2011; Custódio *et al*., 2012; Ibtissem *et al*., 2012; Omoruyi *et al*., 2012; Rodrigues *et al*., 2014). It is important to note that the sample analysed is a crude extract, composed by a mixture of several compounds, while BHT is a pure compound. Moreover, some studies showed that BHT, and other synthetic antioxidants, can be toxic and have a carcinogenic activity, and so the importance in search for new natural antioxidants (Williams *et al*., 1999; Suhaj, 2006; Custódio *et al*., 2012; Okoko *et al*., 2012). Compounds with high antioxidant activity have an important role in the protection of cells against injuries caused by oxidative stress (Finkel *et al*., 2000). Since neurodegenerative diseases are potentiated by ROS, it is crucial avoid their formation and accumulation. There is some indications that the intake of this antioxidants can prevent ageing and oxidative stress, leading to a decreased of the risks of stress-related diseases (Vina *et al*., 2004; Dai *et al*., 2010; Falleh *et al*., 2011; Custódio *et al*., 2012).

As showed before, the high levels of metals, like iron and copper, can influence the production of free radicals, and consequently the increase of ROS. Through the iron and copper chelating activity and iron reducing power, we can evaluated the capacity of the extracts to capture or reduced this metals and avoid the increasing of oxidative stress.

In this study, the hexane and dichloromethane extracts of *C. edulis* had a high capacity to chelate iron. This species had previously showed a high ICA, which is correlated with the phenolic content (Omoruyi *et al*., 2012). For copper, the methanol extract had a high activity at 10 mg/mL.

According the results for the iron reducing power, the ethyl acetate extract was the most effective with 76.6% of activity at a concentration at 10 mg/mL, followed by dichloromethane and chloroform extracts at same concentration. In other studies the methanol extract at lowest concentration tested showed a high iron reducing activity, which was not observed in this study (Custódio *et al*., 2012). This result can be explained by the differences between the extractions methods realised.

The protection against iron-induced free radical reactions can be associated with antioxidant properties of flavonoids (Cao *et al*., 1997).

The NO plays an important role in modulation of blood flow, neurotransmission and on the immune system (Moncada *et al*., 2006; Pacher *et al*., 2007). At high concentrations it can be toxic to the cells, and can react with O_2^- producing peroxynitrite (PN) which is an extremely active oxidant agent (Taylor *et al*., 2013). In the search of antioxidant compounds, it was performed the NO-scavenging assay, which showed that methanol extract had the highest NO scavenging activity at the highest concentration tested (10 mg/mL). Another study, with other extracts tested showed that *C. edulis* had a high NO scavenging activity (Omoruyi *et al*., 2011). Banerjee *et al* reported that polar solvents, like ethanol and methanol always show the high activity, probably because of their high phenolic contents (Martins *et al*., 2011).

4.2 Phytochemical analysis

Phenolics compounds have multiple biological properties, including antioxidant activity (Kahkonen *et al*., 1999; Falleh *et al*., 211). Previous studies indicates the presence of phenolic, flavonoids and tannins compounds in *C. edulis* (Hanen *et al*., 2009; Falleh *et al*., 211; Custódio *et al*., 2012; Omoruyi *et al*., 2012), and probably is the main cause of the several properties assigned to this species, like antimicrobial, anti-infective and antifungal (Falleh *et al*., 2011; 2013). The methanol extract had a strong antioxidant activity and this one can be related to a high content of phenolic compounds (Falleh *et al*., 2013). Previous report identify several compounds in this species, such as rutin, neohesperidin, hyperoside, cactichin and ferulic acid (Custódio *et al*., 2012). In this work the extracts revealed a high content of total phenolics, flavonoids and condensed tannin compounds which can explain the high antioxidant activity. The methanol extract had a high value of total flavonoid content (587.05 mg RE/g DW), which has been reported to display anti-tumoral, anti-inflammatory activity and inhibition of several enzymes, such as lipoxygenase, cyclooxygenase, monooxygenase,

xanthine oxidase, mitochondrial succinoxidase and NADH-oxidase, phospholipase A_2, and protein kinases (Cao *et al*., 1997).

4.3. Enzyme inhibition assays

4.3.1. Cholinesterase inhibition

The actual drugs administrated in AD patients, like tacrine, donepezil and the natural products rivastigmine and galanthamine, have the function to block the action of both cholinesterases, AChE and BChE, increasing the levels of this neurotransmitter (Zarotsky et al 2003; Natarajan *et al*., 2009). Some of this drugs have adverse side-effects and are toxic, so it is important the search for natural solutions which reduce the toxicity and the side-effects, or at least reduced at a minimum level ((Hansen *et al*., 2008; Natarajan *et al*., 2009)). The AChE and BChE inhibitory activities were classified as potent (> 50% inhibition), moderate (30-50% inhibition), low (<30% inhibition) or nil (< 5% inhibition) (Vinutha *et al*., 2007). According to this classification the ethyl acetate and the methanol extracts had potent AChE inhibitory activity. In a general way all extracts tested have a potent or moderate BChE inhibitory activity, depending on the concentration tested. This extracts might be a source for compounds with the capacity to increase cholinergic activity through the inhibition of AChE and BChE. Some studies show that inhibition of ChE by this compounds not only rise the ACh levels, but also avoid the development of Aβ plaques, since ChEIs can activate α-secretase, which acts on the Aβ precursor and lead to Aβ plaques formation (Mesulam *et al*., 2002; Natarajan *et al*., 2009; Custódio *et al*., 2012). This compounds can have an important role in the prevention of neuronal death caused by AD (Giacobini 2004; Custódio *et al*., 2012). The ChE inhibitory activity of *C. edulis* show that it can be a source of compounds with therapeutic uses in ND, like AD and PD (Williams *et al*., 2011; Custódio *et al*., 2012). In another study, it has been shown that flavonoids can inhibit some enzymes, such as monooxygenase, by reacting with free radicals generated at the enzyme's active site (Cao et al, 1997).

4.3.2. Tyrosinase inhibition

The inhibitory activity of tyrosinase is important in disorders, like hyperpigmentary diseases and post-inflammatory hyperpigmentation (Kolbe *et al*., 2012). The tyrosinase also contribute to neuromelanin formation, which is found in nigral dopaminergic cells (Hasewaga 2010). Previous studies showed that the

expression of this enzyme increases the neuronal susceptibility to oxidizing conditions (Tessari *et al*., 2013). In this work methanol extract had moderate TYRO activity, while the other extracts had, generally, low activity. In 2004, *Kang et al* identify the *Ecklonia stolonifera*, a brown algae, as had tyrosinase inhibitory activity. They show that active ethyl acetate fraction of the methanol extract of *E. stolonifera* exhibit a high tyrosinase inhibitory activity, higher than the arbutin used as positive control. The phloroglucinol (phenol derivate), eckstolonol (phlorotannin), eckol (phlorotannin), phlorofucofuroeckol A (phlorotannin), and dieckol (phlorotannin) compounds were identify as the responsible for the tyrosinase inhibitory activity in this algae (Kang *et al*, 2004). Other compound, oxyresveratrol, show to have a dose-dependent and potent inhibitory effect on tyrosinase activity (Kim *et al*, 2002).

4.4 Neuroprotective activity

Oxidative stress is the major factor implicated in neurodegenerative diseases (like AD and PD). Previous studies showed that ROS induces cell dead and the H_2O_2 can cause injuries in several cell types (Gao *et al*., 2001; Kwon *et al*., 2011). It was also demonstrated that the effect of ROS can be blocked by the addiction of antioxidants (Kwon *et al*., 2011). In this work it was assed the capacity of the extracts to protect *in vitro* neuronal cells against oxidative stress induced by H_2O_2. The methanol extract protected the cells against the injury. Possibly we can related this good results with the high value of antioxidant activity and the higher concentration of phenolic compounds of this extract, particularly the flavonoids content. Other studies have been done on the protective effect of flavonoids on neurons, and showed that high levels in scavenging free-radicals could protect neuronal cells from oxidative stress (Gao *et al*., 2001) We have not found any previous work for this assay on this species, but comparing with another plants (Custodio *et al*., 2013) the C. edulis showed to have better protective effect than the results for the cork aok and holm oak, for example. *Gao et al*, in 2001, showed that the root of *Scutellaria baicalensis Georgi*, a traditional Chinese herb medicine and used as an anti-inflammatory drug, had a high flavonoid content and showed a high scavenge hydroxyl radicals, DPPH radicals and enzymatic or non-enzymatic inhibition induced by mitochondria lipid peroxidation.

4.5. Anti-inflammatory activity on LPS-stimulated microglia cells

The methanol extract of *C. edulis* reduced the NO production *in vitro* by microglia cells by 77%, when compared to LPS-stimulated cells, thus suggesting that this extracts has compounds with anti-inflammatory properties. Microglial cells can remove infectious agents, release trophic factors, remove damaged cells and produce cytokines, which make the communication among neuronal and other glial cells (Bodrato *et al*., 2009). Despite its capacity to tissue repair and neuroprotection, when this cells are constantly activated, lead to a chronic inflammation, producing neurotoxins, like pro-inflammatory cytokines and NO, contributing for the pathogenesis of neurodegenerative diseases (Emerit *et al*., 2004; Bodrato *et al*., 2009). It becomes increasingly important to find substances that had anti-inflammatory activity for potential treatment for these diseases (Falleh *et al*., 2011). Other study shows that *Salicornia bigelovii*, another halophyte, suppress the increasing production of NO on LPS-stimulated BV-2 microglial cells (Kang *et al*, 2013). This study suggests that *S. bigelovii* can be an effective anti-inflammatory agent (Kang *et al*, 2013). They connected this activity with high antioxidant activity of this species. *S. bigelovii* have active compounds such as triterpenoids, flavones, glycosides, saponins, vitamins and minerals (Kang *et al*, 2013). Another study shows that flavonoids have an anti-inflammatory activity, which may explain the high activity of methanol extract, along with high flavonoid content of this extract (Talhouk *et al*, 2007).

5. Conclusion and future work

This study shows the potential of halophytes as sources of antioxidant compounds in particular the *Carpobrotus edulis* species. This species had a high antioxidant activity, was rich in phenolic compounds, display a high capacity to inhibit AChE and BChE enzymes and a good neuroprotective and anti-inflammatory activity using neuronal and microglia cells as *in vitro* models.

Future studies should be performed, in order to identify and isolate the bioactive compounds present in the methanol extract, and determine its mode of action.

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