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The role of aromatic plant compounds in the prevention and treatment of neurological disorders

Patrícia Filipa Santos Costa

Tese para obtenção do grau de Doutor em Ciências Biológicas
(Especialidade em Biotecnologia)

Trabalho efetuado sob a orientação de Professora Doutora Anabela Romano e coorientação de Doutora Sandra Gonçalves

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Declaração de autoria de trabalho

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

"A Ciência não pode resolver o mistério final da Natureza.

E isto porque, em última análise, somos parte do mistério que tentamos resolver."

Max Planck

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OUTLINE OF THE THESIS

This thesis is divided in seven chapters. The first chapter corresponds to a general literature review of the main subjects of the thesis. Chapters 2 to 6 describe the experimental work and the main results attained, and finally, Chapter 7 includes a general discussion of the obtained results and the main conclusions. This thesis was designed to be based on a number of papers that have been published, accepted or submitted for publication, and that will provide an account of the research that underpins this thesis. All the publications presented were written to stand alone and, therefore, the reader may find some repetition in parts of the manuscript.

ABSTRACT

This thesis aims the discovery of valuable plant compounds and to provide a deep and detailed scientific perspective of the following Portuguese aromatic plants: *Lavandula viridis* L'Hér, *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco and *Thymus lotocephalus* G. López & R. Morales. Volatile and phenolic compounds were recovered by different techniques and extraction solvents and their antioxidant and anti-cholinesterase activities were investigated (Chapters 2 and 3). *In vitro* cultures were used as alternative sources of phytochemicals, and the results were compared with wild plants. Furthermore, the effect of cyclodextrins on the antioxidant activity and storage stability of the essential oils was evaluated (Chapter 4). Finally, the neuroprotective potential (Chapters 5) and the influence of *in vitro* digestion on the biological activity of *L. viridis* extracts and rosmarinic acid (Chapter 6) were investigated.

The supercritical fluid extraction technique generated the highest extraction yields in comparison with hydrodistillation, but a larger number of components were identified by hydrodistillation. The chemical analysis highlighted the abundance of oxygen-containing monoterpenes in all the essential oils and in almost all supercritical fluid extracts, and allowed the identification of phenolic acids and flavonoids in the polar extracts. The water:ethanol mixture was the most efficient solvent for the extraction of phenolic compounds followed by infusion. The *in vitro* cultures accumulated much higher contents of rosmarinic acid than wild plants. The polar extracts were the most efficient free radical scavengers, Fe^{2+} chelators and inhibitors of lipid peroxidation, while the essential oils were the most active against cholinesterases. Cyclodextrins produced a remarkable enhancement on the antioxidant activity and storage stability of the essential oils. *L. viridis* extracts attenuated the neurotoxicity induced by H_2O_2 and their biological activity was assured after *in vitro* digestion. This study provides relevant knowledge about the chemical composition and biological activity of three Portuguese species.

Keywords: *Lavandula viridis*, *Lavandula pedunculata* subsp. *lusitanica*, *Thymus lotocephalus*, natural compounds, biological activity, *in vitro* cultures

RESUMO

A doença de Alzheimer é uma doença neurodegenerativa caracterizada pela deterioração progressiva e irreversível de diversas faculdades cognitivas e que afeta cerca de 35,6 milhões de pessoas em todo o mundo. Até ao momento, a doença permanece sem cura. A terapia disponível concentra-se apenas na atenuação dos sintomas característicos e a complexidade da patogénese constitui um obstáculo para o desenvolvimento de novos fármacos. Para além do défice progressivo e irreversível do sistema colinérgico, são claras as evidências do envolvimento do stresse oxidativo na doença. As plantas produzem compostos com interesse medicinal, e são uma fonte infindável de compostos biologicamente ativos. A Flora Portuguesa é, neste sentido, particularmente interessante pela sua riqueza em espécies aromáticas e medicinais. Esta tese pretende contribuir para a pesquisa de compostos biologicamente ativos a partir das seguintes espécies aromáticas de Portugal: *Lavandula viridis* L'Hér, *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco e *Thymus lotocephalus* G. López & R. Morales. Estas espécies são usadas como condimentos ou para fins terapêuticos na medicina tradicional, no entanto, o seu potencial biológico ainda não foi totalmente explorado.

A primeira fase deste trabalho, descrita nos Capítulos 2 e 3, visou avaliar o efeito das técnicas e solventes de extração no rendimento e atividade biológica das espécies em estudo. Existem várias técnicas disponíveis para a extração de compostos vegetais as quais têm que ser adaptadas aos metabolitos de interesse. Neste trabalho a fração volátil foi obtida por hidrodestilação e extração por fluidos supercríticos, e os compostos fenólicos foram extraídos por maceração, infusão e soxhlet. Para além do material vegetal colhido no habitat natural, foram também utilizadas culturas *in vitro* de *L. viridis* e *T. lotocephalus*. Os resultados indicam que a extração por fluidos supercríticos permitiu maiores rendimentos de extração comparativamente à hidrodestilação, e no que diz respeito aos extratos polares, os rendimentos mais elevados foram obtidos usando água e a mistura água:etanol (1:1) como solventes. Recorrendo às técnicas cromatográficas foi possível atestar que os óleos essenciais e a grande maioria dos extratos supercríticos são predominantemente constituídos por monoterpenos oxigenados. Registaram-se diferenças qualitativas e quantitativas na composição dos óleos essenciais e dos extratos supercríticos obtidos no segundo separador (compostos mais voláteis), tendo-se identificado um maior número de compostos por hidrodestilação. Os extratos polares são ricos em ácidos hidroxicinâmicos (ácidos 3-*O*-cafeoilquínico, 4-*O*-cafeoilquínico, 5-*O*-cafeoilquínico, cafeico e rosmarínico) e flavonoides

(luteolina, apigenina e pinocembrina), tendo o ácido rosmarínico sido identificado como o composto maioritário na maioria dos extratos, incluindo os obtidos das culturas *in vitro*. Comparando os materiais vegetais estudados, as condições usadas *in vitro* foram as mais favoráveis à acumulação de ácido rosmarínico.

Atualmente existe um interesse crescente na utilização de antioxidantes naturais, particularmente de origem vegetal, na prevenção e/ou tratamento de algumas patologias. A implicação do stresse oxidativo no progresso da doença de Alzheimer sugere os antioxidantes naturais como potenciais agentes preventivos. A aplicação destes metabolitos ativos é imensa; também na indústria alimentar, e a potencial toxicidade dos antioxidantes sintéticos estimula a sua substituição por antioxidantes naturais. Neste contexto, as espécies vegetais em estudo foram testadas quanto ao seu potencial antioxidante designadamente a capacidade de (a) captar diferentes radicais (ORAC - oxygen radical antioxidant capacity, DPPH - 2,2-diphenyl-1-picrylhydrazyl e TEAC - Trolox equivalent antioxidant capacity); (b) quelar o ferro e (c) inibir a peroxidação lipídica em homogeneizados de cérebro de ratinho. Os extratos hidroalcoólicos e infusões foram, geralmente, os sequestradores de radicais mais eficientes (Capítulos 2 e 3). No que diz respeito aos extratos supercríticos, a pressão de extração afetou a capacidade dos extratos colhidos no primeiro separador de captar o radical peroxilo, sendo que a atividade mais elevada se verificou com o incremento da pressão. Os extratos aquosos, hidroalcoólicos e infusões foram os mais ativos na quelação do ferro e inibição da peroxidação lipídica. Ambos os materiais vegetais estudados (selvagem e micropropagado) demonstraram capacidade na quelação de ferro e os extratos etanólicos das culturas *in vitro* foram os mais eficientes na inibição da peroxidação lipídica. A presença de ácidos hidroxicinâmicos e flavonoides nos extratos polares poderá, em parte, justificar a atividade antioxidante observada. O solvente de extração influenciou significativamente a recuperação de compostos com potencial antioxidante, sendo a mistura água:etanol (1:1) a mais adequada para estas espécies.

Os óleos essenciais estão entre os compostos mais valiosos produzidos pelas plantas com aplicações que se estendem às mais diversas áreas. Contudo, a baixa solubilidade em sistemas aquosos e a elevada volatilidade são alguns dos fatores que condicionam a sua aplicação. Ao longo dos anos vários sistemas de encapsulação têm sido desenvolvidos para ultrapassar estas limitações, nomeadamente as ciclodextrinas. Estas são particularmente interessantes pelo caráter anfifílico característico, e foram por isso usadas neste estudo como agentes solubilizantes, concretamente, a *β*-ciclodextrina e hidroxipropil-*β*-ciclodextrina. Os resultados

apresentados no Capítulo 4 indicam que as ciclodextrinas, nomeadamente a hidroxipropil-*β*-ciclodextrina, produziram um aumento significativo na atividade antioxidante dos óleos essenciais, provavelmente consequência do aumento da solubilidade de alguns compostos menos polares. Para além do aumento da atividade antioxidante, esta manteve-se estável ao longo de trinta dias.

A estratégia mais utilizada atualmente no tratamento da doença de Alzheimer compreende a melhoria da função colinérgica que é clinicamente conseguida através do uso de inibidores colinesterásicos. Embora estes fármacos licenciados sejam eficientes na atenuação dos sintomas, apresentam efeitos secundários e não impedem o progresso da doença. Estas limitações representam simultaneamente um grande desafio na pesquisa de novos fármacos, e foi com este objetivo que neste trabalho as espécies em estudo foram testadas quanto ao potencial anti-colinesterásico (acetilcolinesterase e butirilcolinesterase). Os óleos essenciais apresentaram, geralmente, as inibições mais promissoras, juntamente com o extrato metanólico de *L. viridis*, que foi também ativo na inibição enzimática *in vivo*. A atividade anti-colinesterásica dos óleos essenciais poderá refletir a presença de compostos como 1,8-cineol, α-pineno, mirtenal e viridiflorol. No entanto, é de salientar que a atividade biológica de misturas complexas, como os óleos essenciais e extratos, poderá resultar do somatório das atividades individuais dos seus constituintes, e também da presença de efeitos de sinergismo e antagonismo. Relativamente aos extratos supercríticos, apenas os obtidos no primeiro separador apresentaram inicialmente atividade inibitória; contudo, a indicação da presença de falsos positivos sugere que estes resultados poderão estar sobrestimados.

L. viridis foi a espécie mais consistente e promissora em termos de atividade biológica. Os compostos bioativos desta espécie são antioxidantes naturais capazes de atuar como sequestradores de radicais livres, agentes quelantes de metais e inibidores da peroxidação lipídica. Além disso, combinam várias formas de prevenção de doenças multifatorais como a doença de Alzheimer, agindo também como inibidores colinesterásicos. Por estas razões, apenas esta espécie foi testada nos ensaios de neuroproteção (Capítulo 5) e digestão *in vitro* (Capítulo 6). Os extratos de *L. viridis* e o seu composto predominante, o ácido rosmarínico, protegeram as células A172 dos efeitos negativos gerados pelo peróxido de hidrogénio. Todos os extratos, com exceção do extrato metanólico na concentração mais baixa, e o ácido rosmarínico atenuaram a neurotoxicidade induzida pelo peróxido de hidrogénio. Além disso, o extrato hidroalcoólico, infusão, e o ácido rosmarínico reduziram a acumulação intracelular

de espécies reativas de oxigénio. A capacidade de eliminar radicais livres previamente demonstrada explica, em parte, o efeito protetor observado.

A presença de compostos com potencial biológico em determinadas espécies, como no caso de *L. viridis*, por si só não é garantia de que após o seu consumo continuem ativos no momento em que alcançam os tecidos-alvo; é essencial que estes estejam biodisponíveis. Neste sentido, os extratos mais promissores de *L. viridis* foram submetidos a condições de digestão *in vitro*, e as atividades antioxidante e anti-colinesterásica dos extratos metabolizados avaliada (Capítulo 6). O ácido rosmarínico puro foi também estudado para averiguar a influência da matriz vegetal na biodisponibilidade dos compostos. Efetivamente, a matriz do extrato metanólico não influenciou a digestão de ácido rosmarínico, ao contrário do hidroalcoólico que protegeu o composto maioritário da digestão ácida. Estes resultados corroboram a influência da matriz vegetal na biodisponibilidade dos seus constituintes. A atividade antioxidante do extrato metanólico e ácido rosmarínico puro foi assegurada depois dos processos digestivos *in vitro*. No que respeita à atividade anti-colinesterásica dos extratos metabolizados, apenas a inibição da butirilcolinesterase foi afetada. Estes resultados sugerem que os extratos de *L. viridis* atravessam o trato gastrointestinal sem comprometer grandemente a sua atividade biológica.

Esta tese proporcionou um conhecimento mais alargado da composição química e da atividade biológica das espécies *L. viridis*, *L. pedunculata* subsp. *lusitanica* e *T. lotocephalus* utilizadas tradicionalmente. Todas as espécies, em particular *L. viridis*, são ricas em antioxidantes e anti-colinesterásicos naturais capazes de participar ativamente na prevenção e/ou tratamento da doença de Alzheimer, ou outras patologias associadas ao stresse oxidativo. Além disso, *L. viridis* e *T. lotocephalus* sintetizam compostos bioativos *in vitro* representando uma alternativa eficiente à obtenção de metabolitos de elevado valor.

Palavras-chave: *Lavandula viridis*, *Lavandula pedunculata* subsp. *lusitanica*, *Thymus lotocephalus*, compostos naturais, atividade biológica, culturas *in vitro*

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ABBREVIATIONS

AAPH	2,2'-Azobis(2-methylpropionamidine) dihydrochloride
ABTS	2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ATCI	Acetylthiocholine iodide
AUC	Area under the curve
Aβ	Amyloid-β
BChE	Butyrylcholinesterase
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BTCI	Butyrylthiocholine chloride
CD	Cyclodextrin
CAT	Catalase
ChAT	Choline acetyltransferase
ChE	Cholinesterase
CI	Chemical ionization
CoA	Coenzyme A
CO_2	Carbon dioxide
COX	Cytochrome *c* oxidase
DAD	Diode-array detection
DAHP	3-Deoxy-D-*arabino*-heptulosonate 7-phosphate
DCFH	2',7'-Dichlorofluorescein
DCFH-DA	2',7'-Dichlorofluorescein diacetate
DMAPP	Dimethylallyl diphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FID	Flame ionization detector
GC	Gas chromatography
GPx	Glutathione peroxidase
GRAS	Generally Recognized As Safe
HAT	Hydrogen-atom transfer
HD	Hydrodistillation
HBSS	Hank's balanced salt solution
HNE	4-Hydroxynonenal
$HO^{\bullet}$	Hydroxyl radical
$HOO^{\bullet}$	Hydroperoxyl radical
HPLC	High-performance liquid chromatography
HP-*β*-CD	2-Hydroxypropyl-*β*-cyclodextrin
H_2O_2	Hydrogen peroxide
IPP	Isopentenyl diphosphate
MDA	Malondialdehyde
MEP	2-2-Methyl-D-erythritol-4-phosphate

MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVA	Mevalonate
m/z	Mass-to-charge ratio
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
Nrf2	Nuclear factor erythroid-derived 2-related factor 2
$^{\bullet}NO$	Nitric oxide
$ONOO^{-}$	Peroxynitrite anion
ORAC	Oxygen radical absorbance capacity
$O_2^{-\bullet}$	Superoxide anion radical
$^1O_2$	Singlet oxygen
PAL	Phenylalanine ammonia lyase
PKC	Protein kinase C
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSS	Reactive sulfur species
SET	Single-electron transfer
SFE	Supercritical fluid extraction
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	*tert*-Butylhydroquinone
TEAC	Trolox equivalent antioxidant capacity
Trolox	6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
UV	Ultraviolet
VIS	Visible
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

Dementia is a real concern worldwide.

According to the 2012 report, "*Dementia: a public health priority*", developed by the World Health Organization (WHO) and Alzheimer's Disease International, the number of people globally affected by dementia in 2011 was estimated to be 35.6 million and it will continue to grow in an alarming rate. It is expected an increasing to 65.7 million in 2030 and 115.4 million in 2050 (WHO, 2011). Alzheimer's disease (AD) is the most common form of dementia and accounts to 60-70% of cases. In Portugal, there are about 153 thousand people living with dementia, 90 thousand of which have AD (Bial, 2012).

1.1. Alzheimer's disease

AD was described in 1906 by Alois Alzheimer during the autopsy of Auguste Deter who died with severe cognitive failings (Alzheimer, 1907; Williams et al., 2011). Despite the huge effort made over the decades to resolve this alarming disease, AD remains without cure; therapies available are only designed to decrease the progression of the cognitive decline. This age-related neurodegenerative disorder is neuropathologically characterised by the occurrence of extracellular amyloid-β (Aβ) peptide deposited in senile plaques and intracellular neurofibrillary tangles mainly composed of hyperphosphorylated tau protein (Markesbery, 1997; Choi et al., 2012; Aso and Ferrer, 2013). AD is also associated with multiple genetic defects. Mutations in the presenilin 1 and presenilin 2, and amyloid precursor protein (APP) genes can cause early-onset familial AD (Xu et al., 2013). On the other hand, apolipoprotein E type ϵ4 allele, is associated with both early- and late-onset familial AD (Xu et al., 2013). Increasing age is another risk factor for AD; the incidence almost doubles with every 5 years of age after aged 65 (Xu et al., 2013).

1.1.1. Cholinergic hypothesis

AD is marked by a neurotransmitter dysfunction being the cholinergic system one of the most affected (Francis et al., 1999; Aso and Ferrer, 2013). Brain regions that are associated with memory, learning, behavior and emotional responses, particularly the neocortex and hippocampus, are the most affected by the characteristic pathology of AD (Anand and Singh, 2013). Acetylcholine (ACh) was the first neurotransmitter to be discovered and is widely distributed in the nervous system (Mesulam, 2009). ACh is known to be a neurotransmitter at the neuromuscular junction, at peripheral ganglia and a mediator of the parasympathetic actions of the autonomic nervous system (Fisher and Wonnacott, 2012). ACh is synthesised from acetyl coenzyme A (CoA) and choline by the action of the enzyme choline acetyltransferase (ChAT) (Wilkinson et al., 2004). After its synthesis, the vesicular acetylcholine transporter concentrates ACh into synaptic vesicles. Upon nerve stimulation, depolarization and calcium ion entry, the vesicles fuse with the nerve membrane and release ACh in the synaptic cleft (Fisher and Wonnacott, 2012). ACh may then interact with nicotinic and muscarinic receptors on presynaptic and postsynaptic cells (Wilkinson et al., 2004). The activation of muscarinic and nicotinic receptors leads to the activation of biochemical pathways or depolarization of the target cells and, thus, the propagation of the nerve impulse

(Wilkinson et al., 2004). ACh is degraded rapidly by the hydrolytic activity of cholinesterases (ChEs) (Wilkinson et al., 2004). Presynaptic and postsynaptic components of cholinergic pathways contain acetylcholinesterase (AChE), and the adjacent neuroglia contains butyrylcholinesterase (BChE) (Mesulam et al., 2002; Mesulam, 2004). In the human brain, AChE is the most prominent enzyme involved in this hydrolysis, but evidence suggests the involvement of BChE in this process (Mesulam et al., 2002). Postmortem studies of the brains in AD patients revealed low levels of ChAT and ACh (Francis et al., 1999; Park, 2010). Therefore, inhibition of ChEs increases the ACh concentration in the synaptic cleft thus contributing to the enhancement of cholinergic function (Mesulam et al., 2002). Other cholinergic strategies include the use of ACh precursors, muscarinic and nicotinic agonists and AChE releasers (Aso and Ferrer, 2013). Nowadays, the treatment approved to reduce the cognitive symptoms of AD is based on the use of ChEs inhibitors (Galantamine, Donepezil, Rivastigmine and Tacrine) and N-methyl-*D*-aspartate-receptor antagonist (Memantine) (Park, 2010). However, none of these drugs is able to break the progressive deterioration of cognitive functions. For this reason, extensive research has been directed towards the discovery of new and improved ChEs inhibitors. Plants have been explored in this sense because they present a huge number of active compounds able to reduce ChEs activity. A good example of natural product-derived compounds able to improve the cholinergic system is Galantamine, found in the *Amaryllidaceae* family (Park, 2010). This alkaloid is a selective, reversible and competitive AChE inhibitor which is licensed in Europe for AD treatment since 2001 (Houghton and Howes, 2005). Huperzine A, also an alkaloid, has been marketed in China as a new ChE inhibitor for AD treatment and ZT-1, a semi-synthetic derivative of huperzine A, is being developed as anti-AD new drug candidate both in China and Europe (Ma et al., 2007; Saklani and Kutty, 2008). Other phytochemicals, such as phenolic compounds (Ebrahimi and Schluesener, 2012) and terpenes (Dohi et al., 2009), have also demonstrated anti-ChEs properties.

1.1.2. Oxidative stress signaling in Alzheimer's disease

As a multifactorial disease, AD has different events accounting to the neurodegeneration process in addition to the cholinergic deficit, namely, mitochondrial dysfunction, neuroinflammation and oxidative stress. Accumulated evidences suggest the involvement of oxidative stress, the unbalance between reactive species production and the antioxidant

defense, in neuronal dysfunction and degeneration (Markesbery, 1997; Aso and Ferrer, 2013) (Fig. 1.1). If neuronal cells have defense systems to protect them against reactive species, why are they so susceptible to oxidative processes? Although cells have specific mechanisms to neutralize reactive species, including enzymatic [e.g. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and non-enzymatic (e.g. vitamin A, C and E), they seem to be not enough to avoid oxidative modifications in lipids, proteins, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Giorgio et al., 2007). In addition, brain has a high consumption of the total oxygen required to metabolic activities, high concentrations of polyunsaturated fatty acids (which are potential substrates for lipid peroxidation) and high levels of transition metals (Halliwell, 1992; Barnham et al., 2004).

Among all the cellular sources of reactive species, mitochondria appears as the most important one (Bolisetty and Jaimes, 2013). Mitochondrial dysfunction, particularly, a deficiency in cytochrome *c* oxidase (COX) was observed in AD brains (Hirai et al., 2001; Müller et al., 2010). Cardoso et al. (2004) proved that COX deficit is associated with the production of increased reactive oxygen species (ROS). In addition, COX dysfunction can also reduce energy storage and disturbs energy metabolism (Mutisya et al., 1994; Müller et al., 2010). Another source of ROS is Aβ peptide itself (Behl, 1997). Aβ peptide is the major constituent of the senile plaques derived from the larger APP, a ubiquitously expressed transmembrane glycoprotein (Behl, 1997). Aβ induces the activation of microglial cells and astrocytes, lipid peroxidation, protein oxidation and reactive species production (Varadarajan et al., 2000; Butterfield and Lauderback, 2002; Heneka et al., 2010).

Neuroinflammation is characterised by the activation of microglia cells and, consequently, by production of a number of potentially neurotoxic molecules including reactive oxygen and nitrogen species, proinflammatory cytokines and other inflammatory mediators (Streit, 2004; Heneka et al., 2010; Michael et al., 2010). Moreover, it has been postulated that Aβ promotes the production of 4-hydroxynonenal (HNE) and acrolein (Mark et al., 1997). The presence of neurotoxic aldehydes as HNE and acrolein in AD brains is an indicator of the occurrence of lipid peroxidation in the brains of AD patients. HNE, in particular, is able to react with proteins forming covalent adducts to histidine, lysine and cysteine residues through Michael addition (Butterfield and Lauderback, 2002). Furthermore, it can also inhibit the synthesis of DNA, RNA and proteins (Esterbauer et al., 1991). The presence of protein carbonyls in AD brains is a marker of protein oxidation which can be done by free radicals-mediated oxidation of side chains of some amino acid residues, or introduced into proteins by glycation and

reactions with glycoxidation and lipid peroxidation products (Aksenov et al., 2001; Butterfield and Lauderback, 2002). In addition, increased levels of 8-hydroxy-2'-deoxyguanosine, the most abundant oxidized DNA base product from hydroxyl radical ($HO^{\bullet}$) attack, were found in AD brain (Mecocci et al., 1994).

Growing evidence supports the fact that Aβ peptide generates ROS in combination with metal ions (Bondy et al., 1998; Huang et al., 1999). Iron is the most abundant metal in the brain and it is vital for normal neuronal metabolism (Thompson et al., 2001). It is a central component of the heme in hemoglobin, myoglobin, cytochromes and a crucial cofactor for non-heme enzymes, such as ribonucleotide reductase, and for the synthesis of neurotransmitters (Thompson et al., 2001; Duce and Buch, 2010). However, high contents of this biometal in tissues can augment neurodegeneration through free radical processes by different mechanisms, including the participation in Fenton's reaction (Halliwell, 2001; Fraga and Oteiza, 2002).

Taking into account all the issues reported above, it is easy to understand that despite the multiple initiating causes of neurodegeneration we can found a common final pathway: generation of reactive species. As a consequence, different approaches for protection using antioxidants have emerged.

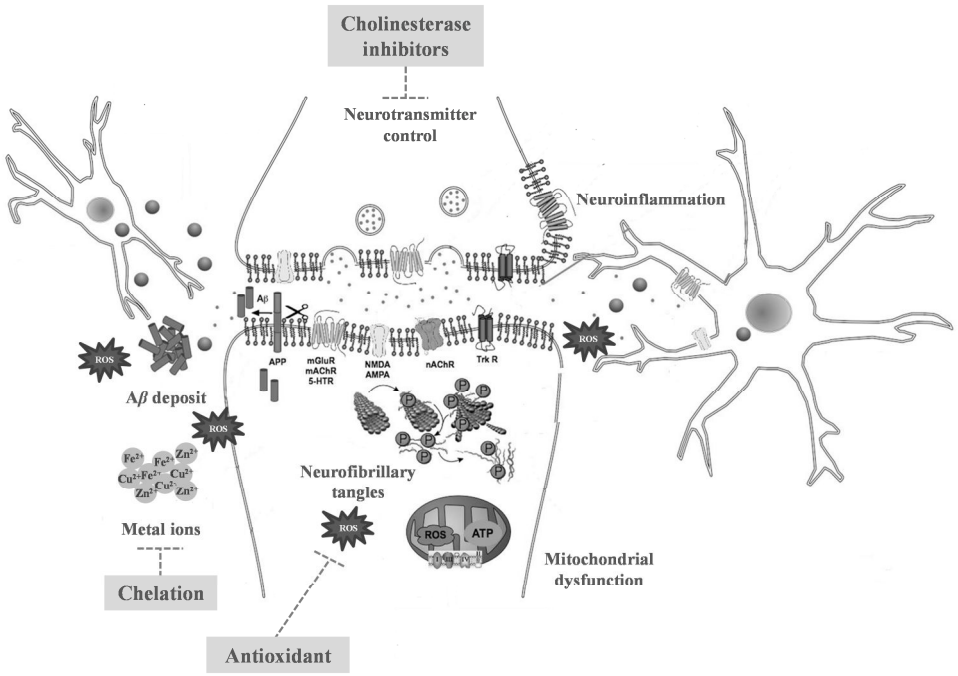

Figure 1.1. Oxidative stress signaling in Alzheimer's disease. Grey boxes represent the preventive and/or therapeutic strategies focused in the present study. Adapted from Aso and Ferrer (2013).

1.2. Reactive species and antioxidants: what are they and how to measure them

Free radicals are atoms, molecules or ions with unpaired electrons that are very unstable and available to react quickly with other molecules (Carocho and Ferreira, 2013). Normally, free radicals can arise during metabolism (e.g. mitochondrial respiration chain, cellular inflammation and phagocytosis), but environmental factors (e.g. pollution, radiation, cigarette smoke) can also promote their production. They derive from oxygen, nitrogen and sulfur, thus creating ROS [e.g. superoxide anion radical ($O_2^{-\bullet}$), $HO^{\bullet}$, hydroperoxyl radical ($HOO^{\bullet}$) and non-radicals, namely hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$)], reactive nitrogen species (RNS) [e.g. nitric oxide ($^{\bullet}NO$) and peroxynitrite anion ($ONOO^-$)], and reactive sulfur species (RSS) (formed by the reaction of ROS with thiols) (Lobo et al., 2010; Carocho and Ferreira, 2013).

Antioxidants are defined as "*any substance that delays, prevents or removes oxidative damage to a target molecule*'' (Halliwell, 2007). They can neutralize reactive species through different mechanisms: by inhibiting reactive species that initiate peroxidation (preventive oxidants); by converting hydroperoxides into low energy products; breaking the autoxidation chain reaction (chain breaking antioxidants); and acting as singlet oxygen quenchers by deactivating high energy species. They can also act as reducing agents, metal chelators and, finally, as inhibitors of pro-oxidative enzymes (lipooxigenases) (Singh and Singh, 2008; Carocho and Ferreira, 2013).

Cells trigger different defense systems which are able to protect them against oxidative stress namely, enzymatic and non-enzymatic endogenous antioxidant defenses. Enzymatic antioxidants can be divided into primary and secondary enzymatic defenses. GPx, CAT and SOD are the major primary endogenous antioxidant enzymes (Rahman, 2007; Carocho and Ferreira, 2013). On the other hand, the secondary enzymatic defense includes glutathione reductase and glucose-6-phosphate dehydrogenase which are important to maintain a steady supply of metabolic intermediates, such as glutathione and reduced nicotinamide adenine dinucleotide phosphate (NADPH), needed for the normal functioning of primary antioxidants (Singh and Singh, 2008; Carocho and Ferreira, 2013). The non-enzymatic antioxidant defenses include vitamins, enzyme cofactors, nitrogen compounds and peptides (Carocho and Ferreira, 2013).

Despite the great efficiency of endogenous antioxidants, they are sometimes insufficient to counteract the oxidative damage on biological targets. In this context, it has been suggested

that an intake of a rich antioxidant diet can prevent or reduce the risk of oxidative stress-related diseases (Landete, 2013). Plants are also subjected to oxidative stress and as a defense mechanism they have developed antioxidant systems capable of efficiently neutralize reactive species. Flavonoids, phenolic acids, carotenoids, tocopherols and essential oils can avoid oxidative stress *via* a number of different mechanisms, such as free radical scavenging, transition metal chelation and interactions with lipid membranes, proteins and nucleic acids (Dai and Mumper, 2010; Miguel, 2010; Brewer, 2011). They can also indirectly exert its antioxidant potential *via* activation of important signaling pathways as the transcription nuclear factor erythroid-derived 2-related factor 2 (Nrf2), which mediates generation of antioxidant enzymes (Jung and Kwak, 2010). Thus, attention has been recently put on searching for natural antioxidants from plant origin to replace synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ), with known toxicity and risk to health (Liu et al., 2011; Karre et al., 2013).

1.2.1. Methods to determine the antioxidant activity

A vast number of methods have been developed to estimate the antioxidant potential of natural compounds based on different strategies and providing relevant information about interactions between reactive species and natural products (López-Alarcón and Denicola, 2013). However, none of them is able to determine the antioxidant potential accurately and quantitatively; in a complex system it is rather difficult to reflect all of the radical sources and antioxidants with distinct physical and chemical characteristics (Prior et al., 2005; López-Alarcón and Denicola, 2013). In this study, the antioxidant assays used are based on different strategies designed to determinate the capacity of antioxidants to chelate metal ions; the consumption of stable free radicals; the ability to protect a target molecule exposed to a free radical source; and the capacity to inhibit the oxidation of lipid substrates.

Regarding the radical chain breaking antioxidant activity, methods can be divided into two groups, depending on the following chemical reactions: assays based on hydrogen-atom transfer (HAT) and assays based on single-electron transfer (SET), although the two reactions can occur simultaneously. HAT-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation and SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound (Prior et al., 2005;

Miguel, 2010). The structure and solubility of the antioxidant, the partition coefficient and the solvent polarity will determine the mechanism. The oxygen radical absorbance capacity (ORAC) assay, Trolox equivalent antioxidant capacity (TEAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays are frequently used to assess the free radicals scavenging activity of plants. ORAC assay is based on HAT mechanism and measures the fluorescence loss of a probe (fluorescein) in the presence or absence of an antioxidant. The colorimetric TEAC and DPPH assays are based on SET mechanism; the TEAC assay measures the ability of antioxidants to reduce the radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), while the DPPH assay is based on a colour change produced by the reduction of the DPPH radical (Prior et al., 2005; Miguel, 2010).

In addition to chemical-based assays, it is essential to evaluate the antioxidant activity at a cellular level to comprise some features of the bioavailability which are critical to the success of the antioxidant activity *in vivo*, such as uptake, metabolism and partitioning in membranes (López-Alarcón and Denicola, 2013). The fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA) has been used to estimate the ability of natural compounds to reduce intracellular ROS production. After diffusion through the cell membrane, DCFH-DA is deacetylated by esterases to form 2',7'-dichlorofluorescein (DCFH), which is then oxidized by ROS generating a fluorescent compound (Wang and Joseph, 1999; Gomes et al., 2005).

1.3. Plant compounds

Throughout the years, humans have extensively used plants for their everyday health and food care needs. From 1991 to 2003 the exportation of pharmaceutical plants amounted an average of 467,000 tonnes/year (US$ 1.2 billion), with China, India, Mexico, Bulgaria, Chile, Egypt, Morocco and Albania being the highest exporter countries (Lange, 2006). The 2005 world market of plant-derived drugs was estimated to worth $18 billion (McWilliams, 2006) and current market research indicates that these values continue to increase, exciding $20 billion in 2012 (BBC Research, 2012).

More than half of the world population uses plants for their health care and only 5-15 % of the approximately 250,000 species of higher plants have been investigated for the presence of bioactive compounds. Therefore, there is still much research to be done in this respect (Lange, 2006). In plants we can found an incredible diversity of compounds with complex structures and diverse functional groups, and modern techniques for separation, structure elucidation,

screening and combinatorial synthesis have stimulated the search for new plant-derived drugs (McChesney et al., 2007; Saklani and Kutty, 2008). Natural products significantly contribute to the basis of new drugs, new drug leads and new chemical entities to the treatment or prevention of infectious, neurological, cardiovascular, inflammatory and oncological diseases (Mishra and Tiwari, 2011). Specific relevant cases are khellin, from *Ammi visnaga* (L.) Lamk., which originated the development of chromolyn (in the form of sodium chromoglycate) as a bronchodilator; galegine, from *Galega officinalis* L., which was the model for the synthesis of metformin and other bisguanidine-type antidiabetic drugs; papaverine from *Papaver somniferum* L. which formed the basis for verapamil used in the treatment of hypertension; and the antimalarial drugs, particularly quinine from the bark of *Cinchona* species and artemisinin from *Artemisia annua* L. (Cragg and Newman, 2013).
A total of 26 plant-based drugs were approved during 2000-2006, including novel molecule-based drugs (Saklani and Kutty, 2008), and from the year 2005 to 2010, 19 natural and semi-synthetic products-based drugs were accepted for marketing worldwide (Mishra and Tiwari, 2011). Therefore, the application of plant-based systems continues to have an essential role in healthcare (Cragg and Newman, 2013).

1.3.1. Aromatic plants: a special case

Aromatic plants arouse particular interest for their capacity to synthesise valuable compounds, such as essential oil components and phenolic compounds (Springob and Kutchan, 2009). They have a very impressive set of characteristics highly appreciated by food (e.g. soft drinks, food and confectionary), pharmaceutical (functional properties, e.g. antimicrobial and antioxidant) and cosmetic (e.g. perfumes, skin and hair care products) industries (Lubbe and Verpoort, 2011).

1.3.1.1. Essential oils

Essential oils are volatiles with a strong odor which are synthesised by different plant organs in specialized anatomical structures, namely secretory idioblasts and cavities or ducts (internal secretory structures), and glandular trichomes (external secretory structures) which can be divided in two types, peltate and capitate trichomes. Such structures are common in Lamiaceae family. Peltate trichomes consist of a basal epidermal cell, a short neck-stalk cell

and a secreting head with 4 to 16 cells, depending on the species. The secreted product is accumulated in a large subcuticular space on the apex and it is released after cuticle rupture. The capitate trichomes are composed by one basal cell, and possess only 1 to 4 secreting cells and a small subcuticular space where the secreted material is accumulated (Figueiredo et al., 2008; Kubeczka, 2010).

Essential oils comprise crucial ecological functions in plants because they provide them protection against pathogens and attract some pollinators and seed dispersing animals (Bakkali et al., 2008). Essential oils contain mainly monoterpenes and sesquiterpenes and their oxygenated derivatives, such as alcohols, aldehydes, ketones, acids, phenols, ethers, and esters (Fornari et al., 2012). Lamiaceae is one of the most important family of aromatic plants producing essential oils mainly constituted by mono- and sesquiterpenes. They are synthesised through condensations of the universal five-carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which derive from two independent biosynthetic pathways: the cytosolic mevalonate (MVA) and the 2-2-methyl-D-erythritol-4-phosphate (MEP) [also called 1-deoxy-D-xylose-5-phosphate pathway] localized in plastids (Fig. 1.2). In cytosol, IPP is synthesised *via* the MVA pathway from the condensation of three molecules of acetyl-CoA, while in plastids it is formed from pyruvate and glyceraldehyde-3-phosphate, through the MEP pathway (Dudareva et al., 2005). The first and second steps of the MVA pathway are catalysed by 3-hydroxy-3-methylglutartl-CoA synthase and 3-hydroxy-3-methylglutartl-CoA reductase, respectively, and the MEP pathway is controlled in part by 1-deoxy-D-xylulose-5-phosphate synthase which is the first enzyme of the pathway (Lane et al., 2010). Geranyl pyrophosphate synthase and farnesyl pyrophosphate synthase catalyse the condensation of IPP and DMAPP for the formation of geranyl diphosphate and farnesyl diphosphate, precursors for monoterpenes and sesquiterpenes, respectively (Ajikumar et al., 2008).

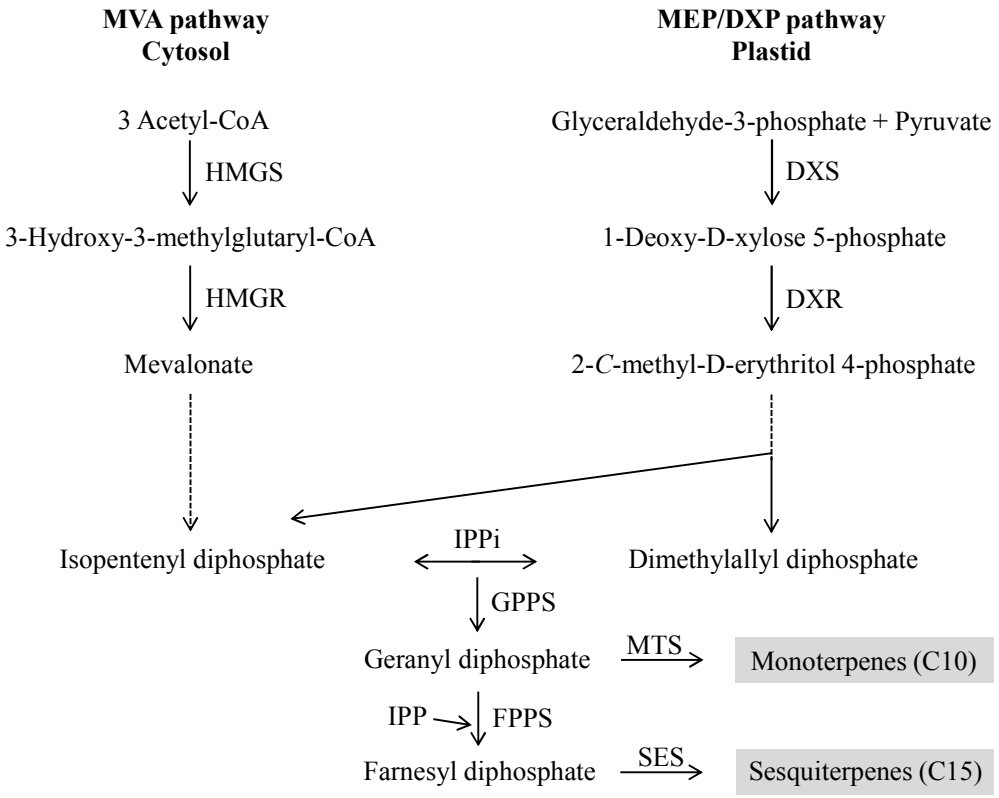

Figure 1.2. The MVA and MEP pathways of mono- and sesquiterpenes biosynthesis in plants. MVA, mevalonate; MEP, 2-2-methyl-D-erythritol-4-phosphate; DXP, 1-deoxy-D-xylose-5-phosphate; HMGS, 3-hydroxy-3-methylglutaryl CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductase; IPP, isopentenyl diphosphate; IPPi, isopentenyl diphosphate isomerase; GPPS, geranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; MTS, monoterpene synthase; SES, sesquiterpene synthase. Multiple steps are indicated by dashed lines. Adapted from Gonçalves and Romano (2013).

Linalool, thymol, eugenol, carvone, cinnamaldehyde, vanillin, carvacrol, citral and limonene have been accepted by the European Commission for their intended use as flavourings in food products. The United States Food and Drug Administration (FDA) also classify them as Generally Recognized As Safe (GRAS) (Hyldgaard et al., 2012). Essential oils are valuable natural products and are considered to be a promising source of antiparasitic, insecticidal, antiviral, antifungal and antioxidant agents (Bakkali et al., 2008; Hussain et al., 2011). Furthermore, some studies have shown that several constituents of essential oils possess anticancer, antimutagenic (Woronuk et al., 2011), and anti-ChEs (Dohi et al., 2009) properties. Additionally, their positive effects on the central nervous system have also been demonstrated (Hudson, 1996; Holmes et al., 2002; Perry et al., 2003; Elliot et al., 2007; Dobetsberger and Buchbauer, 2011). Essential oils are also used in aroma-therapeutic practices due to their calming and sedative effects. For instance, the essential oils from

Lamiaceae family, namely *Melissa officinalis* L. and *Lavandula angustifolia* Mill. demonstrated calming and sedative properties and apparently improve the memory of people with dementia (Elliot et al., 2007). They can be absorbed into the human body through the respiratory system, transdermally (*via* direct contact) or by oral ingestion (Perry and Perry, 2006).

1.3.1.2. Phenolic compounds

Phenolic compounds are a chemically heterogeneous group (simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans and lignins) and are usually accumulated in different plant tissues and cells. They can be found in the wall and vacuole of epidermal cells, and in the leaf interior, both in the palisade and spongy mesophyll cells. In mesophyll cells, phenolic compounds can be located in various compartments, including the nucleus, the chloroplast and the vacuole (Hutzler et al., 1998; Agati et al., 2012; Agati et al., 2013). In plants they are involved in different functional roles including plant resistance against microbial pathogens and animal herbivores, such as insects, and protection against solar radiation (Quideau et al., 2011).

Phenolic compounds are biochemically synthesised *via* the shikimate and/or acetate pathways (Fig. 1.3). The shikimate pathway participates in the biosynthesis of most plant phenols and begins with the condensation of erythrose 4-phosphate and phosphoenol pyruvate to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) in a reaction mediated by the enzyme DAHP synthase (Dewick, 2002; Maeda and Dudareva, 2012). Elimination of phosphoric acid from DAHP followed by an intramolecular aldol reaction generates 3-dehydroquinate. The third and fourth enzymatic reactions in the shikimate pathway include the dehydration of 3-dehydroquinate to 3-dehydroshikimate to introduce the first double bond in the ring, and the reversible reduction of 3-dehydroshikimate into shikimate by NADPH. These reactions are catalysed by 3-dehydroquinate dehydratase and shikimate dehydrogenase (also referred to as shikimate:$NADP^+$ oxidoreductase) respectively (Maeda and Dudareva, 2012). Shikimate is further metabolised to chorismate *via* a new condensation with phosphoenol pyruvate, and the biosynthetic pathways of the three aromatic amino acids, L-phenylalanine, L-tyrosine and L-tryptophan, diverge at this point (Dewick, 2002; Maeda and Dudareva, 2012).

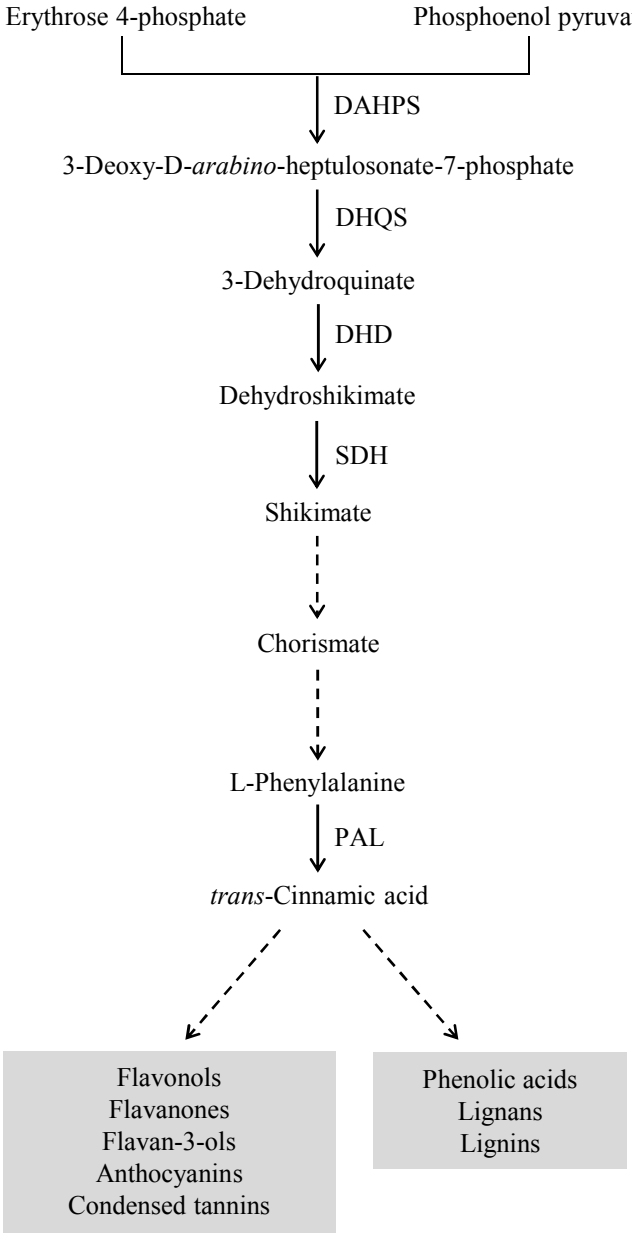

Figure 1.3. Biosynthesis of phenolic compounds in plants. DAHPS, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; DHQS, 3-dehydroquinate synthase; DHD, 3-dehydroquinate dehydratase; SDH, shikimate dehydrogenase; PAL, phenylalanine ammonia lyase. Multiple steps are indicated by dashed lines. Adapted from Tzin et al. (2012).

L-Phenylalanine derives from the conversion of chorismate to prephenate *via* a Claisen rearrangement catalysed by chorismate mutase; decarboxylation then yields phenylpyruvate which, upon amination and reduction, leads to L-phenylalanine, the most common precursor of different phenolic compounds. Phenylalanine ammonia lyase (PAL) catalyses the removal of the amino group of L-phenylalanine to produce *trans*-cinnamic acid, a precursor for most phenylpropanoids, which include phenolic acids, lignans and lignins. Then, *trans*-cinnamic

acid is hydroxylated by a P450 cinnamate-4-hydroxylase to *p*-coumaric acid and the carboxyl group of the latter is activated with CoA by *p*-coumaroyl:CoA ligase (Dewick, 2002; Ferrer et al., 2008; Fraser and Chapple, 2011), which catalyses the conversion of *p*-hydroxycinnamic acids to their corresponding CoA thiol esters. These activated esters are precursors in the biosynthesis of different phenolic compounds, including flavonoids, by condensation with three acetate units as malonyl-CoA (acetate pathway) (Ehlting et al., 2001).

In the flavonoid family, the A ring usually arises from a molecule of resorcinol or phloroglucinol synthesised in the acetate pathway and has a characteristic hydroxylation pattern at the 5 and 7 position, whereas the B ring comes from the shikimate pathway and is usually 4'-, 3',4'-, or 3',4',5'-hydroxylated (Fig. 1.4) (Ross and Kasum, 2002).

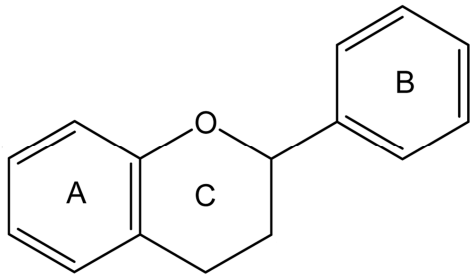

Figure 1.4. Basic structure of flavonoids.

Phenolic compounds exhibit a wide range of beneficial properties to health, such as antibacterial, antiviral, antifungal (Daglia, 2012), chemopreventive (Kang et al., 2011), antithrombotic, anti-inflammatory and vasodilator (Quiñones et al., 2013) effects. Phenolics are also recognized as strong natural antioxidants, which are able to protect neurons from oxidative damage by directly scavenging pathological concentrations of reactive species, chelating transition metal ions or interacting with cellular signaling pathways involved in neurodegeneration (Ebrahimi and Schluesener, 2012). The presence of a phenyl ring, bearing hydroxyl groups in their chemical structure, constitutes an amphiphilic moiety that combines the hydrophobic character of its planar aromatic nucleus with the hydrophilic character of its polar hydroxyl substituent; this allow them to act either as a hydrogen-bond donor or as an acceptor (Quideau et al., 2011).

Phenolics have been reported to act at phosphoinositide 3-kinase, Akt/protein B kinase, tyrosine kinases, protein kinase C (PKC) and mitogen activated protein kinase signaling

cascades. They are able to alter the phosphorylation mode and expression level of targeted molecules within these pathways, affecting cellular function (Williams et al., 2004). PKC signaling pathways are involved in associative memory storage and regulate important molecular events in AD (Alkon et al., 2007).

Phenolic compounds are able to increase endogenous antioxidant enzymes *via* activation of important signaling pathways such as the Nrf2 (Jung and Kwak, 2010). In addition, they can inhibit Aβ aggregate and fibril formation (Harvey et al., 2011), protect against genomic instability events in different somatic tissues of a transgenic mouse model for AD (Thomas et al., 2009), and reduce ChEs activity (Zhang et al., 2009).

1.4. Extraction of natural compounds from aromatic plants

Different methods to extract natural products are described in the literature and efforts have been made to develop new techniques or to improve some already existent, in order to increase their extraction efficiency. In this study, conventional and non-conventional techniques were used, which are discussed in the following sections.

1.4.1. Essential oils

An essential oil can be obtained by distillation processes, such as hydrodistillation (HD) and steam distillation, or by cold pressing in the case of the peel oils from citrus fruits. Basically, in HD the plant material is immersed in water which is then boiled. The volatile components are carried by the steam to a condenser, where a distillate (mixture of water and oil) is formed, and then separated into two layers from which the oil and the water can be separately withdrawn (Kubeczka, 2010; Azmir et al., 2013). Although HD is widely used to isolate essential oils, it does not prevent hydrolysis reactions that can degrade thermolabile compounds. For instance, during HD, tertiary esters are generally hydrolised to the corresponding acids and alcohols by the release of H^+ ions from water (Babu and Kaul, 2005).

In recent years, new techniques have appeared, such as microwave assisted extraction, ultrasound-assisted extraction, pressurized fluid extraction, subcritical water extraction and supercritical fluid extraction (SFE) (Dai and Mumper, 2010). The later one has deserved much attention by the food, pharmaceutical and cosmetic industries because it provides many benefits for the recovery of natural products. SFE produces solvent-free extracts at low

temperatures thus avoiding the degradation of active compounds maintaining the original volatile fraction of an aromatic plant (Reverchon and de Marco, 2006; Pereira and Meireles, 2009). Carbone dioxide (CO_2) (critical conditions: T = 30.9 ºC and p = 73.8 bar) is widely used as supercritical fluid because it is non-toxic, chemically stable, environmentally acceptable, easily separated from the extract since it exists as a gas under normal atmospheric conditions and GRAS by FDA and European Food Safety Authority (Reverchon and de Marco, 2006; Pourmortazavi and Hajimirsadeghi, 2007; Herrero et al., 2010). A supercritical fluid is a compound at a temperature and pressure above the critical values (above critical point) (Brunner, 2005). The main drawback of SFE using CO_2 is its low polarity; however, this limitation can be overcome using polar modifiers (co-solvents) to change the polarity of the supercritical fluid and, consequently, to increase its solvating power (Herrero et al., 2010). SFE provides the opportunity to obtain a selective and enhanced recovery of target compounds by optimizing operation parameters as the plant matrix, pressure and temperature, extraction time and flow rate of the supercritical fluid (Pourmortazavi and Hajimirsadeghi, 2007; Herrero et al., 2010). The nature of the matrix, porosity, surface area, particle size and interactions between solutes and active sites of the matrix influence the SFE results. For instance, the decrease of the particle size represents an increase of the surface area, making extraction more efficient. However, the reabsorption of the analytes on the matrix surface can occur (Pourmortazavi and Hajimirsadeghi, 2007). The increase of contact time between the supercritical fluid and the sample is an important factor contributing to enhance the efficiency of recovery. Solubility of a sample depends on its volatility and on the solvating effect of the supercritical fluid and it can be controlled by selecting the extraction pressure and temperature. The efficiency of extraction is also influenced by the effect of velocity of the supercritical fluid flowing through the cell; the slower the fluid velocity the deeper it penetrates the matrix (Pourmortazavi and Hajimirsadeghi, 2007).

Basically, a SFE system is composed by the compression, the extraction and the separation units. In the compression unit, the liquid CO_2 is refrigerated in an ice cooler, compressed with a circulating pump, and then warmed in a heat exchanger until reaching the desired supercritical conditions. Subsequently, the solvent enters the extraction vessel, where the plant material is placed; the extraction vessel is equipped with temperature controllers and pressure valves at both inlet and outlet to keep the desired extraction conditions. The extraction vessel is pressurized with the fluid by a pump, which is also necessary for the circulation of the fluid in the system (Wang and Weller, 2006). At the exit of the extractor the

supercritical fluid with the extracted solutes flows through a depressurization valve to the corresponding separators where, due to the lower pressure, the extracts are separated from the gaseous solvent and collected (Fornari et al., 2012). In the first separator heavy compounds are deposited while the volatile oil is collected in the second separator.

1.4.2. Phenolic compounds

There is no standardized method to extract phenolic compounds because each plant material has their own properties. In addition, their recovery is hampered by the fact that these secondary metabolites are not homogenously distributed in the plant and their stability varies significantly. For this reason, procedures have to be adjusted to each sample as well as to the purpose of the study (Santos-Buelga et al., 2012). Previous studies confirm that the chemical composition of phenolic extracts and the extraction yields depend on the nature of the tissue matrix, temperature and extraction time, and on the polarity of the solvent system used (Naczk and Shahidi, 2004; Sultana et al., 2009; Trabelsi et al., 2010; Chew et al., 2011; Santos-Buelga et al., 2012). An increase in the extraction temperature can enhance analyte solubility by increasing both solubility and mass transfer rate (Dai and Mumper, 2010). Water, methanol, ethanol, acetone and ethyl acetate have been used for the extraction of phenolic compounds (Sultana et al., 2009). Furthermore, aqueous organic solvents mixtures allow higher yields of phenolic compounds than the corresponding pure organic solvents as reported by Sultana et al. (2009).

Soxhlet extraction, maceration, infusion, ultrasound-assisted extraction, microwave-assisted extraction and pressurized liquid extraction are some of the most common techniques used (Dai and Mumper, 2010). In this study, the phenolic compounds were extracted by the conventional extraction methods, Soxhlet extraction, maceration and infusion. Soxhlet extraction is a standard technique widely used in the extraction of plant compounds due to its convenience. In a Soxhlet system, the plant material is placed in a thimble-holder and a suitable solvent is added. After reaching to an overflow level, the solution of the thimble-holder is aspirated by a siphon and unloads it into the distillation flask, carrying extracted solutes into the bulk liquid. The solvent is separated from the solute in the solvent flask by distillation and the condensed solvent passes back into the plant solid bed. The process runs repeatedly until the extraction is completed (Wang and Weller, 2006; Azmir et al., 2013). The main advantage of Soxhlet extraction is that it is a continuous process (Seidel, 2006).

However, this technique presents substantial disadvantages: damage of thermolabile compounds because extraction generally occurs at the boiling point of the solvent, formation of artifacts, the use of large volumes of hazardous organic solvents and long extraction times (Seidel, 2006; Khoddami et al., 2013).

Maceration is the simplest form of solid-liquid extraction from plants and is carried out at room temperature by soaking the material with the solvent with eventual stirring to facilitate mass-transfer (Azmir et al., 2013; Bucar et al., 2013). This method is more suitable for the extraction of thermolabile compounds. The use of moderate extraction conditions is an advantage of this technique but the high solvent consumption, long extraction times and low extraction yields represent some drawbacks (Bucar et al., 2013). Infusions are simply prepared by macerating the plant material for a short period in cold or boiling water (Seidel, 2006).

1.5. Analytical techniques

The characterisation of metabolites in complex mixtures, like plant extracts, requires sophisticated techniques, which should provide respectable sensitivity and selectivity as well as structural information of the constituents of interest (Marston and Hostettmann, 2009). Chromatography combined with a suitable detection technique offers a powerful tool for separating the individual compounds and developing a characteristic profile of the plant material (Tistaert et al., 2011). Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the two most commonly applied methods to characterise natural compounds (Khoddami et al., 2013).

1.5.1. Gas chromatography

GC is an excellent analytical technique frequently used for the identification and characterisation of volatile compounds (Tistaert et al., 2011). Gas chromatographic techniques coupled with mass spectrometry (GC-MS), combines one of the most powerful separation techniques, GC, with the high degree of structural information provided by MS. This is a potent tool in structure elucidation. The GC setup comprises an injection port, an oven and a column, while the MS comprises an ionization source, the mass analyser and a detection chamber. In GC, the sample is injected through a septum into a heated port which

vaporizes the sample, being then swept through the column by inert gases such as helium, nitrogen, or hydrogen (called *carrier gases*). The separated solutes flow through a detector, which is connected to a recording system. The column consists of a coil of stainless steel, glass or fused silica (quartz) tubing and is enclosed in a thermostatically controlled oven to ensure operation under reproducible conditions. Capillary columns are the most widely used because of their superior resolving power for complex mixture compared to that of a packed column (Fifield and Kealey, 2000). The analytes are separated according to their volatility before reaching the detector. Flame ionization detector (FID) is a universal detector and one of the most common coupled to GC. It is based on the detection of ions produced during combustion of most carbon-containing molecules in a hydrogen-air flame. The FID system is commonly mentioned to as a "carbon counting device" since the magnitude of its response is proportional to the number of carbon atoms (Holm, 1999; von Mühlen et al., 2006).

In any typical MS detector the separated compounds are ionized to generate charged molecules or molecule fragments and separated according their mass-to-charge ratios (*m/z*). Although different ionization methods are available, electron impact (EI) ionization and chemical ionization (CI) are the most common (Sarker and Nahar, 2012). EI involves the bombardment of sample molecules with a high-energy electron beam (~70 eV) leading to their excitation, fragmentation and ionization. In CI, the analytes are ionized by gas-phase ion-molecule reactions (Kellner et al., 1997; Fifield and Kealey, 2000). After ionization and fragmentation of the sample, ions travel through an electromagnetic field that filters the ions based on *m/z*. The final chamber within the MS is the detector, which counts the number of ions with a specific mass. Among the diverse types of mass analysers, quadrupole, ion trap and time-of-flight represent the most common (Kellner et al., 1997). The identification of compounds is carried out through the analysis of the obtained molecule fragments by comparison with library databases, Kovats indices, linear retention indices, relative retention time and standards (Rubiolo et al., 2010).

1.5.2. High-performance liquid chromatography

HPLC is one of the most powerful tools in analytical chemistry with the ability to separate water-soluble, thermally-labile and nonvolatile compounds (Marston and Hostettmann, 2009). This is an easy to operate, fully automatable technique with high resolution, selectivity and sensitivity (Tistaert et al., 2011). HPLC is the preferred technique for both separation and

quantification of phenolic compounds (Khoddami et al., 2013). HPLC offers a unique chance to separate simultaneously all the analysed components together with their possible derivatives or degradation products (Stalikas, 2007). The principle of HPLC setup consists on the use of a two-phase system, namely the stationary phase and the mobile phase, to separate compounds according to specific molecular properties. Chromatography is generally carried out in the reverse-phase mode, on octadecyl carbon chain (C18) bonded silica columns (Khoddami et al., 2013). Isocratic and gradient elution can be applied for analyses of phenolic compounds, and the choice depends on the number and type of the analytes and the nature of the matrix (Stalikas, 2007). However, a gradient elution system is more commonly applied than an isocratic elution system (Khoddami et al., 2013). The recommended pH range of the mobile phase is 2-4 to avoid the ionization of phenolics during identification; aqueous acidified mobile phases predominantly contain acetic, formic, phosphoric, and most rarely, perchloric acids are used to control the pH value (Sarker and Nahar, 2006; Stalikas, 2007; Khoddami et al., 2013). Phenolics are frequently identified using different detection systems as ultraviolet (UV) and diode-array detection (DAD), evaporative light scattering detection and chemiluminescence detection, nuclear magnetic resonance (NMR), and MS (Ignat et al., 2011; Tistaert et al., 2011). Due to the presence of conjugated double and aromatic bonds, all phenolic compounds absorb in the UV or ultraviolet-visible (UV-VIS) region, and therefore this detector is commonly used to identified these natural compounds (Stalikas, 2007). DAD-UV detection is advantageous since all wavelengths are stored during analysis, and thus multiple wavelengths can be monitored at the same time for detection of different classes of compounds (de Rijke et al., 2006).

1.6. Encapsulation of natural metabolites

Natural products, particularly, essential oils, have limited water solubility, are volatile and sensitive to oxygen, light and heat. All these issues can considerably decrease essential oils bioavailability and, consequently, restrict their applications. In recent years, a vast role of molecules has been extensively used as complexing agents to improve the solubility, chemical stability and bioavailability of poorly soluble compounds, such as chitosan (Zivanovic et al., 2005), proteins (Baranauskienė et al., 2006), gums (Chang et al., 2006) and starch-based compounds such as cyclodextrins (CDs) (Petrović et al., 2010).

1.6.1. Cyclodextrins: structure and physicochemical properties

Why are CDs so interesting? Firstly isolated in 1891 by Villiers (1891), as degradation products of starch from a medium of *Bacillus amylobacter*, CDs provide unique structural features and amphiphilic properties. The cavity is lined by the hydrogen atoms and the glycosidic oxygen bridges. The nonbonding electron pairs of the glycosidic oxygen bridges are directed toward the inside of the cavity, producing a high electron density and lending it some Lewis base character. As a result of this spatial arrangement of the functional groups in the CDs molecules, the cavity is relatively hydrophobic while the external surfaces are hydrophilic (Fig. 1.5) (Connors, 1997; Astray et al., 2009).

CDs are natural cyclic oligosaccharides with a truncated cone shape containing 5 or more D-glucopyranose units netted through covalent bonds by C1 and C4 carbons. The most common natural CDs consist of six (α-CD), seven (β-CD) and eight (γ-CD) glucopyranose units. Worldwide, about 30 different pharmaceutical formulations containing CDs are on the market (Loftsson et al., 2005). β-CD is the most commercially attractive (more than 95% of CDs produced and consumed) due to its simple synthesis, availability and price. Nevertheless, β-CD has a relatively low solubility in water when compared to the other CDs possibly due to the strong binding of the β-CD molecules in the crystal state (Szente and Szejtli, 1999). To improve the solubility of the β-CD, chemical modifications can be made introducing other functional groups than the native hydroxyls (e.g. alkyl, hydroxyalkyl, carboxyalkyl, amino, thio, tosyl, glucosyl, maltosyl, ethers and esters) (Szejtli, 1997). This random substitution transforms the crystalline CDs into amorphous derivatives with improved water solubility (Loftsson et al., 2005). CDs derivatives with pharmacological importance include the hydroxypropyl derivatives of β- and α-CD, the randomly methylated β-CD, sulfobutylether β-CD, and the glucosyl-β-CD (Loftsson et al., 2005).

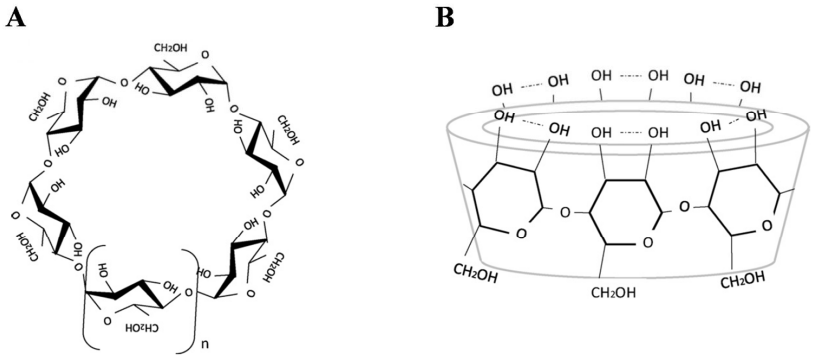

Figure 1.5. Chemical structure (A) and schematic representation of the truncated conical shape (B) of cyclodextrins. The "n" represents the number of repeating sugar units. Adapted from Pazos et al. (2010).

CDs have been used to stabilize fragrances, flavours and vitamins, to protect labile guests against decomposition, oxidation, hydrolysis or loss by evaporation, to reduce or prevent skin irritation and to control volatility and sublimation (Astray et al., 2009; Marques, 2010). CDs have been widely used to improve the solubility, chemical stability and bioavailability of natural compounds, including essential oils and phenolic compounds (Mercader-Ros et al., 2010; Çelik et al., 2011; Lucas-Abellán et al., 2011; Liang et al., 2012; Ceborska et al., 2013); their antioxidant and antimicrobial activities could be also enhanced as reported by Mercader-Ros et al. (2010) and Liang et al. (2012), respectively. Therefore, CDs have been widely applied in foods (Astray et al., 2009), pharmaceuticals (Martin Del Valle, 2004), cosmetics (Schmann and Schollmeyer, 2002), environment protection (Lezcano et al., 2002), bioconversion (Dufosse et al., 1999) and textile industry (Hedges, 1998).

1.6.1.1. Formation of inclusion complexes

Inclusion complexes can be formed either in solution or in the crystalline state, using different methods (Singh et al., 2002). In solution phase, an excess of amount of guest molecules is added to an aqueous CD solution under agitation and at a desired temperature. Then, the suspension is filtered or centrifuged to obtain a clear guest-host complex solution (Singh et al., 2002). For the preparation of solid complexes, normally the water and, when necessary, other co-solvents (e.g. used to dissolve the guest molecule or to obtain limpid solutions before drying), are removed from the aqueous host-guest solution by evaporation (e.g. spray-drying) or sublimation (e.g. lyophilization) (Loftsson and Brewster, 1996; Singh et al., 2002).

1.6.1.2. Mechanism of inclusion complex formation

The inclusion complex formation is a dynamic equilibrium process, where the host molecule (CD) includes, totally or partly, the guest molecules. The driving forces responsible for the complex formation include electrostatic interactions, van der Waals contributions, hydrogen bonding, release of conformational strain and charge-transfer interactions (Brewster and Loftsson, 2007). In aqueous solution, the polar-apolar interactions between entrapped water molecules and the CD cavity are not energetically favorable. Complexation is seen as an entropically driven process where water molecules from the CD cavity are released to the bulk (Górnas et al., 2009). This release of enthalpy-rich water molecules to the bulk increases the entropy of water and, consequently, the entropy of the system. At the same time, less polar guest molecules in the solution are encapsulated in the CD cavity. The overall encapsulation process is therefore spontaneous (i.e. Gibbs free energy is negative) due to an entropy gain (i.e. water release from CD cavity) and enthalpy gain (i.e. new hydrogen-bonds formed after water release from CD cavity and favorable hydrophobic interactions between CD interior and apolar guest molecule) (Brewster and Loftsson, 2007; Górnas et al., 2009).

1.6.1.3. Evidence and characterisation of inclusion complexes

Inclusion in CDs exerts a profound effect on the physicochemical properties of guest molecules. Changes in solubility, chemical reactivity, UV-VIS absorbance, fluorescence, compound retention, pKa values, potentiometric measurements and chemical stability, NMR chemical shifts and effects on drug permeability through artificial membranes are among the effects which have been reported (Loftsson et al., 2005; Marques, 2010). Therefore, any methodology sensitive enough to measure these changes can be used to confirm and characterise the occurrence of inclusion complexes. NMR is a powerful technique for the characterisation of complexes in solution. If the guest molecule is entrapped into the CD cavity, considerable changes in the chemical shift values of the CD protons, namely H_3 and H_5 located inside the cavity, are observed (Marques, 2010; Duchêne, 2011). On the other hand, UV-VIS spectrometry is a simple method where the presence of guest-host complex is detected by changes on the absorbance as result of the perturbation of the chromophore electrons of the guest by its inclusion in the CD (Duchêne, 2011). Additionally, chromatographic techniques can also be applied since during the inclusion process the hydrophobic guest is altered to hydrophilic molecules (Szejtli, 1997). Through differential

scanning calorimetry analysis it is possible to verify the occurrence of inclusion complex because when the guest molecule is entrapped into the CD cavity its melting, boiling and sublimation points shift to a different temperature (Marques, 2010; Duchêne, 2011). Powder x-ray diffractometry is used to measure the crystallinity of a product. The intensity of diffraction peaks is indicative of the crystalline character of the product (Marques, 2010; Duchêne, 2011).

1.7. Production of secondary metabolites using *in vitro* techniques

The production of phytochemicals is affected by environmental and physiological conditions and their chemical synthesis is complex due to their structures and specific stereo-chemical characteristics. In addition, some species which are known to produce valuable phytochemicals are considered to be critically endangered and legally protected. This means that the use of wild plants is highly limited and restricted as a source of bioactive compounds. Biotechnological approaches, as micropropagation, allow the production of plant material with a significant potential for the accumulation of high-value phytochemicals allowing to circumvent the above mentioned limitations (Matkowski, 2008; Karuppusamy, 2009).

Micropropagation is the process by which a single cell can be divided and differentiated to originate a whole plant (totipotency), in a defined growth medium under aseptic and controlled environment conditions (George and Debergh, 2008). There are two methods of *in vitro* propagation: propagation from axillary or terminal buds, which requires pre-existing meristems in the explants; and propagation by the formation of adventitious shoots or somatic embryos either directly, from explant tissues without previously-formed callus (direct organogenesis or direct embryogenesis), or indirectly, when shoots or embryos regenerate on previously-formed callus or in cell culture (indirect organogenesis or indirect embryogenesis) (George and Debergh, 2008). The *in vitro* propagation process is developed in five stages: plant selection and preparation, establishment of aseptic culture, shoots multiplication, root induction, and acclimatization (George and Debergh, 2008). The *in vitro* propagation process is initiated by mother plant selection and preparation under hygienic and controlled conditions (temperature, light, photoperiod and growth regulators) to avoid contamination problems and allow to obtain an explant more suitable or more reliable as starting material. *In vitro* cultures will be then established into aseptic conditions to avoid contamination and providing a considerable amount of explants (Debergh and Zimmerman, 1991). The purpose of the next

stage is the multiplication of shoots to a number required for subsequent rooting. Multiplication of shoots is achieved by subculturing them in medium containing a specific cytokinin concentration at regular intervals that may vary from two to eight weeks depending on the shoot development (Hartmann et al., 1997). The third stage consists in the elongation of the produced shoots and their rooting (Debergh and Zimmerman, 1991). The last stage involves the acclimatization of *in vitro* produced plants to the *ex vitro* environment (Hartmann et al., 1997).

Plant tissue culture techniques allow the selective, rapid and effective production of secondary metabolites under controlled environmental conditions with no seasonal constraints and independent of geographical and soil conditions (Matkowski, 2008; Karuppusamy, 2009). Moreover, *in vitro* cultures allow bioactive compounds to be extracted without a negative impact on natural habitats (Matkowski, 2008; Karuppusamy, 2009). Therefore, these techniques offer the rare opportunity to promote the accumulation of a target metabolite by manipulating the chemical and physical conditions of the environment (Karuppusamy, 2009).

Secondary metabolites are produced by plants as a part of defense response against insects, herbivores and pathogens and adaptation to other biotic and abiotic stresses (e.g. heavy metals), which are triggered and activated by elicitors, the signal compounds of plant defense responses. Thus, elicitation is a suitable approach to enhance valuable compounds production (Zhao et al., 2005; Namdeo, 2007; Smetanska, 2008; Karuppusamy, 2009). The biosynthetic pathways of many secondary metabolites are easily altered by external factors such as nutrient levels, stress factors, light and growth regulators, by the addition of precursors, selection of highly productive cell lines and biotransformation (Rao and Ravishankar, 2002; Canhoto, 2010).

Because secondary metabolites are not homogeneously distributed in plants, the use of biotechnological tools for the production of secondary metabolites in differentiated tissues is advantageous. For instance, in *Salvia officinalis* L. (Grzegorczyk et al., 2007) and *Rosmarinus officinalis* L. (Caruso et al., 2000) *in vitro* cultures, the abietane diterpene antioxidants (carnosol and carnosic acid) are present only in shoot cultures and not in callus, suspension or hairy roots. In the particular case of essential oils, they are only produced in specialized plant tissues or glands. Therefore, the use of differentiated plantlets or organ cultures is required for their production (Gonçalves and Romano, 2013).

The ability of cultured plant cells, tissues and organs to produce identical chemical compounds as the parental plant encourages the use of these *in vitro* techniques (Karuppusamy, 2009). For instance, Nogueira and Romano (2002) and Gonçalves et al. (2008) compared the chemical profiles of essential oils and volatiles from *Lavandula viridis* L'Hér plants in the field, *in vitro* cultures and micropropagated plants, finding no significant compositional variations among the three sources. Due to its huge economic value, essential oils mainly from Lamiaceae family have been produced using *in vitro* cultures. Recently, Gonçalves and Romano (2013) reviewed the different *in vitro* propagation techniques that have been established in several *Lavandula* spp., and the use of *in vitro* plant cells and tissues for the biosynthesis of high-value compounds.

Given the capacity of natural antioxidants to prevent or treat oxidative stress-related diseases many different approaches have been used to increase their biosynthesis in plant cell cultures. Matkowski (2008) revised the most active antioxidants derived from plant tissue cultures. Among them, rosmarinic acid widespread in Lamiaceae family and with known health-promoting effects has been extensively produced using plant tissue culture techniques, for instance, suspension cell cultures of *Lavandula vera* (Georgiev et al., 2006) and *Salvia officinalis* (Hippolyte et al., 1992), and callus transformed with *Agrobacterium tumefaciens* of *Coleus blumci* Benth (Bauer et al., 2004).

1.8. Plants description

1.8.1. Lamiaceae

The Lamiaceae family is one of the most important and valuable aromatic species and consists of about 252 genera and more than 6700 species (Hedge, 1992). *Lavandula* and *Thymus* are two genera of this family important to the food, cosmetic, perfumery and pharmaceutical industries. *Lavandula* and *Thymus* species have also been used as culinary herbs, flavouring ingredients in food and traditional medicines (Cavanagh and Wilkinson, 2002; Upson and Andrews, 2004).

1.8.2. The genus *Lavandula*

Lavandula species are endemic to the Mediterranean region, Canary, Cape Verde and Madeira Islands, the Arabian Peninsula, Asia and India. There are 39 species, numerous

hybrids and nearly 400 registered cultivars. They are widely used as ornamental and melliferous plants (Upson and Andrews, 2004). The high industrial and commercial value of these species is mainly because of their essential oils (Büyükokuroğlu et al., 2003), which are strictly regulated under international ISO standards (ISO/TC 54-ISO/CD 8902, 2007; ISO/TC 54 N-ISO/WD 4719, 2009). In the food industry, *Lavandula* essential oils are used in beverages, confectionary and ice creams, and in the fragrance industry they are used in perfumes, skin lotions, soaps and other cosmetics. They have also been used in aromatherapy products (Boelens, 1995; Kim and Lee, 2002). Several oils from this genus have been reported to possess antibacterial, antifungal and anti-ChE effects (Cavanagh and Wilkinson, 2002; Dohi et al., 2009; Hanamanthagouda et al., 2010). In addition to the oils, the neuroprotective, anti-inflammatory and antioxidant properties of *Lavandula* extracts were also proved (Büyükokuroğlu et al., 2003; Hajhashemi et al., 2003; Kovatcheva-Apostolova et al., 2008).

Algarve, the southern region of Portugal, has a wide range of aromatic plants (Franco, 1971). The present study was focused on two *Lavandula* species which are little explored regarding their biological potentialities: *Lavandula viridis* L'Hér and *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco.

1.8.2.1. *Lavandula viridis* L'Hér

Lavandula viridis L'Hér is a xerophytic aromatic shrub naturally distributed in Madeira Island, south Portugal and south-west Spain. In Portugal, we can find this species growing in dry conditions and in nutrient poor and degraded soils of the Algarve and Alentejo regions (Fig. 1.6) (Franco, 1971). *L. viridis* grows up to 30-50 cm with leaves broadly linear-lanceolate. Inflorescences are spike up to 8 cm and contain fertile bracts obovate (Fig. 1.7A and B), that blossom from April to May. *L. viridis*, commonly known as 'Rosmaninho-verde', is distinctly different due to the presence of amazing yellow-green flowers (Upson, 2002).

The scientific literature for *L. viridis* mainly focus on the chemical composition of its essential oil (Nogueira and Romano, 2002; Gonçalves et al., 2008) and their antioxidant (Matos et al., 2009) and antifungal (Zuzarte et al., 2011) activities. *In vitro* regeneration of *L. viridis* from single node explants was previously described by Dias et al. (2002). In addition, Nogueira and Romano (2002), and later Gonçalves et al. (2008), using headspace solid phase micro-extraction, studied the chemical composition of the essential oils from

field-grown *L. viridis* plants, as well as *in vitro* shoot cultures and micropropagated plants produced from the same clone. They identified 1,8-cineole as the major compound in all the essential oils, followed by camphor.

1.8.2.2. *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco

Lavandula pedunculata (Miller) Cav. is an aromatic shrub with purple bracts and flowers arranged in light-green long-stalked spikes (up to 50 cm), that blooms in the beginning of spring (Matos et al., 2009). It is commonly found in the Iberian Peninsula and traditionally used in Portuguese medicine and as ornamental plants. Infusions prepared from flowered aerial parts are traditionally consumed to treat anxiety, insomnia, anorexia, cough and bronchitis (Proença da Cunha et al., 2003; Salgueiro, 2004). Franco (1971) considered three subspecies for *L. pedunculata*: subsp. *pedunculata* in northwest Portugal, subsp. *sampaiana* in north and central Portugal and subsp. *lusitanica* in central and south Portugal (Fig. 1.6, Fig. 1.7C and D).

The essential oils from *L. pedunculata* have been studied by several authors in the last decades. For instance, the essential oils of *L. pedunculata* harvested in north and central Portugal are characterised by a high amount of oxygenated monoterpenes with 1,8-cineole, fenchone and camphor as the most abundant with important antifungal activity (Zuzarte et al., 2009). In addition, the anti-AChE activity of the essential oil and extracts of *L. pedunculata* from eastern Portugal was previously described (Ferreira et al., 2006). Regarding the *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco, collected in south Portugal, Matos et al. (2009) analysed the chemical profile and antioxidant activity of its essential oils and found fenchone and camphor as the majority compounds.

1.8.3. The genus *Thymus*

Thymus is a complex genus of aromatic plants that is widely distributed in the Mediterranean region. Around 110 species of *Thymus* occur in the Mediterranean area (Morales, 1997). In Portugal, we can found 11 *Thymus* species (Figueiredo et al., 2001). *Thymus* species are traditionally used as culinary spices, herbal medicines, ornamental plants and flavouring agents (Figueiredo et al., 2001; Figueiredo et al., 2008). Infusions and the essential oil of *Thymus* are traditionally used in treating abscesses, cutaneous ulcers, dermatitis, illnesses of

the respiratory tract and gastrointestinal troubles (Zarzuelo and Crespo, 2002). Due to their savoury taste and preservative qualities, *Thymus* species are appreciated and widely used as condiment in Mediterranean gastronomy for seasoning food (Figueiredo et al., 2008). Furthermore, the huge economic importance of *Thymus* spp. is also associated to applications in perfumes and in the composition of cosmetic creams, toothpaste and mouthwashes or for flavouring chocolates (Zarzuelo and Crespo, 2002; Figueiredo et al., 2008). Their essential oils are of great economic value and, therefore, strictly regulated by international ISO standards (ISO 14715, 2010).

1.8.3.1. *Thymus lotocephalus* G. López & R. Morales

Thymus lotocephalus G. López & R. Morales is a dwarf undershrub (up to 30 cm) with characteristic terminal conspicuous stout inflorescences (Fig. 1.7E and F) that blossoms from April to June. The ascending branches bear axillary clusters of small linear ciliate leaves. The bracts are large, ovate and purplish, surrounding the whorls of purplish two-lipped flowers (Figueiredo et al., 2001). *T. lotocephalus*, commonly known as "tomilho cabeçudo" or "erva-ursa", is an endemic species restricted to the Algarvian Barrocal (Fig. 1.6), found in dry open places and also in dry scrub. *T. lotocephalus* is used for flavouring culinary dishes in some areas of the Algarve. The distribution of *T. lotocephalus* is confined to a few populations; the species is considered to be critically endangered by the increase of the tourism pressure, agriculture and construction and is legally protected by both the European Habitats Directive (92/43/CEE) and Portuguese law (Reference 140/99, April 24 1999; ICN, 2006). Recently, Coelho et al. (2012a) developed a micropropagation protocol for this endangered species and studied the germination and cryopreservation tolerance of seeds (Coelho et al., 2012b).

The chemical composition of *T. lotocephalus* essential oils collected in different populations has been reported (Salgueiro, 1992; Figueiredo et al., 1993; Salgueiro et al., 2000; Faleiro et al., 2003). These results showed some important differences between populations from relatively close localities. In fact, the large chemical polymorphism of *T. lotocephalus* essential oil makes it difficult to obtain general characterisation of its composition (Figueiredo et al., 2008). Faleiro et al. (2003) reported the antimicrobial activity of the essential oils collected at Campus de Gambelas (Algarve).

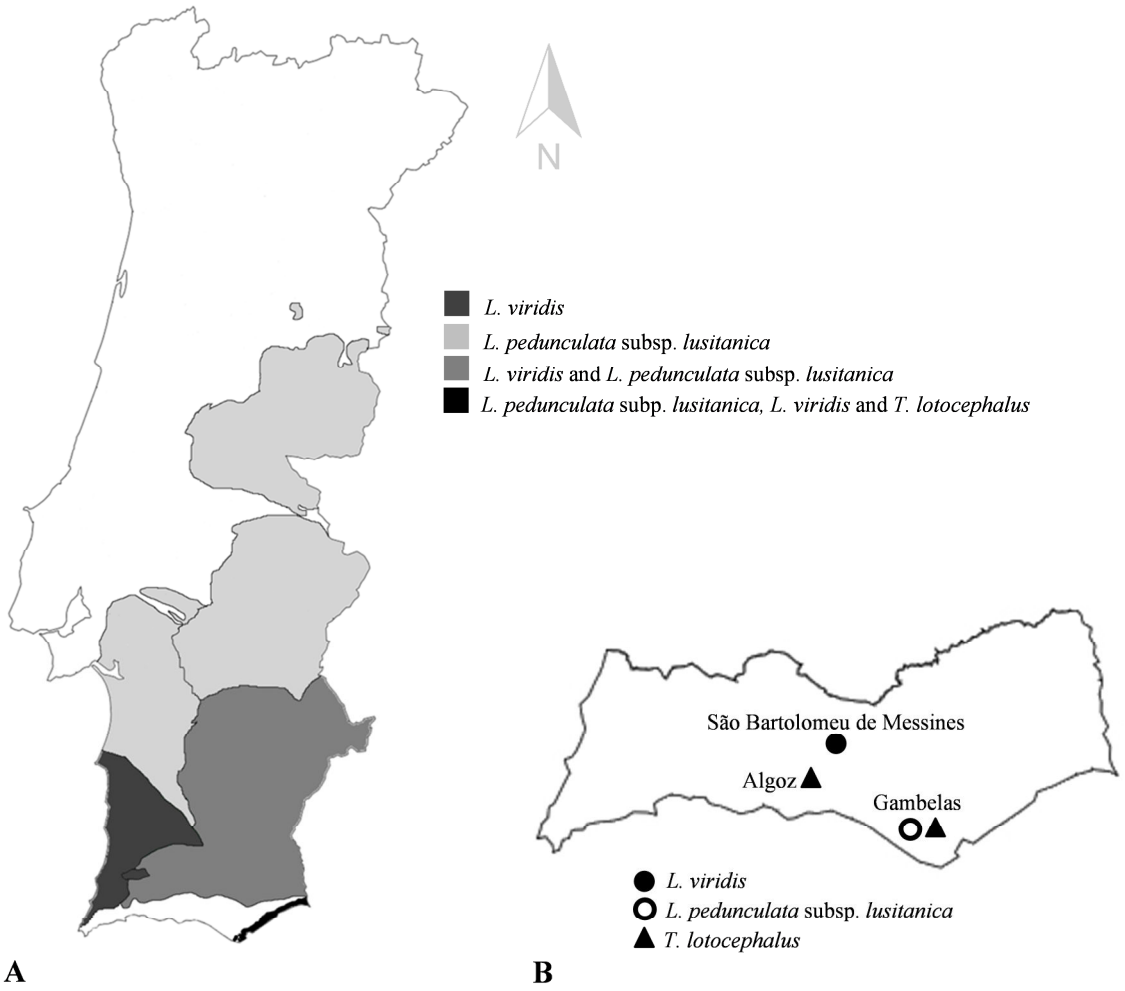

Figure 1.6. (A) Distribution of *Lavandula viridis*, *Lavandula pedunculata* subsp. *lusitanica* and *Thymus lotocephalus* in continental Portugal. Adapted from Franco (1971). (B) Algarve region: location of studied populations.

Figure 1.7. Aspect of the sampled population and flower detail of the aromatic plants *Lavandula viridis* (A and B), *Lavandula pedunculata* subsp. *lusitanica* (C and D) and *Thymus lotocephalus* (E and F).

1.9. Main aims of this study

Neurodegenerative diseases, particularly AD, are a current concern throughout the world. Based on the traditional knowledge and scientific studies conducted over the years, it is recognized that plants produce a wide range of biologically active compounds which may be a valid alternative for the prevention and/or treatment of AD. Many species traditionally used either as condiments or as therapeutics, are not explored in its full biological potential and have not yet the scientific background to support their benefits. Therefore, this study intends to elucidate the biological potential of three aromatic species from the Algarve region (*L. viridis*, *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus*) belonging to the Lamiaceae family.

In this study, it is proposed to:

- Isolate essential oils and phenolic compounds from plant materials by different procedures and evaluate their chemical profiles by chromatographic techniques;
- Evaluate the antioxidant and anti-ChE activities of the essential oils and phenolic extracts;
- Isolate bioactive metabolites from *in vitro* produced cultures and study their chemical profile and biological activity in comparison with wild plants;
- Assess the effects of encapsulation systems on the biological activity and storage stability of the essential oils;
- Investigate the neuroprotective effect against H_2O_2-induced toxicity in A172 human astrocyte cell line;
- Understand the *in vitro* absorption and transport mechanisms of plant compounds through the gastrointestinal tract.

It is expected that this thesis provides important information about the chemical composition and biological activities of the studied species. It is also intended that the results obtained contribute to understand the role of aromatic plants on the prevention of neurodegenerative diseases.

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CHAPTER 2

CHEMICAL CHARACTERISATION AND BIOLOGICAL ACTIVITY

Costa P, Gonçalves S, Grosso C, Andrade PB, Valentão P, Bernardo-Gil G, Romano A (2012). Chemical profiling and biological screening of *Thymus lotocephalus* extracts obtained by supercritical fluid extraction and hydrodistillation. Industrial Crops and Products, 36, 246-256.

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Chemical profiling and biological screening of *Thymus lotocephalus* extracts obtained by supercritical fluid extraction and hydrodistillation

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ABSTRACT

Essential oil and extracts from the aerial parts of *Thymus lotocephalus* were obtained by hydrodistillation (HD) and supercritical fluid extraction (SFE) in two different collectors, respectively. SFE was conducted at 40 °C and a working pressure of 12 or 18 MPa. The chemical profiles were determined using GC-FID and GC–IT–MS. Oxygen-containing monoterpenes were the primary constituents in the essential oil and SFE extracts collected in the second separator, while the extracts obtained in the first separator were predominantly oxygen-containing sesquiterpenes. A large number of compounds were identified by hydrodistillation and, in contrast, the highest extraction yields were obtained using SFE. Linalool (10.43 ± 1.63%) was the main component in essential oil, whereas camphor (7.91 ± 0.84%) and *cis*-linalool oxide (7.25 ± 1.45%) were the major compounds in the extracts-2nd separator obtained at pressures of 12 and 18 MPa, respectively. Caryophyllene oxide was the primary constituent identified in the extracts-1st separator (4.34 ± 0.51 and 4.41 ± 1.25% obtained at 12 and 18 MPa, respectively). The antioxidant activity was assessed by ORAC and DPPH assays, and the anti-cholinesterase activity was evaluated *in vitro* using Ellman's method. The essential oil and SFE extracts (first separator) of *T. lotocephalus* possessed antioxidant activity and strongly inhibited cholinesterases. We also demonstrated that the acetylcholinesterase and butyrylcholinesterase inhibitory activities of the essential oil could be attributed to 1,8-cineole and caryophyllene oxide, respectively.

1. Introduction

Thymus lotocephalus G. López & R. Morales is a species endemic to the Algarve region that is typically found in dry open places and scrublands. The genus *Thymus* (Lamiaceae), which is widely distributed in the Mediterranean region, includes a vast group of aromatic plants with great value as culinary herbs, ornamental plants and flavouring agents (Figueiredo et al., 2001, 2008). In addition, these plants possess an array of biological properties that positively impact human health. Infusions prepared from the *Thymus* species have been used in folk medicine for gastrointestinal complaints and as effective therapies for irritating coughs and bronchitis because of their expectorant and antitussive properties (Figueiredo et al., 2008). Moreover, these plants have dermatological benefits against acne, and more recently, anti-platelet, antimicrobial and antioxidant activities have also been demonstrated (Okazaki et al., 2002; Baranauskienė et al., 2003; Faleiro et al., 2003; Hazzit et al., 2009; Oh et al., 2009; Dandlen et al., 2010; Grosso et al., 2010). There are few reports specifically concerning the biological properties of *T. lotocephalus*; however, the essential oil isolated from its flowering aerial parts was shown to possess antimicrobial activity (Faleiro et al., 2003).

It has been demonstrated that an overproduction of reactive oxygen species (ROS), generated during biological processes, might contribute to the pathogenesis of many diseases, such as Alzheimer's disease (AD). Conversely, natural compounds might act as antioxidants and consequently augment cellular

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; ATCI, acetylthiocholine iodide; BChE, butyrylcholinesterase; BTCI, butyrylthiocholine chloride; ChE, cholinesterase; CO_2, carbon dioxide; DTNB, 5,5′-dithiobis[2-nitrobenzoic acid]; DPPH, 2,2-diphenyl-1-picrylhydrazyl; BHT, butylated hydroxytoluene; SET, single electron transfer; GC-FID, gas chromatography-flame ionisation detector; GC–IT–MS, gas chromatography–ion trap–mass spectrometry; HAT, hydrogen atom transfer; HD, hydrodistillation; ORAC, oxygen radical absorbance capacity; ROS, reactive oxygen species; SFE, supercritical fluid extraction; TE, trolox equivalents; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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defences against damage caused by ROS (Frank and Gupta, 2005). Alzheimer's disease is an irreversible and progressive neurodegenerative disorder characterised by the loss of memory and cognitive impairment. The human brain is particularly susceptible to oxidative processes because it has high energy requirements and an increased rate of oxygen consumption (Behl, 2005). In addition to the neuropathologic hallmarks of this disease, AD is marked by a deficit in the cholinergic system (Francis et al., 1999). The use of cholinesterase (ChE) inhibitors is an effective approach for the treatment of AD, contributing to the enhancement of acetylcholine (ACh) levels in the synaptic cleft and, consequently, to the improvement of the cholinergic system (cholinergic hypothesis) (Francis et al., 1999).

Essential oils are odiferous, concentrated mixtures of volatile aromatic compounds produced by plants as secondary metabolites (Anitescu et al., 1997; Bakkali et al., 2008). Hydrodistillation (HD) is a conventional technique extensively used to extract essential oils from plant material, but this process does not avoid the potential hydrolysis or thermal degradation of the most sensitive compounds (Reverchon and de Marco, 2006; Herrero et al., 2010). In contrast, supercritical fluid extraction (SFE) enables shorter extraction times, reduces the consumption of hazardous organic solvents and avoids the degradation of active compounds, thus preserving the original composition of the extracted volatiles (Reverchon and de Marco, 2006; Chen et al., 2009). Carbon dioxide (CO_2) is widely used as supercritical fluid because it is nontoxic, chemically stable, environmentally acceptable and easily separated from the extract because of its gaseous standard state (Al-Marzouqi et al., 2007; Herrero et al., 2010).

The purpose of this study is twofold: (i) to obtain extracts from *T. lotocephalus* by HD (essential oil) and SFE (under different conditions) and determine their chemical profiles using gas chromatography techniques (GC-FID and GC–IT–MS); and (ii) to evaluate the influence of the extraction technique and different SFE pressures and separators on antioxidant and anti-ChE activities.

2. Materials and methods

2.1. Standards and reagents

Reference and alkane standard solutions and butyrylthiocholine chloride (BTCI) were acquired from Fluka (Steinheim, Germany). Fluorescein and ethanol were obtained from Panreac (Barcelona, Spain). Acetylthiocholine iodide (ATCI), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), acetylcholinesterase (AChE) (Electric-eel, EC 3.1.1.7, Type VI-S), butyrylcholinesterase (BChE) (horse-serum, EC 3.1.1.8), caryophyllene oxide, galanthamine hydrobromide, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and methanol were purchased from Sigma–Aldrich (Steinheim, Germany). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and butylated hydroxytoluene (BHT) were purchased from Acros Organics (Geel, Germany). Carbon dioxide (CO_2) was obtained from Air Liquide (Lisboa, Portugal).

2.2. Plant material

The aerial parts (flowers and leaves) of *T. lotocephalus* were collected during the flowering period at Campus de Gambelas (Algarve, Portugal). A voucher specimen was deposited in the herbarium of the University of Algarve under the number ALGU 8081. The plant material was dried at room temperature, ground to a powder using a blender and stored at −20 °C prior to use.

2.3. HD and SFE extraction

The essential oil was obtained by a 3 h HD of 50 g of plant material in a Clevenger-type apparatus (Rao et al., 2005). The SFE extracts were obtained using 10 g of plant material and an extraction apparatus with a fixed tubular extractor of 9.4 cm in height and 2.13 cm in internal diameter, as described by Esquível and Bernardo-Gil (1993). Briefly, CO_2 (99.5%, w/w pure) was delivered from a standard cylinder and compressed to an extraction pressure of 12 or 18 MPa by an air-driven liquid pump after cooling at a flow rate of 0.3 kg/h. The CO_2, together with the sample material, entered the extraction cell through a heat exchanger, where it reached an extraction temperature of 40 °C. Two different types of SFE products were obtained in two different separators under the same conditions of pressure and temperature; the heaviest components were collected in the first separator (extracts-1st separator), and the volatiles were collected in the second separator (extracts-2nd separator).

2.4. GC-FID analysis

Quantitative analyses of the essential oil and SFE extracts were performed in a Finnigan Focus GC (Thermo Electron Corporation) equipped with a flame ionisation detector (FID) and a VF-5 ms (30 m × 0.25 mm × 0.25 μm) column (VARIAN). The oven temperature was set at 40 °C for 2 min, followed by an increase of 3 °C/min to 250 °C and 5 °C/min to 300 °C. The injector and detector temperatures were 300 and 310 °C, respectively. Helium C-60 was used as a carrier gas at a constant flow of 1.5 ml/min, and the samples were injected using a split ratio of 1:20. The volume of injection was 1 μl. The percentage composition of the products was calculated by normalisation of the GC peak areas without using response factors.

2.5. GC–IT–MS analysis

The essential oil and SFE extracts were analysed using a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 mass selective detector (USA), a VF-5 ms (30 m × 0.25 mm × 0.25 μm) column (VARIAN) and a Saturn GC/MS workstation software version 6.8. The injector port was heated to 300 °C and the samples were injected using a split ratio of 1:40. Helium C-60 (Gasin, Portugal) was used as a carrier gas at a constant flow of 1.5 ml/min. The oven temperature was set at 40 °C for 2 min, followed by an increase of 3 °C/min to 250 °C and 5 °C/min to 300 °C. All mass spectra were acquired in electron impact (EI) mode. The ionisation was turned off during the first 4 min to avoid solvent overloading. The settings on the Ion Trap detector were programmed as follows: transfer line, manifold and trap temperatures at 280, 50 and 180 °C, respectively. The mass ranged from 50 to 600 *m*/*z* with a scan rate of 6 scans/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to the auto tune procedure. The maximum ionisation time was 25,000 μs, with an ionisation storage level of 35 *m*/*z*. The injection volume for liquid extracts was 1 μl, and the analysis was performed in the full-scan mode. The components were identified according to their retention indices relative to C_8–C_{20} *n*-alkanes and mass spectra, which were compared with those of the NIST 05 MS Library Database (Match and R.Match >80%), pure standards analysed under the same conditions and the NIST Chemistry WebBook and with bibliography (Salgueiro et al., 2000; Figueiredo et al., 2001; Baranauskienė et al., 2003; Tzakou and Constantinidis, 2005; Paaver et al., 2008; Hazzit et al., 2009; Jovanovic et al., 2009; Soković et al., 2010).

2.6. Oxygen radical absorbance capacity (ORAC) assay

The total antioxidant capacity was evaluated as described by Gillespie et al. (2007). Fluorescein was used as the fluorescent probe and AAPH as a peroxyl radical generator. A black microplate (NUNC, Rochester, NY, USA) was loaded with 150 μl of fluorescein (0.08 μM) and 25 μl of diluted sample (0.05–10 mg/ml), a Trolox standard (6.25–50 μM) or phosphate buffer (blank), and after a 10 min incubation period at 37 °C, the reaction was initiated by adding 25 μl of AAPH (150 mM) to each well. The fluorescence was measured every minute for 90 min at an excitation wavelength of 485 nm with emission detected at 530 nm, and the decrease in fluorescence was determined. The ORAC value for each SFE extract/essential oil was calculated using the respective area under the curve (AUC) and the regression equation between Trolox equivalents (TE) and the net AUC. The results were expressed as TE per gram of extract.

2.7. Reduction of DPPH radical

The ability of the SFE extracts/essential oil to scavenge DPPH radicals was determined according to the procedure described by Soler-Rivas et al. (2000) with slight modifications. A total of 100 μl of 90 μM DPPH methanolic solution was added to 10 μl of sample solution at different concentrations (0.25–40 mg/ml), and the mixture was diluted with 190 μl of methanol. For the control, the SFE extract/essential oil was replaced with solvent, and for the blank probe control, the methanol (290 μl) and the SFE extract/essential oil (10 μl) were mixed. After 1 h, the reduction of DPPH radicals was measured at an absorbance of 515 nm. Butylated hydroxytoluene (BHT) was used as the reference antioxidant at different concentrations (1–4 mg/ml). The antioxidant activity was expressed as a percentage of inhibition of DPPH radicals reduction. All experiments were performed in triplicate.

2.8. In vitro AChE and BChE inhibition

The AChE and BChE activities were measured using a 96-well microplate reader, according to Ellman's method (Ellman et al., 1961). A total of 125 μl of 3 mM DTNB, 25 μl of 15 mM substrate (ATCI or BTCI), 50 μl of 100 mM phosphate buffer (pH 8.0) and 25 μl of SFE extract/essential oil (0–10 mg/ml) or chemical compounds (linalool, caryophyllene oxide, camphor, 1,8-cineole and α-pinene), buffer or galanthamine (reference compound) (2.5–60 μg/ml) was mixed. The chemical compounds were dissolved in 86% ethanol, and a control mixture was prepared with 86% ethanol. Finally, 25 μl of AChE or BChE (0.28 U/ml) were added and the absorbance was measured at 405 nm for 5 min. The enzymatic activity was calculated as a percentage of the velocities in the reaction mixtures compared with that of the control reactions. The inhibitory activity was calculated by subtracting the percentage of enzyme activity from 100%. The IC_{50} values were given as the means ± standard error (SE) of three individual determinations each performed in triplicate.

2.9. Statistical analysis

The data were presented as the mean ± SE of triplicate experiments. The results were subjected to one-way analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA). Significant differences between mean values were determined using Duncan's New Multiple Range Test except anti-ChE assay (extracts-2nd separator and IC_{50} of terpenoids). The dose–response curves were obtained by plotting the percentage of inhibition versus the concentrations.

Table 1
Extraction yield (%, w/w) achieved by hydrodistillation (HD) and supercritical fluid extraction (SFE).

	Essential oil	Extracts	
		1st separator	2nd separator
HD	0.30 ± 0.02	–	–
SFE 12 MPa	–	2.43 ± 0.14 a	6.02 ± 0.20 b
SFE 18 MPa	–	2.24 ± 0.10 a	7.76 ± 0.56 a

Values are expressed as mean ± SE ($n = 3$). Values followed by different letters within the same column are significantly different at $p < 0.05$.

3. Results and discussion

3.1. Extraction yield

The extraction yield (Table 1) (expressed as weight of extract relative to the weight of the starting plant material) of the oil obtained from the aerial parts of *T. lotocephalus* by HD was 0.30 ± 0.02% (w/w), and the yield of the SFE extracts obtained from the second separator was 6.02 ± 0.20 and 7.76 ± 0.56% (w/w) at extraction pressures of 12 and 18 MPa, respectively. The extraction yield of the extracts obtained in the first separator was lower (2.43 ± 0.14 and 2.24 ± 0.10% (w/w) at 12 and 18 MPa, respectively) (Table 1).

To obtain an efficient supercritical extraction of the volatile compounds, it is necessary to optimise some parameters according the purpose of the research, such as the extraction pressure (Pourmortazavi and Hajimirsadeghi, 2007). In this work, the SFE was conducted at two different pressures (12 or 18 MPa) to assess their influence on the extraction yield. The increase of the extraction pressure had a significant ($p < 0.05$) positive effect on the yield of extracts obtained in the second separator (Table 1). The results also showed that this alternative technique (SFE) generates high extraction yields. These results could be due to the co-extraction of other compounds, namely waxes, during supercritical extraction (Reverchon, 1997).

3.2. GC-FID and GC–IT–MS analysis

The chemical profiles of the essential oil and SFE extracts obtained from *T. lotocephalus* were compared by traditional (HD) and alternative (SFE) techniques (Table 2). Forty-four constituents were identified in the oil extracted by HD, representing 70.49% of their total components, whereas by SFE, at 12 and 18 MPa, 19 and 14 constituents were identified, respectively (47.44% and 40.79% of the total extracts-2nd separator composition, respectively). In the extracts-1st separator, 9 and 10 compounds were identified (11.94% and 12.77%, respectively). The broader range of relative percentages observed for SFE is a consequence of the different pressure conditions (12 versus 18 MPa). The oxygen-containing monoterpenes were the primary constituents in the essential oil and extracts collected in the second separator at the pressures of 12 and 18 MPa, respectively, ranging from 40.30 to 48.39% of the total compounds identified. The extracts obtained in the first separator, at the pressures of 12 and 18 MPa, consisted primarily of the oxygen-containing sesquiterpenes (9.12 and 10.81% of the total identified constituents, respectively). These results confirm that the heaviest compounds were collected in the first separator.

Although the volatiles must be collected in the second separator, there were significant differences between the chemical profiles of the SFE extracts obtained in the second separator and the essential oil (Figs. 1, 2B and 3B, and Table 2), with HD allowing the characterisation of a higher number of compounds. Linalool (10.43 ± 1.63%) was the main component in the essential oil, whereas camphor (7.91 ± 0.84%) and *cis*-linalool oxide (7.25 ± 1.45%) were the major compounds in the extract-2nd

Table 2
Chemical composition (relative %) of the essential oil obtained by hydrodistillation (HD) and of the extracts (first and second separator) obtained by supercritical fluid extraction (SFE) isolated from *T. lotocephalus*.[a]

	Compounds	*m/z*	RI	RI[b]	HD	SFE 12 MPa		SFE 18 MPa	
					essential oil	1st separator	2nd separator	1st separator	2nd separator
1	α-Pinene[c,d,e,f,g,h,i]	51/67/73/79/91/93/105/111/121/129/135	936	934	0.16 ± 0.01	–	–	–	–
2	Camphene[c,d,e,f,g,h,i]	53/67/73/79/93/107/121/136	953	949	0.15 ± 0.01	–	–	–	–
3	2,4(10)-Thujadiene[e]	51/65/77/91/105/119/133	956	954	0.18 ± 0.02	–	–	–	–
4	Sabinene[d,e,f,g,h]	51/69/79/82/93/107/121/136	975	969	0.05 ± 0.01	–	–	–	–
5	β-Pinene[c,d,e,f,g,h]	55/58/67/73/79/87/93/96/109/121/128/136	980	975	0.03 ± 0.00	–	–	–	–
6	1-Octen-3-ol[e,f,h,i]	29/31/43/55/57/63/68/72/81/85/95/99/109/127	992	986	0.82 ± 0.06	–	–	–	–
7	α-Terpinene[c,d,e,f,g,h,i]	53/58/65/74/79/87/91/93/105/121/132/136	1020	1015	0.28 ± 0.03	–	–	–	–
8	*p*-Cymene[d,e,f,g,h,i]	51/65/77/91/103/119/134	1029	1027	0.45 ± 0.04	–	–	–	–
9	1,8-Cineole[c,d,e,f,g,h]	55/67/81/93/108/125/139/148/154	1037	1031	3.82 ± 0.26	–	1.70 ± 0.23	–	1.24 ± 0.98
10	*trans*-β-Ocimene[d,e,f,g,h,i]	51/67/79/93/105/121/136	1050	1052	0.33 ± 0.05	–	–	–	–
11	3-Carene[e]	27/41/53/65/77/91/93/105/121/136	1055	1016	0.02 ± 0.00	–	–	–	–
12	γ-Terpinene[c,d,e,f,g]	51/65/77/93/105/115/121/136	1061	1060	0.48 ± 0.01	–	–	–	–
13	*cis*-Linalool oxide[c,d,e,g]	55/59/67/71/79/93/97/109/119/126/136/143/150/159	1075	1078	1.00 ± 0.06	0.34 ± 0.20	4.47 ± 0.27	0.25 ± 0.20	7.25 ± 1.45
14	*trans*-Linalool oxide[c,d]	55/59/67/79/93/109/119/137/155	1091	1087	1.06 ± 0.29	–	4.23 ± 0.27	–	5.53 ± 0.82
15	Linalool[c,d,e,f,g,h,i]	55/71/81/93/107/121/136/154	1103	1105	10.43 ± 1.63	–	2.35 ± 0.67	–	1.12 ± 0.17
16	α-Campholenal[d,e,f]	53/67/81/93/108/117/133/151	1132	1128	1.11 ± 0.12	0.30 ± 0.18	–	0.21 ± 0.12	–
17	*p*-Mentha-1,3,8-triene[f]	41/51/65/77/91/93/105/117/119/134	1143	1113	0.26 ± 0.06	–	–	–	–
18	*cis*-Sabinol[g]	55/79/92/105/119/134/149	1148	1143	1.41 ± 0.05	–	2.07 ± 0.16	–	–
19	Camphor[c,d,e,f,g,h]	55/67/69/72/81/83/89/91/93/95/108/119/125/137/152	1156	1147	7.97 ± 0.47	1.49 ± 0.29	7.91 ± 0.84	1.19 ± 0.16	6.37 ± 1.59
20	Pinocarvone[d,e]	53/55/67/73/79/81/91/108/122/125/135/150	1170	1165	0.37 ± 0.01	–	–	–	–
21	Borneol[c,d,e,f,g,h,i]	55/59/63/67/71/79/81/95/103/110/115/121/136	1180	1199	5.62 ± 0.06	–	7.50 ± 1.05	–	6.15 ± 0.52
22	Terpinen-4-ol[d,e,f,g,h,i]	55/67/71/81/86/91/93/107/111/115/121/136/147/154	1191	1171	2.20 ± 0.21	–	3.97 ± 0.34	–	2.39 ± 0.41
23	*p*-Cymen-8-ol[e,g]	50/65/77/91/95/105/119/135/149	1193	1185	0.70 ± 0.10	–	2.47 ± 0.11	–	2.86 ± 0.16
24	α-Terpineol[c,d,e,f,g,h,i]	53/59/67/71/81/93/107/121/136	1201	1189	4.46 ± 0.12	–	1.82 ± 0.16	–	1.16 ± 0.43
25	Verbenone[c,d,e,f]	53/67/79/91/95/107/122/135/150	1217	1204	0.78 ± 0.03	0.69 ± 0.40	5.42 ± 1.18	0.30 ± 0.03	5.43 ± 0.85
26	*cis*-Carveol[c]	55/59/67/73/74/84/87/91/105/109/119/137/147	1229	1220	0.49 ± 0.03	–	0.98 ± 0.25	–	0.80 ± 0.46
27	*cis*-Geraniol[c,d,e,g,h]	53/59/69/81/84/93/111/121/131/139/147	1233	1229	0.78 ± 0.25	–	–	–	–
28	Carvone[c,e,f,g]	53/67/79/82/93/108/121/135/150	1250	1246	0.21 ± 0.06	–	–	–	–
29	Bornyl acetate[c,d,e,f,g,h]	55/67/80/95/108/121/136/143/154/191	1294	1287	3.52 ± 0.15	–	1.64 ± 0.10	–	–
30	Geranyl acetate[c,d,e,g]	53/69/81/84/93/107/121/136/149/164/177	1381	1385	2.20 ± 0.57	–	–	–	–
31	*trans*-α-Bergamotene[e,f]	55/67/79/91/105/119/134/151/189	1424	1441	0.35 ± 0.04	–	–	–	–
32	β-Caryophyllene[d,e,f,g,h,i]	55/67/79/91/105/120/133/147/161/175/189/204	1434	1419	0.08 ± 0.08	–	–	–	–
33	γ-Gurjunene[h]	55/67/79/91/105/119/133/147/162/175/189/204	1457	1472	0.21 ± 0.04	–	–	–	–
34	*allo*-Aromadendrene[d,e,f,g,h,i]	55/67/79/91/105/119/133/148/161/175/189/204	1469	1458	0.38 ± 0.05	–	–	–	–
35	Valencene[d]	53/67/73/79/91/107/119/133/147/161/175/189/204	1512	1490	0.33 ± 0.00	–	–	–	–
36	Calamenene[d]	50/63/75/91/105/115/131/144/159/173/185/201	1529	1516	0.35 ± 0.01	–	–	–	–
37	α-Calacorene[f]	51/57/63/69/79/91/107/115/128/142/157/169/179/187/200	1552	1540	0.31 ± 0.04	–	–	–	–
38	Palustrol[e]	55/67/77/91/98/111/122/133/147/161/175/189/200	1575	1567	0.41 ± 0.04	–	–	–	–
39	Spathulenol[e,f,g,h]	55/67/79/91/105/119/131/147/159/179/183/205/217	1594	1578	0.97 ± 0.15	–	0.08 ± 0.05	0.71 ± 0.06	–
40	Caryophyllene oxide[c,d,e,f,g,h,i]	53/69/81/95/107/119/131/147/161/177/203	1600	1592	8.71 ± 1.56	4.34 ± 0.51	0.19 ± 0.04	4.41 ± 1.25	0.07 ± 0.03
41	Globulol[d,e]	55/67/71/79/93/105/121/135/147/161/175/189/204/222	1605	1585	3.70 ± 0.53	2.21 ± 0.21	0.06 ± 0.02	2.32 ± 0.44	0.08 ± 0.10
42	Viridiflorol[d,f,g]	55/67/81/91/107/121/133/147/161/175/189/204/222	1615	1589	2.69 ± 0.39	1.66 ± 0.13	0.06 ± 0.02	1.69 ± 0.31	0.34 ± 0.16
43	Cubenol[f]	55/67/81/95/105/119/135/147/161/179/189/204	1624	1642	0.47 ± 0.01	0.49 ± 0.06	0.24 ± 0.06	0.51 ± 0.06	–
44	Juniper camphor[i]	55/58/67/71/81/85/95/105/125/147/161/175/189/204/221	1635	1690	0.18 ± 0.02	0.41 ± 0.05	0.26 ± 0.17	1.18 ± 0.12	–

Table 2 (*Continued*)

Compounds *m/z*	RI	RI[b]	HD essential oil	SFE 12 MPa		SFE 18 MPa	
				1st separator	2nd separator	1st separator	2nd separator
Identified compounds (%)			70.49	11.94	47.44	12.77	40.79
Monoterpene hydrocarbons			2.13	0.00	0.00	0.00	0.00
Oxygen-containing monoterpenes			48.39	2.82	46.54	1.95	40.30
Sesquiterpene hydrocarbons			2.01	0.00	0.00	0.00	0.00
Oxygen-containing sesquiterpenes			17.14	9.12	0.90	10.81	0.49
Others			0.83	0.22	0.00	0.03	0.00

[a] Results are expressed as mean ± standard deviation of three injections.
[b] Retention indices from NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/name-ser.html).
[c] Compounds identified by comparison with authentic standard and with NIST 05 library.
[d] Compounds tentatively identified by comparison with NIST 05 library and Figueiredo et al. (2001).
[e] Compounds tentatively identified by comparison with NIST 05 library and Hazzit et al. (2009).
[f] Compounds tentatively identified by comparison with NIST 05 library and Tzakou and Constantinidis (2005).
[g] Compounds tentatively identified by comparison with NIST 05 library and Paaver et al. (2008).
[h] Compounds tentatively identified by comparison with NIST 05 library and Baranauskienė et al. (2003).
[i] Compounds tentatively identified by comparison with NIST 05 library and Jovanovic et al. (2009).

separator extracted at pressures of 12 and 18 MPa, respectively. Linalool is the main constituent in thyme oils, which have great importance for the flavour and perfume industries (Figueiredo et al., 1993). Furthermore, linalool possesses several biological and pharmacological properties, including antimicrobial (Vila et al., 2010) and antioxidant activities (Çelik and Özkaya, 2002) and central nervous system depressant actions (Silva Brum et al., 2001). Recent studies indicate that camphor also possesses antimicrobial and antioxidant activities (Ortet et al., 2010; Zaouali et al., 2010).

The chemical profiles of the SFE extracts (first separator) obtained at different pressures were similar, with caryophyllene oxide as the main constituent (4.34 ± 0.51 and $4.41 \pm 1.25\%$ at 12 and 18 MPa, respectively). Upon analysis of the GC-FID chromatograms of the SFE extracts (first separator) (Figs. 2A and 3A), it is clear that a considerable number of compounds were not identified. Reverchon (1997) reported that at the highest extraction densities ($T = 40–50\,^{\circ}C$ and $p = 10–20$ MPa), large quantities of paraffins and steroptens are co-extracted with the SFE products, which affects the results. The large chemical polymorphism of *T. lotocephalus* essential oil makes it difficult to obtain a general characterisation of its composition (Figueiredo et al., 2008). Several studies have demonstrated the influence of the collection sites, sample types and developmental stage on the chemical composition of *T. lotocephalus* essential oils. Salgueiro (1992) reported a large amount of 1,8-cineole, camphor, linalool, linalyl acetate and α-pinene in the essential oil of *T. lotocephalus* collected in the Algarve region. Figueiredo et al. (1993) identified the oil constituents isolated from flowers and leaves. The composition of the oil from flowers was dominated by linalyl acetate, while 1,8-cineole was the major compound in the oil from leaves. In addition, Salgueiro et al. (2000) also provided relevant information about four populations of *T. lotocephalus*. In this study, linalool, geranyl acetate and 1,8-cineole were the major constituents identified. These results showed some important differences between populations from relatively close localities. Figueiredo et al. (2001) reported the composition of essential oils collected during both vegetative and flowering phases in the Algarve (South Portugal). The oils isolated from the aerial parts (leaves and flowers) were dominated by oxygen-containing monoterpenes, and the main compounds identified were 1,8-cineole, linalyl acetate and linalool.

3.3. Antioxidant activity

The antioxidant capacity of plant material is strongly influenced not only by chemical composition but also by the test conditions. In this research, the antioxidant activity was assessed by both ORAC and DPPH assays to account for various mechanisms of action based on hydrogen electron transfer (HAT) and on single electron transfer (SET), respectively (Prior et al., 2005). The ORAC assay measures the fluorescence loss of a probe (fluorescein) in the presence or absence of an antioxidant, and the DPPH assay is based on a colour change produced by the reduction of the DPPH radicals, which is determined by measuring the absorbance at 515 nm (Prior et al., 2005; Gelmez et al., 2009). The radical scavenging effects of the essential oil and SFE extracts were compared (Table 3). The essential oil showed the highest value of antioxidant activity determined by the ORAC assay, followed by the extracts-1st separator. In contrast with the extracts obtained in the second separator, the ORAC values exhibited by the extracts-1st separator increased with the increment of pressure.

The free radical scavenging of *T. lotocephalus* in comparison with synthetic BHT ($IC_{50} = 462.24 \pm 0.03$ μg/ml) was assessed using the DPPH assay. The essential oil and extracts-1st separator were the most effective in terms of radical scavenging capacity (Table 3).

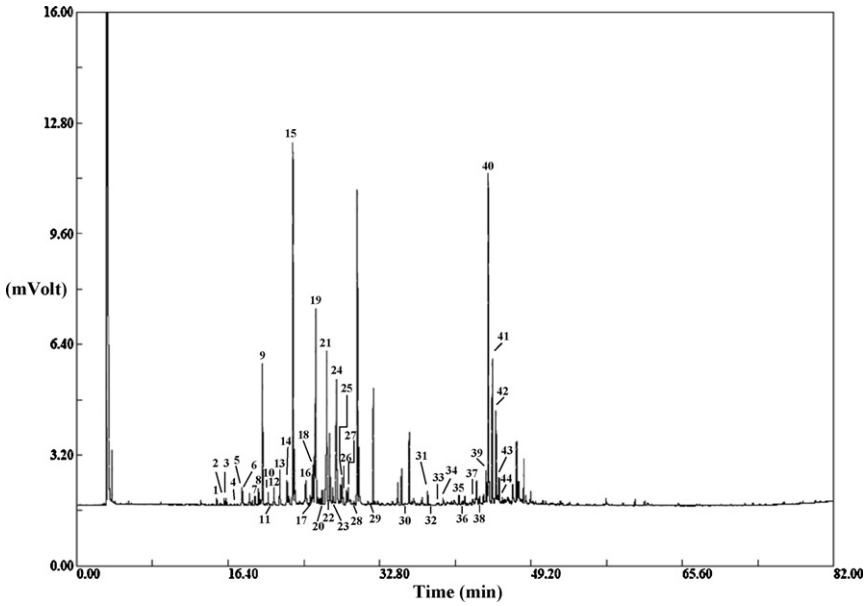

Fig. 1. GC-FID chromatogram of the essential oil obtained from *T. lotocephalus* by hydrodistillation. The identities of the compounds are listed in Table 2.

Furthermore, the results also revealed that the increment of pressure did not affect the radical scavenging properties of the extracts.

The antioxidant activity of the *Thymus* species has been reported in the literature (Mata et al., 2007; Figueiredo et al., 2008; Viuda-Martos et al., 2010; Dandlen et al., 2010). This activity could be attributed to the high content of monoterpenes present in the essential oil, which are known for their increased levels of antioxidant activity (El-Massry et al., 2002; Edziri et al., 2010). Among monoterpenes, previous studies have shown that linalool and camphor possess radical scavenging abilities (Çelik and Özkaya, 2002; Zaouali et al., 2010).

The antioxidant activity of the SFE extracts (first separator) could be attributed to the scavenging properties of caryophyllene oxide (Kaurinovic et al., 2010). Moreover, the high number of unidentified compounds in these extracts suggests that other constituents could contribute to the antioxidant capacity. The results obtained in this study revealed a considerable influence of the extraction technique on the antioxidant activity. To extract more active compounds using the SFE technique, it will be necessary to optimise the operating conditions. It is well established that the purpose of the study determines the selection of the extraction conditions. Reverchon and de Marco (2006) reported that the extraction of volatile components must be performed in the range of 40–50 °C and 8–12 MPa to avoid the thermal degradation and co-extraction of undesired compounds. Different research groups have assessed the composition of the volatiles of the *Thymus* species. Moldão-Martins et al. (2000) obtained extracts of *Thymus zygis* by SFE at 27 and 40 °C and 8 and 15 MPa; Díaz-Maroto et al. (2005) extracted the volatile oils from *T. vulgaris* leaves by SFE at 40 °C and 12 MPa; and Grosso et al. (2010) studied the composition of *T. vulgaris* volatiles at different temperatures (40 and 50 °C) and pressures (9 and 10 MPa). Moreover, other conditions are necessary to isolate antioxidant fractions. Nguyen et al. (1991) observed that temperatures ranging from 90 to 100 °C were the most suitable for the isolation of the antioxidant fraction from Lamiaceae species. In a study by Babovic et al. (2010), the antioxidant fractions of thyme were extracted at 100 °C and 35 MPa. Further, the different antioxidant activities exhibited by the *Thymus* species could also be justified by other factors, such as the different geographic locations, climate, different plant parts studied and the degree of plant maturation (Figueiredo et al., 2001, 2008).

3.4. *In vitro AChE and BChE inhibition assay*

After impulse transmission, the neurotransmitter ACh is degraded by the hydrolytic activity of ChE. AChE is the most prominent enzyme involved in this hydrolysis, and evidence suggests that BChE can also participate in ACh hydrolysis, thus contributing to the enhancement of cholinergic function (Mesulam et al., 2002). The SFE extracts and oils from *T. lotocephalus* were also tested to determine their possible capacity as AChE and BChE inhibitors, and the results are depicted in Table 4. All of the samples tested affected AChE and BChE activities. Nevertheless, the inhibitory effect produced by the essential oil and extracts-1st separator were clearly more pronounced than that of the extracts-2nd separator, which did not reach 50% inhibition of either enzymes at the highest concentration tested (2.5 mg/ml). The essential oil was significantly ($p < 0.05$) more active against BChE than against AChE. The

Table 3
Antioxidant activity of *T. lotocephalus* assessed by oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays.

	Samples	Pressure (MPa)	ORAC ($\mu mol_{TE}/g_{extract}$)	DPPH (% inhibition)[a,*]
Extracts	1st separator	12	84.45 ± 8.07 c	32.43 ± 0.68 a
		18	116.36 ± 4.55 b	34.48 ± 0.91 a
	2nd separator	12	3.90 ± 0.25 d	7.57 ± 2.82 b
		18	2.24 ± 0.05 d	7.51 ± 3.19 b
Essential oil		–	328.47 ± 10.85 a	32.08 ± 1.56 a

Values are expressed as mean ± SE ($n = 3$).
[a] Percent of inhibition at the highest concentration tested (12 mg/ml). Values followed by different letters within the same column are significantly different at $p < 0.05$.
[*] Antioxidant reference (BHT), $IC_{50} = 462.24 \pm 0.03$ μg/ml.

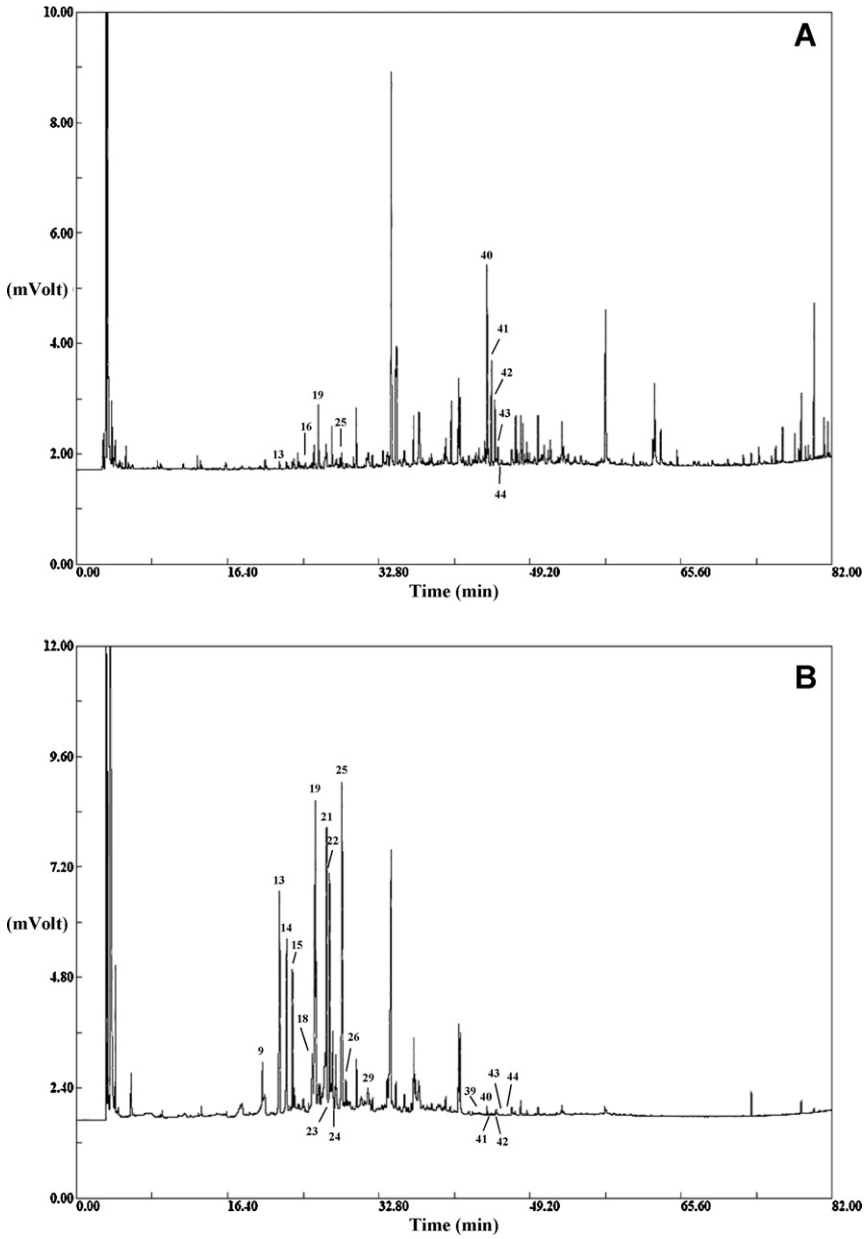

Fig. 2. GC-FID chromatogram of the 1st separator (A) and 2nd separator (B) obtained from *T. lotocephalus* by SFE at 40 °C and 12 MPa. The identities of the compounds are listed in Table 2.

extraction pressure influenced the bioactivity of the SFE extract (first separator) isolated at the lowest pressure, which was the most active in inhibiting AChE (IC_{50} = 1.54 ± 0.04 mg/ml). Caryophyllene oxide, the main constituent identified in this extract, could be responsible for the inhibition of ChEs (Savelev et al., 2003). When comparing both enzymes, the activity of BChE was more affected by the SFE extracts/essential oil than with the reference drug galanthamine, which was more active against AChE. This is not surprising

Table 4
Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition activities, expressed by IC_{50} values, of the essential oil and SFE extracts from *T. lotocephalus* (mg/ml) and galanthamine (μg/ml).

	Samples	Pressure (MPa)	IC_{50} values[a]	
			AChE	BChE
Extracts	1st separator	12	1.54 ± 0.04 b*	0.14 ± 0.02 b
		18	4.27 ± 0.12 a*	0.31 ± 0.03 ab
	2nd separator	12	ND	ND
		18	ND	ND
Essential oil		–	0.90 ± 0.04 c*	0.50 ± 0.12 a
Galanthamine		–	2.20 ± 0.04	11.72 ± 1.09 *

Values are expressed as mean ± SE (n = 3).

[a] Concentration required to inhibit the hydrolysis of the substrate, by 50%. ND, not determined, means the maximum level of inhibition below 50% (2.5 mg/ml). Values followed by different letters within the same column are significantly different at $p < 0.05$. Values marked with * indicate means significantly higher at $p < 0.05$.

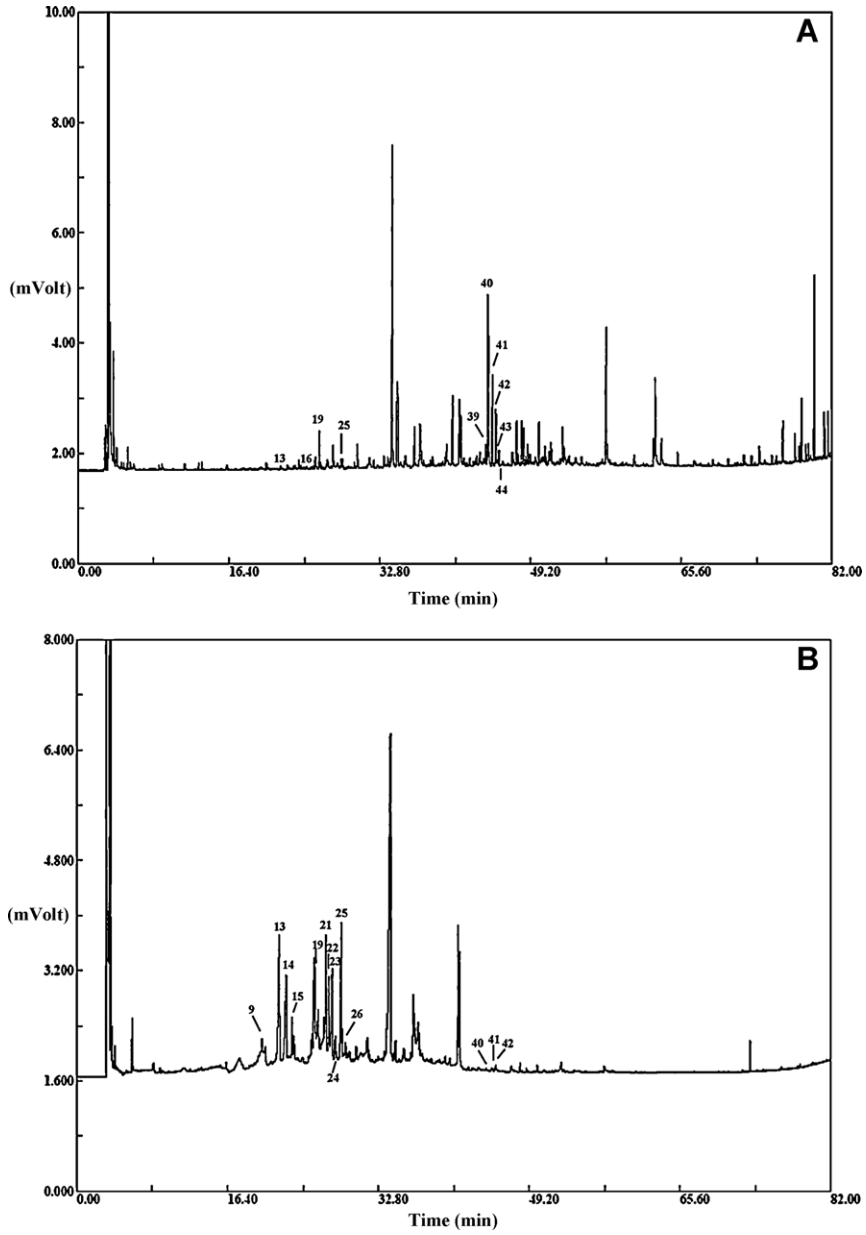

Fig. 3. GC-FID chromatogram of the 1st separator (A) and 2nd separator (B) obtained from *T. lotocephalus* by SFE at 40 °C and 18 MPa. The identities of the compounds are listed in Table 2.

because galanthamine is a selective inhibitor of AChE used in the treatment of AD (Bores and Kosley, 1996).

There are few reports about the anti-ChE activity of the *Thymus* species. Some authors have reported the anti-AChE activity of the essential oils from *T. serpyllum* and *Thymus praecox* subsp. *caucasicus* var. *caucasicus* (Mata et al., 2007; Orhan et al., 2009). *T. vulgaris* also exhibited anti-ChE activity, and the main active phytochemicals were oxygenated compounds (Jukic et al., 2007). Conversely, Sigurdsson and Gudbjarnason (2007) demonstrated that the ethanolic extract from *T. praecox* did not possess an AChE inhibitory effect.

In this study, we also intended to elucidate which compounds in the essential oil were responsible for the anti-ChE activity and, more specifically, to clarify their individual contribution to the essential oil activity. Essential oils have gained importance due to their AChE inhibitory activity, attributed mainly to terpenoids (Savelev et al., 2003; Miyazawa and Yamafuji, 2005, 2006; Dohi et al., 2009). The main constituents in the essential oil used for the analysis of ChEs inhibition were linalool (10.43 ± 1.63%), caryophyllene oxide (8.71 ± 1.56%), camphor (7.97 ± 0.47%), 1,8-cineole (3.82 ± 0.26%) and α-pinene (0.16 ± 0.01%) (Table 2). Fig. 4 shows the dose–response curves used to predict the IC_{50} values of 1,8-cineole, α-pinene and caryophyllene oxide. As expected, 1,8-cineole and α-pinene were the most potent against AChE (Savelev et al., 2003; Dohi et al., 2009), and caryophyllene oxide was the most active against BChE (Table 5). The remaining compounds were less potent and did not reach 50% enzyme inhibition at the highest concentration tested (2.5 mg/ml) (Table 5).

Table 6 shows the contribution of some terpenoids to AChE and BChE inhibitory activity, which was determined by their naturally occurring concentrations in the *T. lotocephalus* essential oil. The AChE inhibitory activity exhibited by the essential oil seems to be primarily due to 1,8-cineole. Although it was not the major component present in the oil, this terpene accounted for approximately 20% of the half inhibition demonstrated by the whole oil, whereas the remaining compounds showed moderate

Table 5
Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory effect of terpenoids present in the *T. lotocephalus* essential oil.

	IC_{50} (mg/ml)[a]		Inhibition (%)[b]	
Compound	AChE	BChE	AChE	BChE
Linalool	–	–	12.47 ± 1.63 b	30.64 ± 1.99 a
Caryophyllene oxide	–	0.42 ± 0.05	25.81 ± 1.83 a	–
Camphor	–	–	14.96 ± 1.07 b	20.34 ± 1.25 b
1,8-Cineole	0.28 ± 0.02 b	–	–	24.70 ± 1.71 b
α-Pinene	0.45 ± 0.01 a	–	–	31.30 ± 1.92 a

Values are expressed as mean ± SE ($n = 3$).
[a] Concentration of terpenoids responsible for 50% enzyme inhibition calculated from the dose–response curve equations.
[b] Percent inhibition obtained at final concentration of 1.25 mg/ml. Values followed by different letters within the same column are significantly different at $p < 0.05$.

Table 6
Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory effect of terpenoids based on the concentrations present in the *T. lotocephalus* essential oil.

Compound	Percentage in oil	AChE		BChE	
		mg/ml[a]	Inhibition (%)	mg/ml	Inhibition (%)
Linalool	10	0.09	2.21 ± 0.71 b	0.05	5.78 ± 0.97 bc
Caryophyllene oxide	9	0.08	4.32 ± 1.72 b	0.04	16.57 ± 0.94 a
Camphor	8	0.07	6.00 ± 0.53 b	0.04	6.61 ± 1.15 bc
1,8-Cineole	4	0.03	19.82 ± 0.74 a	0.02	4.86 ± 1.32 c
α-Pinene	0.2	1.44×10^{-3}	5.40 ± 1.46 b	7.92×10^{-3}	8.84 ± 0.93 b

[a] It corresponds to the concentration that individual compounds represent in the essential oil composition, estimated from the IC_{50} values of the whole oil displayed in Table 4. IC_{50} values of the essential oil were taken as 100% for the convenience of calculations. Values are expressed as mean ± SE ($n = 3$). Values followed by different letters within the same column are significantly different at $p < 0.05$.

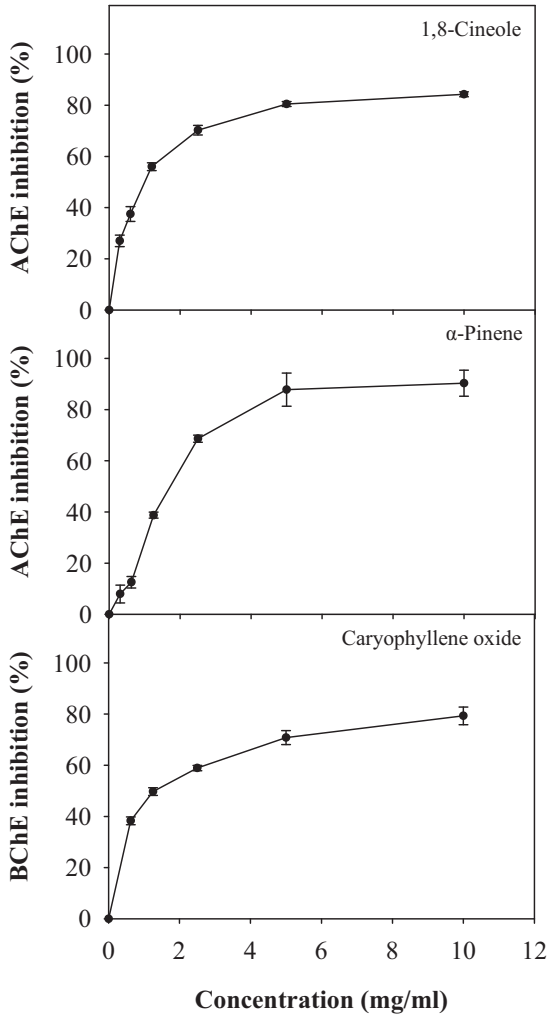

Fig. 4. The dose–response curves used to calculate the IC_{50} values of 1,8-cineole, α-pinene and caryophyllene oxide, respectively. Each point represents the mean of the values obtained from three independent experiments performed in triplicate.

contributions. In contrast, 1,8-cineole represents only about 5% of the observed BChE inhibitory effect. These results are consistent with the studies showing that 1,8-cineole is not a selective inhibitor for BChE (Yu et al., 1999; Savelev et al., 2003). Caryophyllene oxide is the second major compound present in the essential oil of *T. lotocephalus*, and the most active against BChE, contributing to approximately 17% of the half inhibition displayed by the whole oil. Camphor does not significantly contribute to the anti-ChE activity but, interestingly, α-pinene, representing only 0.2% of the essential oil, showed an acceptable inhibition of AChE. The chemical analysis of the essential oil from *T. lotocephalus* revealed that linalool was the major constituent; however, this compound showed a weak contribution to the biological effect (2.21 ± 0.71 and 5.78 ± 0.97% to AChE and BChE inhibition, respectively). These results prove that although there is a tendency to attribute the biological effect to the main constituents, the potential role of minor compounds must be considered (Nakatsu et al., 2000). Thus, other compounds present in the oil could also contribute to the anti-ChE activity, and therefore, the activity exhibited might result from the collective activities of different constituents (Dohi et al., 2009). Furthermore, synergistic and antagonistic interactions between some terpenoids have been demonstrated. 1,8-Cineole and α-pinene exhibited slight synergistic interactions, whereas 1,8-cineole and camphor show antagonism. Borneol and caryophyllene oxide, also present in *T. lotocephalus* oil, could be AChE inhibitors. Moreover, synergism was observed between caryophyllene oxide and 1,8-cineole (Savelev et al., 2003). Therefore, interactions among compounds are not to be excluded.

4. Conclusions

The results show that a higher number of compounds were identified by hydrodistillation, and in contrast, the highest extraction yields were obtained through SFE. The chromatographic analysis indicated that the chemical compositions of the essential oil and extracts-2nd separator differ qualitatively and quantitatively. Overall, the chemical profile was not influenced by the extraction pressure used in this research, and in contrast with the extracts obtained in the 2nd separator, the pressure did not affect the

extraction yield of extracts obtained in the 1st separator. *T. lotocephalus* essential oil and SFE extracts showed acceptable antioxidant activity, acting preferentially via a hydrogen atom transfer mechanism. Concerning anti-ChE activity, the essential oil and extracts-1st separator were the most active, mainly against the BChE enzyme, which is supported by their chemical profiles. It was shown that the AChE and BChE inhibitory activities exhibited by the essential oil are primarily due to 1,8-cineole and caryophyllene oxide, respectively. Overall, the essential oil obtained by HD was the most active. This research suggests *T. lotocephalus* as a source of bioactive compounds with different targets and pathways.

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Supercritical fluid extraction and hydrodistillation for the recovery of bioactive compounds from *Lavandula viridis* L'Hér

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ABSTRACT

The chemical profiles of bioactive essential oil and extracts obtained by hydrodistillation (HD) and supercritical fluid extraction (SFE), respectively, from *Lavandula viridis* were compared. The SFE was performed at 40 °C and at extraction pressures of 12 or 18 MPa in two different separators. Evaluation of the essential oil and SFE extracts by GC–FID and GC–IT–MS revealed that oxygen-containing monoterpenes were the major constituents in both cases, but there were important differences between the chemical profiles produced by the different extraction techniques. More compounds were isolated by HD but higher yields were achieved by SFE. Camphor was the main component identified in the essential oil (31.59 ± 1.32%), and in extracts from the first (1.61 ± 0.34%) and second SFE separators (22.48 ± 1.49%) at 12 MPa. In contrast, the first separator SFE extract at 18 MPa (heavy compounds) was dominated by myrtenol (5.38 ± 2.04%) and camphor (4.81 ± 1.93%), whereas the second separator SFE extract (volatiles) was dominated by verbenone (13.97 ± 5.27%). The essential oil and heavy compound extracts from the first separator possessed antioxidant and anti-cholinesterase activities. Our data show that phytochemicals from the aerial parts of *L. viridis* could be developed as natural antioxidant and anti-cholinesterase drugs, with particular applications in the symptomatic treatment of Alzheimer's disease.

1. Introduction

Lavandula spp. (family Lamiaceae) are valued as medicinal plants because of the economic importance of their health-promoting essential oils (Büyükokuroğlu, Gepdiremen, Hacimüftüoğlu, & Oktay, 2003), which are strictly regulated under international standards (ISO TC 54-ISO/CD 8902, 2007; ISO TC 54 N-ISO/WD 4719, 2009). In the food industry, *Lavandula* essential oils are used in beverages, confectionary and ice cream, and in the fragrance industry they are used in perfumes, skin lotions, soaps and other cosmetics, as well as more recently in aromatherapy products (Boelens, 1995; Kim & Lee, 2002). Several *Lavandula* essential oils have antimicrobial properties and/or can inhibit cholinesterases (Dohi, Terasaki, & Makino, 2009; Hanamanthagouda et al., 2010).

Lavandula viridis L'Hér, an aromatic species traditionally used in Portuguese folk medicine, is endemic to the South–West Iberian Peninsula (Franco, 1971). Phytochemical studies of its essential oils have revealed the presence of antioxidants (Gonçalves et al., 2008; Matos et al., 2009; Nogueira & Romano, 2002; Zuzarte et al., 2011) and antifungal compounds (Zuzarte et al., 2011). Recently, *L. viridis* methanol extracts have been shown to contain antioxidants and to inhibit Fe^{2+}-induced lipid peroxidation and cholinesterase activity (Costa, Gonçalves, Andrade, Valentão, & Romano, 2011).

Plant-derived drugs that inhibit cholinesterase can be used for the symptomatic treatment of Alzheimer's disease (AD) because acetylcholine (ACh) levels in the brains of AD patients are often lower than normal (Gomes, Campos, Órfão, & Ribeiro, 2009; Wilkinson, Francis, Schwam, & Payne-Parrish, 2004). Oxidative stress is also involved in AD pathogenesis, so plant-derived antioxidants are useful for the treatment of AD because they can help to remove free radicals and prevent oxidative damage (Valko et al., 2007).

Abbreviations: AAPH, 2,2'-azobis(2-methylpropionamidine) dihydrochloride; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; ATCI, acetylthiocholine iodide; BChE, butyrylcholinesterase; BTCI, butyrylthiocholine chloride; ChE, cholinesterase; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; BHT, butylated hydroxytoluene; SET, single electron transfer; GC–FID, gas chromatography–flame ionisation detector; GC–IT–MS, gas chromatography–ion trap–mass spectrometry; HAT, hydrogen atom transfer; HD, hydrodistillation; ORAC, oxygen radical absorbance capacity; SFE, supercritical fluid extraction; TE, trolox equivalents; TLC, thin layer chromatography; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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Hydrodistillation (HD) is a popular approach for the recovery of bioactive compounds from plants, although it does not prevent hydrolysis reactions that can degrade thermolabile compounds. An alternative is supercritical fluid extraction (SFE) using CO_2 as the supercritical fluid, which produces solvent-free extracts at low temperatures thus avoiding the degradation of active compounds (Reverchon & de Marco, 2006). CO_2 is widely used because it is non-toxic, chemically stable, environmentally acceptable and easily separated from the extract since it exists as a gas under normal atmospheric conditions (Al-Marzouqi, Rao, & Jobe, 2007; Herrero, Mendiola, Cifuentes, & Ibáñez, 2010). SFE is an important process in the food, pharmaceutical and cosmetic industries and provides many benefits for the recovery of natural products because it maintains the original composition of volatile compounds (Pereira & Meireles, 2009; Reverchon & de Marco, 2006).

It was aimed to find the chemical profiles of *L. viridis* essential oil obtained by HD and extracts obtained by SFE under different conditions using gas chromatography (GC–FID and GC–IT–MS). The influence of the extraction technique and SFE conditions (extraction pressure and separator type) on the antioxidant and anti-cholinesterase activities of each extract was also assessed. The false-positive effects in acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition assays were evaluated.

2. Materials and methods

2.1. Standards and reagents

Standards, alkane standard solution and butyrylthiocholine chloride (BTCI), were acquired from Fluka (Steinheim, Germany). Fluorescein, ethanol absolute and ethyl acetate were obtained from Panreac (Barcelona, Spain). Acetylthiocholine iodide (ATCI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), acetylcholinesterase (AChE) (Electric-eel, EC 3.1.1.7, Type VI-S), butyrylcholinesterase (BChE) (horse-serum, EC 3.1.1.8), galanthamine hydrobromide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol and toluene ($\geqslant$99.5%) were purchased from Sigma–Aldrich (Steinheim, Germany). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and butylated hydroxytoluene (BHT) were purchased from Acros Organics (Geel, Germany). Carbon dioxide (CO_2) was obtained from Air Liquide (Lisbon, Portugal). Silica gel 60 plates were obtained from Merck (Darmstadt, Germany).

2.2. Plant material

The aerial parts of *L. viridis* plants were collected during flowering at São Bartolomeu de Messines (Algarve, Portugal). A voucher specimen was deposited in the herbarium of the Botanical Garden of the University of Lisbon, as referenced by Nogueira and Romano (2002). The plant material was dried at room temperature, ground to powder in a blender (mean particle size <2 mm) and stored at −20 °C until required.

2.3. HD and SFE extraction

Essential oil was obtained by HD for 3 h, using 50 g of plant material in a Clevenger-type apparatus (Rao, Kaul, Syamasundar, & Ramesh, 2005). SFE extracts were obtained from 10 g of plant material using an extraction apparatus with a fixed tubular extractor 9.4 cm in height and 2.13 cm internal diameter, as described by Esquível and Bernardo-Gil (1993). Briefly, CO_2 N45 grade (99.5% w/w pure) was delivered at a flow rate of 0.3 kg/h from a standard cylinder, and was compressed to an extraction pressure of 12 or 18 MPa using an air-driven liquid pump after cooling. The CO_2 was delivered to the extraction cell via a heat exchanger to maintain the extraction temperature at 40 °C. Under each pressure/temperature extraction condition (12 MPa/40 °C and 18 MPa/40 °C), two separators were used in order to obtain two different extracts: heavy components in the first separator and volatiles in the second separator.

2.4. GC–FID analysis

Quantitative analysis of the essential oil and SFE extracts was carried out using a Finnigan Focus GC (Thermo Electron Corporation) equipped with a flame ionisation detector (FID) and a VF-5 ms (30 m × 0.25 mm × 0.25 μm) column (VARIAN). The oven temperature was set at 40 °C for 2 min, increasing by 3 °C/min to 250 °C and then 5 °C/min to 300 °C. The injector and detector temperatures were 300 and 310 °C, respectively. Helium C-60 (Gasin, Portugal) was used as a carrier gas at a constant flow rate of 1.5 ml/min, and 1 μl samples were injected using a split ratio of 1:20. The percentage composition of the products was calculated by normalisation of the GC peak areas without response factors.

2.5. GC–IT–MS analysis

The essential oil and SFE extracts were analysed using a Varian CP-3800 gas chromatograph (USA) equipped with a VARIAN Saturn 4000 mass selective detector (USA), a VF-5 ms (30 m × 0.25 mm × 0.25 μm) column (VARIAN) and Saturn GC/MS workstation software version 6.8. The injector port was heated to 300 °C and the samples were injected using a split ratio of 1:40, with helium C-60 as the carrier gas at a constant flow rate of 1.5 ml/min. The oven temperature was set at 40 °C for 2 min, increasing by 3 °C/min to 250 °C and then 5 °C/min to 300 °C. All mass spectra were acquired in electron impact (EI) mode. Ionisation was turned off for the first 4 min to avoid solvent overloading. The settings on the ion trap detector were programmed as follows: transfer line, manifold and trap temperatures at 280, 50 and 180 °C, respectively. The mass ranged from 50 to 600 m/z with a scan rate of six scans per second. The emission current was 50 μA, and the electron multiplier was set in relative mode to the auto tune procedure. The maximum ionisation time was 25,000 μs, with an ionisation storage level of 35 m/z. The injection volume was 1 μl, and the analysis was performed in the full-scan mode. The components were identified according to their retention indices relative to C_8–C_{20} *n*-alkanes and mass spectra, which were compared with those of the NIST 05 MS Library Database (Match and R. Match >80%), pure standards analysed under the same conditions and the NIST Chemistry WebBook and with bibliography (Bousmaha, Boti, Bekkara, Castola, & Casanova, 2006; Giray et al., 2008; Gonçalves et al., 2008; Gören et al., 2002; Hassiotis, Tarantilis, Daferera, & Polissiou, 2010; Imelouane, Elbachiri, Wathelet, Dubois, & Amhamdi, 2010; Nogueira & Romano, 2002; Palá-Paúl, Brophy, Goldsack, & Fontaniella, 2004; Soković, Glamočlija, Marin, Brkić, & van Griensven, 2010).

2.6. Oxygen radical absorbance capacity (ORAC) assay

The total antioxidant capacity was evaluated as described by Gillespie, Chae, and Ainsworth (2007). Fluorescein was used as the fluorescent probe and AAPH as a peroxyl radical generator. A black microplate (NUNC, Rochester, New York, USA) was loaded with 150 μl of fluorescein (0.08 μM) and 25 μl of diluted sample (0.05–10 mg/ml), a Trolox standard (6.25–50 μM) or phosphate buffer (blank). After a 10 min incubation period at 37 °C, the reaction was initiated by adding 25 μl AAPH (150 mM) to each well. The fluorescence was measured every minute for 90 min at an excitation wavelength of 485 nm with emission detected at

530 nm, and the decrease in fluorescence was determined with an Infinite 200 microplate reader (Tekan, Grödig, Austria). The ORAC value for each extract was calculated using the respective area under the curve (AUC) and the regression equation between Trolox equivalents (TE) and the net AUC. The results were expressed as TE per gramme of extract. All experiments were performed in triplicate.

2.7. Reduction of DPPH radical

The ability of the SFE extracts/essential oil to scavenge DPPH radicals was determined according to the procedure described by Soler-Rivas, Espín, and Wichers (2000) with slight modifications. 100 μl of 90 μM DPPH methanolic solution was added to 10 μl of sample solution at different concentrations (0.25–40 mg/ml), and the mixture was diluted with 190 μl of methanol in a clear 96-well microplate (NUNC, Rochester, New York, USA). The SFE extracts/essential oil was replaced with solvent for the control, and the methanol (290 μl) and the SFE extract/essential oil (10 μl) were mixed to create a blank probe control. After 1 h, the reduction of DPPH radicals was measured at an absorbance of 515 nm. Butylated hydroxytoluene (BHT) was used as the reference antioxidant at different concentrations (1–4 mg/ml). The antioxidant activity was expressed as a percentage of DPPH reduction. All experiments were performed in triplicate.

2.8. In vitro AChE and BChE inhibition

AChE and BChE activities were measured using a 96-well microplate reader, according to Ellman, Courtney, Andres, and Featherstone (1961). The following were mixed: 125 μl of 3 mM DTNB, 25 μl of 15 mM the substrate ATCI or BTCI, 50 μl of 100 mM phosphate buffer (pH 8.0) and 25 μl of SFE extract/essential oil (0–10 mg/ml) or chemical standards (camphor, 1,8-cineole and α-pinene), buffer or the reference compound galanthamine (2.5–60 μg/ml). These chemical compounds were dissolved in 86% ethanol and a control mixture was prepared with 86% ethanol alone. Finally, 25 μl 0.28 U/ml AChE or BChE was added and measured the absorbance at 405 nm for 5 min. The enzymatic activity was calculated as a percentage of the velocities in the reaction mixtures compared with that of the control reactions. The IC_{50} values were presented as the means ± standard error (SE) of three individual determinations each performed in triplicate.

2.9. Qualitative determination of false-positive effects in AChE and BChE inhibition assay

AChE and BChE inhibition assays were carried out by thin layer chromatography (TLC) as described by Rhee, van Rijn, and Verpoorte (2003) with slight modifications. A Silica gel 60 plate (stationary phase) was loaded with 5 μl of each sample (40 mg/ml dissolved in 86% ethanol) or the reference galanthamine (0.5 mg/ml in 100 mM phosphate buffer, pH 8.0) and the plate was developed in toluene–ethyl acetate (93:7). The plate was dried at room temperature and sprayed with 3 mM DTNB and 15 mM ATCI or BTCI until saturation. After drying, at room temperature, the plate was sprayed with 3 U/ml AChE or BChE resulting in the development of a yellow background with white bands indicating AChE or BChE-inhibiting compounds. False-positive effect reactions were carried out to verify whether the positive results were due to enzymatic or chemical inhibition between DTNB and the product from the enzyme-substrate reaction (Rhee et al., 2003). The plate was sprayed with 3 mM DTNB followed by the enzyme-substrate mixture (at the same concentrations as before) previously incubated for 15 min at 37 °C. The appearance of white bands on the yellow background indicated a false-positive effect. These results were compared with those obtained with the conventional TLC assay.

2.10. Statistical analysis

The data were presented as the mean ± standard error from triplicate experiments. The results were processed by one-way analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA). Significant differences between mean values were determined using Duncan's New Multiple Range Test, except the anti-cholinesterase activity in the extracts from the second separator.

3. Results and discussion

3.1. Extraction yield

Table 1 shows the yields of *L. viridis* essential oil and SFE extracts, expressed as the weight of extract relative to the weight of the starting plant material. The highest extraction yields corresponded to SFE extracts collected in the second separator (9.27 ± 0.47 and 8.80 ± 0.69% at extraction pressures of 12 and 18 MPa, respectively). The yields of SFE extracts from the first separator were 3.45 ± 0.27 and 3.41 ± 0.57% at 12 and 18 MPa, respectively. The increase in extraction pressure from 12 to 18 MPa therefore did not affect the yield of the SFE extracts. The lowest extraction yield was obtained by HD (0.53 ± 0.04%).

3.2. GC–FID and GC–IT–MS analysis

The qualitative and quantitative chemical profiles of the *L. viridis* essential oil and the two SFE extracts are shown in Figs. 1–3, and are summarised in Table 2. There were considerable differences among the chemical profiles generated by the traditional and alternative techniques. Oxygen-containing monoterpenes were the predominant compounds in all three experiments. We identified 32 compounds in the essential oil derived by HD, representing 76.14% of its constituents. SFE allowed the identification of 19 constituents at an extraction pressure of 12 MPa, and 8 compounds at an extraction pressure of 18 MPa, obtained in both separators. Although the most volatile compounds are, theoretically, collected in the second separator, some amount could also be retained in the first separator.

Camphor was the main component of the essential oil (31.59 ± 1.32%), and of the SFE extracts at 12 MPa (1.61 ± 0.34% in the first separator extract, 22.48 ± 1.49% in the second separator extract). In contrast, the first separator extract at 18 MPa was dominated by myrtenol (5.38 ± 2.04%) and camphor (4.81 ± 1.93%), whereas the second separator extract was dominated by verbenone (13.97 ± 5.27%).

Table 1
Extraction yield (%, w/w) achieved by hydrodistillation (HD) and supercritical fluid extraction (SFE).

	Essential oil	Extracts	
		First separator	Second separator
HD	0.53 ± 0.04	–	–
SFE 12 MPa	–	3.45 ± 0.27 a	9.27 ± 0.47 a
SFE 18 MPa	–	3.41 ± 0.57 a	8.80 ± 0.69 a

Values are expressed as mean ± SE ($n = 3$). Values followed by the same letter within the same column are not significantly different at $p \geqslant 0.05$.

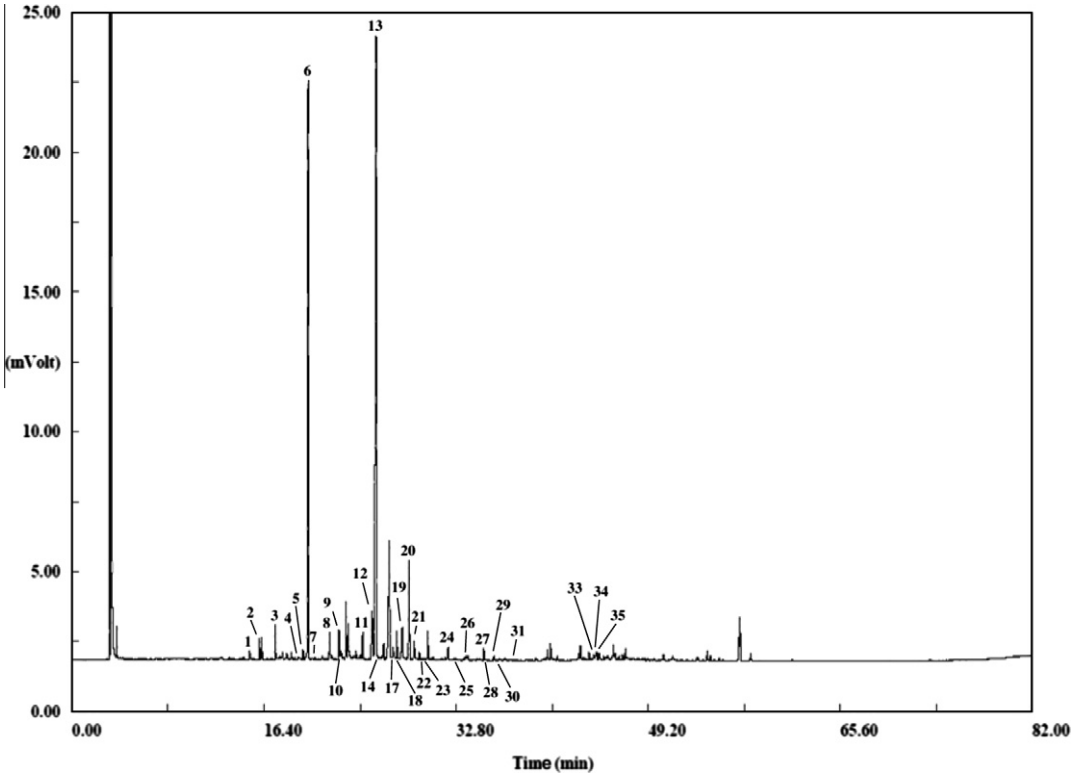

Fig. 1. GC–FID chromatogram of the *L. viridis* essential oil obtained by hydrodistillation. The identity of the compounds is listed in Table 2.

The lower extraction pressure allowed the isolation of a larger number of volatiles in the second separator, which demonstrates the selectivity of the method and its ability to target high-value compounds such as camphor, an important flavour and fragrance (Bauer, Garbe, & Surburg, 2001) as well as a topical analgesic (Burkhart & Burkhart, 2003). Other advantage of SFE extraction is to obtain extracts that resemble more the original flavour of the aromatic plant than the essential oil (Damjanović, Lepojević, Živković, & Tolić, 2005), since SFE extraction allows a higher recovery of oxygenated components, compared to that of the mono and sesquiterpene hydrocarbons. This was observed in the present study since some lighter monoterpene hydrocarbons, such as α-pinene, camphene, β-pinene, α-terpinene are absent from the SFE extracts, while the SFE extracts from the second separator, which usually are more close to the essential oil, are richer in *cis*- and *trans*-linalool oxide, epoxy-linalool, borneol, terpinen-4-ol and verbenone than the essential oil.

The SFE extracts from the first separator also contained several unidentified compounds (Fig. 2A and 3A). Previously, it has been shown that large quantities of paraffins and steroptens are co-extracted with SFE products at high extraction densities (T = 40–50 °C and p = 10–20 MPa) (Reverchon, 1997). To the best of our knowledge, this is the first report describing the use of SFE to isolate *L. viridis* extracts, but SFE has been used to obtain specific compounds from other *Lavandula* spp. (Da Porto, Decorti, & Kikic, 2009; Topal, Sasaki, Motonobu, & Otles, 2008). Topal et al. (2008) studied the composition of essential oils from the dried leaves of *Lavandula stoechas* spp. *stoechas* L., obtained by SFE and steam distillation. GC–MS analysis showed that the chemical composition of the essential oils obtained by SFE was richer than that obtained by steam distillation. Da Porto et al. (2009) recovered flavour compounds from *Lavandula angustifolia* using three different extraction techniques (SFE, ultrasound extraction and HD) and found that the SFE extracts contained more compounds, particularly those concerned with flavour quality and stability. In addition, only a small amount of high-molecular-weight compounds was co-extracted at 12 MPa and 40 °C.

The chemical composition of *L. viridis* essential oil we report in this article is in partial agreement with previous studies, which likewise show the predominance of oxygen-containing monoterpenes albeit with quantitative differences in the levels of different compounds (Gonçalves et al., 2008; Matos et al., 2009; Nogueira & Romano, 2002; Zuzarte et al., 2011). Camphor was found to be the main constituent of the essential oil (31.59 ± 1.32%), followed by 1,8-cineole (21.31 ± 0.78%). However, previous reports have shown that 1,8-cineole is the most abundant compound (Gonçalves et al., 2008; Matos et al., 2009; Nogueira & Romano, 2002; Zuzarte et al., 2011).

Nogueira and Romano (2002) studied the composition of essential oil from field-grown *L. viridis* plants collected at São Bartolomeu de Messines (Algarve, Portugal) during the flowering phase, as well as *in vitro* shoot-cultures and micropropagated plants produced from the same clone. The major compound identified in all the essential oils by GC and GC–MS was 1,8-cineole (18.2–25.1%), followed by camphor (9.1–15.7%). Gonçalves et al. (2008) reported the chemical composition of volatiles from *in vitro* shoot-cultures, micropropagated and field-grown plants collected at the same location. The results obtained by headspace solid phase microextraction followed by GC–MS again showed 1,8-cineole as the predominant component (51.9–74.0%) followed by camphor (2.9–15.3%). The discrepancies noted above therefore do not relate to the geographical origin or development stage of the plants, but is likely to reflect the well-recognised phenomenon that the chemical composition of essential oils is sensitive to variations in environmental conditions as well as genetic factors (Figueiredo et al., 2008).

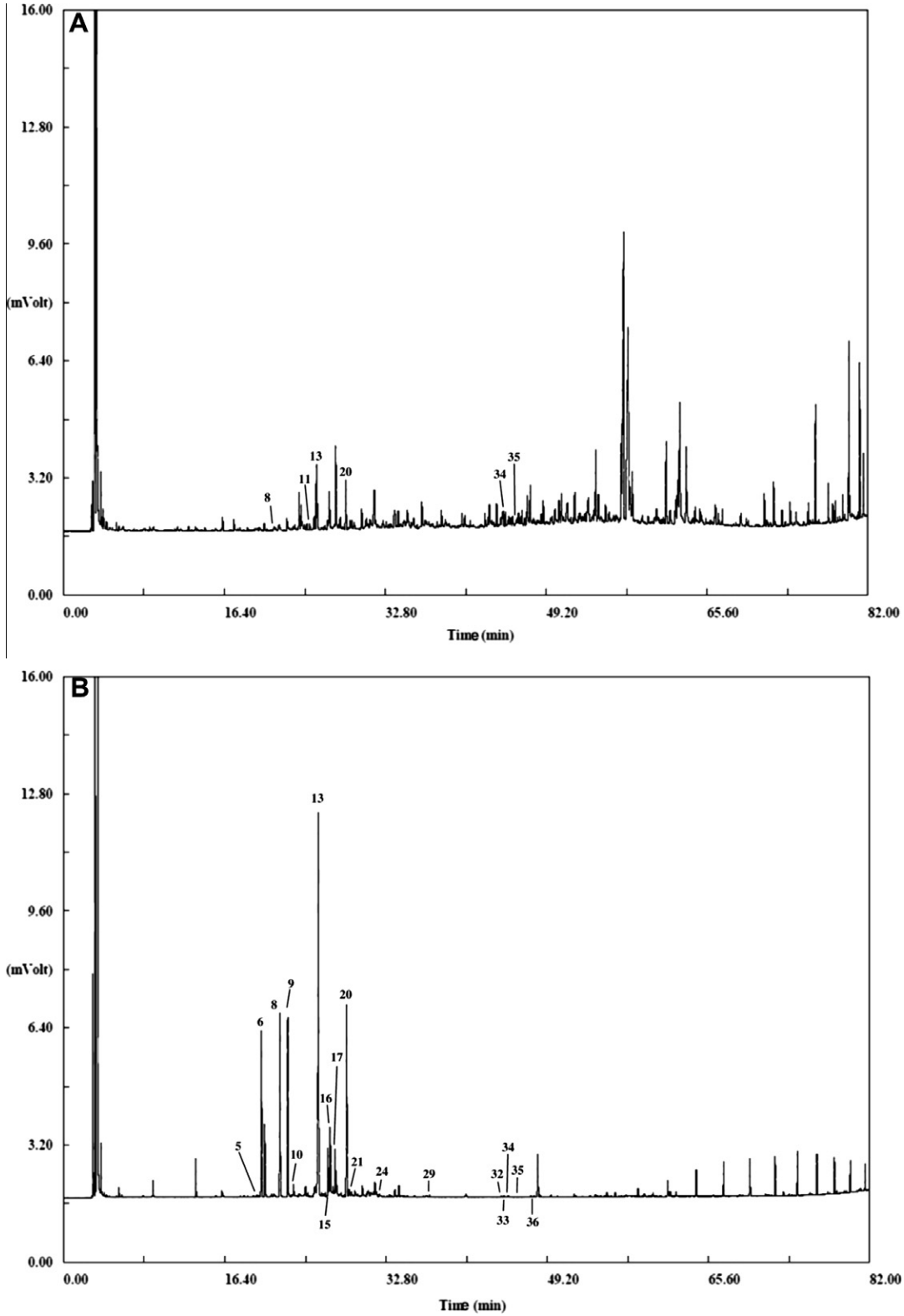

Fig. 2. GC–FID chromatogram of the first (A) and second separator (B) obtained from *L. viridis* by SFE at 40 °C and 12 MPa. The identity of the compounds is listed in Table 2.

3.3. Antioxidant activity

Any imbalance between the antioxidant defence system and free radical formation in the human body can damage biological target molecules, affecting their function and integrity. In this respect, free radical scavengers can be used as a preventive intervention in free radical-mediated diseases such as Alzheimer's disease (Valko et al., 2007). The free radical-scavenging capacity of *L. viridis* essential oil and SFE extracts was assessed by two methods, oxygen radical absorbance capacity (ORAC), which is

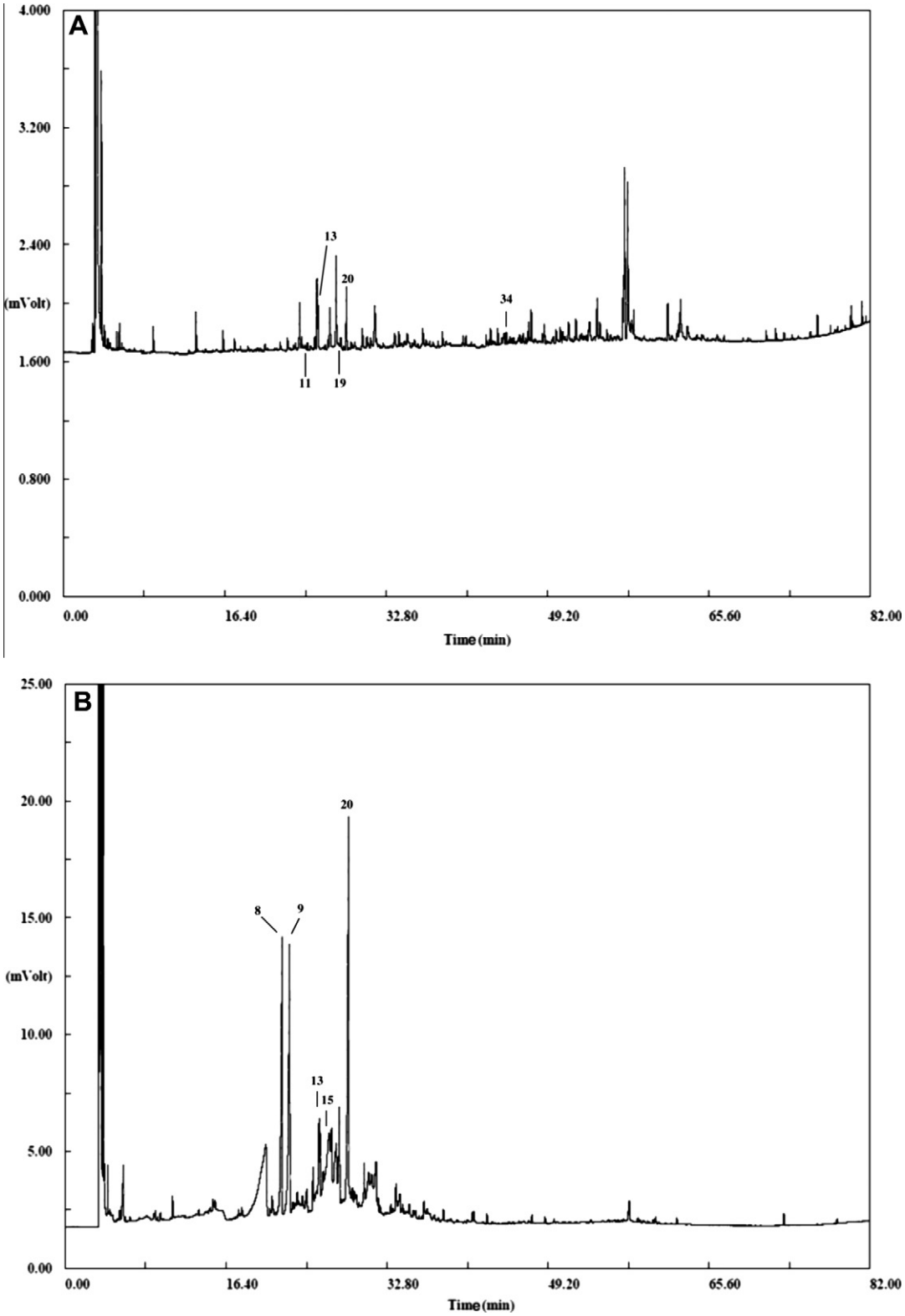

Fig. 3. GC–FID chromatogram of the first (A) and second separator (B) obtained from *L. viridis* by SFE at 40 °C and 18 MPa. The identity of the compounds is listed in Table 2.

based on the measurement of hydrogen atom transfer (HAT), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, which is based on single electron transfer (SET) (Prior, Wu, & Schaich, 2005).

Table 3 shows the free radical-scavenging capacity of the *L. viridis* essential oil and SFE extracts. The essential oil displayed the highest ORAC values (468.85 ± 7.42 $\mu mol_{TE}/g_{extract}$), followed by the SFE extracts from the first separator at 18 MPa (134.06 ± 13.69 $\mu mol_{TE}/g_{extract}$). The antiradical activity of the essential oil probably reflects the high content of monoterpenes (Miguel, 2010) including camphor, which is known to act as an antioxidant (Zaouali, Bouzaine, & Boussaid, 2010). The ORAC value of the

Table 2
Chemical composition (relative %) of the essential oil obtained by hydrodistillation (HD) and of the extracts (first separator and second separator) obtained by supercritical fluid extraction (SFE) isolated from *L. viridis*.[a]

	Compounds	m/z	RI	RI[b]	HD essential oil	SFE 12 MPa		SFE 18 MPa	
						First separator	Second separator	First separator	Second separator
1	α-Pinene[c,d,e,f,g,h,i,j,k,l]	53/59/63/67/73/77/84/89/91/93/105/112/121/129/136	936	934	0.30 ± 0.01	–	–	–	–
2	Camphene[c,d,e,f,g,h,i,k]	53/58/65/67/77/79/81/91/93/95/107/115/121/127/136	953	949	0.62 ± 0.03	–	–	–	–
3	β-Pinene[c,d,f,g,h,i,k,l]	51/55/67/71/79/85/91/93/100/107/121/127/136	980	975	1.18 ± 0.07	–	–	–	–
4	α-Terpinene[c,f,h,i]	53/58/65/69/74/77/81/85/91/93/105/121/129/136	1020	1015	0.13 ± 0.01	–	–	–	–
5	*p*-Cymene[d,f,h,k,l]	51/56/63/67/71/77/81/85/89/91/103/119/128/134	1029	1027	0.33 ± 0.02	–	0.16 ± 0.02	–	–
6	1,8-Cineole[c,d,e,f,g,h,i,j,k]	53/63/69/81/95/108/111/115/125/129/139/143/154	1037	1031	21.31 ± 0.78	–	7.81 ± 0.70	–	–
7	*trans*-β-Ocimene[f,l]	51/55/58/67/71/77/84/89/93/98/105/121/136	1050	1052	0.10 ± 0.01	–	–	–	–
8	*cis*-Linalool oxide[c,d,e,i,k]	55/59/67/71/81/94/97/111/123/135/143/153	1075	1078	1.31 ± 0.69	0.44 ± 0.36	7.93 ± 0.02	–	10.49 ± 3.10
9	*trans*-Linalool oxide[c,d,e,f,i,k]	55/59/67/77/81/91/94/109/119/125/137/143/155	1091	1087	1.78 ± 0.21	–	7.86 ± 0.03	–	13.17 ± 0.85
10	Linalool[c,f,g,i,k,l]	55/59/71/81/93/109/121/136/151	1103	1105	1.75 ± 0.00	–	0.93 ± 0.02	–	–
11	α-Campholenal[e,k]	53/67/69/81/93/108/119/125/133/142/150	1132	1128	0.84 ± 0.04	0.14 ± 0.04	–	0.25 ± 0.12	–
12	Norinone[k]	53/55/65/67/69/73/79/83/91/95/97/105/109/123/129/134	1150	1142	2.12 ± 0.08	–	–	–	–
13	Camphor[c,d,e,f,g,i,j,k]	53/63/69/74/81/91/95/103/108/119/125/137/146/152	1156	1147	31.59 ± 1.32	1.61 ± 0.34	22.48 ± 1.49	4.81 ± 1.93	4.23 ± 2.83
14	Pinocarvone[e]	51/53/55/65/69/74/77/81/91/93/107/122/135/150	1170	1165	0.70 ± 0.03	–	–	–	–
15	Epoxy linalool[g]	51/55/59/68/71/77/81/94/109/119/137/143/155	1176	1080	–	–	0.25 ± 0.03	–	6.80 ± 4.53
16	Borneol[c,d,e,f,g,h,i,j,k]	55/59/63/67/71/79/81/95/103/110/115/121/136	1180	1199	–	–	2.07 ± 0.42	–	–
17	Terpinen-4-ol[d,e,f,g,i,j]	55/67/71/81/86/91/93/107/111/115/121/136/147/154	1191	1171	0.49 ± 0.02	–	3.55 ± 0.61	–	–
18	Myrtenal[i,k]	51/59/65/73/79/91/95/107/117/135/150	1193	1193	1.22 ± 0.04	–	–	–	–
19	Myrtenol[c,e,g,k]	51/59/67/69/77/79/83/91/93/108/119/127/133/139/145	1203	1212	1.77 ± 0.07	–	–	5.38 ± 2.04	–
20	Verbenone[c,d,e,g]	53/65/77/83/91/94/99/107/115/122/129/135/140/150	1217	1204	3.54 ± 0.13	0.84 ± 0.20	12.14 ± 0.87	2.61 ± 1.15	13.97 ± 5.27
21	*cis*-Carveol[c,h,i]	53/55/67/72/79/84/93/95/109/119/137/145/152	1229	1220	0.93 ± 0.03	–	0.18 ± 0.04	–	–
22	*trans*-Pinocarvyl acetate[j]	55/67/79/84/91/108/119/134/149/157/166/176/185	1245	1297	0.11 ± 0.00	–	–	–	–
23	Carvone[c,g,i,k]	54/58/67/71/79/82/91/93/95/99/108/122/135/142/150	1250	1246	0.07 ± 0.00	–	–	–	–
24	Isobornyl formate[f]	55/67/81/84/95/108/121/115/136/147/154/162/169/179	1299	1236	0.55 ± 0.04	–	0.09 ± 0.01	–	–
25	Carvacrol[h,l]	51/65/77/84/91/93/107/115/127/135/141/150	1314	1295	0.15 ± 0.01	–	–	–	–
26	Myrtenyl acetate[c,g]	55/67/79/91/108/119/125/134/142/153/163/175/183/194	1331	1328	0.48 ± 0.07	–	–	–	–
27	Eugenol[c]	55/65/73/77/91/93/103/121/131/149/153/164	1363	1359	0.59 ± 0.01	–	–	–	–
28	Neryl acetate[c,f,j]	53/69/80/84/93/107/121/136/149/164/175/183/191	1371	1367	0.02 ± 0.01	–	–	–	–
29	Geranyl acetate [c,e,f,j]	53/59/69/81/85/93/107/121/136/148/156/164/182/190	1381	1385	0.17 ± 0.00	–	0.03 ± 0.01	–	–
30	β-Bourbonene[l]	55/67/81/91/105/123/133/145/161/189/204	1387	1382	0.04 ± 0.00	–	–	–	–
31	*trans*-α-Bergamotene[f,i]	55/67/79/91/105/119/134/151/189	1428	1441	0.06 ± 0.00	–	–	–	–
32	Spathulenol[h,l]	53/67/79/91/105/119/133/149/159/187/205/218	1594	1578	–	–	0.08 ± 0.01	–	–
33	Caryophyllene oxide[c,g,h,i,k]	57/70/79/93/105/119/136/159/177/203/220	1596	1592	1.21 ± 0.05	–	0.06 ± 0.01	–	–
34	Globulol[e,g]	55/67/71/79/93/105/121/135/147/161/175/189/204/222	1605	1585	0.47 ± 0.02	0.37 ± 0.03	0.18 ± 0.04	0.47 ± 0.06	–
35	Viridiflorol[g]	55/67/79/91/109/119/133/147/161/175/189/205/222	1615	1589	0.27 ± 0.01	0.50 ± 0.05	0.06 ± 0.01	–	–
36	Juniper camphor[e]	55/58/67/71/81/85/95/105/125/147/161/175/189/204/221	1635	1690	–	–	0.06 ± 0.00	–	–
	Identified compounds (%)				76.14	3.90	65.81	13.52	48.66
	Monoterpene hydrocarbons				2.65	0.00	0.16	0.00	0.00
	Oxygen-containing monoterpenes				70.87	3.02	65.29	13.05	48.66
	Sesquiterpene hydrocarbons				0.10	0.00	0.00	0.00	0.00
	Oxygen-containing sesquiterpenes				1.94	0.87	0.43	0.47	0.00
	Others				0.59	0.00	0.00	0.00	0.00

[a] Results are expressed as mean ± standard deviation of three injections.
[b] Retention indices from NIST Chemistry WebBook (http://webbook.nist.gov/chemistry/name-ser.html).
[c] Compounds identified by comparison with authentic standard and with NIST 05 library.
[d] Compounds tentatively identified by comparison with NIST 05 library and Gonçalves et al. (2008).
[e] Compounds tentatively identified by comparison with NIST 05 library and Nogueira and Romano (2002).
[f] Compounds tentatively identified by comparison with NIST 05 library and Hassiotis et al. (2010).
[g] Compounds tentatively identified by comparison with NIST 05 library and Giray et al. (2008).
[h] Compounds tentatively identified by comparison with NIST 05 library and Gören et al. (2002).
[i] Compounds tentatively identified by comparison with NIST 05 library and Imelouane et al. (2010).
[j] Compounds tentatively identified by comparison with NIST 05 library and Soković et al. (2010).
[k] Compounds tentatively identified by comparison with NIST 05 library and Bousmaha et al. (2006).
[l] Compounds tentatively identified by comparison with NIST 05 library and Palá-Paúl et al. (2004).

Table 3
Antioxidant activity of *L. viridis* assessed by oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays.

Samples		Pressure (MPa)	ORAC ($\mu mol_{TE}/g_{extract}$)	DPPH (% Inhibition)[a,b]
Extracts	First separator	12	68.18 ± 7.30c	24. 61 ± 0.44b
		18	134.06 ± 13.69b	23.91 ± 0.83b
	Second separator	12	5.16 ± 0.74d	8.70 ± 3.81c
		18	1.71 ± 0.27d	7.47 ± 2.34c
Essential oil		–	468.85 ± 7.42a	56.03 ± 1.91a

Values are expressed as mean ± SE ($n = 3$).
[a] Percent of inhibition at the highest final concentration tested (12 mg/ml). Values followed by different letters within the same column are significantly different at $p < 0.05$.
[b] Antioxidant reference (BHT), IC_{50} = 462.24 ± 33.94 μg/ml.

extract from the first separator in SFE increased with the higher extraction pressure.

The results from the DPPH assay (Table 3) are expressed as the percentage inhibition of the highest concentration tested, which corresponds to 12 mg/ml in the final reaction volume. The essential oil and first separator extracts showed a weak capacity to scavenge DPPH radicals compared to the reference BHT (IC_{50} = 462.24 ± 33.94 μg/ml), whereas the second separator extracts showed the lowest antioxidant levels assessed with both the ORAC and DPPH assays. The results indicate that the bioactive compounds from *L. viridis* essential oil and first separator SFE extracts have the ability to quench free radicals preferentially by hydrogen donation.

The antioxidant activity of *Lavandula* spp. extracts has been reported previously (Hussain, Anwar, Iqbal, & Bhatti, 2011; Matos et al., 2009). Matos et al. (2009) observed that the essential oil of *L. viridis*, collected in the Algarve region, has free radical scavenging properties. In addition, Costa et al. (2011) reported the antioxidant activity of the methanol extract from *L. viridis* determined by the ORAC assay under the same experimental conditions. It is evident the stronger antioxidant activity displayed by the methanol extract (2858.39 ± 70.97 $\mu mol_{TE}/g_{extract}$) compared to that shown by the essential oil (468.85 ± 7.42 $\mu mol_{TE}/g_{extract}$). Furthermore, this extract was able to scavenge free radicals, chelate Fe^{2+} and reduce the malondialdehyde content of mouse brains *in vitro*.

3.4. In vitro AChE and BChE inhibition assay

After impulse transmission, the neurotransmitter acetylcholine is degraded, predominantly by acetylcholinesterase (AChE) but with butyrylcholinesterase (BChE) playing a minor role (Mesulam, Guillozet, Shaw, & Quinn, 2002). The *L. viridis* essential oil and SFE extracts were tested to evaluate their anti-cholinesterase activities (Table 4), revealing that the essential oil was the most effective AChE inhibitor (IC_{50} = 411.33 ± 72.73 μg/ml) and that the first separator SFE extracts were the most effective BChE inhibitors (IC_{50} = 215.56 ± 13.60 and IC_{50} = 204.76 ± 22.86 μg/ml at extraction pressures of 12 and 18 MPa, respectively).

The inhibitory activity displayed by the essential oil probably reflects its high content of terpenoids, which are known as potent cholinesterase inhibitors (Dohi et al., 2009; Savelev, Okello, Perry, Wilkins, & Perry, 2003). The second separator extracts did not achieve 50% inhibition even at the highest final concentration tested (2.5 mg/ml). Although these extracts have more compounds in common with the essential oil (mainly that one obtained at 12 MPa), there are differences in acetylcholinesterase inhibition that can be explained by the relative content of the components. Moreover, most of the main compounds are absent from the first separator extracts although they were more active than those collected in the second one. This reflects the importance of the minor

Table 4
Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition activities, expressed by IC_{50} values[a] (μg/ml), of the essential oil and SFE extracts from *L. viridis* and galanthamine.

Samples		Pressure (MPa)	AChE (μg/ml)	BChE (μg/ml)
SFE extracts	First separator	12	3524.51 ± 465.82[a*]	215.56 ± 13.60b
		18	1975.16 ± 187.91b*	204.76 ± 22.86b
	Second separator	12	ND	ND
		18	ND	ND
Essential oil		–	411.33 ± 72.73c	748.69 ± 105.42a
Galanthamine		–	2.20 ± 0.04	11.72 ± 1.09*

Values are expressed as mean ± SE ($n = 3$). Values followed by different letters within the same column are significantly different at $p < 0.05$. Values marked with * indicate means significantly higher at $p < 0.05$.
ND, not determined, means the maximum level of inhibition below 50% (2.5 mg/ml).
[a] Final concentration required to inhibit the hydrolysis of the substrate, by 50%.

Table 5
Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory effect of terpenoids based on the final concentrations present in the *L. viridis* essential oil.

Compound	Percentage in the essential oil	AChE		BChE	
		μg/ml[a]	Inhibition (%)	μg/ml	Inhibition (%)
Camphor	31.59	160.00	14.37 ± 1.74b	240.00	19.14 ± 1.61a
1,8-Cineole	21.31	105.00	35.92 ± 1.37a	160.00	8.75 ± 0.90b
α-Pinene	0.30	1.50	17.27 ± 1.61b	2.25	10.13 ± 1.13b

[a] It corresponds to the final concentration that individual compounds represent in the essential oil composition, estimated from the IC_{50} values of the whole oil displayed in Table 4. IC_{50} values of the essential oil were taken as 100% for the convenience of calculations. Values are expressed as mean ± SE ($n = 3$). Values followed by different letters within the same column are significantly different at $p < 0.05$.

components for the overall bioactivity. For instance, Miyazawa, Watanabe, Umemoto, and Kameoka (1998) reported that viridiflorol, present in both essential oil and extracts from first separator (at 12 MPa), is almost twice more potent than 1,8-cineole. Furthermore, the interaction between components of the extracts (Savelev et al., 2003) and the non-identified compounds found in the several extracts (Figs. 1–3) could also influence the observed activities.

To determine which components of the essential oil were responsible for the observed anti-cholinesterase activity and to measure their individual contributions we evaluated certain terpenoids based on their natural concentrations in the essential oil (Table 5). The main test compounds were camphor, 1,8-cineole and α-pinene, which are present in the essential oil at relative levels of 31.59 ± 1.32%, 21.31 ± 0.78% and 0.30 ± 0.01%, respectively (Table 2). The most active compound was 1,8-cineole, accounting for ~36% of the AChE inhibitory activity of the whole oil, whereas camphor accounted for ~19% of the BChE inhibitory activity. Despite representing only 0.3% of the essential oil, α-pinene had an important inhibitory effect on both AChE (17.27 ± 1.61%) and BChE (10.13 ± 1.13%). Nevertheless, additional minor constituents and complex interactions among compounds in the essential oil could also play a major role in this activity (Dohi et al., 2009; Nakatsu, Lupo, Chinn, & Kang, 2000).

3.5. Qualitative determination of false-positive effects in the AChE and BChE inhibition assays

The Ellman method (Ellman et al., 1961) has been useful for the identification of new cholinesterase inhibitors, but it does not distinguish between enzymatic inhibition and loss of activity caused by the reaction between thiocholine and DTNB, which would result in a false-positive effect (Wang, Zhou, Gao, Wang, & Yao, 2007). TLC assay was carried out to measure the false-positive effect in *in vitro*. Only the second separator extracts did not affect the activity of both enzymes, and the reference compound galanthamine showed inhibition in the enzymatic plate but not in the chemical reaction between DTNB and thiocholine, as anticipated. The concentrations tested were much higher than those used in the microplate assay in order to ensure their detection.

The first separator extracts generated many white bands on the false-positive detection plate that were more pronounced against the inhibition of AChE, possibly reflecting the co-extraction of undesirable compounds during SFE. Few false-positive effects were detected in the essential oil, in either the AChE or BChE inhibition assays. This confirms the original quantitative data and shows that *L. viridis* essential oil is a potent inhibitor of anti-cholinesterase activity which confirmed that our original results were genuine and it was not a chemical artefact. The combination of TLC and the microplate assay allows enzymatic anti-cholinesterase activity to be separated from chemical artefacts and provides support for further purification steps as necessary to isolate bioactive compounds.

4. Conclusions

It was shown that SFE achieves the highest yield of bioactive compounds from *L. viridis* but that an increase in the extraction pressure does not produce any further benefits. The chemical analysis of *L. viridis* essential oil and SFE extracts highlighted the abundance of oxygen-containing monoterpenes but revealed significant differences in the chemical profiles. More compounds were identified following the extraction of *L. viridis* essential oil by HD than by SFE. The essential oil and first separator SFE extracts possessed antioxidant activity and a remarkable capacity to inhibit cholinesterases.

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Inhibitory effect of *Lavandula viridis* on Fe^{2+}-induced lipid peroxidation, antioxidant and anti-cholinesterase properties

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ABSTRACT

In this research, the total phenolic content of a *Lavandula viridis* methanol extract was evaluated and the phenolic identification and quantification was assessed. Rosmarinic acid and luteolin-7-*O*-glucoside were the two major compounds identified by HPLC–DAD (ca. 39 and 13 g/kg, respectively). This extract showed a strong antioxidant activity in ORAC (2858.39 ± 70.97 $\mu mol_{TE}/g_{extract}$) and TEAC (967.18 ± 22.57 $\mu mol_{TE}/g_{extract}$) assays, as well as Fe^{2+} chelating and ·OH scavenging abilities. Furthermore, the extract prevented Fe^{2+}-induced lipid peroxidation, by reducing MDA content in mouse brains (*in vitro*), and inhibited AChE and BChE activities both *in vitro* and *in vivo*. These findings demonstrate that the methanol extract from *L. viridis* is a potential source of natural antioxidants and cholinesterase inhibitors.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, characterised by the loss of memory and the progressive decline of cognitive abilities. Clear evidence exists supporting the involvement of free radicals, which play an important role in neuronal dysfunction and degeneration, in the pathogenesis of AD (Markesbery, 1997; Uttara, Singh, Zamboni, & Mahajan, 2009). The human brain is particularly susceptible to oxidative processes due to its high concentration of polyunsaturated fatty acids, which are potential substrates for lipid peroxidation, its low levels of antioxidant defence enzymes, its high consumption of total oxygen and its elevated levels of transition metals that can catalyse radical formation (Floyd & Hensley, 2002; Halliwell, 1992; Markesbery, 1997). AD is neuropathologically characterised by the occurrence of β-amyloid plaques and neurofibrillary tangles. Growing evidence supports the fact that biometals play an important role in amyloid β-protein (Aβ) accumulation and neuronal degeneration (Valko et al., 2007). Iron is the most important metal in the brain and is vital for normal neuronal metabolism (Thompson, Shoham, & Connor, 2001). However, high contents of iron in tissues can augment neuron degeneration through free radical processes by different mechanisms, including participation in Fenton's reaction, as well as the development of membrane lipid peroxidation, which leads to cell membrane fluidity, thereby jeopardising cell viability (Fraga & Oteiza, 2002; Halliwell, 2001).

Additional pathological mechanisms of AD may include defects in the cholinergic system as a consequence of a gradual loss of neurons from distinct regions of the brain (Francis, Palmer, Snape, & Wilcock, 1999). Recent attempts to treat this disease have focused on enhancing cholinergic function using cholinesterase inhibitors (ChEIs), in order to enhance the concentration of acetylcholine (ACh) in the synaptic cleft (Francis et al., 1999). The human brain contains the two following cholinesterases: acetylcholinesterase (AChE), which is more abundant in the brain and specifically hydrolyses ACh, and butyrylcholinesterase (BChE), which hydrolyses ACh as well as many other esters. Contemplating the complexity of AD, a combination of different therapeutic agents may result in the most effective strategy for its treatment (Doraiswamy, 2002).

Abbreviations: $ABTS^{\cdot+}$, 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid radical cation; ACh, Acetylcholine; AChE, Acetylcholinesterase; AD, Alzheimer's disease; ATCI, Acetylthiocholine iodide; Aβ, Amyloid β-protein; BChE, Butyrylcholinesterase; BTCI, Butyrylthiocholine; ChEIs, Cholinesterase inhibitors; DS, Detergent soluble; DTNB, 5,5′-dithiobis [2-nitrobenzoic acid]; ET, Single-electron transfer; F–C reagent, Folin–Ciocalteu reagent; GAE, Gallic acid equivalents; HAT, Hydrogen-electron transfer; H_2O_2, Hydrogen peroxide; HPLC–DAD, High-Performance Liquid Chromatography-Diode-Array Detection; MDA, Malondialdehyde; ORAC, Oxygen radical absorbance capacity; SS, Salt soluble; TBA, Thiobarbituric acid; TCA, Trichloroacetic acid; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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Since ancient times, therapeutic records have existed for several species of *Lavandula* (Lamiaceae) and is one of the most popular medicinal plants with high economic value (Büyükokuroğlu, Gepdiremen, Hacimüftüoğlu, & Oktay, 2003). Several oils from this genus have been reported to possess antibacterial, antifungal and anti-cholinesterase effects (Cavanagh & Wilkinson, 2002; Dohi, Terasaki, & Makino, 2009; Hanamanthagouda et al., 2010). In addition to the oils, the neuroprotective, anti-inflammatory and antioxidant properties of *Lavandula* extracts were also demonstrated (Büyükokuroğlu et al., 2003; Hajhashemi, Ghannadi, & Sharif, 2003; Kovatcheva et al., 2001).

Lavandula viridis L'Hér, traditionally used in Portuguese folk medicine, is a xerophytic aromatic shrub endemic to the southwest Iberian Peninsula, which grows in dry conditions and in nutrient poor and degraded soils of the Algarve and Alentejo regions (Franco, 1971). The essential oils of this species were already analysed, and their antioxidant activity proven (Matos et al., 2009; Nogueira & Romano, 2002). However, there are few scientific records on the chemical composition and biological properties of *L. viridis* extracts. Thus, this study was conducted to assess the antioxidant and anti-cholinesterase abilities of an extract from aerial parts of *L. viridis*. Its protective activities against Fe^{2+}-induced lipid peroxidation in mouse brain homogenates were also evaluated. Furthermore, the identification and quantification of phenolic compounds present in this extract were carried out by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC–DAD).

2. Materials and methods

2.1. Standards and reagents

5-*O*-Caffeoylquinic and rosmarinic acids and luteolin-7-*O*-glucoside were purchased from Extrasynthése (Genay, France). Pinocembrin was purchased from Fluka (Steinheim, Germany) and caffeic acid was purchased from Sigma–Aldrich (Steinheim, Germany). Formic acid and acetonitrile were acquired from Merck (Darmstadt, Germany). Folin–Ciocalteu's phenol reagent (F–C reagent), gallic acid, sodium carbonate (Na_2CO_3) and iron(III) chloride ($FeCl_3$) were purchased from VWR (Leuven, Belgium). Butyrylthiocholine chloride (BTCI) and sodium chloride (NaCl) were acquired from Fluka (Steinheim, Germany). Fluorescein, 1,10-phenanthroline and trichloroacetic acid (TCA) were obtained from Panreac (Barcelona, Spain). 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, potassium persulfate, thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), acetylthiocholine iodide (ATCI), 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB), acetylcholinesterase (AChE) (Electric-eel, EC 3.1.1.7, Type VI-S), butyrylcholinesterase (BChE) (horse-serum, EC 3.1.1.8), galanthamine hydrobromide, bovine serum albumin (BSA), Triton X-100 and methanol were purchased from Sigma–Aldrich. Sodium dodecyl sulphate (SDS), potassium ferricyanide ($K_3Fe(CN)_6$), deoxyribose, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Acros Organics (Geel, Germany). Iron(II) sulphate ($FeSO_4$) and acetic acid were purchased from Merck. Hydrochloric acid (HCl) was obtained from BDH (Poole, England) and Dulbecco's phosphate-buffered saline (D-PBS) was obtained from Gibco (Carlsbad, USA).

2.2. Plant material and extraction procedure

Aerial parts of *L. viridis* were collected during the flowering period at São Bartolomeu de Messines, Algarve, Portugal. The *L. viridis* voucher has been previously deposited in the Herbarium of the Museu, Laboratório e Jardim Botânico de Lisboa, as referenced by Nogueira and Romano (2002).

The plant material was dried at 40 °C and finely powdered using a blender. The plant material (10 g) was then extracted with methanol (150 ml), at room temperature for 5 h, in a Soxhlet apparatus. Removal of the solvent was carried out with a rotary evaporator at 40 °C. The extraction yield was 28.75% (w/w, in terms of initial dried material) and the extract was stored at −20 °C until future use.

2.3. Animals

Mice, C57BL/6 Black, male, 25–30 g, 3–4 month-old, were acquired from the Animal House of the Centre for Molecular and Structural Biomedicine, University of Algarve, Portugal. The studies involving animal experiments were conducted in accordance with the ethical issues for clinical research and EU guidelines for animal research. Mice were kept in polyacrylic cages and housed in a room under a controlled temperature between 20 and 23 °C and a relative humidity between 40% and 55%. Mice had *ad libitum* access to food.

2.4. Identification and quantification of phenolic compounds by HPLC–DAD

Twenty microlitres of the *L. viridis* extract that was redissolved in methanol (10 mg/ml) was analysed using an HPLC unit (Gilson, Villiers le Bel, France) and a Spherisorb ODS2 column (4.6 × 250 mm, 5 μm particle size). The solvent system used was a gradient of water/formic acid (19:1) (A) and acetonitrile (B). The gradient was as follows: 0 min – 17% B; 40 min – 23% B; 57 min – 49% B; 59 min – 100% B; 60 min – 100% B. Elution was performed at a solvent flow rate of 1 ml/min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were accumulated in the range of 200–400 nm, and chromatograms were recorded at 280, 320 and 350 nm, for flavanones, phenolic acids and flavones, respectively. The data were processed on a Unipoint Software system (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked using the software contrast facilities.

The quantification of phenolic compounds was achieved by comparison of their absorbance with that of commercially available external standards. The caffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid and the unidentified flavanone as pinocembrin. Caffeic and rosmarinic acids, luteolin-7-*O*-glucoside and pinocembrin were determined as themselves. The detection limits were calculated as the concentration corresponding to three times the standard deviation of the background noise and varied between 0.13 and 3.99 μg/ml. Reproducibility was confirmed by analysing the sample five times, by the same analyst, within the same day. The coefficients of variation ranged from 1.5% to 8.2%, indicating that the reproducibility of the procedure was good. The intermediate precision was determined by different analysts on three separate days (three injections a day) and was also found to be satisfactory, with coefficients of variation ranging from 2.7% to 13.0%.

2.5. Total phenolic content

The F–C colorimetric method was performed as described by Ainsworth and Gillespie (2007) with slight modifications (Gonçalves, Xavier, Alberício, Costa, & Romano, 2010). A standard curve was calculated using gallic acid concentrations ranging from 4 μM to 0.5 mM and the results were expressed as gallic acid equivalents (GAE) per gram of extract.

2.6. Oxygen radical absorbance capacity (ORAC) assay

The total antioxidant capacity was evaluated as described by Gillespie, Chae, and Ainsworth (2007). Fluorescein was used as the fluorescent probe and AAPH as the peroxyl radical generator. The decrease in fluorescence was determined by collecting readings for excitations at 485 nm and emissions at 530 nm every minute, for 90 min. The ORAC value for each extract was calculated using the respective area under the curve (AUC) and the regression equation between trolox equivalents and the net AUC. The results were expressed as trolox equivalents (TE) per gram of extract.

2.7. Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant activity was determined by the TEAC assay using the radical cation $ABTS^{\cdot+}$, according to the procedure proposed by Re et al. (1999). The $ABTS^{\cdot+}$ stock solution (7 mM) was prepared using the recommendations described, using potassium persulfate as the oxidant agent. The absorbance was read at 734 nm, 1 min after initial mixing. The sample dilution that produced 20–80% inhibition of the blank absorbance was used for TEAC calculation and the results were expressed as TE per gram of extract.

2.8. Preparation of brain homogenates

Mice were killed by CO_2 asphyxiation and the cerebral tissue (whole brain) was rapidly dissected, placed on ice and weighed. Tissues were immediately homogenised in cold 0.1 M Tris–HCl (pH 7.4) (1/10, w/v). The homogenate was centrifuged (Eppendorf, Centrifuge 5804R, Hamburg, Germany) for 10 min at 3000*g* to yield a pellet that was discarded and a low-speed supernatant (S1) mainly containing water, proteins, lipids, DNA and RNA, which was used for the lipid peroxidation assay (Bellé, Dalmolin, Fonini, Rubin, & Rocha, 2004).

2.9. Lipid peroxidation and thiobarbituric acid reactions

The lipid peroxidation assay was determined by measuring thiobarbituric acid-reactive substances at 532 nm, as described by Ohkawa, Ohishi, and Yagi (1979). The brain homogenate was incubated at 37 °C for 1 h in a medium containing 0.1 M HCl buffer (pH 7.4), extract (0.25–2 mg/ml), 250 μM freshly prepared $FeSO_4$ and distilled water. After incubation, the reaction mixture was stopped by adding 8.1% SDS, acetic acid/HCl (pH 3.4) and 0.8% TBA.

2.10. Fe^{2+} chelation assay

The ability of the extract to chelate ferrous ions was measured using the method of Minotti and Aust (1987), which was modified by Puntel, Nogueira, and Rocha (2005).

2.11. Degradation of deoxyribose (Fenton's reaction)

The degradation of deoxyribose by hydroxyl radicals was evaluated using the method of Halliwell and Gutteridge (1981), with slight modifications. Briefly, 200 mM deoxyribose, 0.1 M phosphate buffer (pH 7.4), 20 mM H_2O_2, 16 mM $FeSO_4$ and extract (1.5–10 mg/ml) were mixed. After incubation at 37 °C for 30 min, 2.8% TCA and 0.6% TBA solution were added. Malondialdehyde (MDA)-like products of deoxyribose damage were quantified at 532 nm.

2.12. In vitro AChE and BChE inhibition assay

The evaluation of AChE and BChE inhibitory activities was based on Ellman's method (Ellman, Courtney, Andres, & Featherstone, 1961) as described by Chattipakorn et al. (2007). The reaction enzyme activity was calculated as a percentage of the velocities, compared to that of the assay, using buffer without any inhibitor. The IC_{50} (the concentration required to inhibit AChE or BChE activity by 50%) values were calculated as the mean ± SE of three individual determinations, each performed in triplicate.

2.13. In vivo AChE and BChE activities determination

For the evaluation of AChE and BChE activities *in vivo*, 18 animals were divided into 3 groups ($n = 6$) that were treated with PBS, 5 mg/kg of galanthamine or 50 mg/kg of extract (concentration selected based on preliminary assays). The injection volume was 100 μl for all treatments. The animals were sacrificed 120 min after intraperitoneal injection and the cerebral tissue (whole brain) was rapidly dissected, placed on ice and weighted. Tissues were immediately homogenised in 1/10 (w/v) 100 mM phosphate buffer (pH 7.6). The homogenate was centrifuged at 4000 rpm at 4 °C for 30 min to recover a salt-soluble (SS) fraction. The pellets were re-extracted with an equal volume of phosphate buffer containing 1% (v/v) Triton X-100, and the suspensions were centrifuged at 4000 rpm at 4 °C for 30 min to recover a detergent soluble (DS) fraction. The supernatants that were collected served as the enzyme source (Chattipakorn et al., 2007; Papandreou et al., 2009). Protein concentrations were determined by the Bradford assay using BSA as the standard (0.1–1 mg/ml) (Bradford, 1976).

The AChE and BChE activities were determined by the method previously described by Ellman et al. (1961) and were adapted in order to determine the enzyme activities in the supernatants of mouse cortical homogenates by Chattipakorn et al. (2007). The AChE and BChE activities were expressed as mol/min/g of tissue protein. The final results were expressed as percentage (%) inhibition relative to the group without any treatment.

2.14. Statistical analysis

Data were presented as the mean ± standard error of triplicate determinations. The results were subjected to one-way analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA). Significant differences between mean values were determined using Duncan's New Multiple Range Test.

3. Results and discussion

3.1. Determination of total phenolic content

The influence of phenolic content on the antioxidant activity of plant extracts has been demonstrated in previous studies (Jagtap, Panaskar, & Bapat, 2010; Li, Wong, Cheng, & Chen, 2008). Therefore, in this work, the total phenolic content of the *L. viridis* extract was evaluated by a colorimetric assay, using the F–C reagent ($893.01 \pm 17.09\ \mu mol_{GAE}/g_{extract}$) (Table 1).

Despite being the most popular method to evaluate total phenolic content, it is necessary to take some care in interpreting results obtained from the F–C method, due to its poor specificity. This assay is not specific to polyphenols, but also indicates the presence of other substances, including nonphenolic organic substances and some inorganic substances, that could be oxidised by the F–C reagent (Prior, Wu, & Schaich, 2005).

3.2. Identification and quantification of phenolic compounds

The HPLC–DAD analysis (Fig. 1) of the extract from the aerial part of *L. viridis* allowed for the identification and quantification

Table 1
Total phenolic content and antioxidant activity of *L. viridis* extract. The total phenolic content was assessed by Folin–Ciocalteau (F–C) method and antioxidant activity by trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays.

Method	Values
F–C ($\mu mol_{GAE}/g_{extract}$)	893.01 ± 17.09
TEAC ($\mu mol_{TE}/g_{extract}$)	967.18 ± 22.57
ORAC ($\mu mol_{TE}/g_{extract}$)	2858.39 ± 70.97

Values are expressed as mean ± SE (n = 3).

of the following phenolic acids and flavonoids: 3-*O*-caffeoylquinic acid (1), 4-*O*-caffeoylquinic acid (2), 5-*O*-caffeoylquinic acid (3), caffeic acid (4), luteolin-7-*O*-glucoside (5), rosmarinic acid (6) and pinocembrin (7) (Fig. 2). Another flavanone (a) was also present in the extract but has not yet been identified. The phenolic profile we obtained revealed that rosmarinic acid (ca. 39 g/kg) was the major compound, followed by luteolin-7-*O*-glucoside (ca. 13 g/kg) (Table 2). These results are in accordance with those obtained for other related species. Rosmarinic and caffeic acids were previously described in other *Lavandula* species (Areias et al., 2000; Proestos, Sereli, & Komaitis, 2006). Luteolin-7-*O*-glucoside was also reported to occur in leaves of *L. viridis* (Upson et al., 2000). As far as we know, the other compounds are described for the first time in this species.

3.3. Antioxidant capacity

No single method is adequate to estimate the total antioxidant capacity of a sample, due to the variability of extract composition and the conditions of the test used. Antioxidant capacity methods

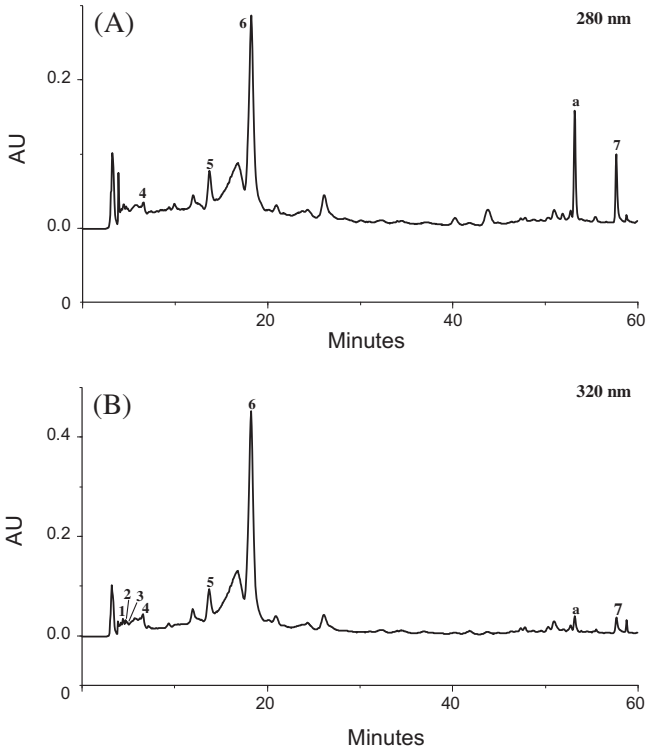

Fig. 1. An HPLC–DAD chromatogram of *L. viridis* extract. Detection at (A) 280 and (B) 320 nm: (1) 3-*O*-caffeoylquinic acid; (2) 4-*O*-caffeoylquinic acid; (3) 5-*O*-caffeoylquinic acid; (4) caffeic acid; (5) luteolin-7-*O*-glucoside; (6) rosmarinic acid; (7) pinocembrin; (a) unidentified flavanone.

can be divided into two groups depending on the following two chemical reactions: assays based on hydrogen-electron transfer (HAT) and assays based on single-electron transfer (ET). For that reason, we combined two methods based on the measurement of the capacity of an antioxidant to prevent the consumption of fluorescein by the peroxyl radical and the measurement of the scavenging effect on the $ABTS^{\cdot+}$ radical (Prior et al., 2005). It was observed that the compounds present in the *L. viridis* extract reacted preferentially via an HAT (2858.39 ± 70.97 $\mu mol_{TE}/g_{extract}$) mechanism instead of an ET (967.18 ± 22.57 $\mu mol_{TE}/g_{extract}$) mechanism (Table 1).

Phenolic acids and flavonoids are secondary metabolites that are synthesised by plants during development, which possess an array of health-promoting benefits (Stalikas, 2007). Rosmarinic acid, the major compound present in the methanolic extract of *L. viridis*, is an ester of caffeic and 3,4-dihydroxyphenyl lactic acids (Pereira et al., 2005). This compound has several interesting biological activities, including its role as an antioxidant (Petersen & Simmonds, 2002). Moreover, the radical scavenging capacity of luteolin-7-*O*-glucoside has been previously demonstrated (Goulas, Papoti, Exarchou, Tsimidou, & Gerothanassis, 2010). Thus, it is probable that rosmarinic acid and luteolin-7-*O*-glucoside may contribute to the antioxidant activity of the *L. viridis* extract observed in this work. Additionally, 5-*O*-caffeoylquinic acid and its isomers have already been reported as radical scavengers (Fukumoto & Mazza, 2000). Although caffeic acid is present in low quantities in the extract compared to the major compounds, its antioxidant potential was previously demonstrated, suggesting the possibility that this compound also contributes to the antioxidant activity displayed by the extract (Chen & Ho, 1997).

3.4. Lipid peroxidation and thiobarbituric acid reactions

Lipid peroxidation is associated with a loss of membrane fluidity and an increase of membrane permeability, causing a decrease in physiological performance (Balu, Sangeetha, Haripriya, & Panneerselvam, 2005). The inhibitory effect of the *L. viridis* extract on Fe^{2+}-induced lipid peroxidation in mouse brain homogenates is shown in Fig. 3. The results clearly indicate that Fe^{2+} is a potent pro-oxidant agent, causing an increase in the MDA content (312.16 ± 9.20%) in the mouse brains when compared with basal brain tissue (without extract and pro-oxidant). This result is in accordance with previous studies (Oboh, Puntel, & Rocha, 2007). The chemical structure of iron, and its capacity to drive one-electron reactions, makes iron a key factor in the formation of free radicals (Fraga & Oteiza, 2002). The *L. viridis* extract protects against lipid peroxidation induced by Fe^{2+}, considerably reducing MDA content in a dose-dependent manner (Fig. 3). Interestingly, other species belonging to the Lamiaceae family have revealed protective effects against Fe^{2+}-induced lipid peroxidation (Barros, Heleno, Carvalho, & Ferreira, 2010).

To explain the mechanism through which the *L. viridis* extract inhibited Fe^{2+}-induced lipid peroxidation, the Fe^{2+} chelating and $^{\cdot}OH$ scavenging abilities of the extract were determined. The extract showed a strong Fe^{2+} chelating capacity in all of the concentrations tested (1.5–10 mg/ml), with a complete chelating capacity at the highest dose (Fig. 4). This capacity could be important because Fe^{2+} can stimulate free radical production; thus, when complexes are formed between the extract and Fe^{2+}, reducing the concentration of free Fe^{2+}, Fe^{2+}-induced lipid peroxidation could be prevented or reduced. The metal-chelating properties of rosmarinic acid have already been demonstrated and its presence in this extract could contribute to the Fe^{2+} chelating ability (Psotová, Lasovský, & Vičar, 2003). The extract was able to scavenge the $^{\cdot}OH$ radical produced during Fe^{2+}-catalysed decomposition of hydrogen peroxide (Fig. 4). The interactions between iron and hydrogen

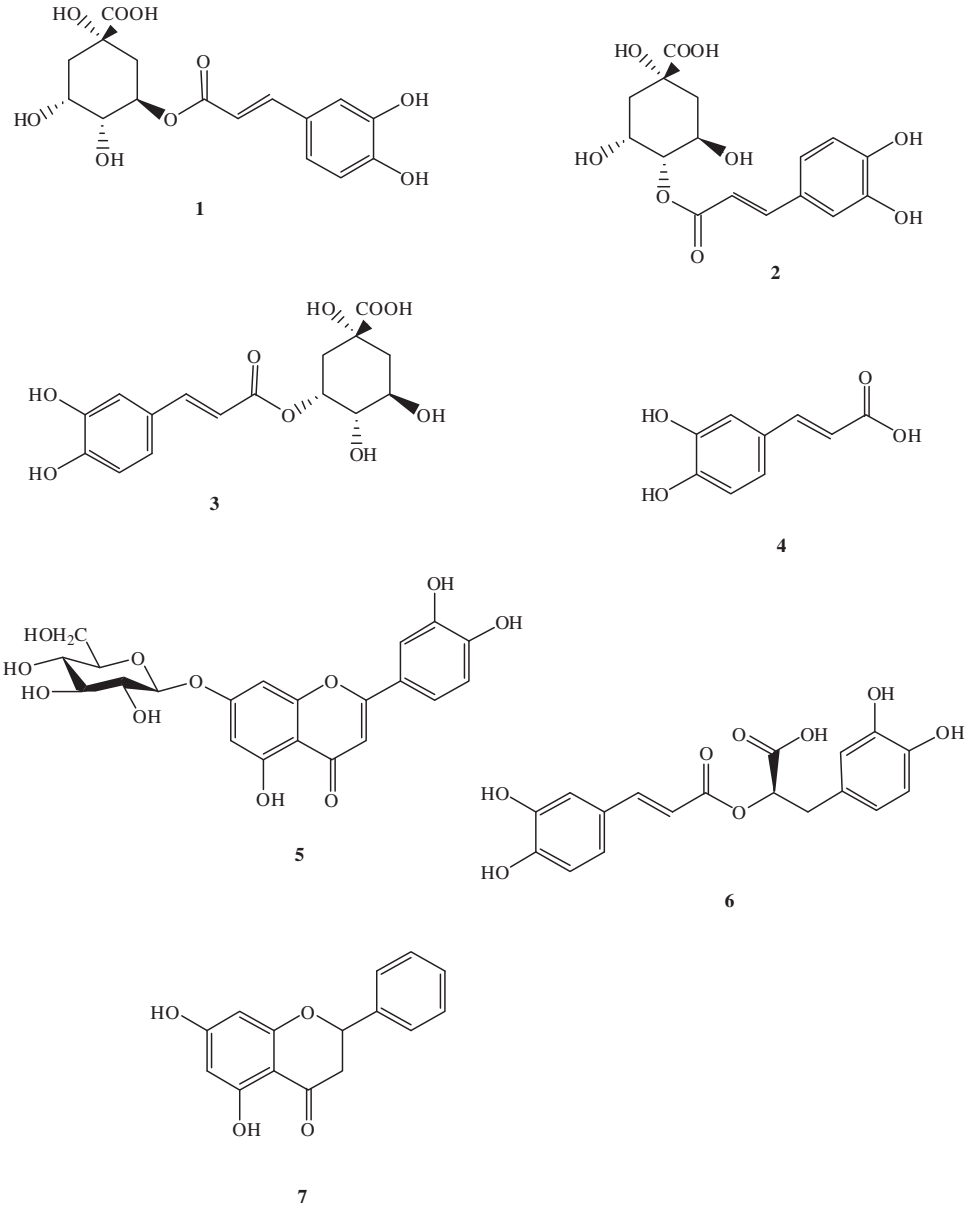

Fig. 2. Chemical structures of the phenolic compounds identified in *L. viridis* extract. (1) 3-*O*-caffeoylquinic acid; (2) 4-*O*-caffeoylquinic acid; (3) 5-*O*-caffeoylquinic acid; (4) caffeic acid; (5) luteolin-7-*O*-glucoside; (6) rosmarinic acid; (7) pinocembrin.

Table 2
Quantification of phenolic compounds in *L. viridis* extract.

	Compound	mg/kg[a]
1	3-*O*-caffeoylquinic acid	2468.2 (16.7)
2	4-*O*-caffeoylquinic acid	1628.5 (81.2)
3	5-*O*-caffeoylquinic acid	845.1 (14.7)
4	Caffeic acid	2631.1 (80.8)
5	Luteolin 7-*O*-glucoside	13404.9 (196.7)
6	Rosmarinic acid	38765.1 (1482.8)
a	Unidentified flavanone	4550.5 (66.9)
7	Pinocembrin	2697.3 (62.0)
	Σ	66990.7

[a] Values are expressed as mean ± SD (n = 3).

peroxide (Fenton's reaction) generate the highly reactive hydroxyl radical, which initiates a process of membrane lipid peroxidation that could lead to alterations in cell structure and function (Halliwell, 2001; Molina-Holgado, Hider, Gaeta, Williams, & Francis, 2007). The preventative effects demonstrated by the extract could be due to the presence of antioxidant compounds (Oboh et al., 2007). In fact, caffeic and rosmarinic acids have already exhibited inhibitory effects against the lipid peroxidation induced by iron/cysteine and the haemolysis of erythrocytes that is induced by hydrogen peroxide (Jiang et al., 2005).

3.5. In vitro and in vivo AChE and BChE inhibition assays

The inhibitory effects of *L. viridis* extract on AChE and BChE activities are listed in Table 3. The *L. viridis* extract showed a strong inhibitory effect on both enzymes, although the activity of AChE was affected more compared to that of BChE (IC_{50} of 244.55 ± 16.26 and 285.28 ± 15.97 μg/ml, respectively). Additionally, galanthamine was more active in inhibiting AChE (IC_{50} of 1.56 ± 0.004 μg/ml) than BChE (3.60 ± 0.45 μg/ml). Galanthamine is a selective, reversible, competitive acetylcholinesterase inhibitor used for the treatment of AD and was used as the standard

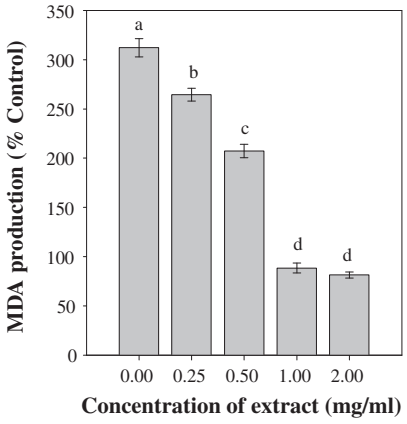

Fig. 3. The inhibition of Fe^{2+}-induced lipid peroxidation by the *L. viridis* extract in mouse brain homogenates. The percent inhibition of malondialdehyde (MDA) production was expressed with respect to basal brain tissue (without extract and pro-oxidant). Values represent the mean ± SE ($n = 3$). Mean values with different letters are significantly different at $p < 0.05$.

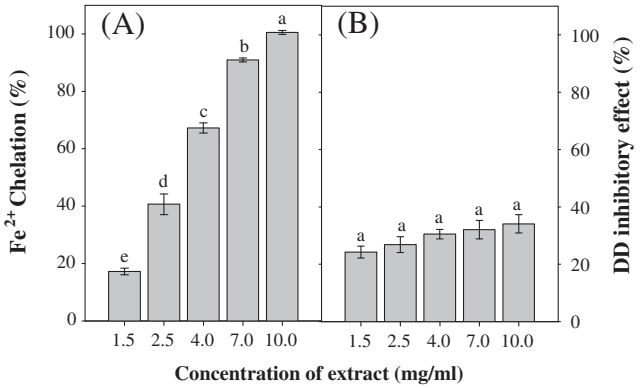

Fig. 4. (A) Fe^{2+} chelating ability and (B) Inhibition of deoxyribose decomposition (DD) by *L. viridis* extract. Values are expressed as the mean ± SE ($n = 3$). In each graph mean values with different letters are significantly different at $p < 0.05$.

inhibitor (Bores & Kosley, 1996). The anti-cholinesterase activity of other *Lavandula* species was previously reported (Adsersen, Gauguin, Gudiksen, & Jäger, 2006; Dohi et al., 2009; Ferreira, Proença, Serralheiro, & Araújo, 2006).

The inhibition of AChE and BChE by the *L. viridis* extract (50 mg/kg) and galanthamine (5 mg/kg) was also evaluated *in vivo*, using mouse brain homogenates as the source of the enzyme. The percent inhibition of AChE and BChE activities, 120 min after galanthamine injection, was similar to that of extract administration (Table 4). The results obtained in this study suggest that this extract could inhibit both enzymes *in vitro* and *in vivo*. The *in vitro* inhibitory effect of the extract was much lower than that of galanthamine. In contrast, in the *in vivo* study, the effects were similar. These differences can be explained by variations in the bioavailability of the active compounds in the extract and galanthamine, for the *in vitro* and *in vivo* models (Chattipakorn et al., 2007). The extract administration resulted in a significant decrease in AChE and BChE activities and no significant differences ($p \geqslant 0.05$) were found between SS and DS fractions. In this study, it was not possible to investigate which brain areas were affected by the plant compounds. Moreover, it is not known if the biological compounds reach the brain intact or whether they are metabolised. To clarify these issues, additional studies are necessary, such as histological and enzymatic analysis, which could establish the regional distribution in the brain, as well as determine which compounds or metabolites reach the brain (Perry, Houghton, Jenner, Keith, & Perry, 2002).

Ellman's method (Ellman et al., 1961) has been useful in the identification of new ChEIs. However, this method does not distinguish between whether the inhibition is at an enzymatic level or a result of the chemical reaction between thiocholine and DTNB, which would result in a false positive (Wang, Zhou, Gao, Wang, & Yao, 2007). Therefore, in the future, it will be important to investigate this phenomenon.

The ability of natural antioxidants, namely phenolic compounds, to protect cells from oxidative stress has been proven (Choi et al., 2010). Rosmarinic acid has several biological activities, namely anti-inflammatory, antibacterial, antiviral activities, and exhibits a strong scavenging activity for free radicals like $ONOO^-$ (Alkam, Nitta, Mizoguchi, Itoh, & Nabeshima, 2007; Tepe, 2008). Furthermore, it can protect against the reactive oxygen species induced by Aβ (Iuvone, De Filippis, Esposito, D'Amico, & Izzo, 2006). To understand the possible contribution of this compound to anti-cholinesterase inhibition, further studies, such as the evaluation of the activities of isolated compounds, would be necessary. The protective properties of caffeic acid, a compound also identified in this extract, against β-amyloid-induced neurotoxicity by the inhibition of calcium influx and tau phosphorylation was reported by Sul et al. (2009). It is known that preventive and symptomatic treatment of AD must include a multi-target drug strategy (Perry, Bollen, Perry, & Ballard, 2003). In this way, the results exhibited by the *L. viridis* extract seem to be promising because, apart from its antioxidant capacity, the extract simultaneously showed *in vitro* and *in vivo* anti-cholinesterase capacity.

Table 3
In vitro acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory effect by *L. viridis* extract and galanthamine (standard inhibitor).

	IC_{50} (μg/ml)[a]	
	AChE	BChE
Methanol extract	244.55 ± 16.26	285.28 ± 15.97
Galanthamine	1.56 ± 0.004	3.60 ± 0.45

Values are expressed as mean ± SE ($n = 3$).
[a] Concentration required to inhibit AChE or BChE activity by 50%.

Table 4
Effect of intraperitoneal administration of *L. viridis* extract (50 mg/kg) and galanthamine (standard inhibitor) (5 mg/kg) on *in vivo* acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities in mice brain.

Enzyme	Fraction	Inhibition (%)	
		Methanol extract	Galanthamine
AChE	SS	87.37 ± 2.25	63.22 ± 18.44
	DS	86.09 ± 7.43	91.34 ± 4.28
BChE	SS	68.02 ± 16.78	72.95 ± 11.83
	DS	73.23 ± 13.04	82.25 ± 12.31

SS – Salt soluble; DS – Detergent soluble. Values are expressed as mean ± SE ($n = 6$).

4. Conclusions

The present study demonstrated that the methanolic extract of *L. viridis* possesses a strong antioxidant activity through different mechanisms, which could be derived from compounds like phenolic acids and flavonoids, which were identified in the extract by HPLC–DAD. These results provide evident indicators that the free radical scavenging and strong metal-chelating effects of this extract may contribute to the prevention of Fe^{2+}-induced lipid peroxidation. Furthermore, this extract was efficient in inhibiting AChE and BChE activities *in vitro* and *in vivo*. The results indicate that

several targets relevant to the treatment of AD could be found in the *L. viridis* extract.

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Metabolic profile and biological activities of *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco: Studies on the essential oil and polar extracts

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ABSTRACT

We investigated the metabolic profile and biological activities of the essential oil and polar extracts of *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco collected in south Portugal. Gas chromatography–mass spectrometry (GC–MS) analysis revealed that oxygen-containing monoterpenes was the principal group of compounds identified in the essential oil. Camphor (40.6%) and fenchone (38.0%) were found as the major constituents. High-performance liquid chromatography with diode array detection (HPLC–DAD) analysis allowed the identification of hydroxycinnamic acids (3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic and rosmarinic acids) and flavones (luteolin and apigenin) in the polar extracts, with rosmarinic acid being the main compound in most of them. The bioactive compounds from *L. pedunculata* polar extracts were the most efficient free-radical scavengers, Fe^{2+} chelators and inhibitors of malondialdehyde production, while the essential oil was the most active against acetylcholinesterase. Our results reveal that the subspecies of *L. pedunculata* studied is a potential source of active metabolites with a positive effect on human health.

1. Introduction

Aromatic plants synthesize a diverse array of organic compounds with important ecological and physiological functions in plant-environment interactions. Essential oils contain secondary metabolites that, together with phenolic compounds, are among the most important ones produced by aromatic plants (Osbourn & Lanzotti, 2009). Monoterpenes and sesquiterpenes are the main constituents of essential oils. They are synthesized through condensations of the universal five-carbon precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which derive from two independent pathways (Dudareva et al., 2005). Phenolic compounds are a chemically heterogeneous group (simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans and lignins) biochemically synthesized *via* the shikimate and/or acetate pathways (Garcia-Salas, Morales-Soto, Segura-Carretero, & Fernandez-Gutierrez, 2010; Singer, Crowley, & Thompson, 2003).

Nowadays, the demand for plant-derived compounds by food, pharmaceutical and cosmetic industries has increased, because they are accepted by consumers as relatively safe and healthy compared to their synthetic counterparts. *Lavandula* species (Lamiaceae) are some of the most popular aromatic plants and are widely used in food, perfume and pharmaceutical industries (Boelens, 1995; Kim & Lee, 2002). Many *Lavandula* species produce compounds with antimicrobial (Hanamanthagouda et al., 2010; Zuzarte et al., 2009), antioxidant and anti-cholinesterases properties (Costa, Gonçalves, Andrade, Valentão, & Romano, 2011; Costa et al., 2012a; Matos et al., 2009). Natural antioxidants can efficiently protect cells against oxidative stress, because they can act as free radical scavengers, reducing agents, hydrogen donators

Abbreviations: Aβ, amyloid beta; AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; $ABTS^{\cdot+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; ATCI, acetylthiocholine iodide; AUC, area under the curve; BChE, butyrylcholinesterase; BHT, butylated hydroxytoluene; BTCI, butyrylthiocholine chloride; DTNB, 5,5′-dithiobis [2-nitrobenzoic acid]; EDTA, ethylenediaminetetraacetic acid; HD, hydrodistillation; HPLC–DAD, high-performance liquid chromatography–diode array detection; GC–MS, gas chromatography–mass spectrometry; MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; SDS, sodium dodecylsulphate; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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and transition metals chelators (Dai & Mumper, 2010). Based on the premise that free radicals are involved in neurodegenerative diseases, such as Alzheimer's disease (AD) (Andersen, 2004), natural antioxidants are of all interest. AD has a complex pathogenesis, being characterised by a deficit in the cholinergic system. The use of cholinesterase inhibitors is an efficient strategy for the treatment of AD, contributing to the enhancement of acetylcholine (ACh) levels in the synaptic cleft (cholinergic hypothesis) (Wilkinson, Francis, Schwam, & Payne-Parrish, 2004). Plant-derived compounds are used in the treatment of AD and this is a positive indicator that natural product discovery is important for AD therapy (Saklani & Kutty, 2008). *Lavandula pedunculata* (Miller) Cav. is an aromatic shrub common in the Iberian Peninsula and traditionally used in Portuguese medicine and as ornamental plant. Franco (1984) considered three subspecies for *L. pedunculata*: subsp. *pedunculata* in northwest Portugal, subsp. *sampaiana* in north and central Portugal, and subsp. *lusitanica* in central and south Portugal. Infusions prepared from flowered aerial parts are traditionally consumed to treat anxiety, insomnia, anorexia, cough and bronchitis (Proença da Cunha, Pereira da Silva, & Roque, 2003; Salgueiro, 2004). The essential oils from *L. pedunculata* have been studied by several authors in the last decades. For instance, the essential oils of *L. pedunculata* harvested in north and central Portugal are characterised by a high amount of oxygenated monoterpenes with 1,8-cineole (2.4–55.5%), fenchone (1.3–59.7%), and camphor (3.6–48.0%) as the most abundant and having an important antifungal activity (Zuzarte et al., 2009). In addition, the anti-acetylcholinesterase activity of the essential oil and extracts of *L. pedunculata* from eastern Portugal has been previously described (Ferreira, Proença, Serralheiro, & Araújo, 2006). In this work, *L. pedunculata* subsp. *lusitanica* (Chaytor) Franco [=*Lavandula* stoechas subsp. *lusitanica* (Chaytor) Rozeira] collected in south Portugal was studied because there are few reports concerning the biological potential of this subspecies. Only Matos et al. (2009) analysed the chemical profile and antioxidant activity of its essential oil. Therefore, we describe the metabolic profile and biological activity of the essential oil and polar extracts from *L. pedunculata* subsp. *lusitanica*. The qualitative and quantitative analysis of the essential oil and extracts was performed by gas chromatography coupled to mass spectrometry (GC–MS) and by high-performance liquid chromatography with diode-array detection (HPLC–DAD), respectively. The antioxidant activity was evaluated by measuring the Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), Fe^{2+}-chelation activity and the inhibition of Fe^{2+}-induced lipid peroxidation in mouse brain homogenates (*in vitro*) and the anti-cholinesterases activity was assessed by the Ellman's method. To our knowledge this is the first report about the phenolic profile and biological potential of extracts from this subspecies and investigations on its essential oil are still scarce.

2. Materials and methods

2.1. Standards and reagents

Apigenin, 5-*O*-caffeoylquinic acid, luteolin and rosmarinic acid were purchased from Extrasynthése (Genay, France). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, potassium persulphate ($K_2S_2O_8$), thiobarbituric acid (TBA), trizma base (Tris), acetylthiocholine iodide (ATCI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), acetylcholinesterase (AChE) (Electric-eel, EC 3.1.1.7, Type VI-S), butyrylcholinesterase (BChE) (horse-serum, EC 3.1.1.8), galantamine hydrobromide, malondialdehyde tetrabutylammonium salt (MDA) and butyrylthiocholine chloride (BTCI) were purchased from Sigma–Aldrich (Steinheim, Germany). Formic acid, methanol and iron(II) sulphate ($FeSO_4$) were acquired from Merck (Darmstadt, Germany). Fluorescein, 1,10-phenanthroline and absolute ethanol were obtained from Panreac (Barcelona, Spain). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), butylated hydroxytoluene (BHT) and sodium dodecylsulphate (SDS) were purchased from Acros Organics (Geel, Germany). Qualitative filter paper was purchased from VWR (Leuven, Belgium). Ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) were purchased from Fluka (Steinheim, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Plant material and extraction procedure

The aerial parts of *L. pedunculata* subsp. *lusitanica* plants were collected during the flowering period at Campus de Gambelas (Algarve, south Portugal). A voucher specimen was deposited in the herbarium of the University of Algarve under the number ALGU 8080. The plant material was dried at room temperature, ground to powder in a blender to achieve a mean particle size lower than 2 mm and stored at −20 °C until required.

Essential oil was obtained by hydrodistillation (HD) for 3 h, using 50 g of plant material in a Clevenger-type apparatus. Extraction of phenolic compounds was performed using water and ethanol separately and in a 1:1 mixture. The plant material (10 g) was soaked overnight at room temperature in 200 ml of each solvent and the resulting extract was filtered through a 5–13 μm membrane. An infusion was also prepared by homogenizing 1 g of the plant material in 20 ml hot water (90 °C) for 5 min. Finally, the extracts were concentrated to dryness (the water extracts were lyophilized and the ethanol and water:ethanol extracts were dried in a rotary evaporator) and stored at −20 °C. The extraction yields (w/w, in terms of initial dried material) were 1.1% for the essential oil and 22.5%, 22.4%, 19.4% and 19.6% for infusion, water, water:ethanol and ethanol extracts, respectively.

2.3. GC–MS analysis

The essential oil was analysed using an Agilent 6890 Series gas chromatograph equipped with an Agilent 7683 automatic liquid sampler coupled to an Agilent 5973N mass selective detector (Agilent Technologies, Little Falls, DE, USA). A programmed temperature vaporisation injector with a liner filled with glass wool was used operating in the split mode injection (1:100) heated at a constant temperature of 275 °C. The samples were injected using a volume of 1 μl. Helium was used as a carrier gas at a constant pressure mode (13.9 psi). GC analysis was performed on a TRB-5MS (30 m × 0.25 mm ID, 0.25 μm film thickness) capillary column (5% diphenyl, 95% dimethylpolysiloxane; Teknokroma, Spain). The oven temperature was set at 50 °C for 1 min, followed by an increase of 5 °C/min to 240 °C in a 39 min total run. All mass spectra were acquired in electron impact (EI) mode. The transfer line, ion source, and quadrupole analyser temperatures were 280, 230, and 150 °C, respectively, and a solvent delay of 3 min was selected. Fullscan mode electron ionisation mass spectra were recorded, in the range 35–550 Da at 70 eV electron energy, with an ionisation current of 34.6 μA. Data recording and instrument control were performed by the MSD ChemStation software (G1701CA; Version C.00.00; Agilent Technologies). Repeatability was verified by analysing the sample three times and the components were identified by comparison of their retention index, relative to a standard mixture of n-alkanes (Adams, 2001) and by comparison with the Wiley's library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies).

2.4. HPLC–DAD analysis

The extracts were analysed on a HPLC unit (Gilson), using a Spherisorb ODS2 column (4.6 × 250 mm, 5 μm, particle size). The injection volume was 20 μl. The solvent system used was of a gradient of water–formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% of B at 3 min, 25% of B at 13 min, 30% of B at 25 min, 35% of B at 35 min, 45% of B at 39 min, 45% of B at 42 min, 50% of B at 44 min, 55% of B at 47 min, 70% of B at 50 min, 75% of B at 56 min and 80% of B at 60 min, at a solvent flow rate of 0.9 ml/min. Detection was achieved with a Gilson Diode Array Detector (DAD). Spectral data from all peaks were accumulated in the range 200–400 nm, and chromatograms were recorded at 320 and 350 nm for phenolic acids and flavones, respectively. The data were processed on an Unipoint® System software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was determined using the software contrast facilities. The compounds in each extract were identified by comparing their retention times and UV–Vis spectra in the 200–400 nm range with authentic standards and with the library of spectra, previously compiled by the authors. Quantification of phenolic compounds was achieved by the absorbance recorded in the chromatograms relative to external standards. 3-*O*-Caffeoylquinic and 4-*O*-caffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid and the other compounds were quantified as themselves.

The detection limit values were calculated as the concentration corresponding to three times the standard deviation of the background noise and varied between 1.8 and 9.7 μg/ml. The quantification limits, determined as the concentration corresponding to 10 times the standard deviation of the background noise, ranged between 6.0 and 32.2 μg/ml.

Repeatability was checked by analysing the same sample three times, by the same analyst, within the same day. The coefficients of variation ranged from 0.1% to 1.1%, indicating that the repeatability of the procedure was good. Intermediate precision determined by different analysts on three separate days was also found to be satisfactory (coefficients of variation ranging from 1.4% to 2.2%).

2.5. Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant activity was determined by the TEAC assay using the radical cation $ABTS^{\cdot+}$, according to the procedure proposed by Re et al. (1999). The $ABTS^{\cdot+}$ stock solution (7 mM) was prepared following the described recommendations, using potassium persulphate as the oxidant agent. The absorbance was read at 734 nm, 1 min after the initial mixing. The sample dilution that produced 20–80% inhibition of the blank absorbance was used for TEAC calculation and the results were expressed as Trolox equivalents (TE) per gram of extract.

2.6. Oxygen radical absorbance capacity (ORAC) assay

Total antioxidant capacity was evaluated as described by Gillespie, Chae, and Ainsworth (2007). Fluorescein was used as the fluorescent probe and AAPH as the peroxyl radical generator. The decrease in fluorescence was determined by collecting readings for excitation at 485 nm and emission at 530 nm every minute, for 90 min. The ORAC value for each extract was calculated using the respective area under the curve (AUC) and the regression equation between Trolox equivalents and the net AUC. The results were expressed as TE per gram of extract.

2.7. Fe^{2+} chelation assay

The ability of the essential oil and extracts of *L. pedunculata* to chelate ferrous ions was measured using the method proposed by Minotti and Aust (1987), which was modified by Puntel, Nogueira, and Rocha (2005). Briefly, 500 μM of freshly-prepared $FeSO_4$ was added to a reaction mixture comprising 0.1 M Tris–HCl (pH 7.4), 0.9% NaCl and the extract or 0.1 M Tris–HCl (blank) or reference (EDTA). After a 5 min incubation time at room temperature, 0.35% 1,10-phenanthroline (w/v) was added and the absorbance at 510 nm was determined.

2.8. Lipid peroxidation assay

The mice brain homogenates were obtained as described previously (Costa et al., 2011) and lipid peroxidation was estimated by measuring thiobarbituric acid-reactive substances (TBARS), as reported by Ohkawa, Ohishi, and Yagi (1979). The brain homogenate was incubated at 37 °C, for 1 h in a medium containing 0.1 M Tris–HCl buffer (pH 7.4), sample or references (Trolox and BHT), 250 μM of freshly-prepared $FeSO_4$ and distilled water. After incubation, the reaction was stopped by adding 8.1% SDS, acetic acid/HCl (pH 3.4) and 0.8% TBA. TBARS were measured by determining the absorbance at 532 nm and using a standard curve of MDA.

Table 1
Chemical composition (relative %) of the essential oil obtained by hydrodistillation (HD) from the aerial parts of *Lavandula pedunculata* subsp. *lusitanica*.

Components	RI[a]	%[b]
α-Pinene	929	0.1
Camphene	933	tr
Oct-1-en-3-ol	974	tr
p-Cimene	991	tr
1,8-Cineole	995	0.9
cis-β-Ocimene	1022	tr
cis-Linalool oxide	1040	0.8
Fenchone	1055	38.0
Linalool	1073	2.0
α-Fenchol	1085	2.6
α-Campholenal	1092	0.1
Camphor	1112	40.6
cis-Verbenol	1117	0.8
Pinocarvone	1127	0.1
Borneol	1135	0.8
Terpinene-4-ol	1145	0.3
p-Cymen-8-ol	1163	1.3
Myrtenal	1169	tr
Verbenone	1179	1.5
trans-Carveol	1197	0.4
Carvone	1218	0.5
Bornyl acetate	1259	0.4
Lavandulyl acetate	1272	0.2
β-Caryophyllene	1394	tr
Germacrene D	1440	tr
Eremophilene	1465	1.4
δ-Cadinene	1502	0.1
α-Calacorene	1520	tr
Caryophyllene oxide	1557	0.1
Globulol	1569	0.2
Viridiflorol	1582	0.2
Muurolol	1617	0.1
α-Cadinol	1630	1.3
Monoterpene hydrocarbons		0.1
Oxygen-containing monoterpenes		90.6
Sesquiterpene hydrocarbons		1.6
Oxygen-containing sesquiterpenes		2.0
Others		0.6
Identified compounds (%)		94.9

[a] RI, retention index.
[b] Normalized peak area abundances without using the correction factors; tr, traces (<0.05%).

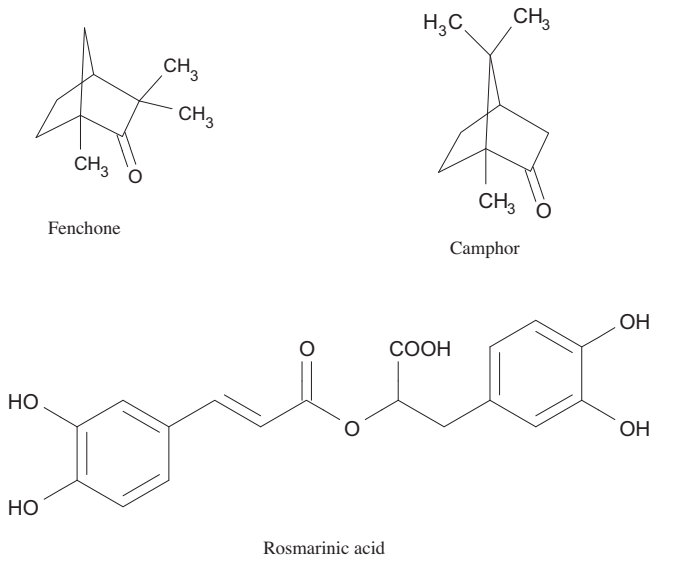

Fig. 1. Structures of the main compounds found in the essential oil and polar extracts from *Lavandula pedunculata* subsp. *lusitanica*.

2.9. In vitro AChE and BChE inhibition

AChE and BChE activities were measured using a 96-well microplate reader, according to Ellman, Courtney, Andres, and Feather-Stone (1961). We mixed 3 mM DTNB, 15 mM substrate, ATCI or BTCI, 100 mM phosphate buffer (pH 8.0) and sample, buffer or the reference compound galantamine. Finally, we added 0.28 U/ml AChE or BChE and measured the absorbance at 405 nm for 5 min. The enzymatic activity was calculated as a percentage of the velocities in the reaction mixtures compared with that of the control reactions.

2.10. Statistical analysis

The data were presented as the mean ± standard error from triplicate experiments. The results were processed by one-way analysis of variance (ANOVA) to assess treatment differences using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA). Significant differences between mean values were determined using Duncan's New Multiple Range Test.

3. Results and discussion

3.1. Chemical analysis

The essential oil was analysed by gas chromatography–mass spectrometry (GC–MS) and the compounds are listed in Table 1, following their order of elution on the TRB-5MS capillary column. Thirty-three constituents were identified, which represents 94.9% of the total essential oil analysed. Oxygen-containing monoterpenes (90.6%) were found to be the main group of compounds, followed by oxygen-containing sesquiterpenes (2.0%), sesquiterpene hydrocarbons (1.6%) and monoterpene hydrocarbons (0.1%). The major compounds identified were camphor (40.6%) and fenchone (38.0%) (Fig. 1). The chemical profile found is in partial agreement with previous analysis, which likewise shows the abundance of oxygen-containing monoterpenes, although with quantitative differences in what concerns to the levels of several compounds (Matos et al., 2009; Zuzarte et al., 2009).

As expected, the extraction of phenolic compounds is influenced by the nature and polarity of the solvent system, extraction time and temperature, as well as by the characteristics of the plant material (Garcia-Salas et al., 2010). In this study, we obtained polar extracts by using environmental friendly solvent systems. The chemical analysis by high-performance liquid chromatography–diode array detection (HPLC–DAD) (Table 2), revealed the presence of hydroxycinnamic acids (3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic and rosmarinic acids) and flavones (luteolin and apigenin). These compounds were found in all extracts, excepting 3-*O*-caffeoylquinic acid, which was not present in water:ethanol and ethanol extracts. To the best of our knowledge, this is the first report describing the phenolic content of *L. pedunculata* subsp. *lusitanica* extracts. The water:ethanol extract showed the highest phenolic content (14,256 mg/kg), followed by the infusion (12,850 mg/kg), ethanol (7248 mg/kg) and water (997 mg/kg) extracts. Recent reports suggest water:ethanol (1:1) as a suitable solvent for the extraction of phenolic compounds from Lamiaceae species (Costa et al., 2012b; Miron, Plaza, Bahrim, Ibanez, & Herrero, 2011). As previously described in other *Lavandula* species (Costa et al., 2011), rosmarinic acid was the main compound (Fig. 1), excepting in the water extract, in which 4-*O*-caffeoylquinic acid was dominant. The ethanol was the least efficient solvent in extracting caffeoylquinic acids derivatives.

3.2. Biological activities

The radical scavenging effects of the essential oil and polar extracts from *L. pedunculata* subsp. *lusitanica* were assessed by Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays and the results are listed in Table 3 The infusion was the most efficient at neutralizing both $ABTS^{\cdot+}$ and peroxyl radicals, in contrast with the essential oil, which was the least active. The weak antioxidant activity of the essential oil has been described previously using other methods (Matos et al., 2009). The high antiradical activity of the extracts could be attributed, at least partially, to the presence of rosmarinic and 5-*O*-caffeoylquinic acids (Fukumoto & Mazza, 2000; Petersen & Simmonds, 2003). Their high redox potential allows them to act as hydrogen donors and singlet oxygen quenchers (Miguel, 2010).

Other important characteristic of natural antioxidants is their capacity to chelate transition metals (Sahreen, Khan, & Khan,

Table 2
Phenolic compounds in polar extracts from *Lavandula pedunculata* subsp. *lusitanica* (mg/kg)[a].

	Rt (min)	Infusion	Water	Water:ethanol	Ethanol
3-*O*-Caffeoylquinic acid	10.4	n.q.	11.9 ± 0.5	n.d.	n.d.
4-*O*-Caffeoylquinic acid	15.2	53.3 ± 2.7 c	692 ± 24.3 a	173 ± 6.4 b	n.q.
5-*O*-Caffeoylquinic acid	15.9	1232 ± 19.1 a	130 ± 8.9 c	299 ± 12.4 b	n.q.
Rosmarinic acid	38.0	11,454 ± 32.0 a	150 ± 0.4 c	6073 ± 44.3 b	6246 ± 386 b
Luteolin	51.8	112 ± 0.4 b	12.5 ± 0.5 b	4975 ± 270 a	234 ± 3.6 b
Apigenin	54.2	n.q.	n.q.	2736 ± 54.7 a	768 ± 31.5 b
Σ		12,850 b	997 d	14,256 a	7248 c

[a] Values are expressed as mean ± standard deviation (SD) ($n = 3$); n.d.: not detected; n.q.: not quantified. Values followed by different letters within the same line are significantly different ($p < 0.05$).

Table 3
Antiradical activities of the essential oil and polar extracts from *Lavandula pedunculata* subsp. *lusitanica*, assessed by Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays.

Extract	TEAC ($\mu mol_{TE}/g_{extract}$)	ORAC ($\mu mol_{TE}/g_{extract}$)
Infusion	866 ± 12.5 a	3018 ± 91.1 a
Water	569 ± 1.99 c	1530 ± 121 c
Water:ethanol	688 ± 10.59 b	2567 ± 151 b
Ethanol	224 ± 6.41 d	861 ± 6.00 d
Essential oil	NA	157 ± 10.6 e

Values are expressed as mean ± SE ($n = 3$). NA – Not active. Values followed by different letters within the same column are significantly different at $p < 0.05$.

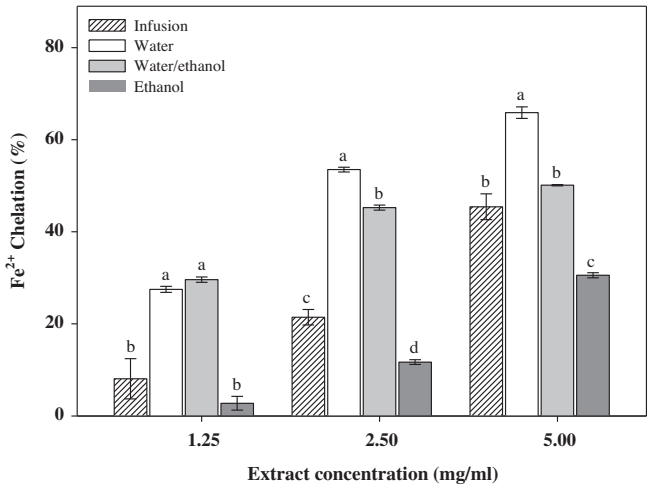

Fig. 2. Chelating effects of the polar extracts from *Lavandula pedunculata* subsp. *lusitanica*. Values represent the mean ± SE ($n = 3$). For each concentration, values followed by different letters are significantly different ($p < 0.05$). Reference (EDTA) = 98.1 ± 0.25% (0.30 mg/ml).

2010). Iron is crucial for normal cellular functions, but in increased amounts it can induce damage in lipids, proteins and nucleic acids

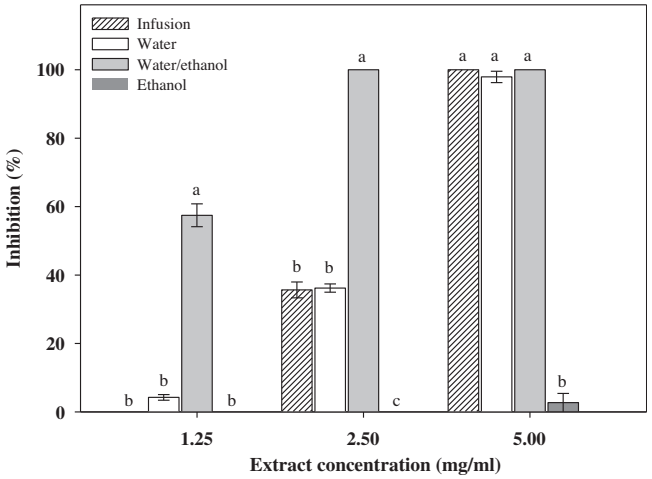

Fig. 3. Effects of the polar extracts of *Lavandula pedunculata* subsp. *lusitanica* on Fe^{2+}-induced lipid peroxidation in mouse brain homogenates (*in vitro*). The inhibition of malondialdehyde (MDA) production was expressed with respect to basal brain tissue (in the presence of the pro-oxidant and without extract) that was taken as 100%. The essential oil was not able to inhibit MDA production. Antioxidant references: BHT = 64.2 ± 4.47% (0.30 mg/ml) and Trolox = 53.3 ± 7.37% (0.20 mg/ml). Values represent the mean ± SE ($n = 3$). For each concentration, values followed by different letters are significantly different ($p < 0.05$).

(Britton, Leicester, & Bacon, 2002). Thus, the depletion of free iron by natural antioxidants can attenuate iron-promoted radical production. In addition, it has been suggested that amyloid beta (Aβ) peptide, in the presence of biometals and oxygen, generates reactive oxygen species in neuronal cell membranes (Butterfield & Lauderback, 2002; Rauk, 2008). In this sense, chelation therapy can be an efficient strategy against AD, by decreasing Aβ-mediated oxidative damage (Bandyopadhyay, Huang, Lahiri, & Rogers, 2010). Therefore, the Fe^{2+}-chelating ability of *L. pedunculata* subsp. *lusitanica* essential oil and polar extracts was assessed. Overall, the capacity of the extracts to chelate Fe^{2+} increased in a dose-dependent manner, water (65.9 ± 1.27%) and water:ethanol (50.1 ± 0.14%) extracts showing the best results (Fig. 2). On the contrary, the essential oil had no positive effect.

Lipid peroxidation is a free radical-induced process that compromises structural and protective cell membrane functions, thereby jeopardizing its viability (Niki, Yoshida, Saito, & Noguchi, 2005). In this study, the extent of lipid peroxidation was determined by measuring the production of malondialdehyde (MDA), which can be quantified colorimetrically following its controlled reaction with thiobarbituric acid (TBA). The capacity of the *L. pedunculata* subsp. *lusitanica* to inhibit Fe^{2+}-induced lipid peroxidation in homogenized mouse brain tissue (Fig. 3) was compared with that of BHT (64.2 ± 4.47%; 0.30 mg/ml) and Trolox (53.3 ± 7.37%; 0.20 mg/ml). At the highest concentration tested (5 mg/ml), the infusion, water and water:ethanol extracts completely prevented MDA production.

Our results reveal that the polar extracts were more effective than the essential oil, which was not able to inhibit MDA production. It is well known that phenolic compounds are able to donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first ignition step (Miguel, 2010). However, in this work we observed that the water extract even possessing low phenolic content demonstrated a high capacity to inhibit MDA production (Table 2, Fig. 3), suggesting that other compounds may also play an important role in the antioxidant activity of the extracts. Our results indicate that active metabolites from *L. pedunculata* subsp. *lusitanica* can prevent cells from Fe^{2+}-induced lipid peroxidation, acting as Fe^{2+} chelators and/or free radicals scavengers.

The essential oil and polar extracts were also tested as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors, and the results are listed in Table 4. All the extracts and the essential oil were active against cholinesterases, with the essential oil being the most effective inhibitor against AChE (57.2 ± 0.43%). This fact may be explained by the presence of terpenes in the essential oil, which represent one of the major classes of compounds reported as effective cholinesterases inhibitors (Mukherjee, Kumar, Mal, & Houghton, 2007). Some of the compounds identified in the essential oil (Table 1), as α-pinene and 1,8-cineole, are potent cholinesterases inhibitors even at low concentrations and probably contribute for this activity (Dohi, Terasaki, & Makino, 2009; Savelev, Okello, Perry, Wilkins, & Perry, 2003). However, we cannot

Table 4
In vitro cholinesterase inhibitory activity of the essential oil and polar extracts from *Lavandula pedunculata* subsp. *lusitanica*.

Extract	Inhibition (%)[a]	
	AChE	BChE
Infusion	31.7 ± 1.36 d	18.4 ± 0.65 b
Water	29.1 ± 0.40 e	36.9 ± 2.90 a
Water:ethanol	39.1 ± 0.36 c	45.5 ± 5.24 a
Ethanol	44.5 ± 0.60 b	46.9 ± 4.91 a
Essential oil	57.2 ± 0.43 a	48.2 ± 0.42 a

Reference (Galantamine, 5 μg/ml): AChE = 67.78 ± 4.36% and BChE = 40.16 ± 0.76.
[a] Percent inhibition at the final concentration of 2.5 mg/ml. Values followed by different letters within the same column are significantly different at $p < 0.05$.

ignore the synergistic and antagonistic interactions between some terpenes and, therefore, the effect exhibited might result from the collective activities of different constituents present in the essential oil (Dohi et al., 2009). The anti-AChE activity of the essential oil and extracts of *L. pedunculata* from eastern Portugal has been previously described (Ferreira et al., 2006). However, since the author did not specified the subspecies used, we cannot directly compare the results.

We observed that the extracts possess a higher antioxidant activity than the essential oil, but in contrast the essential oil showed the best capacity to inhibit cholinesterases, particularly, AChE. Because Alzheimer's disease (AD) is a multifactorial disease, natural products with different targets and pathways may have a greater potential for its preventive and/or therapeutic success by acting as antioxidant agents and/or cholinesterases inhibitors.

4. Conclusions

In this study, we describe the metabolic profile and biological activity of the essential oil and polar extracts from *L. pedunculata* subsp. *lusitanica* in south Portugal. Because free radicals are involved in AD, we investigated the potential of metabolites from *L. pedunculata* to act as antioxidants and cholinesterases inhibitors. We found that the extracts were the most efficient free-radical scavengers, Fe^{2+} chelators and inhibitors of MDA production, but the essential oil was the most active against AChE. Regarding the extracts, the results indicated that the extraction solvent influenced the recovery of target active metabolites – the water and aqueous:organic solvent mixture were the most suitable choice. This research suggests *L. pedunculata* subsp. *lusitanica* as a source of natural compounds able to prevent neurodegenerative diseases.

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CHAPTER 3

BIOCOMPOUNDS FROM WILD PLANTS AND *IN VITRO* CULTURES

Costa P, Gonçalves S, Valentão P, Andrade PB, Coelho N, Romano A (2012). *Thymus lotocephalus* wild plants and *in vitro* cultures produce different profiles of phenolic compounds with antioxidant activity. Food Chemistry, 135, 1253-1260.

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Thymus lotocephalus wild plants and *in vitro* cultures produce different profiles of phenolic compounds with antioxidant activity

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ABSTRACT

We compared the phenolic metabolites and antioxidant activities of *Thymus lotocephalus* G. López & R. Morales wild plants and *in vitro* cultures using different extraction solvents. HPLC–DAD analysis allowed the identification and quantification of phenolic (caffeic and rosmarinic) acids and flavones (luteolin and apigenin) in extracts from both sources. The *in vitro* cultures accumulated large amounts of rosmarinic acid. However, extracts from both sources were able to neutralise free radicals in different test systems (TEAC and ORAC assays), to form complexes with Fe^{2+} and to protect mouse brains against Fe^{2+}-induced lipid peroxidation. The solvent significantly influenced the phenolic content and antioxidant activity of the extracts, water/ethanol being the most efficient for the extraction of antioxidant phytochemicals. We conclude that *in vitro* cultures of *T. lotocephalus* represent a promising alternative for the production of valuable natural antioxidants and an efficient tool for the *in vitro* biosynthesis of rosmarinic acid, therefore avoiding the need to exploit populations of wild plants.

1. Introduction

Free radicals are an integral component of many redox processes in eukaryotic cells, which maintain homeostasis by synthesising antioxidant enzymes and metabolites. Excess free radicals are produced under stress and in response to pathogens, often resulting in oxidative damage to lipids, proteins and nucleic acids. In humans, this damage is implicated in ageing and in numerous diseases (Valko et al., 2007). Synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been used in food processing and preservation (Botterweck, Verhagen, Goldbohm, Kleinjans, & van den Brandt, 2000; Hinneburg, Dorman, & Hiltunen, 2006). However, these chemicals are toxic and their risk to health has increased the demand for natural antioxidants (Liu et al., 2011).

Phenolic compounds can prevent oxidative damage via a number of different mechanisms, such as free radical scavenging, transition metal chelation and interactions with lipid membranes, proteins and nucleic acids (Dai & Mumper, 2010). The spectrum of phenolic compounds isolated from plants depends on several factors, such as the nature of the tissue matrix, the extraction time and temperature, and the polarity of the solvent system (Dai & Mumper, 2010). Water, ethanol, methanol, acetone and aqueous/organic solvent mixtures are frequently used to extract phenolic compounds from plants (Chew et al., 2011; Sultana, Anwar, & Ashraf, 2009; Trabelsi et al., 2010).

Plants of the mint family (Lamiaceae) produce many metabolites with bioactive properties, but large-scale extraction is challenging because the family is genetically heterogeneous (Shetty, 1997). For this reason, there is great interest in the production of specific metabolites under controlled environmental conditions that maintain a given phytochemical profile (Makunga & van Staden, 2008; Pérez-Tortosa, López-Orenes, Martínez-Pérez, Ferrer, & Calderón, 2012; Shetty, 1997; Zuzarte, Dinis, Cavaleiro, Salgueiro, & Canhoto, 2010).

Thymus is a taxonomically complex genus of aromatic plants that is widely distributed in the Mediterranean region and

Abbreviations: AAPH, 2,2'-azobis (2-methylpropionamidine) dihydrochloride; $ABTS^{\cdot+}$, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation; BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; F–C reagent, Folin–Ciocalteu reagent; GAE, gallic acid equivalents; HAT, hydrogen atom transfer; HPLC–DAD, high-performance liquid chromatography–diode array detection; MDA, malondialdehyde; MS, Murashige and Skoog medium; ORAC, oxygen radical absorbance capacity; SET, single electron transfer; TBA, thiobarbituric acid; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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traditionally used as culinary herbs, ornamental plants and flavouring agents (Figueiredo, Miguel, Duarte, Barroso, & Pedro, 2001; Figueiredo et al., 2008). *Thymus* spp. also produce secondary metabolites that can be used as antioxidants, expectorants, antitussives, antiplatelet drugs, antimicrobials and drugs for the treatment of skin disorders (Dandlen et al., 2010; Faleiro et al., 2003; Figueiredo et al., 2008; Hazzit, Baaliouamer, Veríssimo, Faleiro, & Miguel, 2009; Oh et al., 2009; Okazaki, Kawazoe, & Takaishi, 2002). *Thymus lotocephalus* G. López & R. Morales is an aromatic species endemic to the Algarve region, which blossoms from April to June and is typically found in dry open areas and scrublands (Figueiredo et al., 2001, 2008). It produces phytochemicals with antimicrobial, antioxidant and anticholinesterase activities, although only for a limited time (Costa et al., 2012; Faleiro et al., 2003). The species is considered to be critically endangered and is legally protected by both the European Habitats Directive (92/43/CEE) and by Portuguese law (Reference 140/99, April 24 1999; ICN, 2006). These restrictions mean that natural populations cannot be exploited as a source of bioactive compounds and alternatives are required.

Biotechnology can be used to facilitate the large-scale production of plant material without a negative impact on natural habitats (Matkowski, 2008; Zuzarte et al., 2010). In particular, *in vitro* culture under controlled environmental conditions allows bioactive compounds to be extracted throughout the year, with no seasonal constraints (Dias, Barros, Sousa, & Ferreira, 2011; Shaik, Singh, & Nicholas, 2011; Zuzarte et al., 2010). We have therefore recently developed a micropropagation protocol for *T. lotocephalus* that could be used to replenish natural populations and also to generate *in vitro* cultures for the extraction of phytochemicals (Coelho, Gonçalves, González-Benito, & Romano, 2012). The suitability of the latter approach depends on the ability of micropropagated plants to synthesise useful phenolic profiles, but the capacity of both wild plants and *in vitro* cultures to produce phenolic molecules is not understood in detail.

We therefore set out to compare the phenolic profiles of wild plants and *in vitro* cultures, and to evaluate the effect of different solvents on the recovery of phenolic compounds with antioxidant activity by measuring the Trolox equivalent antioxidant capacity, oxygen radical absorbance capacity, Fe^{2+} chelation activity and the inhibition of Fe^{2+}-induced lipid peroxidation in mouse brains. The phenolic compounds present in *T. lotocephalus* extracts were identified and quantified by high-performance liquid chromatography with diode-array detection (HPLC–DAD).

2. Materials and methods

2.1. Standards and reagents

Apigenin, luteolin and rosmarinic acid were purchased from Extrasynthése (Genay, France). Caffeic acid, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, potassium persulphate, thiobarbituric acid (TBA) and Triton X-100 were purchased from Sigma–Aldrich (Steinheim, Germany). Formic acid, methanol, iron(II) sulphate ($FeSO_4$) were acquired to Merck (Darmstadt, Germany). Fluorescein, 1,10-phenanthroline and absolute ethanol were obtained from Panreac (Barcelona, Spain). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), butylated hydroxytoluene (BHT) and sodium dodecylsulphate (SDS) were purchased from Acros Organics (Geel, Germany). Qualitative filter paper, Folin–Ciocalteu (F–C) reagent, gallic acid and sodium carbonate were purchased from VWR (Leuven, Belgium). Ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) were purchased from Fluka (Steinheim, Germany).

2.2. Plant material

Aerial parts from *T. lotocephalus* were collected from wild plants growing in a natural population in Algoz (Algarve region, Portugal) during the flowering period. The *T. lotocephalus* voucher was previously deposited in the Herbarium of the University of Algarve (number ALGU 8081).

In vitro cultures of *T. lotocephalus* were produced as previously described (Coelho et al., 2012). Briefly, plants were cultured in MS medium (Murashige & Skoog, 1962) supplemented with 2% (w/v) sucrose and 1% (w/v) agar. The cultures were incubated at 25 ± 2 °C with a 16-h photoperiod (cool white fluorescent lamps, 40 μmol m^{-2} s^{-1}) and were subcultured every 6 weeks.

2.3. Extraction procedure

Material from wild plants and *in vitro* cultures was dried at 40 °C and powdered in a blender to achieve a mean particle size less than 2 mm. We used water and ethanol separately and in a 1:1 mixture to generate extracts with different compositions. The plant material (10 g) was soaked overnight at room temperature in 200 ml of each solvent. After soaking, the mixture was filtered through a membrane with a retention size of 5–13 μm; the extract was concentrated to dryness (the water extract was lyophilised and the ethanol and water/ethanol extracts were dried in a rotary evaporator) and the residue stored at −20 °C.

2.4. Identification and quantification of phenolic compounds by HPLC–DAD

The extracts were analysed by HPLC (Gilson, Villiers le Bel, France) using a Spherisorb ODS2 column (4.6 × 250 mm, 5 μm particle size) and a solvent system comprising solvent A (19:1 water/formic acid) and solvent B (methanol). The gradient comprised 5% solvent B at 0 min increasing to 15% at 3 min, 25% at 13 min, 30% at 25 min, 35% at 35 min, 45% at 39 min and 42 min, 50% at 44 min, 55% at 47 min, 70% at 50 min, 75% at 56 min and 100% at 60 min. The injection volume was 20 μl and the flow rate was 0.9 ml/min. Detection was achieved with a Gilson diode array detector. Spectral data from all peaks were collected in the 200–400 nm range, and chromatograms were recorded at 320 and 350 nm for phenolic acids and flavones, respectively. The data were processed using the Unipoint Software platform (Gilson Medical Electronics). Peak purity was determined using the software contrast facilities. Phenolic compounds were quantified by comparison with standards.

2.5. Total phenolic content (F–C assay)

The Folin–Ciocalteu (F–C) colorimetric method was carried out as described by Ainsworth and Gillespie (2007), with slight modifications. Briefly, 10% (v/v) F–C reagent was added to each sample, standard or phosphate buffer (blank) in a microtube. A 700 mM sodium carbonate solution was added to each microtube and incubated for 2 h at room temperature, then 200 μl were transferred to a clear 96-well microplate (NUNC, Rochester, NY) and the absorbance at 765 nm was determined using an Infinite 200 microplate reader (Tekan, Grödig, Austria). The standard curve was calculated using gallic acid and the results were expressed as gallic acid equivalents (GAE) per gram of extract. All experiments were carried out in triplicate.

2.6. Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant activity was determined using the TEAC assay with the radical cation $ABTS^{\cdot+}$ as described by Re et al. (1999). The 7 mM

$ABTS^{\cdot+}$ stock solution was prepared using potassium persulphate as the oxidising agent. The absorbance was determined at 734 nm, 1 min after mixing. The sample dilution that achieved 20–80% inhibition of the blank absorbance was used to calculate the TEAC values and the results were expressed as Trolox equivalents (TE) per gram of extract. All experiments were carried out in triplicate.

2.7. Oxygen radical absorbance capacity (ORAC) assay

Antioxidant capacity was evaluated as described by Gillespie, Chae, and Ainsworth (2007) using fluorescein as the fluorescent probe and AAPH as a peroxyl radical generator. A black microplate (NUNC) was loaded with 0.08 μM fluorescein plus the sample, Trolox standard or phosphate buffer (blank), and the reaction was initiated by adding 150 mM AAPH to each well after incubating for 10 min at 37 °C. The reduction in fluorescence was determined by reading fluorescein excitation at 485 nm and emission at 530 nm every minute for 90 min. The ORAC value for each extract was calculated using the area under the curve (AUC) and the regression equation between the TE and the net AUC. The results were expressed as TE per gram of extract. All experiments were carried out in triplicate.

2.8. Fe^{2+} chelation assay

The ability of extracts to chelate Fe^{2+} was determined as described by Minotti and Aust (1987), with modifications by Puntel, Nogueira, and Rocha (2005). Briefly, 500 μM of freshly-prepared $FeSO_4$ were added to a reaction mixture comprising 0.1 M Tris–HCl (pH 7.4), 0.9% NaCl and the extract or 0.1 M Tris–HCl (blank) or reference (EDTA). After a 5-min incubation at room temperature, we added 0.35% 1,10-phenanthroline (w/v) and determined the absorbance at 510 nm. All experiments were carried out in triplicate.

2.9. Preparation of brain homogenates

C57BL/6 mice (male, 25–30 g, 3–4 months old) were sourced from the animal house at the Centre for Molecular and Structural Biomedicine, University of Algarve, Portugal, and were housed in polyacrylic cages at 20–23 °C and 40–55% relative humidity, with *ad libitum* access to food. The animal experiments conformed to ethical standards for clinical research and EU guidelines. Mice were killed by CO_2 asphyxiation and the cerebral tissue (whole brain) was rapidly dissected, placed on ice and weighed, and then homogenised in 0.1 M Tris–HCl (pH 7.4) (1/10, w/v). The homogenate was centrifuged (Eppendorf, Centrifuge 5804 R, Hamburg, Germany) for 10 min at 3000*g* and the low-speed supernatant was used for the lipid peroxidation assay (Bellé, Dalmolin, Fonini, Rubin, & Rocha, 2004).

2.10. Lipid peroxidation assay

Lipid peroxidation was estimated by measuring thiobarbituric acid-reactive substances at 532 nm, as described by Ohkawa, Ohishi, and Yagi (1979). The brain homogenate was incubated at 37 °C for 1 h in a medium containing 0.1 M Tris–HCl buffer (pH 7.4), sample or references (Trolox and BHT), 250 μM freshly-prepared $FeSO_4$ and distilled water. After incubation, the reaction was stopped by adding 8.1% SDS, acetic acid/HCl (pH 3.4) and 0.8% TBA. All the tests were carried out in triplicate.

2.11. Statistical analysis

All data were expressed as the mean ± SE of triplicate experiments. Statistical analysis was carried out using the SPSS statistical package for Windows, release 18.0 (SPSS Inc., Chicago, IL). Groups were compared by analysis of variance, and additional pairwise comparisons were carried out using Duncan's New Multiple Range Test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Extraction yields

The extraction yields (expressed as weight of extract relative to the weight of the initial plant material sample) ranged from 7.68% to 25.50%, and were influenced by several parameters, including solvent polarity and the chemical composition and physical characteristics of the plant material (Dai & Mumper, 2010). The highest extraction yields were achieved with water (21.01% and 23.60%, for wild plants and *in vitro* cultures, respectively) and water/ethanol (11.04% and 25.50%, for wild plants and *in vitro* cultures, respectively). Zieliński and Kozłowska (2000) proposed that higher extraction yields were achieved with water compared to water/ethanol and ethanol alone because proteins and carbohydrates are more soluble in water than in ethanol and aqueous mixtures of ethanol.

3.2. Phenolic composition

The phenolic composition of the extracts was determined by HPLC–DAD (Fig. 1 and Table 1), revealing the presence of caffeic and rosmarinic acids, luteolin and apigenin, as observed previously in other *Thymus* spp. (Dorman, Bachmayer, Kosar, & Hiltunen, 2004; Kulišić, Dragović-Uzelac, & Miloš, 2006; Özgen et al., 2011). Other less important and unidentified compounds presented UV spectra resembling those of hydroxycinnamic acids and flavonoid derivatives. The solubility of phenolic compounds depends on the chemical nature of the plant tissue and the polarity of the solvent system (Dai & Mumper, 2010). We found that the water/ethanol mixture was the most efficient solvent for the extraction of phenolic compounds from both wild plants and *in vitro* cultures (19.4 and 23.6 g/kg, respectively), followed by ethanol (10.9 and 23.0 g/kg, respectively) then water (0.440 and 0.264 g/kg, respectively). Among organic solvents, ethanol is usually preferred for the recovery of phenolic compounds because of its low toxicity (Sultana, Anwar, & Przybylski, 2007).

The phenolic content of the extracts was also determined using the Folin–Ciocalteu method (Table 2) and the values obtained ranged from 243 to 938 $\mu mol_{GAE}/g_{extract}$. As observed when using the HPLC–DAD method, the water/ethanol mixture was the most efficient at extracting phenolic compounds from both wild plants and *in vitro* cultures (938 and 539 $\mu mol_{GAE}/g_{extract}$, respectively; $p < 0.05$). However, the total phenolics content was higher in the water extract from wild plants than in the ethanol extract (562 and 243 $\mu mol_{GAE}/g_{extract}$, respectively), perhaps reflecting the co-extraction of certain phenolic compounds with carbohydrates and proteins that are highly soluble in water (Zieliński & Kozłowska, 2000). Such non-phenolic compounds can also react with the F–C reagent, possibly contributing to the elevated phenolic concentrations (Prior, Wu, & Schaich, 2005). These data highlight the impact of solvent choice on the efficiency of extraction. Sultana et al. (2009) reported that aqueous organic solvents generally yield higher yields of phenolics than the corresponding absolute organic solvents. Trabelsi et al. (2010) showed that the addition of 20% water to methanol, acetone or ethanol can enhance the extraction of antioxidants from *Limoniastrum monopetalum* leaves. Other studies have shown that ethanol concentration, extraction time and temperature affect the recovery of phenolic compounds and the antioxidant capacity of plant extracts (Chew et al., 2011). Furthermore, Miron,

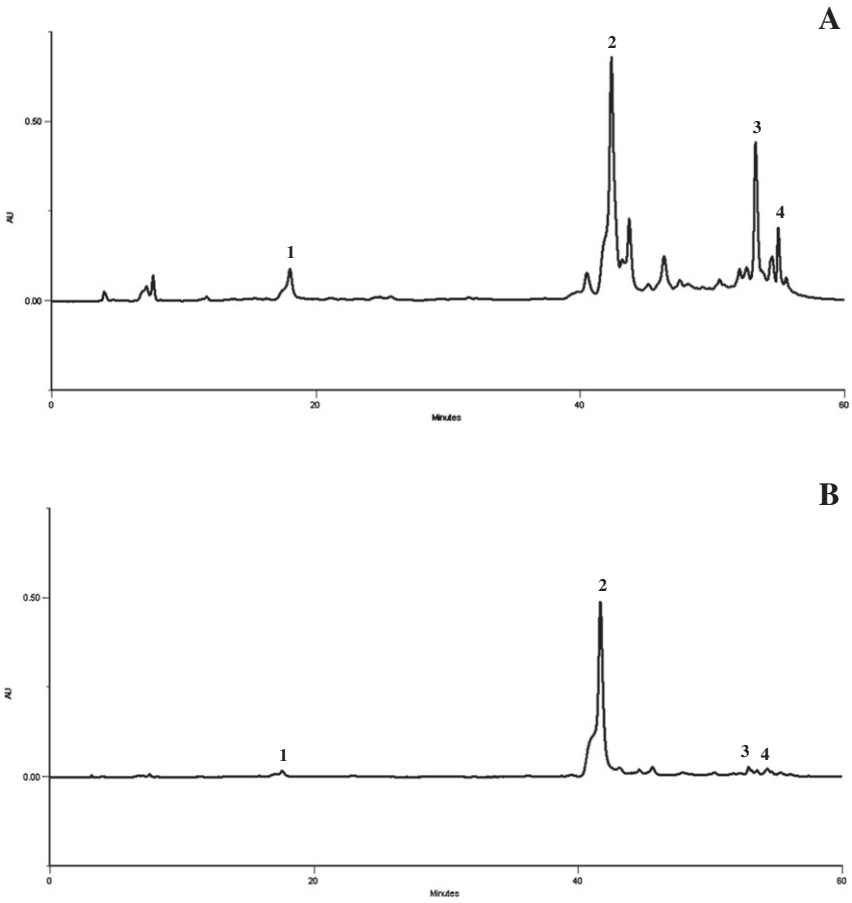

Fig. 1. HPLC–DAD chromatogram of water/ethanol extract from wild plants (A) and *in vitro* cultures (B) of *Thymus lotocephalus*. Detection at 320 nm. (**1**) Caffeic acid; (**2**) rosmarinic acid; (**3**) luteolin and (**4**) apigenin.

Table 1
Quantification of the phenolic compounds identified in *Thymus lotocephalus* extracts (dry basis).

Extract	Caffeic acid		Rosmarinic acid		Luteolin		Apigenin		Σ (mg/kg)
	(mg/kg[a])	(%)	(mg/kg)	(%)	(mg/kg)	(%)	(mg/kg)	(%)	
Wild plants									
Water	65.4 (1.9) d	14.9	322 (4.0) d	73.3	24.1 (0.3) e	5.5	28.0 (1.7) d	6.4	440 e
Water/ethanol	1310 (4.9) a	6.8	12800 (31.9) b	66.2	3770 (9.1) a	19.5	1460 (33.6) a	7.5	19400 c
Ethanol	219 (1.5) b	2.0	10300 (130) c	94.7	177 (3.2) b	1.6	184 (2.2) b	1.7	10900 d
In vitro cultures									
Water	217 (3.8) b	82.4	n.d.	0.0	24.0 (0.2) e	9.1	22.5 (0.6) d	8.5	264 e
Water/ethanol	195 (1.3) c	0.8	23100 (368.2) a	98.2	144 (1.0) c	0.6	93.1 (0.8) c	0.4	23600 a
Ethanol	189 (2.7) c	0.8	22600 (363.1) a	98.3	108 (0.3) d	0.5	101 (2.0) c	0.4	23000 b

n.d.: not detected.
Values followed by different letters within the same column are significantly different at $p < 0.05$.
[a] Values are expressed as mean ± SD ($n = 3$).

Table 2
Phenolic content and free radical scavenging ability of *Thymus lotocephalus* extracts obtained from wild plants and *in vitro* cultures. The phenolic content was determined by Folin-Ciocalteu (F–C) method and antioxidant activity by Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays.

Extract	F–C ($\mu mol_{GAE}/g_{extract}$)		TEAC ($\mu mol_{TE}/g_{extract}$)		ORAC ($\mu mol_{TE}/g_{extract}$)	
	Wild plants	*In vitro* cultures	Wild plants	*In vitro* cultures	Wild plants	*In vitro* cultures
Water	562 ± 17.8 b*	284 ± 8.20 b	297 ± 0.39 b	552 ± 5.56 a*	2080 ± 280 b*	997 ± 27.7 b
Water/ethanol	938 ± 50.9 a*	539 ± 21.3 a	1110 ± 21.0 a*	563 ± 15.6 a	3460 ± 262 a*	2420 ± 276 a
Ethanol	243 ± 2.96 c	305 ± 5.73 b*	302 ± 12.2 b*	263 ± 8.12 b	1300 ± 47.1 c	1190 ± 6.84 b

Values are expressed as mean ± SE ($n = 3$).
Values followed by different letters within the same column are significantly different at $p < 0.05$.
* Indicate significant differences ($p < 0.05$) between wild plants and *in vitro* cultures for each extract.

Plaza, Bahrim, Ibáñez, and Herrero (2011) studied the influence of different ethanol/water mixtures on the extraction of phenolics from *Thymus serpyllum* and observed maximum extraction efficiency with a 1:1 mixture at 100 °C.

Phenolic (rosmarinic and caffeic) acids and flavones (luteolin and apigenin) were found in both wild plants and *in vitro* cultures, albeit with certain quantitative differences (Table 1). Rosmarinic acid was the major compound in most extracts, although the water extract of *in vitro* material was exceptional as it contained more caffeic acid than rosmarinic acid. Kulišić et al. (2006) also found that rosmarinic acid was the major compound present in different *Thymus* spp. extracts.

The production of secondary metabolites is influenced by environmental and physiological conditions (Barros et al. 2012; Ncube, Ngunge, Finnie, & Van Staden, 2011). We found that caffeic acid, luteolin and apigenin were present at higher concentrations in the water/ethanol extract from wild plants than the extracts from *in vitro* cultures (Table 1). Similarly, García-Pérez, Gutiérrez-Uribe, and García-Lara (2012) found higher amounts of luteolin in *Poliomintha glabrescens* wild plants compared to *in vitro* cultures. This might reflect the impact of *in vitro* conditions (such as reduced light intensity) on secondary metabolism. In contrast, we found that rosmarinic acid was significantly ($p < 0.05$) more abundant in the water/ethanol and ethanol extracts from *in vitro* cultures compared to wild plants, and represented up to 98% of the total phenolic content. Wang, Provan, and Helliwell (2004) reported that a solvent comprising 30–60% (v/v) ethanol in water gave the highest yield of rosmarinic and caffeic acids.

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid with known health-promoting effects (Petersen & Simmonds, 2003; Shetty, 1997). This has encouraged research into *in vitro* culture techniques for the synthesis of rosmarinic acid, e.g., using suspension cell cultures of *Lavandula vera* (Georgiev, Pavlov, & Ilieva, 2006) and *Salvia officinalis* (Hippolyte, Marin, Baccou, & Jonard, 1992). In addition, Bauer, Leljak-Levanic, and Jelaska (2004) produced rosmarinic acid using *Coleus blumei* Benth. callus transformed with *Agrobacterium tumefaciens*. The *in vitro* production of rosmarinic acid is stimulated by high levels of sucrose and the addition of phenylalanine to the culture medium (Bauer et al., 2004). Our results suggest that *in vitro* cultures of *T. lotocephalus* can be used as an alternative to wild plants for the efficient, large-scale production of rosmarinic acid and other high-value secondary metabolites.

3.3. Antioxidant activity

3.3.1. Free radical scavenging

The complex chemical composition of plants and the diversity of phenolic compounds demand the evaluation of antioxidant activity under different oxidation conditions and using distinct methods (Frankel & Meyer, 2000). We therefore used Trolox equivalent antioxidant activity (TEAC) and oxygen radical absorbance capacity (ORAC) assays to estimate the antioxidant activity of plant extracts based on single electron transfer (SET) and hydrogen atom transfer (HAT), respectively (Prior et al., 2005). The ORAC assay uses a fluorescent probe to compete with antioxidants for peroxyl radicals generated by the decomposition of AAPH, whereas the colorimetric TEAC assay measures the ability of antioxidants to reduce the radical cation $ABTS^{\cdot+}$ (Dai & Mumper, 2010).

All the extracts we tested displayed antioxidant activity, although extracts from wild plants were more active than extracts from *in vitro* cultures (Table 2). Water/ethanol was the most efficient solvent for the extraction of free radical-scavenging compounds from both materials, in agreement with previous studies showing that the addition of water improves extraction efficiency and increases the antioxidant activity of extracts (Chew et al., 2011; Sultana et al., 2009; Trabelsi et al., 2010). Extracts from wild plants were generally better at neutralising $ABTS^{\cdot+}$ than extracts from *in vitro* cultures, although this was not the case for the water extract. The quantity of phenolic compounds detected by HPLC–DAD was significantly ($p < 0.05$) lower in water extracts than water/ethanol and ethanol extracts (Table 1). The results also indicate that antioxidants in both materials neutralise free radicals preferentially by donating a hydrogen atom. The antioxidant properties of the extracts probably reflect their high content of phenolic acids and flavones (Table 1) (AlGamdi, Mullen, & Crozier, 2011; Chen & Ho, 1997; Petersen & Simmonds, 2003; Özgen et al., 2011). Phenolic hydroxyl groups are excellent hydrogen atom and electron donors to free radicals (Dai & Mumper, 2010) and previous studies have confirmed free radical scavenging by caffeic acid, rosmarinic acid, luteolin and apigenin (AlGamdi et al., 2011; Chen & Ho, 1997; Petersen & Simmonds, 2003; Özgen et al., 2011).

Several strategies have been used to improve the production of antioxidants *in vitro* (Matkowski, 2008). For example, Pérez-Tortosa et al. (2012) investigated the influence of salicylic acid on the antioxidant activity, total phenolic content and rosmarinic acid accumulation of *in vitro* cultivated *Thymus membranaceus* Boiss.

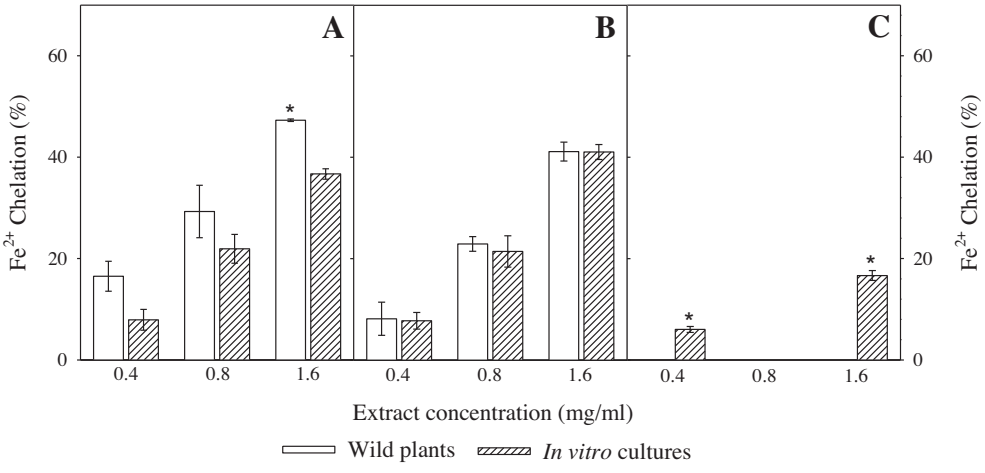

Fig. 2. Fe^{2+} chelating ability of *Thymus lotocephalus* extracts from wild plants and *in vitro* cultures. (A) Water extract; (B) water/ethanol extract and (C) ethanol extract. Values represent the mean ± SE ($n = 3$). Values marked with * indicate significant differences ($p < 0.05$) between wild plants and *in vitro* cultures for each extract concentration. Reference (EDTA) = 98.07 ± 0.25% (0.30 mg/ml).

subsp. *membranaceus* shoots, and showed that salicylic acid induces the synthesis of phenolic compounds including rosmarinic acid. Plant tissue culture techniques allow the chemical profile of plants to be altered by manipulating the chemical and physical environment, which should also allow the phytochemical profile of *T. lotocephalus in vitro* cultures to be modulated by changing the *in vitro* cultivation conditions.

3.3.2. Fe^{2+} chelation

High iron levels may act catalytically to produce reactive oxygen species, with a negative impact on the structure and function of cells (Britton, Leicester, & Bacon, 2002). Iron can promote the formation of hydroxyl radicals and decompose lipid hydroperoxides into highly reactive lipid alkoxyl and peroxyl radicals, which perpetuate the chain reaction of lipid peroxidation (Britton et al., 2002; Minotti & Aust, 1992). The capacity of *T. lotocephalus* extracts to chelate Fe^{2+} is shown in Fig. 2. We found that water (Fig. 2A) and water/ethanol (Fig. 2B) extracts could chelate Fe^{2+} efficiently and therefore reduce the production of free radicals. EDTA was used as reference in this assay and its Fe^{2+}-chelating activity was 98.07 ± 0.25% at 0.30 mg/ml. Water was the most efficient solvent for the extraction of chelating agents followed by water/ethanol ($p < 0.05$), and the activities increased in a dose-dependent manner. It has been reported that the presence of *ortho*-dihydroxyl groups, i.e., molecules bearing catechol or galloyl groups, 5-OH and/or 3-OH in conjunction with C4 keto group and a large number of OH groups, are essential for Fe-binding (Andjelković et al., 2006; Khokhar & Apenten, 2003). In the present study, ethanol extracts showed little Fe^{2+}-chelating activity (Fig. 3C) even if possessing high content of catechol groups. In fact, other organic substances, such as organic acids, amino acids and sugars, with capacity to chelate transition metal ions, may be also co-extracted and contribute to the results observed. Overall, there were no significant differences between extracts from wild plants and *in vitro* cultures in terms of Fe^{2+} chelation.

3.3.3. Inhibition of lipid peroxidation

Lipid peroxidation is a consequence of oxidative damage to membranes (Britton et al., 2002), resulting in the production of malondialdehyde (MDA) which can be detected using a thiobarbituric acid colour reaction (Frankel & Neff, 1983). The inhibition of Fe^{2+}-induced lipid peroxidation in mouse brain homogenates by *T. lotocephalus* extracts is shown in Fig. 3. The results confirm that Fe^{2+} is a strong initiator of lipid peroxidation as previously proved (Costa, Gonçalves, Andrade, Valentão, & Romano, 2011; Oboh, Puntel, & Rocha, 2007). All the *T. lotocephalus* extracts significantly ($p < 0.05$) reduced the MDA content in mouse brain homogenates compared to controls. The water/ethanol extracts were the most potent inhibitors of MDA production ($p < 0.05$). Moreover, no significant differences were found between water and ethanol extracts, and for the latter, the best results were obtained with *in vitro* cultures at the highest concentrations (2.50 and 5.00 mg/ml). The interaction between phenolics and membrane lipids may help to prevent lipid peroxidation (Oteiza, Erlejman, Verstraeten, Keen, & Fraga, 2005). Because antioxidants could be distributed in the lipophilic compartment of membranes or in the aqueous phase (Niki, Yoshida, Saito, & Noguchi, 2005), we used two antioxidant references: BHT (lipophilic) and Trolox (hydrophilic). Both references showed a strong capacity to reduce the Fe^{2+}-induced formation of MDA (BHT = 64.17 ± 4.47% and Trolox = 53.28 ± 7.37%). The capacity to protect lipid membranes against oxidative processes has been demonstrated in other *Thymus* spp. (Dandlen et al., 2010; Hazzit et al., 2009). Our chemical analysis (Table 1) suggests that phenolic compounds from *T. lotocephalus* extracts may contribute to inhibit lipid peroxidation. In addition, interactions between phytochemical compounds, identified and unidentified, could occur and affect the biological activities measured (Lila & Raskin, 2005). Fadel, El Kirat, and Morandat (2011) showed that rosmarinic acid can spontaneously penetrate lipid membranes to prevent lipid peroxidation. Luteolin and caffeic acid (present in all of the extracts) have also been shown to inhibit lipid peroxidation (Khennouf, Amira, Arrar, & Baghiani, 2010; Lee et al., 2003). As discussed above, the bioactive compounds in the water/ethanol extracts were efficient free radical scavengers and Fe^{2+} chelators, suggesting that both activities help to prevent Fe^{2+}-induced lipid peroxidation in mouse brain homogenates *in vitro*.

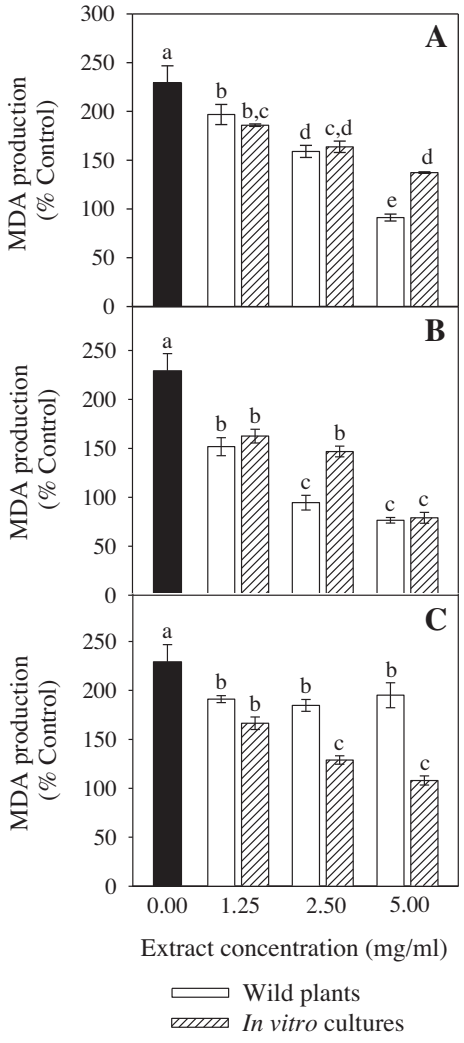

Fig. 3. Effects of *Thymus lotocephalus* extracts from wild plants and *in vitro* cultures on Fe^{2+}-induced lipid peroxidation in mouse brain homogenates (*in vitro*). The percent inhibition of malondialdehyde (MDA) production was expressed with respect to basal brain tissue (without plant extract and pro-oxidant). (A) Water extract; (B) water/ethanol extract and (C) ethanol extract. Antioxidant references: BHT = 64.17 ± 4.47% (0.30 mg/ml) and Trolox = 53.28 ± 7.37% (0.20 mg/ml). Values represent the mean ± SE ($n = 3$). In each graph, mean values with different letters are significantly different at $p < 0.05$.

4. Conclusion

To our knowledge this is the first report characterising the phenolic profiles and antioxidant activities of wild *T. lotocephalus* plants and *in vitro* cultures. The extracts from both sources were able to scavenge free radicals, chelate Fe^{2+} and reduce the MDA content of mouse brain homogenates efficiently, reflecting the presence of caffeic acid, rosmarinic acid, and the flavones, luteolin and apigenin.

We also found that the extraction solvent had a significant impact on the phenolic content and antioxidant activity of different extracts. Overall, the water/ethanol extracts contained the highest levels of antioxidants. The *in vitro* environment was the most favourable for the accumulation of high levels of rosmarinic acid, suggesting that *in vitro* cultures of *T. lotocephalus* could be used as a source of natural antioxidant compounds (particularly rosmarinic acid), thus helping to preserve natural populations.

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Accumulation of phenolic compounds in *in vitro* cultures and wild plants of *Lavandula viridis* L'Hér and their antioxidant and anti-cholinesterase potential

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ABSTRACT

In this study, we evaluated the phenolic profile, antioxidant and anti-cholinesterase potential of different extracts from wild plants and *in vitro* cultures of *Lavandula viridis* L'Hér. The HPLC–DAD analysis allowed the identification and quantification of 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic and rosmarinic acids, and luteolin and pinocembrin. Water/ethanol extract from *in vitro* cultures contained the highest amount of the identified phenolic compounds (51652.92 mg/kg). To investigate the antioxidant activity we used Trolox equivalent antioxidant capacity, oxygen radical absorbance capacity, Fe^{2+} chelation activity and the inhibition of Fe^{2+}-induced lipid peroxidation in mouse brain homogenates (*in vitro*). Overall, all the extracts from both wild plants and *in vitro* cultures exhibited ability to scavenge free radicals, to chelate Fe^{2+} and to protect against lipid peroxidation. In addition, the extracts from *L. viridis* were active in inhibiting both acetylcholinesterase and butyrylcholinesterase (Ellman's method). Our findings suggest that *L. viridis in vitro* cultures represent a promising alternative for the production of active metabolites with antioxidant and anti-cholinesterase activity.

1. Introduction

Alzheimer's disease (AD) is a progressive and complex neurodegenerative disorder characterized by the occurrence of senile plaques and neurofibrillary tangles. Amyloid beta (Aβ) peptide is the main component of senile plaques and is highly involved in the progression of AD. Accumulated evidences suggest that Aβ peptide, in the presence of biometals and oxygen, generates reactive oxygen species and causes lipid peroxidation in neuronal cell membranes (Butterfield and Lauderback, 2002; Rauk, 2008). Nowadays, the most efficient therapeutic approach to AD is based on cholinesterases inhibitors in order to enhance the concentration of acetylcholine (ACh) in the synaptic cleft (Wilkinson et al., 2004). Natural products might slow the progression of AD because they can simultaneously protect neurons from oxidative stress (Ramassamy, 2006) and act as cholinesterases inhibitors (Hostettmann et al., 2006).

The production of phytochemicals is affected by environmental and physiological conditions and their chemical synthesis is complex due to their structures and specific stereo-chemical characteristics (Smetanska, 2008). Biotechnological approaches, particularly plant tissue cultures, are found to have significant potential for the production of high-value phytochemicals. *In vitro* propagation methods allow the selective, rapid and effective production of secondary metabolites with no seasonal constraints and independent of geographical and soil conditions.

Lavandula species (Lamiaceae) produce valuable compounds for food, pharmaceutical and cosmetic industries (Boelens, 1995). Recently, Gonçalves and Romano (2013) reviewed the application of *in vitro* propagation methods to *Lavandula* spp. and demonstrated the usefulness of plant tissue culture technique for the production of valuable secondary metabolites overcoming the limitations of conventional propagation methods. *Lavandula viridis* L'Hér is an aromatic shrub endemic to the southwest Iberian Peninsula that is used in traditional medicine. The essential oil from this species has antioxidant, anti-cholinesterase (Costa et al., 2012a) and

Abbreviations: Aβ, amyloid beta; AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; $ABTS^{\cdot+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; ATCI, acetylthiocholine iodide; AUC, area under the curve; BChE, butyrylcholinesterase; BHT, butylated hydroxytoluene; BTCI, butyrylthiocholine chloride; DTNB, 5,5′-dithiobis [2-nitrobenzoic acid]; EDTA, ethylenediaminetetraacetic acid; HPLC–DAD, high-performance liquid chromatography–diode array detection; MDA, malondialdehyde; MS, Murashige and Skoog medium; ORAC, oxygen radical absorbance capacity; SDS, sodium dodecylsulfate; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid.

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antifungal activities (Zuzarte et al., 2011). In addition to volatiles, *L. viridis* also produces phenolic compounds able to scavenge free radicals by different mechanisms (Costa et al., 2011; Ferreira et al., 2006; Matos et al., 2009), to inhibit lipid peroxidation (Costa et al., 2011; Gonçalves et al., 2013) and cholinesterases activities (Costa et al., 2011; Ferreira et al., 2006). Nogueira and Romano (2002) compared the chemical composition of *L. viridis* essential oils from *in vitro*- and field-grown plant material and no significant differences were found. In this work, we compared, for the first time, the phenolic profile and biological activities of wild plants and *in vitro* cultures of *L. viridis*. The qualitative and quantitative phenolic profiles were determined by high-performance liquid chromatography with diode-array detection (HPLC–DAD) and the anti-cholinesterase activity was assessed by Ellman's method. In addition, the antioxidant activity was evaluated by measuring the Trolox equivalent antioxidant capacity, oxygen radical absorbance capacity, Fe^{2+}-chelation activity and the inhibition of Fe^{2+}-induced lipid peroxidation in mouse brain homogenates (*in vitro*).

2. Materials and methods

2.1. Standards and reagents

5-*O*-caffeoylquinic acid, luteolin and rosmarinic acid were purchased from Extrasynthése (Genay, France). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, potassium persulfate ($K_2S_2O_8$), thiobarbituric acid (TBA), Trizma base (Tris), acetylthiocholine iodide (ATCI), 5,5′-dithiobis [2-nitrobenzoic acid] (DTNB), acetylcholinesterase (AChE) (Electric-eel, EC 3.1.1.7, Type VI-S), butyrylcholinesterase (BChE) (horse-serum, EC 3.1.1.8), galanthamine hydrobromide, malondialdehyde tetrabutylammonium salt (MDA) and butyrylthiocholine chloride (BTCI) were purchased from Sigma–Aldrich (Steinheim, Germany). Formic acid, methanol and iron(II) sulfate ($FeSO_4$) were acquired from Merck (Darmstadt, Germany). Fluorescein, 1,10-phenanthroline and absolute ethanol were obtained from Panreac (Barcelona, Spain). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), butylated hydroxytoluene (BHT) and sodium dodecylsulfate (SDS) were purchased from Acros Organics (Geel, Germany). Qualitative filter paper was purchased from VWR (Leuven, Belgium). Pinocembrin, ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) were purchased from Fluka (Steinheim, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). 3- and 4-*O*-caffeoylquinic acids were not commercially available, so they were prepared by transesterification of 5-*O*-caffeoylquinic acid using tetramethylammonium hydroxide (Clifford et al., 1989a,b).

2.2. Plant material

The aerial parts of *L. viridis* were collected from its natural habitat during flowering period at São Bartolomeu de Messines (Algarve, Portugal). A voucher specimen was deposited at the herbarium of the Botanical Garden of the University of Lisbon, as referred by Nogueira and Romano (2002). *In vitro* cultures were produced in MS medium (Murashige and Skoog, 1962) with macronutrients at half-strength supplemented with 0.67 μM 6-benzyladenine, as previously described by Dias et al. (2002). The cultures were maintained at 25 ± 2 °C with a 16-h photoperiod (cool white fluorescent lamps, 40 μmol m^{-2} s^{-1}) and were subcultured every 6 weeks.

2.3. Extraction procedure

Plant material was dried at 40 °C and powdered in a blender to achieve a mean particle size less than 2 mm. Extraction of phenolic compounds from *L. viridis* was performed using water and ethanol separately and in a 1:1 mixture. The plant material (10 g) was soaked overnight at room temperature in 200 ml of each solvent and the resulting extract was filtered through a 5–13 μm membrane. Finally, the extracts were concentrated to dryness (the water extract was lyophilized and the ethanol and water/ethanol extracts were dried in a rotary evaporator) and stored at −20 °C.

2.4. HPLC–DAD analysis

The extracts were analyzed on an analytical HPLC unit (Gilson), using a Spheri-sorb ODS2 column (4.6 × 250 mm, 5 μm, particle size). The solvent system used was a gradient of water-formic acid (19:1) (A) and methanol (B), starting with 5% methanol and installing a gradient to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min and 80% B at 60 min, at a solvent flow rate of 0.9 ml/min. Detection was achieved with a Gilson Diode Array Detector (DAD). Spectral data from all peaks were accumulated in the range 200–400 nm, and chromatograms were recorded at 280, 320 and 350 nm. The data were processed on an Unipoint® System software (Gilson Medical Electronics, Villiers le Bel, France). The compounds in each extract were identified by comparing their retention times and UV–Vis spectra in the 200–400 nm range with authentic standards and with the library of spectra previously compiled by the authors. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. 3-*O*-Caffeoylquinic and 4-*O*-caffeoylquinic acids were quantified as 5-*O*-caffeoylquinic acid and the other compounds were quantified as themselves.

2.5. Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant activity was determined using the TEAC assay with the radical cation $ABTS^{\cdot+}$, as described by Re et al. (1999). The 7 mM $ABTS^{\cdot+}$ stock solution was prepared using potassium persulfate as the oxidizing agent. The absorbance was determined at 734 nm, 1 min after mixing. The sample dilution that achieved 20–80% inhibition of the blank absorbance was used to calculate the TEAC values and the results were expressed as Trolox equivalents (TE) per gram of extract.

2.6. Oxygen radical absorbance capacity (ORAC) assay

Antioxidant capacity was evaluated as described by Gillespie et al. (2007) using fluorescein as the fluorescent probe and AAPH as peroxyl radical generator. A black microplate (NUNC, Rochester, New York, USA) was loaded with 0.08 μM fluorescein plus the sample, Trolox standard or phosphate buffer (blank), and the reaction was initiated by adding 150 mM AAPH to each well after incubating for 10 min at 37 °C. The reduction in fluorescence was determined by reading fluorescein excitation at 485 nm and emission at 530 nm every minute for 90 min. The ORAC value for each extract was calculated using the area under the curve (AUC) and the regression equation between the TE and the net AUC. The results were expressed as TE per gram of extract.

2.7. Fe^{2+} chelation assay

Fe^{2+}-chelating capacity of the extracts was determined as described by Minotti and Aust (1987) and modified by Puntel et al. (2005). Briefly, 500 μM of freshly-prepared $FeSO_4$ was added to a reaction mixture comprising 0.1 M Tris–HCl (pH 7.4), 0.9% NaCl and the extract or 0.1 M Tris–HCl (blank) or reference (EDTA). After a 5-min incubation at room temperature, 0.35% 1,10-phenanthroline (w/v) was added and the absorbance at 510 nm was determined.

2.8. Lipid peroxidation assay

C57BL/6 mice (male, 25–30 g, 3–4 months old) were sourced from the animal house at the Centre for Molecular and Structural Biomedicine, University of Algarve, Portugal, and were housed in polyacrylic cages at 20–23 °C and 40–55% relative humidity, with *ad libitum* access to food. The animal experiments conformed to ethical standards for clinical research and EU guidelines. Mice were killed by CO_2 asphyxiation and the cerebral tissue (whole brain) was rapidly dissected, placed on ice and weighted, and then homogenized in 0.1 M Tris–HCl (pH 7.4) (1/10, w/v). The homogenate was centrifuged (Eppendorf, Centrifuge 5804 R, Hamburg, Germany) for 10 min at 3000*g* and the low-speed supernatant was used for the lipid peroxidation assay (Bellé et al., 2004).

Lipid peroxidation was estimated by measuring thiobarbituric acid-reactive substances (TBARS), as described by Ohkawa et al. (1979). The brain homogenate was incubated at 37 °C for 1 h in a medium containing 0.1 M Tris–HCl buffer (pH 7.4), sample or references (Trolox and BHT), 250 μM of freshly-prepared $FeSO_4$ and distilled water. After incubation, the reaction was stopped by adding 8.1% SDS, acetic acid/HCl (pH 3.4) and 0.8% TBA. The TBARS were measured by determining absorbance at 532 nm using a standard curve of malondialdehyde (MDA).

2.9. In vitro anti-cholinesterases inhibition assay

The evaluation of AChE and BChE inhibitory activities was based on Ellman's method (Ellman et al., 1961), using a 96-well microplate reader. Firstly, 3 mM DTNB, 15 mM substrate (ATCI or BTCI), 100 mM phosphate buffer (pH 8.0) and extract (2.5 mg/ml), buffer or galanthamine (standard inhibitor) were mixed. Finally, AChE or BChE (0.28 U/ml) were added and the absorbance was read at 405 nm for 5 min. The reaction enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer without any inhibitor. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity.

2.10. Statistical analysis

All the experiments were carried out three times using triplicate samples. The data were expressed as the mean ± standard error and were subjected to one-way analysis of variance (ANOVA). Multiple comparisons of means were carried out

using Duncan's New Multiple Range Test. All statistical analysis was carried out using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Extraction yields and phenolic composition

The extraction yields (expressed as weight of extract relative to the weight of the initial plant material) ranged from 12.80% to 28.36%. The highest extraction yields were achieved with water (22.08% and 18.76%, for wild plants and *in vitro* cultures, respectively) and water/ethanol (21.26% and 28.36%, for wild plants and *in vitro* cultures, respectively). The phenolic profiles of *L. viridis* extracts were determined by HPLC–DAD (Table 1 and Fig. 1). The chemical analysis allowed the identification of hydroxycinnamic acids (3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic and rosmarinic acids), one flavone (luteolin) and one flavanone (pinocembrin). These compounds were previously identified in the methanol extract from the aerial parts of *L. viridis*, as well as in other *Lavandula* species (Areias et al., 2000; Costa et al., 2011; Proestos et al., 2006).

As far as we know, there are no reports regarding the phenolic compounds characterization in extracts from *in vitro* cultures of *L. viridis*. We found quantitative differences between the amounts of the identified phenolics in wild plants and *in vitro* cultures that can be explained by the differences between *in vivo* and *in vitro* growth conditions (e.g. the composition of the culture medium and the controlled environmental conditions). Water/ethanol extract from *in vitro* cultures contained the highest amount of the identified phenolic compounds (51652.92 mg/kg) and the water extract from wild plants the lowest one (3776.61 mg/kg) (Table 1). Rosmarinic acid was the major identified compound in water/ethanol and ethanol extracts, while 4-*O*-caffeoylquinic and 5-*O*-caffeoylquinic acids were present in higher amounts in the water extracts from wild plants and *in vitro* cultures, respectively. Our results indicate that different solvents and solvent mixtures allow the extraction of natural compounds with distinct physicochemical behavior and quantities (Sultana et al., 2009). The highest recovery of rosmarinic acid was obtained from *in vitro* cultures using water/ethanol as extraction solvent (41293.27 mg/kg), followed by ethanol (32045.76 mg/kg). In addition, the ethanol extract from *in vitro* cultures contained higher amounts of the identified caffeoylquinic acid derivatives than that from wild plants (Table 1). Similar results were previously observed in *in vitro* cultures of *Thymus lotocephalus* G. López & R. Morales (Lamiaceae), water/ethanol extract being the most efficient for the recovery of phenolic compounds and rosmarinic acid in higher amounts in *in vitro* cultures compared to wild plants (Costa et al., 2012b). *In vitro* culture techniques represent an efficient alternative for selective and continuous production of secondary metabolites (Karuppusamy, 2009). *Lavandula* cell cultures have been used to produce secondary metabolites, including rosmarinic acid (Gonçalves and Romano, 2013). This compound has important biological properties, namely antibacterial, antiviral, anti-inflammatory, antioxidant and antiproliferative (Yoshida et al., 2005). All these biological effects make it a valuable metabolite for the pharmaceutical, food and cosmetic industries (Parnham and Kesselring, 1985). The *in vitro* production of rosmarinic acid using *Lavandula vera* DC cell suspensions is extensively reported (Georgiev et al., 2007; Ilieva and Pavlov, 1997). By manipulating *in vitro* environment it is possible to produce plant-specific metabolites with high yields and selectivity (Karuppusamy, 2009). For instance, Ilieva and Pavlov (1997) and Georgiev et al. (2007) proved that the accumulation of rosmarinic acid by *L. vera* cells can be induced by modifying the nutrient supply. Other interesting compounds identified in *in vitro* cultures of *L. viridis*, as 5-*O*-caffeoylquinic acid, have been produced using *in vitro* techniques (Kikowska et al., 2012; Siahpoush et al., 2011; Wang et al., 2003). 5-*O*-Caffeoylquinic acid has several biological properties, including antioxidant (Pirker and Goodman, 2010),

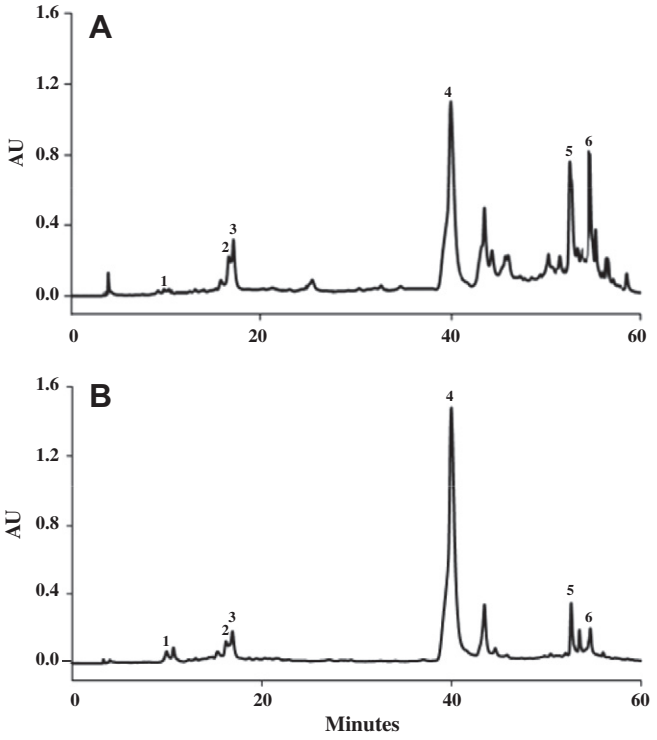

Fig. 1. HPLC–DAD chromatogram of water/ethanol extract from wild plants (A), and *in vitro* cultures (B) of *Lavandula viridis*. Detection at 320 nm. (**1**) 3-*O*-Caffeoylquinic acid; (**2**) 4-*O*-caffeoylquinic acid; (**3**) 5-*O*-caffeoylquinic acid; (**4**) rosmarinic acid; (**5**) luteolin; and (**6**) pinocembrin.

Table 1
Phenolic compounds in extracts from wild plants and *in vitro* cultures of *Lavandula viridis* (mg/kg).[a]

	3-*O*-Caffeoylquinic acid	4-*O*-Caffeoylquinic acid	5-*O*-Caffeoylquinic acid	Rosmarinic acid	Luteolin	Pinocembrin	Σ
Wild plants							
Water	n.q.	1825.10 (70.59) a	605.53 (40.92) d	1345.98 (56.24) e	n.q.	n.q.	3776.61 f
Water/ethanol	96.11 (0.61) d	1334.96 (44.64) b	2331.92 (68.17) a	20714.08 (238.65) c	7086.11 (7.76) a	12744.98 (226.93) a	44308.16 b
Ethanol	n.q.	n.q.	n.q.	16109.58 (74.83) d	175.02 (5.82) d	4933.98 (112.70) b	21218.58 d
In vitro cultures							
Water	2075.37 (33.63) a	121.63 (4.64) d	2450.27 (4.90) a	471.20 (5.26) f	n.q.	n.q.	5118.47 e
Water/ethanol	743.42 (5.16) b	1793.21 (35.01) a	1848.78 (51.07) b	41293.27 (432.13) a	1982.51 (22.31) b	3991.73 (43.16) c	51652.92 a
Ethanol	446.16 (7.47) c	532.26 (129.24) c	1191.05 (360.20) c	32045.76 (37.92) b	1600.63 (27.98) c	3976.54 (13.15) c	39792.40 c

[a] Values are expressed as mean (SD) ($n = 3$); n.q.: not quantified. Values followed by different letters within the same column are significantly different ($p \leqslant 0.05$). Σ, sum of the identified compounds.

Table 2
Free radical scavenging ability of extracts from wild plants and *in vitro* cultures of *Lavandula viridis*, assessed by Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays.

Extract	TEAC ($\mu mol_{TE}/g_{extract}$)	ORAC ($\mu mol_{TE}/g_{extract}$)
Wild plants		
Water	670.95 ± 4.24 b*	1502.22 ± 39.95 b*
Water/ethanol	1149.82 ± 17.31 a*	4030.26 ± 102.40 a*
Ethanol	332.06 ± 2.52 c	1183.95 ± 90.78 c
In vitro cultures		
Water	426.84 ± 0.24 b	1282.20 ± 21.44 b
Water/ethanol	854.12 ± 12.24 a	2837.81 ± 161.96 a
Ethanol	806.30 ± 23.07 a*	1371.13 ± 99.97 b

Values are expressed as mean ± SE ($n = 3$). For each plant material, values followed by different letters within the same column are significantly different at $p \leqslant 0.05$.
* Significant differences ($p \leqslant 0.05$) between wild plants and *in vitro* cultures for each extract.

antiviral, antibacterial and anti-inflammatory activities (Siahpoush et al., 2011).

Our results demonstrate that *in vitro* cultures of *L. viridis* produce high amounts of rosmarinic acid and 5-*O*-caffeoylquinic acid and may be a productive and promising source of natural antioxidants.

3.2. Antioxidant activity

3.2.1. Free radical scavenging

The free radical-scavenging capacity of *L. viridis* extracts was investigated by two methods, Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays. The results are shown in Table 2 and expressed as Trolox equivalents per gram of extract. All of the extracts demonstrated free radical-scavenging ability, but water/ethanol extracts showed the best antioxidant activity in both assays. Water and water/ethanol extracts from wild plants were significantly better ($p \leqslant 0.05$) at neutralizing both peroxyl and $ABTS^{\cdot+}$ radicals than the *in vitro* cultures. In contrast, the ethanol extract from *in vitro* cultures displayed higher $ABTS^{\cdot+}$ radical scavenging capacity. The antioxidant activity of the extracts could be attributed, at least partially, to the scavenging properties of rosmarinic acid (Chen and Ho, 1997), luteolin (Özgen et al., 2011) and 5-*O*-caffeoylquinic acid (Fukumoto and Mazza, 2000). The antioxidant activity of the methanol extract from wild plants of *L. viridis* was previously determined by ORAC and TEAC assays under the same experimental conditions (Costa et al., 2011). The antiradical activity of the methanol extract was higher than that showed by the water and ethanol extracts from wild plants, but lower than water/ethanol extracts (967.18 ± 22.57 and 2858.39 ± 70.97 $\mu mol_{TE}/g_{extract}$, for TEAC and ORAC assays, respectively).

3.2.2. Fe^{2+} chelation

The ability of *L. viridis* extracts to chelate Fe^{2+} is displayed in Fig. 2. Excepting the ethanol extract from wild plants, all the extracts demonstrated Fe^{2+}-chelating activity (Fig. 2). The water extract from wild plants (Fig. 2A) showed the highest capacity to chelate Fe^{2+} ($p \leqslant 0.05$), activity increasing in a dose-dependent manner. We previously reported the influence of the solvent on the capacity of *T. lotocephalus* extracts to chelate Fe^{2+} and, as we observed in the present work, water was the most efficient solvent for the extraction of chelating agents followed by the water/ethanol mixture (Costa et al., 2012b). The Fe^{2+} chelating capacity of the water extract from *L. viridis* is similar to that recently reported for the methanol extract and infusions (Costa et al., 2011; Gonçalves et al., 2013). In some cases (Fig. 2B and C), we observed a decrease in the Fe^{2+} chelation capacity increasing the extract concentration. Chvátalová et al. (2008) reported a dose-dependent increase of Fe^{2+} oxidation in the presence of polyphenols with catechol group. Since the water/ethanol and ethanol extracts from *in vitro* cultures are the richest in phenolic acids with catechol groups (Table 1), we suggest that the low Fe^{2+} chelation capacity observed at highest concentration could be related to the ferroxidase-like activity of phenolic acids.

3.2.3. Inhibition of lipid peroxidation

The capacity of the *L. viridis* extracts to inhibit Fe^{2+}-induced lipid peroxidation in homogenized mouse brain tissue in comparison with synthetic BHT (64.17 ± 4.47%; 0.30 mg/ml) and Trolox (53.28 ± 7.37%; 0.20 mg/ml) was determined by measuring the production of MDA (Fig. 3). Water and water/ethanol extracts showed the highest capacity to protect mouse brain homogenates against lipid peroxidation (Fig. 3A and B). The water/ethanol extract from wild plants was the most efficient, completely inhibiting lipid peroxidation at all concentrations tested (Fig. 3B), while ethanol extract from *in vitro* cultures was more effective in inhibiting MDA production than the extract from wild plants ($p \leqslant 0.05$) (Fig. 3C). The results obtained in this study confirm the high

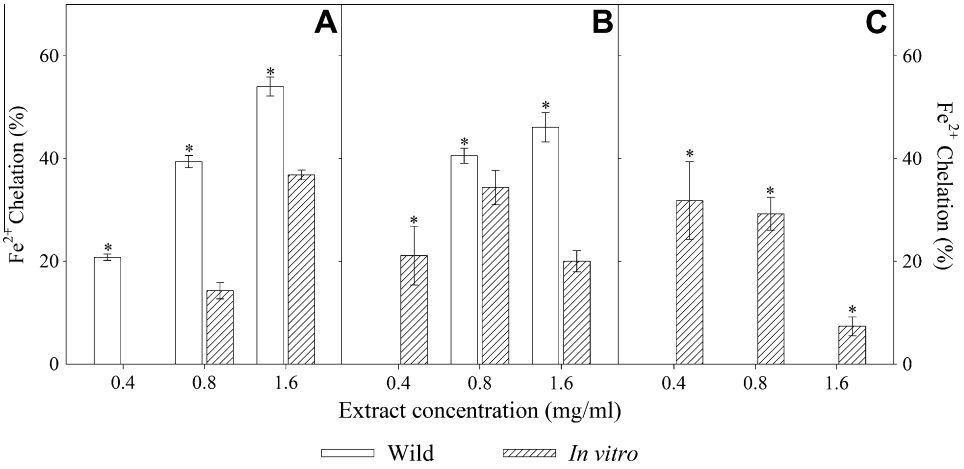

Fig. 2. Chelating effects of *Lavandula viridis* extracts from wild plants and *in vitro* cultures. (A) Water extract; (B) water/ethanol extract; (C) ethanol extract. Values represent the mean ± SE ($n = 3$). In each graph, values marked with * indicate significant differences ($p \leqslant 0.05$) between wild plants and *in vitro* cultures for each extract concentration. Reference (EDTA) = 98.07 ± 0.25% (0.30 mg/ml).

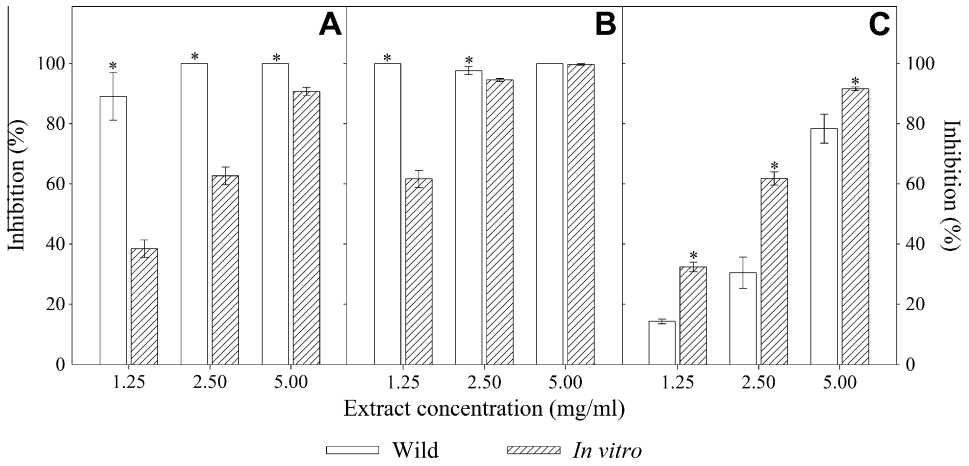

Fig. 3. The inhibition of Fe^{2+}-induced lipid peroxidation by extracts from wild plants and *in vitro* cultures of *Lavandula viridis* in mouse brain homogenates. The percent inhibition of malondialdehyde (MDA) production was expressed with respect to basal brain tissue (in the presence of the pro-oxidant and without extract) that was taken as 100%. (A) Water extract; (B) water/ethanol extract; (C) ethanol extract. Antioxidant references: BHT = 64.17 ± 4.47% (0.30 mg/ml) and Trolox = 53.28 ± 7.37% (0.20 mg/ml). Values represent the mean ± SE (n = 3). In each graph, values marked with * indicate significant differences ($p \leqslant 0.05$) between wild plants and *in vitro* cultures for each extract concentration.

Table 3
In vitro cholinesterase inhibitory activity of extracts from wild plants and *in vitro* cultures of *Lavandula viridis*.

Extract	Inhibition (%)[a]	
	AChE	BChE
Wild plants		
Water	34.53 ± 0.41 b*	32.34 ± 3.03 c*
Water/ethanol	47.09 ± 2.91 a*	63.01 ± 1.84 a*
Ethanol	52.81 ± 1.22 a	51.19 ± 1.52 b
In vitro cultures		
Water	27.40 ± 0.79 c	23.16 ± 0.35 c
Water/ethanol	34.69 ± 3.24 b	51.03 ± 0.80 b
Ethanol	87.69 ± 0.48 a*	61.78 ± 0.48 a*

For each enzyme and plant material (wild plants or *in vitro* cultures), values followed by different letters are significantly different at $p \leqslant 0.05$.
Reference (Galanthamine, 5 μg/ml): AChE = 67.78 ± 4.36% and BChE = 40.16 ± 0.76%.
[a] Percent inhibition at the final concentration of 2.5 mg/ml.
* Significant differences ($p \leqslant 0.05$) between wild plants and *in vitro* cultures for each extract.

protection of *L. viridis* extracts against lipid peroxidation induced by Fe^{2+} (Costa et al., 2011; Gonçalves et al., 2013).

Previous works have demonstrated the preventive effects of rosmarinic acid, luteolin and 5-*O*-caffeoylquinic acid against lipid peroxidation (Fadel et al., 2011; Lee et al., 2003; Ohnishi et al., 1993). The free radical-scavenging and metal-chelating properties demonstrated by *L. viridis* compounds may contribute to the inhibition of lipid peroxidation in mouse brain homogenates (*in vitro*).

3.2.4. In vitro cholinesterases inhibition

The anti-cholinesterase activity of the extracts is shown in Table 3. Water/ethanol and ethanol extracts were the most active in inhibiting AChE and BChE at the final concentration tested (2.5 mg/ml). The ethanol extract from *in vitro* cultures was the most effective AChE and BChE inhibitor (87.69 ± 0.48 and 61.78 ± 0.48%, respectively; $p \leqslant 0.05$). We recently proved that different active compounds, including terpenoids, recovered from the aerial parts of *L. viridis* also have anti-cholinesterase activity (Costa et al., 2011, 2012a). Galanthamine was used as reference inhibitor and was more active against AChE (67.78 ± 4.36%) than BChE (40.16 ± 0.76%), which is not surprising since galanthamine is a selective AChE inhibitor (Bores and Kosley, 1996).

Phenolic compounds are involved in the protective effect of neurodegenerative diseases (Ramassamy, 2006). For example, Hamaguchi et al. (2009) and Iuvone et al. (2006) reported that rosmarinic acid inhibit Aβ aggregation. In addition, previous reports demonstrated the potential of phenolic compounds to inhibit acetylcholineterase activity (Williams et al., 2011). Because phenolic compounds have different intracellular targets they can become an efficient approach to reduce the incidence of AD (Ramassamy, 2006). *In vitro* cultures of *L. viridis* accumulated high contents of phenolic compounds, particularly rosmarinic acid (Table 1). In addition to phenolic compounds, these cultures produce essential oil with identical chemical composition of that isolated from wild plants (Nogueira and Romano, 2002). All these results show the potential of *in vitro* cultures of *L. viridis* to biosynthesize active metabolites with biological effects and economic value.

4. Conclusions

In this study, we found that *in vitro* cultures of *L. viridis* accumulated much higher contents of rosmarinic acid than wild plants. Moreover, the results suggest that compounds from *L. viridis* can avoid neuron degeneration produced by free radical processes, including participation in Fenton's reaction, and inhibit cholinesterases activities. Therefore, this study indicates *L. viridis*, particularly *in vitro* cultures, as a source of valuable active metabolites with antioxidant and anti-cholinesterase activities.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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CHAPTER 4

COMPLEXATION OF ESSENTIAL OILS WITH CYCLODEXTRINS

Costa P, Medronho B, Gonçalves S, Romano A (2013). The effect of cyclodextrins on the antioxidant activity of Lamiaceae essential oils. (submitted)

4.1. Abstract

In the present study, we have investigated the effect of *β*-cyclodextrin (*β*-CD) and 2-hydroxypropyl-*β*-cyclodextrin (HP-*β*-CD) on the antioxidant activity and storage stability of the essential oils from *Lavandula viridis*, *Lavandula pedunculata* subsp. *lusitanica* and *Thymus lotocephalus* (Lamiaceae). The presence of both CDs was found to produce a remarkable enhancement on the antioxidant activity as assessed by the oxygen radical absorbance capacity (ORAC) assay. The highest free radical scavenging effect was observed in the essential oils from *T. lotocephalus*, particularly at the highest concentration of HP-*β*-CD. Moreover, the antioxidant activity of the essential oil/HP-*β*-CD complexes was found to be considerably stable over 30 days possibly due to the higher solubility of the modified *β*-CD. The results obtained suggest that these systems can be very interesting alternatives in food, pharmaceutical and cosmetic formulations as natural source of antioxidants.

4.2. Introduction

Essential oils are natural complex mixtures of volatile components of different functional group classes that are produced as secondary metabolites by aromatic plants that have a great potential for the pharmaceutical, cosmetic and food industries (Bakkali et al., 2008). Many essential oils have already been granted Generally Recognized As Safe (GRAS) status by the Food and Drug Administration (FDA) and, therefore, can be used in food products without further approval (Hyldgaard et al., 2012). Due to their antioxidant and antimicrobial effects, essential oils have, for instance, a great potential as food preservatives reducing the oxidative reactions and microbial contaminations during food handling, processing and storage (Hyldgaard et al., 2012). However, their limited water solubility, volatility and sensitivity to oxygen, light and heat can considerably decrease their bioavailability and, consequently, restrict their applications. For instance, Skold et al. (2002) reported that linalool, frequently incorporated in fragrance materials, oxidizes on air exposure (autoxidation) generating allergenic oxidation products. In addition, chemical reactions between essential oils and the food ingredients must be seriously considered (Hyldgaard et al., 2012).

The complexation of essential oils can be an efficient strategy to ensure the maintenance of their stability and biological activity. For this propose, a vast role of molecules has been extensively used as complexing agents such as chitosan (Zivanovic et al., 2005), proteins (Baranauskienė et al., 2006), gums (Chang et al., 2006) and starch-based compounds (Hadaruga et al., 2007; Ayala-Zavala et al., 2008; Petrović et al., 2010; Ciobanu et al., 2012). Among them, cyclodextrins (CDs) are of particular interest due to their unique structural features and amphiphilic properties. CDs are natural cyclic oligosaccharides with a truncated cone shape containing 5 or more D-glucopyranose units netted through covalent bonds by C1 and C4 carbons (Fig. 4.1A). *β*-CD, with 7 sugar units, has been the most commercially attractive (more than 95% of CDs produced and consumed) due to its simple synthesis, availability and price. Nevertheless, *β*-CD has a relatively low solubility in water (1.8%) when compared to the other CDs and this is believed to be due to the strong binding of the *β*-CD molecules in the crystal state (Szente and Szejtli, 1999). This low solubility has triggered the synthesis of new CD derivatives. Having a safe biological profile, 2-hydroxypropyl-*β*-cyclodextrin (HP-*β*-CD) is a *β*-CD derivative with improved water solubility (above 60%) and the introduction of hydroxypropyl moieties decreases the crystallinity thus enhancing solubility (Szente and Szejtli, 1999). The cavity is lined by the hydrogen atoms and the

glycosidic oxygen bridges. The nonbonding electron pairs of the glycosidic oxygen bridges are directed toward the inside of the cavity, producing a high electron density and lending it some Lewis base character (Astray et al., 2009). As a result of this spatial arrangement of the functional groups in the CDs molecules, the cavity is relatively hydrophobic while the external surfaces are hydrophilic (Fig. 4.1B). Therefore, CDs are expected to considerably change the physicochemical properties of the solutions forming, for instance, inclusion complexes with a wide range of molecules (i.e. flavouring agents, metallic cations, fragrances, pesticides and essential oils) (Marques, 2010).

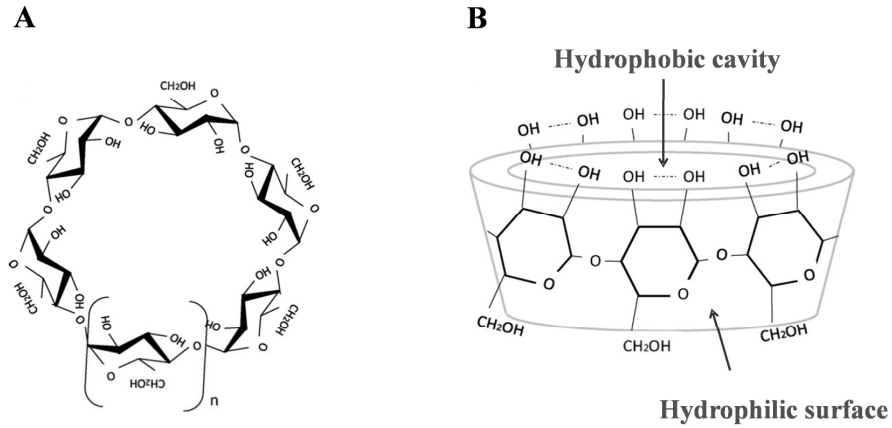

Figure 4.1. Chemical structure (A) and schematic representation of the truncated conical shape (B) of cyclodextrins (adapted from Pazos et al., 2010*)*. The "n" represents the number of repeating sugar units. In the modified HP-*β*-CD, the OH groups at the outer hydrophilic surface are substituted by hydroxypropyl moieties.

Lamiaceae is a diverse and widespread family of plants many of them of great economic importance due to essential oils production. Among others, *Lavandula* and *Thymus* genera comprise popular aromatic plants widely used in different areas such as flavouring agents, pleasant fragrances, culinary and medical field (Boelens, 1995; Zarzuelo and Crespo, 2002). Their essential oils are of great economic valuable and, therefore, strictly regulated by international ISO standards (ISO TC 54-ISO/CD 8902, 2007; ISO TC 54 N-ISO/WD 4719, 2009; ISO 14715, 2010). *Lavandula viridis* L'Hér, *Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco and *Thymus lotocephalus* G. López & R. Morales are important aromatic plants from the Mediterranean region. Previous phytochemical studies of their essential oils have revealed the presence of antioxidants and cholinesterase inhibitors (Costa et al., 2012a; Costa et al., 2012b; Costa et al., 2013). In addition, essential oils from *L. viridis*, *T. lotocephalus* and other subspecies of *L. pedunculata* subsp. *lusitanica* have been shown to

possess remarkable antimicrobial properties (Faleiro et al., 2003; Zuzarte et al., 2009; Zuzarte et al., 2011). This is particularly important for applications in the food preservation field (Singh et al., 2011; Hyldgaard et al., 2012). Therefore, and considering the impressive biological potential of essential oils from the above mentioned plants we intended to investigate, for the first time, the effect of *β*-CD and HP-*β*-CD on their antioxidant activity and storage stability over 30 days.

4.3. Materials and methods

4.3.1. Chemicals

Fluorescein was obtained from Panreac (Barcelona, Spain). 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Acros Organics (Geel, Germany). *β*-Cyclodextrin (*β*-CD) and 2-hydroxypropyl-*β*-cyclodextrin (HP-*β*-CD) were purchased from Sigma-Aldrich (Steinheim, Germany). All chemicals were used as received.

4.3.2. Plant material and preparation of the essential oils

The aerial parts of *L. viridis* plants were collected during the flowering period at São Bartolomeu de Messines (Algarve, Portugal). A voucher specimen was deposited in the herbarium of the Botanical Garden of the University of Lisbon under the number LISU 173992. The aerial parts of *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus* plants were collected during the flowering period at Campus de Gambelas (Algarve, Portugal) and vouchers specimens were deposited in the herbarium of the University of Algarve under the number ALGU 8080 and 8081, respectively. The plant material was dried at room temperature until constant weight, blended to powder to achieve a mean particle size less than 2 mm and stored at -20 ºC until further use. The essential oils were obtained by hydrodistillation (HD). Briefly, 50 g of plant material and 750 ml of water were putted in a Clevenger-type apparatus for 3 h.

4.3.3. Evaluation of antioxidant activity by oxygen radical absorbance capacity (ORAC) assay

Generically, a fixed amount of essential oils from *L. viridis*, *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus* (i.e. 31 μg/ml) was mixed with different concentrations of *β*-CD (i.e. 0, 2.25, 4.5 and 9 mg/ml) and HP-*β*-CD (i.e. 0, 2.25, 4.5, 9 and 18 mg/ml). The mixtures were shaken for 2 h at room temperature on an orbital shake (VWR Collection Tube Rotator, EU plug) and, afterwards, stored at 4 ºC during the test period (30 days). All the essential oils and CDs solutions were prepared in phosphate buffer (75 mM, pH 7.0).

The antioxidant capacity was evaluated as described by Gillespie et al. (2007) using fluorescein as the fluorescent probe and AAPH as a peroxyl radical generator. In this water-based method (ORAC assay), a fluorescent probe is used to compete with antioxidants for peroxyl radicals generated by thermal decomposition of AAPH (Prior et al., 2005). One should mention that this method is limited to hydrophilic compounds, thus excluding lipophilic antioxidants which might be involved in important biological processes (Prior et al., 2005). Briefly, 0.08 μM fluorescein were added to the above-mentioned essential oils/CDs solutions and placed in a black microplate (NUNC, Rochester, New York, USA). After incubating for 10 min at 37 ºC, the oxidation reaction was initiated by adding 150 mM AAPH to each "well" of the microplate. Different calibration solutions of Trolox standard (i.e. 6.25, 12.5, 25 and 50 μM) were used, and blanks were prepared with phosphate buffer and CDs in the absence of the essential oils. The reduction in fluorescence was determined by reading fluorescein excitation at 485 nm and emission at 530 nm, every minute, for 1 h. The essential oils/CDs complex stability was evaluated over 30 days following the alluded procedure. All experiments were carried out in triplicate. The ORAC value for each sample was calculated by subtracting the area under the blank curve from the area under the sample curve to obtain the net area under the curve (Net AUC). Such procedure is illustrated in Fig. 4.2 where one can see in (a) typical fluorescence decay curves, induced by AAPH in the absence (blank) and presence of Trolox (e.g. 50 μM) and (b) the linear regression of Net AUC of Trolox at the different concentrations tested. The results are expressed as Trolox equivalents per gram of essential oil (TE/g_{EO}).

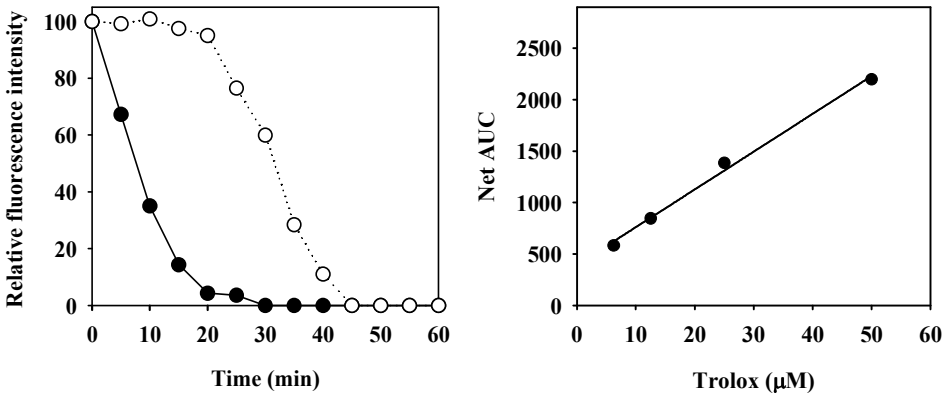

Figure 4.2. (A) Fluorescence decay curves induced by AAPH of blank (black circles) and Trolox (50 μM) (white circles). (B) Linear regression of Net AUC of Trolox at different concentrations tested.

4.3.4. Statistical analysis

The data are expressed as the mean ± standard error and were subjected to one-way analysis of variance (ANOVA). To analyse de effect of CD concentration and storage period on the antioxidant activity, means were compared by Duncan's New Multiple Range Test. All statistical analysis was carried out using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA).

4.4. Results and discussion

4.4.1. Antioxidant activity of Lamiaceae essential oils complexed with CDs

The effect of *β*-CD on the antioxidant capacity of the Lamiaceae essential oils is showed in Fig. 4.3. It is important to note that neither the essential oils nor the CDs alone show free radical scavenging ability at the concentrations tested. In fact, to detect any antioxidant activity in its free state, a much higher concentration of essential oil would be needed (i.e. up to sixteen times higher than the one used in this work) (Costa et al., 2012a; Costa et al., 2012b; Costa et al., 2013). From Fig. 4.3, it is clear that when the essential oils are in the presence of *β*-CD, a remarkable antioxidant potential is observed despite the use of a much less quantity of essential oil. The essential oils from *L. viridis* and *T. lotocephalus* were found to be the most active radical scavengers in the presence of *β*-CD. Moreover, the highest antioxidant activity of *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus* was observed for the higher concentration of *β*-CD used (i.e. 9 mg/ml) whereas no significant effect of the *β*-CD concentration was observed in the *L. viridis* essential oil.

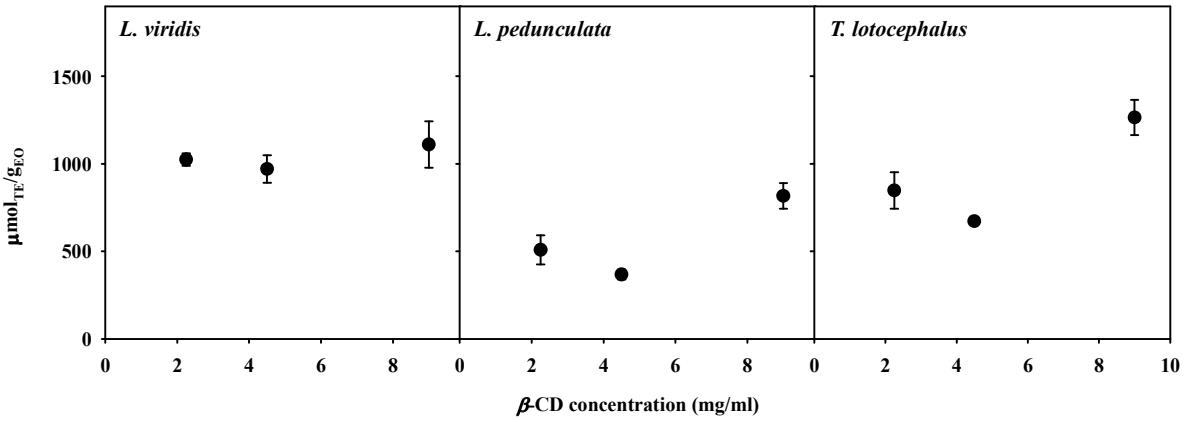

Figure 4.3. Effect of the concentration of *β*-CD on the antioxidant activity of the Lamiaceae essential oils.

Likewise, the activity of all the Lamiaceae essential oils was enhanced by the presence of HP-*β*-CD and, in this case, as can be seen in Fig. 4.4, the effect was significantly ($p < 0.05$) more pronounced in the *T. lotocephalus* essential oil, particularly at the highest concentration of HP-*β*-CD (i.e. 18 mg/ml). In this case, when the concentration of HP-*β*-CD increased from 2.25 mg/ml to 18 mg/ml, the free radical scavenging potential almost doubled reaching the highest ORAC value observed (i.e. 1900 µmol$_{TE}$/g$_{EO}$). Conversely, no significant effect of the HP-*β*-CD concentration was detected in the activity of the *Lavandula* species.

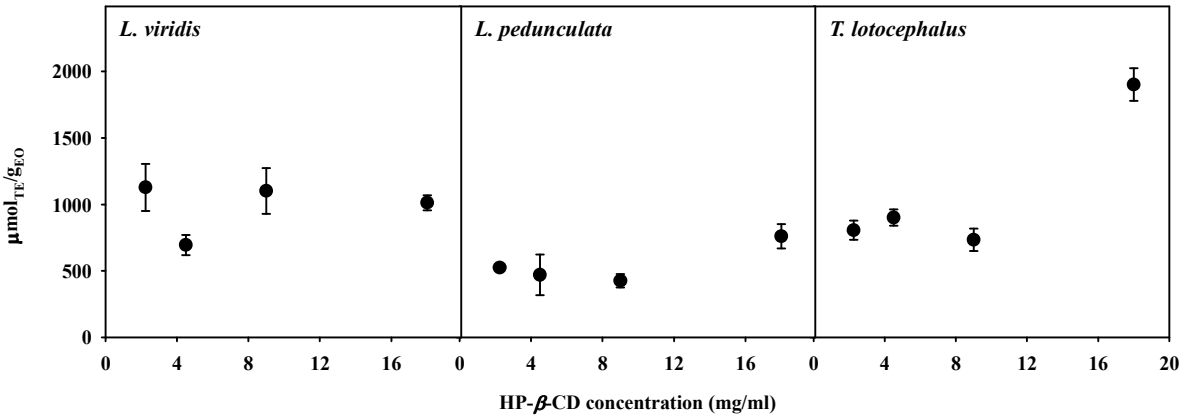

Figure 4.4. Effect of the concentration of HP-*β*-CD on the antioxidant activity of the Lamiaceae essential oils.

As mentioned above, the activity of CDs alone was also investigated and no effect was observed at all the concentrations studied. This lack of antioxidant activity of CDs has also

been reported in previous studies (Lucas-Abellán et al., 2008). These results suggest that the increase on the antioxidant activity is linked to the enhancement of solubility of the essential oils since in its free state, at the same low concentration of 31 μg/ml, have no measurable activity. Furthermore, due to the higher solubility in water, the process of complexation with HP-*β*-CD is much facilitated compared with the native *β*-CD. Different studies have been reported concerning the association constant of volatiles and CDs complexes. For instance, Astray et al. (2010) investigated the binding constants of different flavours, including cineole and camphor, to *α*- and *β*-CDs and reported that the main driving force for CDs-flavour complex formation is related to the hydrophobic and the hydrophilic character of the flavours. It was reported that the majority of volatiles have a 1:1 (volatile:CD) complex formation (Szente and Szejtli, 2004). The inclusion complex ability of CDs depends on the geometric accommodation in the internal cavity and thermodynamic interactions with the guest molecule (Martin Del Valle, 2004). From this point of view, no significant differences are expected between CDs since both possess the same number of sugar units. On the other hand, additionally to the entrapment in the internal cavity of CD, which can occur totally or partially, the hydroxyl groups on the outer surface of the CDs can also interact (e.g. formation of hydrogen bonds) with guest molecules (Loftsson and Duchene, 2007). Both CDs have this capacity, however the chemical structure of the hydroxypropyl moieties in HP-*β*-CD not only makes it more soluble than the native *β*-CD but also changes the surface chemistry. Therefore, we also expect that less polar compounds, which have not been trapped in the HP-*β*-CD cavity (and do not strongly interact with simple hydroxyl groups), can absorb at HP-*β*-CD external surface increasing their solubility thus contributing to the enhancement of the antioxidant activity.

The essential oils of *L. viridis*, *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus* were previously characterised and the main compounds identified are showed in Table 4.1. Camphor was the main constituent identified in the essential oils of *L. viridis* (Costa et al., 2012b) and *L. pedunculata* subsp. *lusitanica* (Costa et al., 2013), whereas linalool was the major compound in the essential oil of *T. lotocephalus* (Costa et al., 2012a).

Table 4.1
Main compounds (relative %) identified in *Lavandula viridis*, *Lavandula pedunculata* subsp. *lusitancia* and *Thymus lotocephalus* essential oils.

L. viridis[a]	*L. pedunculata*[b]	*T. lotocephalus*[c]
Camphor (31.6%)	Camphor (40.6%)	Linalool (10.4%)
1,8-Cineole (21.3%)	Fenchone (38.0%)	Caryophyllene oxide (8.7%)
Verbenone (3.5%)	α-Fenchol (2.6%)	Camphor (8.0%)
Norinone (2.1%)	Linalool (2.0%)	Borneol (5.6%)
trans-Linalool oxide (1.8%)	Verbenone (1.5%)	α-Terpineol (4.5%)
Linalool (1.8%)	Eremophyllene (1.4%)	1,8-Cineole (3.8%)
Myrtenol (1.8%)	α-Cadinol (1.3%)	Globulol (3.7%)
Myrtenal (1.2%)	*p*-Cymen-8-ol (1.3%)	Viridiflorol (2.7%)
Caryophyllene oxide (1.2%)	1,8-Cineole (0.9%)	Geranyl acetate (2.2%)

[a]Costa et al. (2012b)
[b]Costa et al. (2013)
[c]Costa et al. (2012a)

Camphor and linalool are high-value aromatic compounds for many industries and both have been previously demonstrated to have radical scavenging abilities (Çelik and Ozkaya, 2002; Zaouali et al., 2010). Recently, Ciobanu et al. (2012) investigated their interactions with CDs and *β*-CD polymers in *Lavandula angustifolia* essential oil. They reported that *β*-CD have more affinity for linalool and camphor than *α*- or *γ*-CD. Moreover, they found that camphor has apparently a better geometric accommodation inside the CD cavity and, consequently, a greater stability than that of linalool. Nevertheless, there are several examples (Nakatsu et al., 2000; Costa et al., 2012b) proving that the minor compounds cannot be neglected because they determine the final activity of the essential oil. Since the overall effect of the essential oils also results from the symbiotic relationship off all compounds it is not possible to rationalize, in a simple fashion, the higher antioxidant activity generally observed in this work for the essential oil from *T. lotocephalus*.

4.4.2. Storage stability of Lamiaceae essential oils-CD complexes

If care is not taken, storage processes can induce chemical and biological modifications on the activity of volatiles (Marques, 2010). Therefore, it is crucial to investigate the storage stability of any generic formulation. Here we have also investigated the influence of CDs in storage stability of the essential oils, at 4 ºC, over 30 days. Fig. 4.5 represents the effect of *β*-CD at different concentrations (i.e. 2.25 and 9 mg/ml) on the activity of the essential oils.

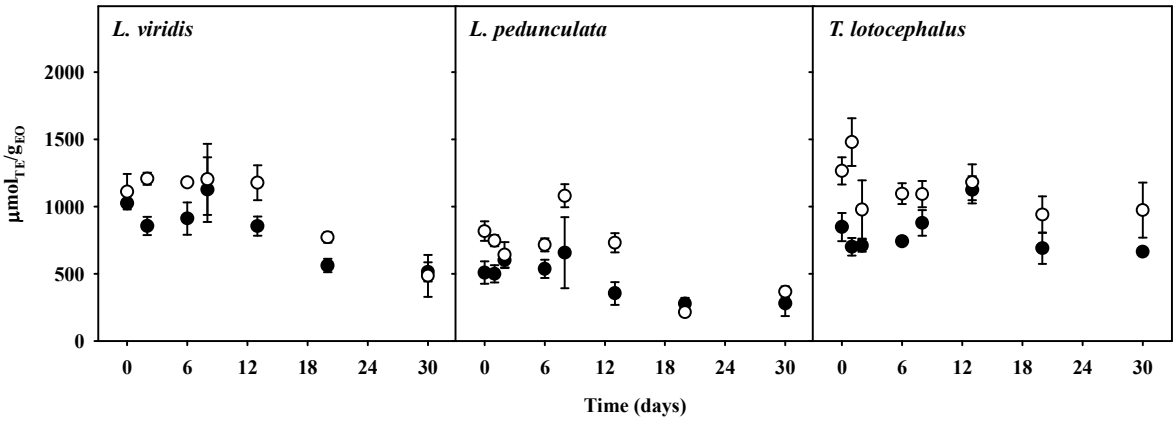

Figure 4.5. Storage stability of the Lamiaceae essential oils for a *β*-CD concentration of 2.25 mg/ml (black circles) and 9 mg/ml (white circles).

After 30 days, regardless the concentration of *β*-CD, the *Lavandula* spp. showed a moderate decrease on the antioxidant activity. On the other hand, the essential oil of *T. lotocephalus* revealed the highest storage stability with no significant change in activity after 30 days, particularly when complexed with *β*-CD at the highest concentration. When HP-*β*-CD is used, all species showed a remarkable stable activity profile over 30 days (Fig. 4.6).

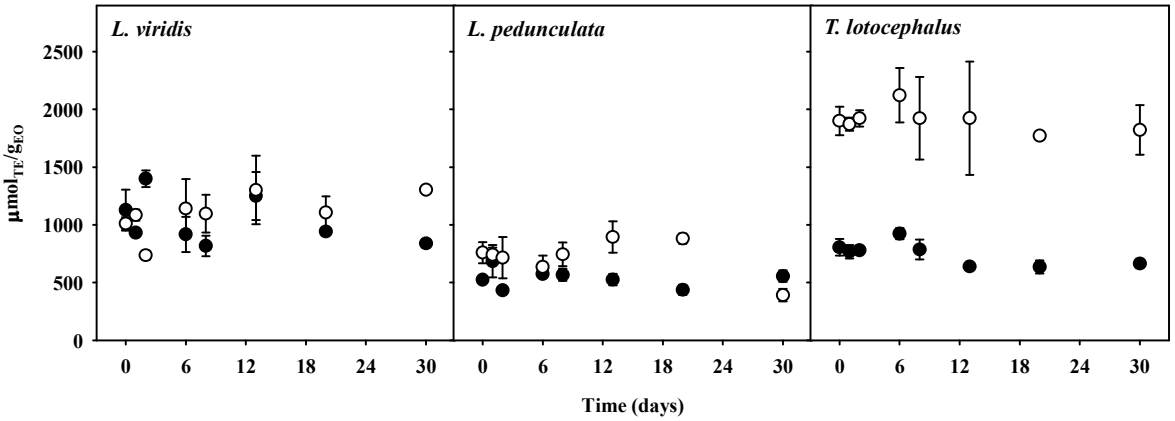

Figure 4.6. Storage stability of the Lamiaceae essential oils for a HP-*β*-CD concentration of 2.25 mg/ml (black circles) and 18 mg/ml (white circles).

Overall, the activity was seen to be more stable in the presence of HP-*β*-CD than with *β*-CD which, again, is not only related with the higher solubility of HP-*β*-CD but also with favored interactions between the hydroxypropyl moieties and the essential oils constituents.

4.5. Conclusions

In this study, we have reported, for the first time, the effect of two CDs, *β*-CD and HP-*β*-CD, on the antioxidant activity of the essential oils from *L. viridis*, *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus*. Alone, both essential oils and CDs did not show any measurable antioxidant capacity for the concentrations investigated. However, when the Lamiaceae essential oils are complexed with *β*-CD or HP-*β*-CD, an impressive antioxidant activity is observed. Must likely, this behavior is due to an enhancement in solubility of some less polar compounds of the essential oils. For the lower concentrations of CDs, little effect on the antioxidant activity of *Lavandula* essential oils is detected. On the other hand, the activity increased for the highest CD concentration tested (i.e. 9 mg/ml for *β*-CD and 18 mg/ml for HP-*β*-CD). The modified CD, HP-*β*-CD, was shown to have a more pronounced effect on essential oils activity, in particular for *T. lotocephalus*.

All formulations with HP-*β*-CD are considerably stable over time presenting a high activity after 1 month of storage. In the case of *β*-CD, the complexes seem less stable and the activity slightly decreased after 1 month of storage time. Nevertheless, even in the worst case, the activity measured at the end of the storage period is still quite appreciable.

We have demonstrated that CDs (particularly HP-*β*-CD) have a tremendous positive impact on the antioxidant activity of essential oils from three Lamiaceae species. Since these essential oils can be seen as a natural source of antioxidants and antimicrobial compounds, we do believe that this system is a good candidate to be used in food, pharmaceutical and cosmetic formulations.

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CHAPTER 5

NEUROPROTECTIVE EFFECT

Costa P, Sarmento B, Gonçalves S, Romano A (2013). Protective effects of *Lavandula viridis* L'Hér extracts and rosmarinic acid against H_2O_2-induced oxidative damage in A172 human astrocyte cell line. Industrial Crops and Products. (under revision)

5.1. Abstract

We investigated the neuroprotective effect of *Lavandula viridis* extracts (infusion, water:ethanol and methanol) and rosmarinic acid, the major compound present in the extracts, against oxidative damage induced by hydrogen peroxide (H_2O_2) in A172 human astrocyte cell line regarding the neurotoxic effect, intracellular reactive oxygen species (ROS) production and the activity of the antioxidant enzyme catalase (CAT). We found that *L. viridis* extracts and rosmarinic acid protected A172 astrocytes against H_2O_2 and reduced intracellular ROS accumulation. Furthermore, the protection effect was not caused by modulation of CAT suggesting that other intracellular mechanisms are involved in the neuroprotective effect. Our results highlight that *L. viridis* extracts and rosmarinic acid have beneficial effects against oxidative damage associated with neurodegenerative diseases.

5.2. Introduction

Neuronal damage resulting from oxidative stress is implicated in the etiology and progression of many neurodegenerative disorders. Due to its high consumption of total oxygen required to metabolic activities, high concentrations of polyunsaturated fatty acids and transition metals and low levels of endogenous antioxidant enzymes, neuronal cells are particularly susceptible to oxidative processes (Halliwell, 1992; Barnham et al., 2004). Hydrogen peroxide (H_2O_2) is a major mediator of oxidative stress. Because its generation in the brain is relatively high, it can easily pass through the biological membranes and be converted into highly toxic reactive oxygen species (ROS) (Finkel, 2003; Milton, 2004). The oxidative stress induced by H_2O_2 can result in mitochondrial dysfunction, calcium imbalance and apoptosis of neuronal cells (Jonas et al., 1989; Halliwell et al., 2000; Giorgio et al., 2007). Although cells have specific mechanisms to neutralize ROS, including enzymatic (e.g. superoxide dismutase, CAT and glutathione peroxidase) and non-enzymatic (e.g. vitamin A, C and E), it cannot be enough to avoid oxidative modifications in lipids, proteins and deoxyribonucleic acid (DNA) (Giorgio et al., 2007). Thus, attention has been recently focused on searching for natural products that can protect cells from oxidative damage. Phenolic compounds are an important class of plant-derived secondary metabolites with recognized neuroprotective ability (Kovacsova et al., 2010). They can act in different molecular targets and by different mechanisms due to their redox potential. They can directly scavenge pathological concentrations of free radicals, chelate transition metal ions or interact with cellular signaling pathways involved in neurodegeneration (Ebrahimi and Schluesener, 2012). Therefore, the use of phenolic compounds appears as an efficient strategy to avoid oxidative damage and to promote neuronal survival signals (Kelsey et al., 2010; Vauzour, 2012).

Lavandula viridis L'Hér (Lamiaceae) is an aromatic shrub endemic to the southwest Iberian Peninsula that is used in traditional medicine. Phytochemical studies from the aerial parts of this species highlighted the presence of different chemical classes of bioactive compounds as terpenes and phenolic compounds, including hydroxycinnamic acids, flavones and one flavanone (Nogueira and Romano, 2002; Costa et al., 2011; Costa et al., 2012; Costa et al., 2013). Recently, we proved through *in vitro* studies the capacity of phenolic extracts as free radical scavengers, metal chelators and cholinesterase inhibitors (Costa et al., 2011; Costa et al., 2013). In this study we studied, for the first time, the capacity of three *L. viridis* extracts

and their main constituent, rosmarinic acid, to protect A172 human astrocyte cell line from H_2O_2-induced oxidative damage.

5.3. Materials and methods

5.3.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), Hank's balanced salt solution (HBSS), penicillin/streptomycin and fetal bovine serum (FBS) (heat inactivated) were purchased from Lonza (Verviers, Belgium). 2',7'-Dichlorofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and rosmarinic acid were acquired from Sigma-Aldrich (Steinheim, Germany). Trypsin/ethylenediaminetetraacetic acid (EDTA) solution was purchased from Biochrom (Berlin, Germany). Hydrogen peroxide (H_2O_2) and L-gluthamine were obtained from VWR (Leuven, Belgium).

5.3.2. Plant material and extracts preparation

The aerial parts of *L. viridis* were collected during flowering period at São Bartolomeu de Messines (Algarve, Portugal). A voucher specimen was deposited at the herbarium of the Botanical Garden of the University of Lisbon under the number LISU 173992. Plant material was dried at 40 ºC and powdered in a blender to achieve a mean particle size less than 2 mm. The plant material (10 g) was then extracted with methanol (150 ml), at room temperature for 5 h in a Soxhlet apparatus. The water:ethanol extract (1:1) was obtained from 10 g of plant material and soaked overnight at room temperature in 200 ml of solvent and the resulting extract was filtered through a 5-13 µm membrane. An infusion was also prepared by homogenizing 1 g of the plant material in 20 ml hot water (90 ºC) for 5 min and filtered (5-13 µm membrane). Finally, the extracts were concentrated to dryness (the methanol and water:ethanol extracts were dried in a rotary evaporator and the infusion was lyophilized) and stored at -20 ºC until future use.

5.3.3. Cell culture

A172 human astrocyte cell line from ATCC was maintained in complete DMEM (without phenol red) supplemented with 10% heat inactivated FBS, 1% L-gluthamine (200 mM) and 1% penicillin/streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin). Cells were maintained at 37 ºC in 5% CO_2 with 95% relative humidity.

5.3.4. Cell viability

The cytotoxicity of the *L. viridis* extracts and rosmarinic acid was evaluated by MTT assay which is based on the cleavage of tetrazolium salts by mitochondrial succinate tetrazolium reductase in viable cells to form formazan dye (Mosmann, 1983). A172 cells were seeded in 96-well plates (NUNC, Rochester, New York, USA) at a density of 5×10^3 cells/well and allowed to attach. After 24 h exposure to the *L. viridis* extracts at different concentrations (125 and 250 µg/ml) or rosmarinic acid (30 µg/ml), the medium was removed and the cells were slightly washed with HBSS. MTT (5 mg/ml) was added, and after 2 h incubation at 37 ºC the resulting formazan crystals were dissolved in DMSO. Cell viability was measured in terms of absorbance at 570 nm in a microplate reader (Infinite 200 Tekan, Grödig, Austria) comparing treated with untreated cells.

5.3.5. Protective effect of *L. viridis* and rosmarinic acid on H_2O_2-induced cell cytotoxicity

A172 cells were seeded at a density of 75×10^3 cells/well in 6-well culture plates and allowed to attach. Then, the medium was removed and cells were washed with HBSS. The cells were treated with H_2O_2 (600 µM) in the presence of different concentrations of extracts (125 and 250 µg/ml) or rosmarinic acid (30 µg/ml) for 24 h at 37 ºC. Then, cell viability was determined by MTT assay as described above. The protective effect was determined comparing treated with untreated cells.

5.3.6. Observations of morphological changes

A172 cells were seeded at a density of 75×10^3 cells/well in 6-well culture plates and allowed to attach. The cells were treated with H_2O_2 (600 µM) in the presence of extracts (250 µg/ml) or rosmarinic acid (30 µg/ml) for 24 h at 37 ºC. The cellular morphology was observed using

an inverted light microscope (Leica DMIL) and photographed by the coupled digital camera (Leica DC 500).

5.3.7. Intracellular reactive oxygen species (ROS) production

The ability of *L. viridis* extracts to reduce intracellular ROS production was estimated using a fluorescent probe (DCFH-DA). DCFH-DA diffused into the cell where cellular esterases cleaved the diacetate moiety to form the more polar 2',7'-dichlorofluorescein (DCFH), which is then oxidized by ROS generating a fluorescent compound (Wang and Joseph, 1999; Gomes et al., 2005). The fluorescence intensity is believed to reflect the amount of ROS formed intracellularly. A172 cells were seeded in a 96-well plate (5×10^3 cells/well), incubated to attach and then pre-incubated with extracts (125 and 250 µg/ml) or rosmarinic acid (30 µg/ml), for 24 h at 37 ºC. Cells were gently washed with HBSS and then incubated with 25 µM DCFH-DA for 30 min at 37 ºC. After incubation, cells were washed with HBSS followed by addition of H_2O_2 (600 µM). The fluorescence was then measured ($\lambda_{exitation}$ = 485 nm; $\lambda_{emission}$ = 530 nm) over 1 h at 37 ºC. ROS generation was calculated as an increase in fluorescent signal between the control and H_2O_2-treated cells.

5.3.8. Determination of endogenous antioxidant catalase (CAT) activity

Cells were seeded in 6-well plates and treated with H_2O_2 (600 µM) in the presence of different concentrations of extracts (125 and 250 µg/ml) or rosmarinic acid (30 µg/ml) for 24 h at 37 ºC. Then, cells were washed with HBSS and lysed with 200 µl of lysis buffer, incubated for 20 min on ice and then centrifuged 15 min at 9000*g*. The CAT activity was determined by the degradation of H_2O_2 using the method of Aebi (1983). The reaction mixture contained 50 mM phosphate buffer (7.0), 40 mM of H_2O_2 and 50 µl of cell lysate. The H_2O_2 decomposition was monitored at 240 nm (ε = 39.4 mM^{-1} cm^{-1}) and the enzyme activity was expressed as units per milligram of protein, based on the measurement of total soluble protein levels according to the method of Bradford (1976).

5.3.9. Statistical analysis

The results reported are the averages of three independent experiments and are represented as the mean ± standard error. We carried out a one-way analysis of variance (ANOVA) on the results to assess treatment differences using the SPSS statistical package for Windows (release 19.0; SPSS Inc., Chicago, IL, USA).

5.4. Results and discussion

Oxidative stress occurs as a result of an imbalance between the antioxidant defense mechanisms and free radical production which can damage biological target molecules affecting their function and integrity. Currently, considerable efforts have been made to search for natural compounds to protect cells from oxidative damage (Almajano et al., 2011; Martín et al., 2011; Kwon et al., 2012). In this study, we investigated for the first time the neuroprotective effect of *L. viridis*, an important aromatic plant, as well as its major compound rosmarinic acid, regarding the neurotoxic effect, ROS production and the activity of the antioxidant enzyme CAT.

The possible neurotoxic effect of the *L. viridis* extracts and rosmarinic acid was evaluated using the MTT assay. As shown in Fig. 5.1, the viability of A172 human astrocyte cell line was not adversely affected by the *L. viridis* extracts and rosmarinic acid at the concentrations tested. Based on these results, we used these concentrations for the subsequent assays.

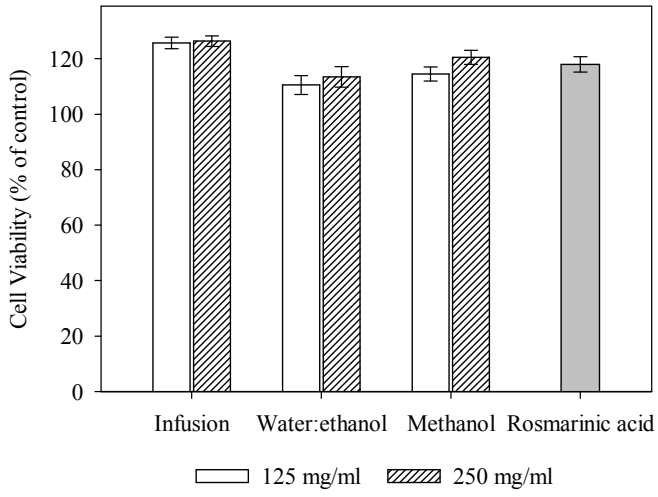

Figure 5.1. Effect of *Lavandula viridis* extracts and rosmarinic acid (30 μg/ml) on the A172 cell viability for 24 h. Cell viability was determined using MTT assay. Data are expressed as the mean ± standard error of three independent experiments.

We investigated the ability of *L. viridis* extracts and rosmarinic acid to protect A172 cells against oxidative stress using H_2O_2 as the free radical generating system (Fig. 5.2). The optimal concentration of H_2O_2 (600 µM) tested was determined based on dose-response curves of H_2O_2-induced cell death (data not shown). Until now, the neuroprotective effect of *L. viridis* had never been studied in a cell model.

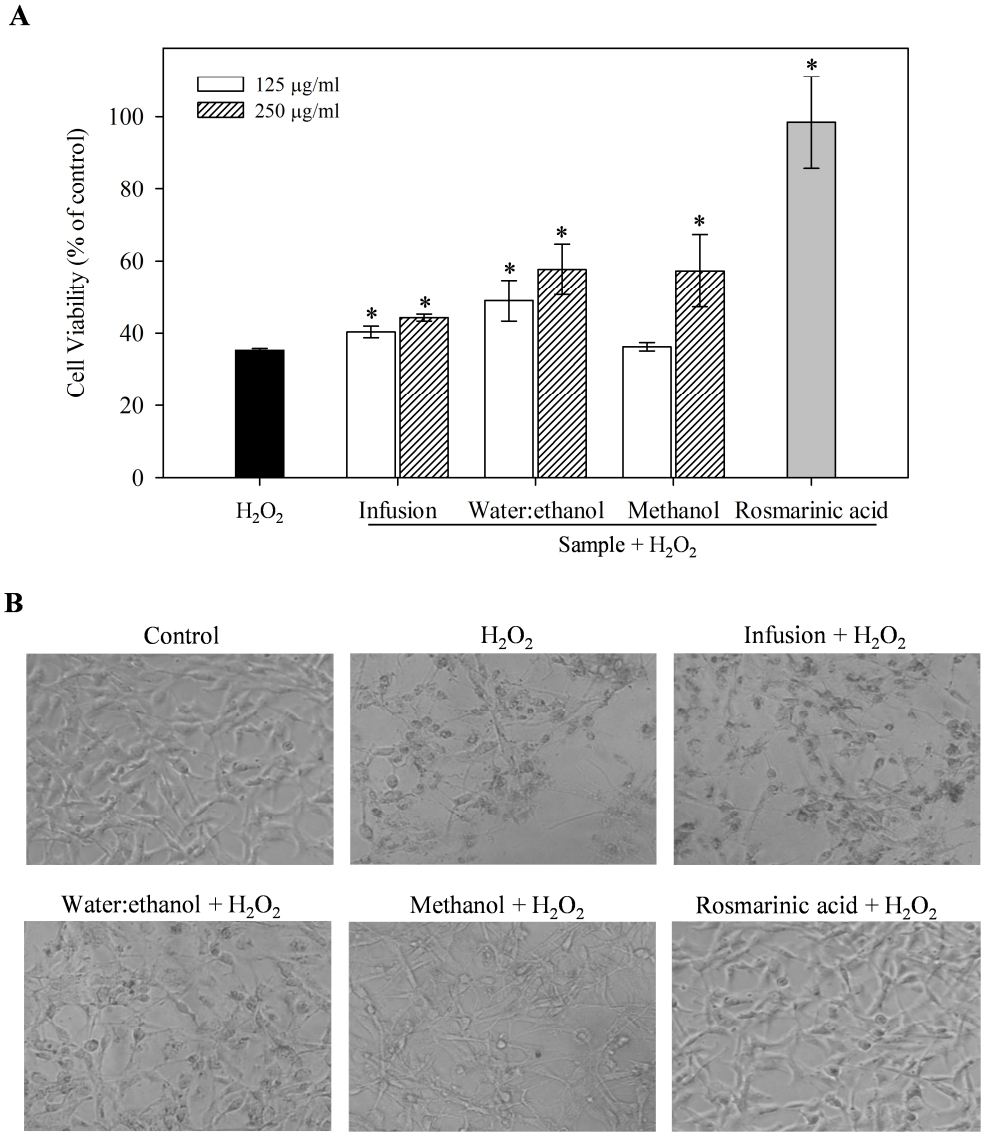

Figure 5.2. (A) Protective effect of *Lavandula viridis* extracts and rosmarinic acid (30 µg/ml) after H_2O_2-induced damage (600 µM) in A172 human astrocyte cell line. Cell viability was determined using MTT assay. Data are expressed as the mean ± standard error of three independent experiments. Values marked with * indicate significant differences ($p < 0.05$) when compared with treated cells with H_2O_2 alone. (B) Effect of *L. viridis* extracts (250 µg/ml) and rosmarinic acid (30 µg/ml) on H_2O_2-induced damage in A172 human astrocyte cell line by inverted light microscope.

As we can see in Fig. 5.2A, cell viability was reduced to 35.31 ± 0.51% after 24 h of exposure to 600 μM H_2O_2 compared to the untreated cells. The results indicated that, except for the lowest concentration of the methanol extract, all the remaining extracts and rosmarinic acid exhibited protective effect against neurotoxicity induced by H_2O_2. Rosmarinic acid showed the greatest neuroprotective effect followed by the water:ethanol and methanol extracts particularly at the highest concentration tested (250 μg/ml); the survival rate was improved to 98.47 ± 12.61%, 57.68 ± 7.03% and 57.29 ± 10.07%, respectively. The protective effect was also confirmed by qualitative morphological observations (Fig. 5.2B). Incubation with 600 μM H_2O_2 induced marked morphological changes manifested by cell shrinkage and a decrease in cell number when compared with control cells. However, the presence of the extracts reduced these morphological alterations.

The ability of the *L. viridis* extracts and rosmarinic acid to prevent or diminish H_2O_2-induced ROS production was investigated using the fluorescence probe DCFH-DA (Fig. 5.3). A172 cells exposed to H_2O_2 showed an increase in the intracellular level of ROS relative to the control cells. However, this effect was attenuated in A172 cells pretreated with the water:ethanol extract, infusion and rosmarinic acid, reducing the ROS levels about 20-30% in comparison with A172 cells treated only with H_2O_2. The methanol extract had no significant ($p \geq 0.05$) effect in the intracellular ROS production at the concentrations tested.

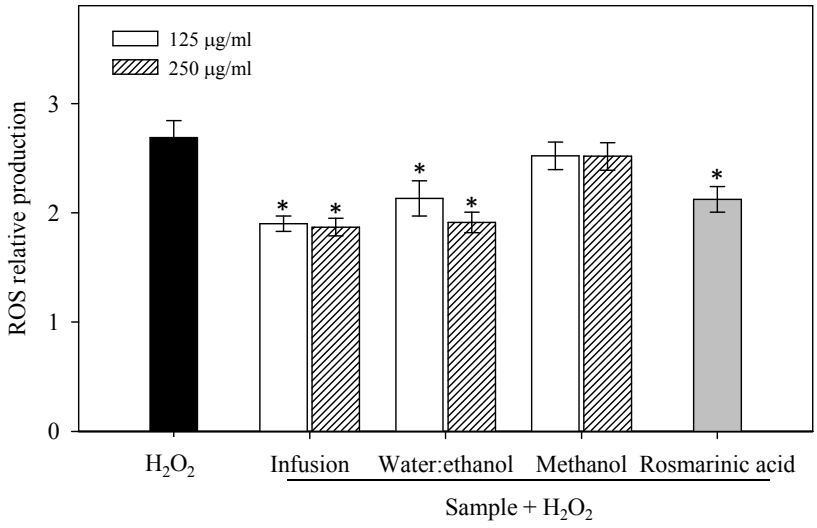

Figure 5.3. Effects of *Lavandula viridis* extracts and rosmarinic acid (30 μg/ml) on intracellular reactive oxygen species (ROS) production induced by H_2O_2 (600 μM) in A172 human astrocyte cell line. ROS generation was calculated as an increase in fluorescent signal between the control and H_2O_2-treated cells. Data are expressed as the mean ± standard error of three independent experiments. Values marked with * indicate significant differences ($p < 0.05$) when compared with cells treated with H_2O_2 alone.

We found that *L. viridis* extracts and rosmarinic acid possess protective effects against oxidative injury caused by exogenous H_2O_2. This effect could be due to the capacity of the extracts to scavenge intracellular ROS production or to neutralize H_2O_2 itself. Since natural polyphenols can regulate the intracellular redox balance by different mechanisms, the protective effect of *L. viridis* probably reflects the presence of phenolic compounds. They can directly act as radical scavengers and metal chelators or indirectly *via* activation of important signaling pathways as the transcription nuclear factor erythroid-derived 2-related factor 2 (Nrf2), which mediates generation of antioxidant enzymes (Jung and Kwak, 2010). The phenolic profile of the three *L. viridis* extracts was investigated by high-performance liquid chromatography with diode-array detection (HPLC-DAD). The water:ethanol extract and infusion were analysed under the same experimental conditions as described in Costa et al. (2013), and the methanol extract as reported in Costa et al. (2011). The chemical analysis revealed the presence of hydroxycinnamic acids (3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, caffeic and rosmarinic acids), flavones (luteolin and luteolin-7-*O*-glucoside) and one flavanone (pinocembrim) (Costa et al., 2011; Costa et al., 2013) (Table 5.1).

Table 5.1
Quantification of phenolic compounds identified in *Lavandula viridis* extracts (dry basis).

(mg/kg)[a]	Methanol[b]	Water: ethanol[c]	Infusion[d]
3-*O*-Caffeoylquinic acid	2468.2 ± 16.7	96.1 ± 0.6	27.9 ± 1.0
4-*O*-Caffeoylquinic acid	1628.5 ± 81.2	1335.0 ± 44.6	403.6 ± 0.7
5-*O*-Caffeoylquinic acid	845.1 ± 14.7	2331.9 ± 68.2	2768.6 ± 2.2
Caffeic acid	2631.1 ± 80.8	-	-
Luteolin 7-*O*-glucoside	13404.9 ± 196.7	-	-
Luteolin	-	7086.1 ± 7.8	1090.6 ± 60.9
Rosmarinic acid	38765.1 ± 1482.8	20714.1 ± 238.6	32905.1 ± 68.0
Pinocembrin	2697.3 ± 62.0	12745.0 ± 226.9	-

[a]Values are expressed as mean ± SD (*n=3*)
[b]Costa et al. (2011)
[c]Costa et al. (2013)
[d]Unpublished data

The protection mechanism of *L. viridis* extracts against ROS generation may be explained, at least partially, by their free radical scavenging activity (Costa et al., 2011; Costa et al., 2013).

By using oxygen radical absorbance capacity (ORAC) assay we found that infusion was the most efficient at neutralizing peroxyl radicals (7014.88 ± 33.60 $\mu mol_{TE}/g_{extract}$) (unpublished data) followed by the water:ethanol (4030.26 ± 102.40 $\mu mol_{TE}/g_{extract}$) and methanol (2858.39 ± 70.97 $\mu mol_{TE}/g_{extract}$) extracts. Regarding the capacity to scavenge the radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$), the tendency observed was similar: infusion (1717.85 ± 13.86 $\mu mol_{TE}/g_{extract}$) > water:ethanol (1148.82 ± 17.31 $\mu mol_{TE}/g_{extract}$) > methanol (967.18 ± 22.57 $\mu mol_{TE}/g_{extract}$). These results could explain the lower capacity of the methanol extract to reduce intracellular ROS production compared to the remaining extracts (Costa et al., 2011; Costa et al., 2013). In addition to the scavenging ability, we also proved their capacity to chelate iron (Costa et al., 2011; Costa et al., 2013). It is well known that high iron levels may act catalytically to produce ROS (Britton et al., 2002). By this way, the depletion of free iron by *L. viridis* extracts can also protect neuronal cells from iron-promoted radical production. Their capacity to inhibit lipid peroxidation in mouse brain homogenates was also demonstrated (Costa et al., 2011; Costa et al., 2013). Therefore, bioactive compounds from *L. viridis* can efficiently targeted different effects of oxidative stress.

Consistent with previous reports, we found that rosmarinic acid is highly active against H_2O_2-induced oxidative damage and able to significantly ($p < 0.05$) reduce the intracellular ROS production (Gao et al., 2005; Qiao et al., 2005). Lee et al. (2008) proved the neuroprotective effect of rosmarinic acid in SH-SY5Y cells; it was able to attenuate apoptotic cell death and to stimulate the antioxidative molecule heme oxygenase-1. Although the protective effect observed by *L. viridis* extracts seems to be primarily due to rosmarinic acid, we cannot ignore the presence of other important phenolic compounds in the extracts which have also revealed neuroprotective effects. For instance, Kim et al. (2012) reported that chlorogenic acid (5-*O*-caffeoylquinic acid), presented in high amounts in the water:ethanol extract and infusion by comparison to the methanol extract, inhibited H_2O_2-induced apoptotic death in primary cortical neurons. They indicated that this protective effect occurred through the upregulation of the anti-apoptotic genes Bcl-2 and Bcl-X_L, as well as through the blockage of H_2O_2-induced cleavage of caspase-3, an important regulator of neuronal apoptosis, and of the DNA repair enzyme pro-poly (ADP-ribose) polymerase. In addition, luteolin has also demonstrated capacity to inhibit H_2O_2-induced apoptosis in SH-SY5Y neuroblastoma cells *via* the interaction with the caspase-3 pathway (Kang et al., 2004).

Central nervous system is protected by endogenous enzymatic antioxidants as superoxide dismutase, CAT and glutathione peroxidase to convert ROS to less reactive molecules (Schreibelt et al., 2007). In this study, we decide to evaluate the CAT activity in H_2O_2-treated A172 cells. CAT is an intracellular antioxidant enzyme that converts H_2O_2 into water and molecular oxygen. We observed that the activity of CAT decreased in cells treated with H_2O_2 as compared with control cells, indicating that a disturbance in the endogenous antioxidant balance occurs (data not shown). However, no increases were observed on the activity of this enzyme when cells were treated with extracts or rosmarinic acid, suggesting that the protective effect might be due to the activity of other intracellular enzymatic systems or through activation of signaling pathways.

5.5. Conclusions

This is the first report about neuroprotective activity of *L. viridis* using *in vitro* neuronal cell model. We proved the protective effect of *L. viridis* extracts and rosmarinic acid against H_2O_2-induced oxidative damage in A172 human astrocyte cell line. The results indicate that *L. viridis* extracts protected A172 cells *via* reduction of intracellular ROS accumulation. However, further investigations are required to fully understand the mechanisms behind the neuroprotective effect of *L. viridis*.

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CHAPTER 6

IN VITRO GASTROINTESTINAL METABOLISM

Costa P, Grevenstuk T, Rosa da Costa AM, Gonçalves S, Romano A (2013). Antioxidant and anti-cholinesterase activities of *Lavandula viridis* L'Hér extracts after *in vitro* gastrointestinal digestion. (submitted)

6.1. Abstract

Lavandula viridis L'Hér is an aromatic shrub rich in phenolic compounds. In this study, we investigated the chemical profile and biological activities [antioxidant and anti-cholinesterase (anti-ChE)] of *L. viridis* extracts, after *in vitro* gastrointestinal digestion. The influence of the *L. viridis* extract matrices on the digestion of the main component rosmarinic acid was also investigated. We observed that the *L. viridis* extracts were more stable in the basic pH of the pancreatic environment than in the acidic pH of the gastric juice. Overall, the antioxidant and anti-ChE activities were assured after *in vitro* gastrointestinal processes and we observed that the *L. viridis* matrices have an important role in the bioactive effects of their secondary metabolites. The *L. viridis* extracts and rosmarinic acid did not show any toxic effect on colon epithelial (Caco-2) cell viability. Finally, the compounds from *L. viridis* extracts were not metabolised by Caco-2 cells and were not able to permeate into them.

6.2. Introduction

Phenolics are one of the most important active compounds synthesised by plants and probably the main candidates contributing to the claimed beneficial effects associated with plant medicines. It has been postulated that dietary intake of phenolic compounds may prevent degenerative and cardiovascular diseases, mostly due to their antioxidant potential (Manach et al., 2004). However, they must be effectively absorbed into the blood stream (bioavailable) to exert their biological function. Thus, several *in vitro* and *in vivo* studies have been performed to understand their bioavailability (Williamson and Manach, 2005; Kountouri et al., 2007; Laurent et al., 2007). Bioavailability is dependent upon their release from the plant matrix (bioaccessibility), their stability during digestion process, and the efficiency of their transepithelial passage (Manach et al., 2004). Absorption *via* the small intestine is influenced by several physicochemical (e.g. molecular size, lipophilicity and solubility) and biological factors (e.g. gastric and intestinal transit time, lumen pH, membrane permeability and biliary excretion after first pass metabolism) (Lin et al., 1999; Stahl et al., 2002). Bioaccessibility is particularly important because phytochemicals must be previously available to exert their biological activities. For instance, interactions between phenolics and other components within the plant matrix, such as polysaccharides, have been observed for some plants (Manach et al., 2004; Pong et al., 2005).

Lavandula viridis L'Hér (Lamiaceae), traditionally used in Portuguese folk medicine, is a xerophytic aromatic shrub naturally distributed in Madeira Island, south Portugal and south-west Spain. In Portugal, we can find this species growing in dry conditions and in nutrient poor and degraded soils of the Algarve and Alentejo regions (Franco, 1971). Phytochemical studies from the aerial parts of this species highlighted the presence of phenolic compounds, including hydroxycinnamic acids (3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, caffeic and rosmarinic acids), flavones (luteolin and luteolin-7-*O*-glucoside) and one flavanone (pinocembrim) (Costa et al., 2011; Costa et al., 2013a). Recently, we proved through *in vitro* studies the capacity of phenolic extracts from this species as free radical scavengers, metal chelators and ChE inhibitors (Costa et al., 2011; Costa et al., 2013a). In addition, they showed neuroprotective effect against hydrogen peroxide-induced oxidative damage in A172 human astrocytes cell line (Costa et al., 2013b).

Rosmarinic acid is abundant in the Lamiaceae family and the main compound identified in the *L. viridis* extracts. It has relevant biological activities, such as antioxidant (Tepe et al., 2007),

antimicrobial (Moreno et al., 2006) and neuroprotective (Ono et al., 2012). There are some data concerning the absorption, metabolism, distribution, and excretion of rosmarinic acid (Baba et al., 2004; Baba et al., 2005; Konishi and Kobayashi, 2005). *In vivo* studies have indicated that orally administered rosmarinic acid exhibited anti-allergenic and anti-carcinogenic effects (Makino et al., 2001; Sanbongi et al., 2004). *L. viridis* extracts are rich in rosmarinic acid and are therefore an interesting bioresource for food and pharmaceutical industries. However, there are no reports showing the influence of *in vitro* gastrointestinal metabolism of *L. viridis* extracts on their biological activity. Therefore, we decided, for the first time, to investigate the chemical profile and biological activities (antioxidant and anti-ChE) of extracts from *L. viridis* after *in vitro* gastrointestinal digestion.

6.3. Materials and methods

6.3.1. Chemicals and reagents

Rosmarinic acid, pepsin from porcine gastric mucosa (CAS: 9001-75-6), pancreatin from porcine pancreas (CAS: 8049-47-6), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, potassium persulfate ($K_2S_2O_8$), acetylthiocholine iodide (ATCI), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), acetylcholinesterase (AChE) (Electric-eel, EC 3.1.1.7, Type VI-S), butyrylcholinesterase (BChE) (horse-serum, EC 3.1.1.8), butyrylthiocholine chloride (BTCI), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Steinheim, Germany). Formic acid was acquired from Merck (Darmstadt, Germany). Absolute ethanol was obtained from Panreac (Barcelona, Spain). Acetonitrile was acquired from Fisher Scientific (Loughbourough, United Kingdom). 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was purchased from Acros Organics (Geel, Germany). Qualitative filter paper was purchased from VWR (Leuven, Belgium). Ethylenediaminetetraacetic acid (EDTA) and sodium chloride (NaCl) were purchased from Fluka (Steinheim, Germany). Dulbecco's Modified Eagle's Medium (DMEM), Hank's balanced salt solution (HBSS), penicillin/streptomycin and fetal bovine serum (FBS) (heat inactivated) were purchased from Lonza (Verviers, Belgium). Trypsin/EDTA and MEM non-essential amino acids solutions were purchased from Biochrom (Berlin, Germany).

6.3.2. Plant material and extracts preparation

The aerial parts of *L. viridis* were collected during flowering period at São Bartolomeu de Messines (Algarve, Portugal). A voucher specimen was deposited at the herbarium of the Botanical Garden of the University of Lisbon under the number LISU 173992. Plant material was dried at 40 ºC and powdered in a blender to achieve a mean particle size less than 2 mm. The plant material (10 g) was then extracted with methanol (150 ml), at room temperature for 5 h, in a Soxhlet apparatus. The water:ethanol extract (1:1) was obtained from 10 g of plant material and soaked overnight at room temperature in 200 ml of solvent, and the resulting extract was filtered through a 5-13 µm membrane. Finally, the extracts were concentrated to dryness in a rotary evaporator and stored at -20 ºC until future use.

6.3.3. *In vitro* gastrointestinal digestion

6.3.3.1. *In vitro* gastric juice digestion

The digestion by gastric juice was assessed as described by Yamamoto et al. (1999) with slight modifications. To prepare a volume of 100 ml simulated gastric juice, 320 mg of pepsin and 200 mg of NaCl were mixed and the pH was adjusted to 1.2 using HCl. Then, gastric juice was added to each *L. viridis* extract (500 µg/ml) or rosmarinic acid solution at the same concentration found in each extract (125 and 250 µg/ml, to water:ethanol and methanol extracts, respectively), and incubated at 37 ºC. The reaction was stopped after 2 h by liquid nitrogen. The samples were then filtered through a 5-13 µm membrane and analysed by high-performance liquid chromatography with diode-array detection (HPLC-DAD). Controls with gastric juice in the absence of samples and vice-versa were subjected to the same experimental conditions to differentiate the effects due to pepsin from those caused by the chemical environment in the assay. All the experiments were performed in triplicate.

6.3.3.2. *In vitro* pancreatic juice digestion

The digestion by pancreatic juice was assessed as described by Yamamoto et al. (1999) with slight modifications. To simulate the pancreatic juice, 250 mg of pancreatin were added to 10 ml of phosphate buffer (50 mM, pH 8.0). Then, pancreatic juice was added to each *L. viridis* extract (500 µg/ml) or rosmarinic acid solution (125 and 250 µg/ml). The reaction mixture was left to incubate at 37 ºC for 2 h. Reaction was stopped by liquid nitrogen and the

samples were filtered through a 5-13 μm membrane and analysed by HPLC-DAD. Controls with pancreatic juice in the absence of samples and vice-versa were subjected to the same experimental conditions to differentiate the effects due to pancreatin from those caused by the chemical environment in the assay. All the experiments were performed in triplicate.

6.3.4. Cell culture

Human colon adenocarcinoma Caco-2 from ATCC was maintained in complete DMEM supplemented with 10% heat inactivated FBS, 1% MEM non-essential amino acids solution and 1% penicillin/streptomycin (10,000 units/ml penicillin and 10 mg/ml streptomycin). Cells were maintained at 37 ºC in 5% CO_2 with 95% relative humidity.

6.3.4.1. Effect of *L. viridis* extracts and rosmarinic acid on Caco-2 cell viability

The cytotoxicity of the *L. viridis* extracts and rosmarinic acid was evaluated by MTT assay which is based on the cleavage of tetrazolium salts by mitochondrial succinate tetrazolium reductase in viable cells to form formazan dye (Mosmann, 1983). A172 cells were seeded in 96-well plates (NUNC, Rochester, New York, USA) at a density of 1×10^5 cells/well, and allowed to attach. After 24 h exposure to the *L. viridis* extracts (500 μg/ml) or rosmarinic acid (125 and 250 μg/ml), the medium was removed and the cells were slightly washed with HBSS. MTT (5 mg/ml) was added and, after 2 h incubation at 37 ºC the resulting formazan crystals were dissolved in DMSO. Cell viability was measured in terms of absorbance at 570 nm in a microplate reader (Infinite 200 Tekan, Grödig, Austria) comparing treated with untreated cells.

6.3.4.2. Metabolism by the Caco-2 cells

Cells were seeded in 6-well plates at a density of 3×10^4 cells/well and allowed to attach. The medium was replaced by the *L. viridis* extracts (500 μg/ml) and rosmarinic acid (125 and 250 μg/ml), and the cells were incubated at 37 ºC. An hundred microliters of each sample of the supernatant were collected at 0; 1; 2; 4 and 6 h and immediately frozen with liquid nitrogen and analysed by HPLC-DAD. Aliquots of each extract and rosmarinic acid in DMEM medium without cells, were also subjected to the same experimental conditions and

analysed by HPLC-DAD. After 6 h the medium was removed and the cells washed with HBSS. Then, the cells were removed enzymatically using trypsin-EDTA (0.25%), centrifuged at 3000 rpm for 3 min, lysed and sonicated for 10 min. Then, the cellular mixture was centrifuged at 3000 rpm for 3 min, and the supernatant solutions analysed by HPLC-DAD.

6.3.5. High-performance liquid chromatography (HPLC) analysis

The methanol and water:ethanol extracts and rosmarinic acid standard solutions were analysed on an Agilent 1100 series LC system (Agilent Technologies, Waldbronn, Germany). Separation was achieved using a Phenomenex Kinetex C18 column (4.6 × 150 mm, 2.6 μm particle size) (Tecnocroma, Caldas da Rainha, Portugal) and a binary solvent consisting of acetonitrile (A) and formic acid aqueous solution (0.1%) with the following gradient: 13% A at 0 min, 35% A at 20 min, 100% A at 25 min and hold at 100% A for 5 min. The flow rate was 0.8 ml/min and the injection volume 20 μl. Spectral data from all peaks were accumulated in the range 200-400 nm, and chromatograms were recorded at 280 nm. The LC3DChemStation software (Agilent Technologies) was used for instrumental control and data processing. Rosmarinic acid quantification was achieved by the absorbance recorded in the chromatograms relative to external standards in the range 62.5-500 μg/ml.

6.3.6. Biological activities

6.3.6.1. Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant activity was determined using the TEAC assay with the radical cation $ABTS^{\bullet+}$ as described by Re et al. (1999). The 7 mM $ABTS^{\bullet+}$ stock solution was prepared using potassium persulfate as the oxidizing agent. The absorbance was determined at 734 nm, 1 min after mixing. Controls with gastric or pancreatic juice in the absence of samples and vice-versa were subjected to the same experimental conditions to differentiate the effects due to pepsin or pancreatin from those caused by the chemical environment in the assay. The sample dilution that achieved 20-80% inhibition of the blank absorbance was used to calculate the TEAC values. All experiments were carried out in triplicate. The antioxidant activity was determined before and after digestion and the free radical scavenging activity was expressed as percentage (%) inhibition relative to the control treatment.

6.3.6.2. *In vitro* anti-cholinesterase inhibition assay

The evaluation of AChE and BChE inhibitory activities was based on Ellman's method (Ellman et al., 1961), using a 96-well microplate reader (Infinite 200 Tekan, Grödig, Austria). Firstly, 3 mM DTNB, 15 mM substrate (ATCI or BTCI), 100 mM phosphate buffer (pH 8.0) and extract (2.5 mg/ml) or buffer were mixed. Finally, AChE or BChE (0.28 U/ml) were added and the absorbance was read at 405 nm for 5 min. Controls with gastric or pancreatic juice in the absence of samples and vice-versa were subjected to the same experimental conditions to differentiate the effects due to pepsin or pancreatin from those caused by the chemical environment in the assay. The reaction enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer without any inhibitor. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. The anti-ChE activity was determined before and after digestion and expressed as percentage (%) inhibition relative to the control treatment.

6.3.7. Statistical analysis

All the experiments were carried out three times. The data were expressed as the mean ± standard error and were subjected to one-way analysis of variance (ANOVA). All statistical analysis was carried out using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA).

6.4. Results and discussion

6.4.1. *In vitro* gastrointestinal digestion of the *L. viridis* extracts and rosmarinic acid

Rosmarinic acid, the main component identified in the *L. viridis* extracts (Costa et al., 2011; Costa et al., 2013a) is abundant in the Lamiaceae family. It has been reported that plant matrix can influence the digestion and bioavailability of bioactive compounds (Manach et al., 2004). Therefore, in this study we evaluated the effect of *in vitro* gastrointestinal digestion on the biological activity of *L. viridis* extracts and the influence of the plant matrix on the digestion of rosmarinic acid. We used pepsin and pancreatin to simulate the gastric and pancreatic digestions, respectively. Table 6.1 presents the concentration of rosmarinic acid either in pure solutions and in *L. viridis* methanol and water:ethanol extracts, before and after *in vitro* gastric and pancreatic digestions. The results show that the concentration of

rosmarinic acid is reduced after gastric digestion. The fact that a lower percentage of rosmarinic acid was detected after gastric digestion at 125 μg/ml (~72%) than at 250 μg/ml (~88%) suggests that the digestion reaction may be limited by the pepsin concentration. No statistical differences were obtained between the reduction of rosmarinic acid in the methanol extract and standard solution at corresponding concentration (250 μg/ml) after gastric digestion, suggesting that this *L. viridis* matrix has no effect on the digestion of rosmarinic acid.

Table 6.1
Rosmarinic acid concentration (μg/ml) quantified in *Lavandula viridis* extracts and pure solutions obtained by HPLC analysis, before and after *in vitro* digestion.

	Gastric digestion			Pancreatic digestion	
	Before digestion	After digestion	Reduction (%)	Before digestion	After digestion
Water:ethanol	118.29 ± 0.45	96.50 ± 2.30*	16.67 ± 1.15	94.47 ± 3.44	94.67 ± 0.68
Standard solution[1]	111.21 ± 0.85	80.50 ± 1.02*	27.59 ± 1.48†	87.50 ± 1.03	89.19 ± 1.41
Methanol	251.57 ± 0.64	223.47 ± 2.97*	11.17 ± 1.29	197.27 ± 3.09	200.22 ± 4.45
Standard solution[1]	244.24 ± 7.91	214.11 ± 0.44*	12.16 ± 2.76	194.71 ± 1.81	195.64 ± 3.41

Values are expressed as mean ± standard error (*n=3*). Values marked with * indicate significant differences ($p < 0.05$) before and after *in vitro* digestion. Values marked with † mean significant differences ($p < 0.05$) between the reduction of rosmarinic acid concentration after *in vitro* digestion in each extract and standard solution at the corresponding concentration. [1] Rosmarinic acid solution at the same concentration found in each extract (125 and 250 μg/ml for water:ethanol and methanol extracts, respectively).

On the contrary, the reduction of rosmarinic acid was ($p < 0.05$) less in the water:ethanol extract (~17%) than in the corresponding rosmarinic acid standard solution (~28%). Our results suggest that the water:ethanol extract contains metabolites that protect/retard the acid digestion of rosmarinic acid. Gião et al. (2012) also found that the *in vitro* digestion of rosmarinic acid was affected by plant matrix as the reduction of rosmarinic acid after gastric digestion was more intense in savory than in sage. The obtained chromatograms indicate that, at 280 nm, no new metabolites resulting from rosmarinic acid digestion could be detected (Fig. 6.1). Apart from the consumption of rosmarinic acid, the only difference in signal intensity that could be observed was a reduction of the more apolar constituents of the *L. viridis* water:ethanol extract under the acidic gastric conditions (Fig. 6.1A).

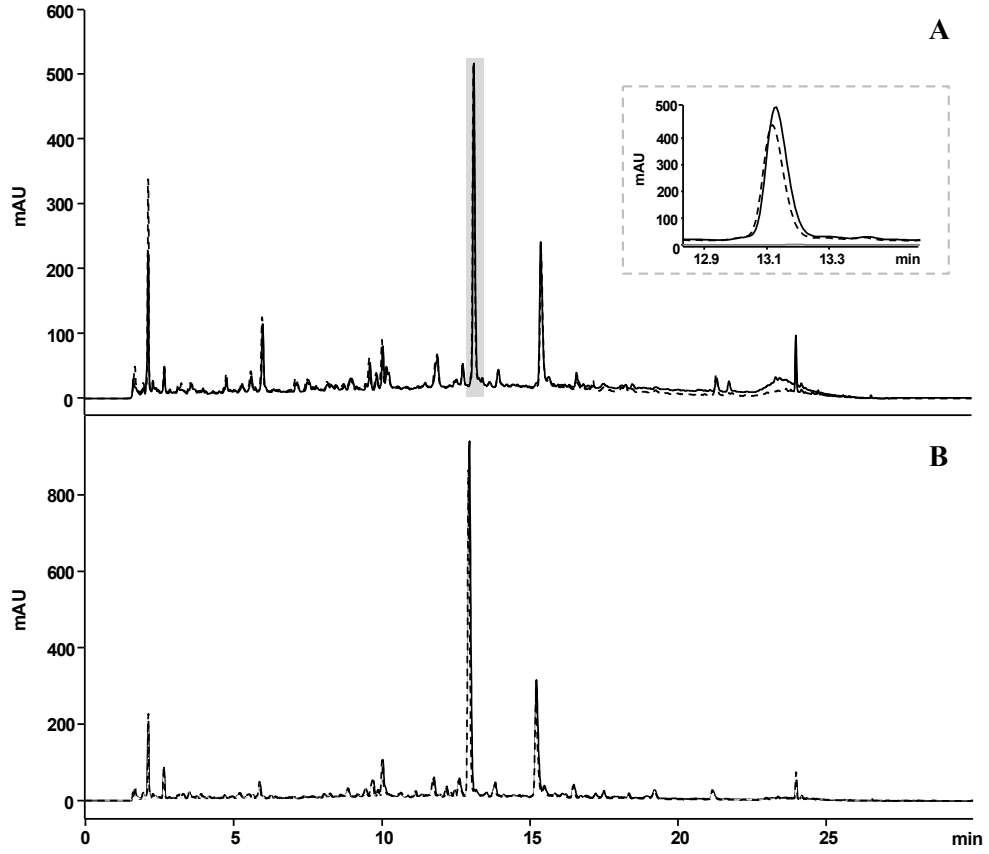

Figure 6.1. HPLC chromatograms of water:ethanol (A) and methanol (B) extracts of *Lavandula viridis* before (bold line) and after gastric (dashed line) digestion procedure. Inset depicts the enlarged signal corresponding to rosmarinic acid.

We found that the *L. viridis* extracts and rosmarinic acid were more stable in the basic pH of the pancreatic environment than in the acidic pH of the gastric juice, since no quantitative and qualitative differences were found in their chemical profiles after *in vitro* pancreatic digestion (Table 6.1). The fact that rosmarinic acid is not consumed during *in vitro* pancreatic digestion is in agreement with results presented by Gião et al. (2012).

Several *in vivo* studies have been performed to understand the metabolism and bioavailability of rosmarinic acid (Baba et al., 2004; Baba et al., 2005). Particularly, Baba et al. (2005) studied the *in vivo* absorption, metabolism, and urinary excretion of rosmarinic acid after ingestion of *Perilla frutescens*. Rosmarinic acid, methylated rosmarinic acid, caffeic acid, ferulic acid and a trace of *m*-coumaric acid were found in the urine after intake of *P. frutescens*. In plasma, rosmarinic acid, methylated rosmarinic acid and ferulic acid were also detected. The results obtained in the present study need to be carefully analysed because *in vitro* digestion models are often different to those found using *in vivo* models due to the

highly complex physicochemical and physiological events occurring in human digestive tracts (Hur et al., 2011). Therefore, it is important to subject the extracts to other digestion conditions to predict more accurately what happens to the extracts during *in vivo* digestion.

6.4.2. Effect of *in vitro* digestion on the biological activities of the *L. viridis* extracts and rosmarinic acid

We recently proved the antioxidant and anti-ChE activities of the extracts from *L. viridis* (Costa et al., 2011; Costa et al., 2013a). The water:ethanol extract showed a strong antioxidant activity in TEAC assay (1149.82 ± 17.31 $\mu mol_{TE}/g_{extract}$) as well as the methanol extract (967.18 ± 22.57 $\mu mol_{TE}/g_{extract}$) (Costa et al., 2011; Costa et al., 2013a). Regarding the ChEs inhibitory activity, both extracts showed a remarkable inhibitory effect on AChE and BChE activities. However, to exert its activity *in vivo*, rosmarinic acid must be effectively absorbed into the blood stream and reach the active site. During digestion processes, bioactive compounds can be structurally and chemically modified and, consequently, their biological activity compromised (Hur et al., 2011). Therefore, in this study, we intended to understand if under *in vitro* gastrointestinal conditions the biological potential of the *L. viridis* extracts and their main component, rosmarinic acid, is maintained. The antioxidant activity of the *L. viridis* extracts and pure rosmarinic acid was determined by the TEAC assay and the results are displayed in Table 6.2. The free radical scavenging activity of the methanol extract from *L. viridis* and rosmarinic acid was not affected by the simulated *in vitro* gastric juice, unlike the water:ethanol extract.

Table 6.2
Effect of *in vitro* digestion on the antioxidant activity (%) of the *Lavandula viridis* extracts and rosmarinic acid, assessed by Trolox equivalent absorbance capacity (TEAC) assay.

	Gastric juice		Pancreatic juice	
	Before digestion	After digestion	Before digestion	After digestion
Water:ethanol	100.00 ± 1.76	77.30 ± 3.00*	100.00 ± 2.45	102.93 ± 3.03
Methanol	100.00 ± 0.95	93.77 ± 2.31	100.00 ± 2.24	122.84 ± 3.87*
Rosmarinic acid	100.00 ± 1.71	98.27 ± 2.45	100.00 ± 0.36	135.14 ± 4.30*

Values are expressed as mean ± standard error (*n=3*). For the calculations, the antioxidant activity before digestion was taken as 100%. Values marked with * indicate significant differences ($p < 0.05$) before and after *in vitro* digestion.

After pancreatic digestion, we observed an increase in the free radical scavenging activity of the methanol extract and rosmarinic acid (Table 6.2). In fact, different factors can explain this increase after *in vitro* simulated digestion. It has been reported that other compounds present in the plant matrix, such as amino acids, sugars and uronic acids can be released after *in vitro* simulated gastrointestinal digestion, and display positive interfering effects in certain antioxidant capacity assays, such as TEAC (Cilla et al., 2011). However, this hypothesis is not valid for the rosmarinic acid standard solution, suggesting that other factors may be responsible for the increased activity.

Regarding the anti-ChE activity of the methanol and water:ethanol extracts (Table 6.3), only the inhibition of BChE was affected after *in vitro* digestion; methanol and water:ethanol extracts showed a 10% and 33% decrease after gastric digestion, and 60% and 20% after pancreatic digestion. The rosmarinic acid was also submitted to the same assay, but it was not active at the studied concentrations (125 and 250 μg/ml), suggesting that the activity displayed by the extracts is not primarily due to the presence of rosmarinic acid.

Table 6.3
Effect of *in vitro* digestion on the anti-cholinesterase activity (%) of the *Lavandula viridis* extracts.

	Gastric juice		Pancreatic juice	
	Before digestion	After digestion	Before digestion	After digestion
Acetylcholinesterase (AChE)				
Water:ethanol	100.00 ± 2.04	86.07 ± 9.25	100.00 ± 13.55	109.29 ± 13.79
Methanol	100.00 ± 7.46	99.31 ± 3.84	100.00 ± 10.38	99.72 ± 16.56
Butyrylcholinesterase (BChE)				
Water:ethanol	100.00 ± 3.10	67.28 ± 1.48*	100.00 ± 2.57	79.56 ± 6.56*
Methanol	100.00 ± 0.41	90.44 ± 1.78*	100.00 ± 1.51	39.99 ± 3.70*

Values are expressed as mean ± standard error ($n=3$). For the calculations, the antioxidant activity before digestion was taken as 100%. Values marked with * indicate significant differences ($p < 0.05$) before and after *in vitro* digestion.

Despite the decrease of the anti-ChE activity we may consider that the activity is ensured after *in vitro* digestion. Controls tested under the same experimental conditions did not show antioxidant and anti-ChE activities indicating that the observed effect is exclusively due to the digestion process.

6.4.3. Metabolism of *L. viridis* extracts and rosmarinic acid by Caco-2 cells

Caco-2 cell culture has been used as an *in vitro* digestion model to predict the absorption and metabolism of bioactive components from plant material (Mukinda et al., 2010; Hur et al., 2011; Qiang et al., 2011; Gião et al., 2012). The cytotoxic effect of the *L. viridis* extracts and rosmarinic acid was evaluated using the MTT assay and the results were expressed as percentage of the control. As shown in Fig. 6.2, the viability of Caco-2 cell line was not affected by the extracts and pure solutions of rosmarinic acid.

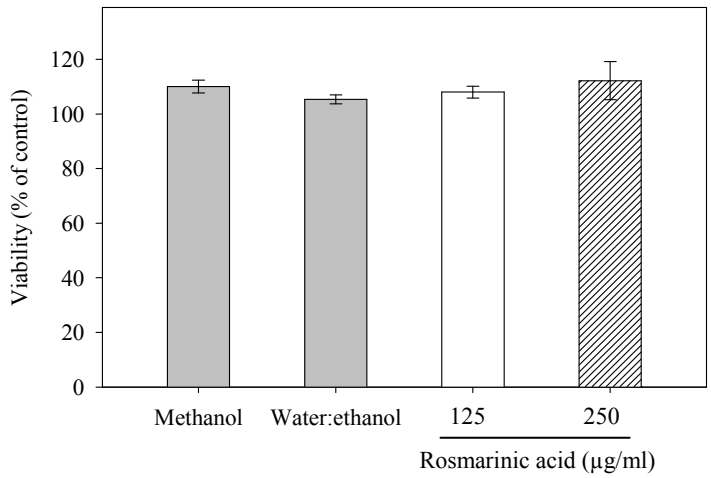

Figure 6.2. Effect of *Lavandula viridis* extracts (500 µg/ml) and rosmarinic acid (125 and 250 µg/ml) on the Caco-2 cell viability for 24 h. Cell viability was determined using MTT assay. Data are expressed as the mean ± standard error of three independent experiments.

We also investigated whether the compounds present in the extracts of *L. viridis,* as well as the rosmarinic acid, are incorporated into Caco-2 cells and if any extracellular protein produced by these cells could change the chemical profile of the *L. viridis* extracts. After 6 h incubation, phenolic compounds from both *L. viridis* extracts and pure solutions of rosmarinic acid were detected in the medium and no changes were found on their chemical profiles.

To investigate the presence of phenolics inside the cells, a homogenised was prepared, as described previously, and then analysed by HPLC. No phenolic compounds were found within the cells (data not shown) suggesting that the compounds were not able to penetrate them. The metabolism was monitored hourly by HPLC and the results were similar to those observed at the end of 6 h. Factors as dilutions of the extracts and rosmarinic acid, as well as interactions with other chemicals within the plant matrices could affect the bioavailability of

phenolic compounds present in the *L. viridis* extracts. Konishi and Kobayashi (2005) reported that the intestinal absorption efficiency of rosmarinic acid, the main compound, is low, being the transport mainly *via* paracellular diffusion. They also found that rosmarinic acid is unsusceptible to hydrolysis by mucosa esterase in Caco-2 cells.

6.5. Conclusions

In this study we reported the effect of *in vitro* digestion on the chemical profiles and biological activities of the *L. viridis* extracts and pure rosmarinic acid solutions. The results showed that gastric juice had a significant reducing effect on the concentration of rosmarinic acid unlike to the pancreatic juice. The antioxidant activity of the methanol extract and pure rosmarinic acid was maintained after *in vitro* gastrointestinal processes. Regarding the anti-ChE activity, only the inhibition of BChE was affected after *in vitro* gastric and pancreatic digestions. However, we can consider that the anti-ChE activity was assured. We found that bioactive compounds from *L. viridis* and pure solutions of rosmarinic acid did not affect the viability of Caco-2 cells and were not able to permeate into them. This result allowed to perceive the effect of *in vitro* digestion on the *L. viridis* extracts, providing a scientific basis for further studies regarding the utilization of this species.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1. General discussion

7.1.1. Chemical characterisation of the essential oils and extracts

Plants are an inexhaustible source of bioactive compounds with different functional groups, many of which with an important role in the prevention and treatment of several diseases. The selection and preparation of plant samples, as well as the correct choice of the extraction technique can determine the success of the recovery of active metabolites. During the collection of the plant material, it is important to select healthy plants and free of contamination, to ensure the original chemical profile (Seidel, 2006). The yield and composition of the essential oils and extracts may also be affected by genetic and environmental factors as well as by the storage conditions to a certain extent (Figueiredo et al., 2008a; Salgueiro et al., 2010). With respect to the essential oils, the type of secretory structure is relevant. Internal secretory structures are less vulnerable to losses or chemical changes of volatiles, whereas external secretory structures can naturally release their secretion by cuticle disruption, thus being more susceptible to mechanical damage (Figueiredo et al., 2008a).

The extraction technique must be appropriated to the classes of compounds of interest and exhaustive, in order to isolate as much of the desired metabolites as possible (Seidel, 2006). Hydrodistillation (HD) is traditionally used for the recovery of essential oils, however the high temperatures applied can affect their quality. Alternatively, supercritical fluid extraction (SFE) can be successfully used for the recovery of volatile compounds from, for instance, Lamiaceae species (Fornari et al., 2012).

In this study, HD and SFE were used to isolate the volatile fraction of *L. viridis*, *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus*, whereas maceration was applied to the extraction of phenolic compounds. Pure water and ethanol separately or in a 1:1 mixture were used as solvent extraction to produce extracts with distinct compositions from the three species. In the particular case of *L. viridis*, the Soxhlet extraction was also applied for the preparation of the methanol extract. The methanol extract of *L. viridis* was studied because it has been previously demonstrated its high capacity to isolate plant bioactive compounds.

The main results concerning the influence of the extraction solvent and technique on the recovery of volatile and phenolic compounds are presented in Chapters 2 and 3. Additionally the antioxidant and anti-cholinesterase (anti-ChE) activities of the extracted volatiles and phenolic compounds are also presented in Chapters 2 and 3. Although the results are not

showed in these Chapters, the SFE extracts from *L. pedunculata* subsp. *lusitanica* were also prepared and chemically analysed; however, data indicated that this technique, under the extraction conditions used, was not as effective as for the remaining studied species. Infusions from all the species and materials (wild plants and *in vitro* cultures) were also prepared, chemically characterised and subjected to the biological assays.

In the case of SFE, the effect of the extraction pressure and separator type was evaluated; the SFE was performed at 40 ºC, at 12 or 18 MPa in two different separators. The extraction conditions were selected to avoid degradation of thermolabile compounds, such as those comprising essential oils and with the aim of extracting volatiles and compare their performance with the essential oils. It is important to note that pressures above 18 MPa are no longer suitable for this purpose. Generally, moderate pressures (i.e. 9-12 MPa) and temperatures (i.e. 35-50 ºC) are sufficient to solubilize volatile components (Fornari et al., 2012). However, high pressures can enhance the mass transfer and the liberation of the volatile fraction from the cell (Fornari et al., 2012). The collection in two separators yielded two different extracts: heavy components in the first separator and volatiles in the second separator.

Based on the results obtained, the SFE technique generated the highest extraction yields in comparison with HD. The increase in extraction pressure from 12 to 18 MPa affected the yield of the SFE extracts of *T. lotocephalus*, collected in the second separator. Other substances, including paraffins and steroptens, could also be co-extracted when extraction pressure is increased (Reverchon, 1997; Fornari et al., 2012). Regarding the extraction yields of the polar extracts, generally the infusions and water extracts allowed the highest recovery of metabolites followed by the water:ethanol and ethanol extracts. The high yields generated by the most polar extracts could also reflect the co-extraction of proteins and carbohydrates (Visht and Chaturvedi, 2012).

The qualitative and quantitative analyses of the essential oils and SFE extracts were performed using gas chromatography with flame ionization detector (GC-FID) and/or gas chromatography coupled to mass spectrometry (GC-MS). A large number of components were identified in the essential oils; oxygen-containing monoterpenes were the primary compounds in all the essential oils and SFE extracts excepted from *T. lotocephalus* extracts collected in the first separator at both pressures. The chemical compositions of the essential oils and extracts collected in the second separator (volatiles) differ qualitatively and

quantitatively; the main compound identified in *Lavandula* spp. essential oils was camphor, whereas linalool was the major constituent in the essential oil from *T. lotocephalus*. Regarding the SFE extracts collected at extraction pressure of 12 MPa, camphor was the main compound in *L. viridis* in both separators. At the highest pressure, the SFE extracts of *L. viridis* were dominated by myrtenol (first separator) and verbenone (second separator). Caryophyllene oxide and camphor were the major compounds found in the SFE extracts of *T. lotocephalus* at the extraction pressure of 12 MPa in the first and second separators, respectively. Caryophyllene oxide and *cis*-linalool oxide were the main components identified in the SFE extracts of *T. lotocephalus* at the highest pressure, obtained in the first and second separators, respectively. The chemical compositions of the essential oils obtained from all the species are in reasonable agreement with previous studies.

The composition of the essential oils from field-grown *L. viridis* plants, collected at São Bartolomeu de Messines (Algarve, Portugal) during the flowering phase, as well as from *in vitro* shoot-cultures and micropropagated plants produced from the same clone, were reported by Nogueira and Romano (2002) and Gonçalves et al. (2008). 1,8-Cineole was found to be the major constituent of all the essential oils, followed by camphor. Changes in environmental conditions as well as genetic factors can explain the few disparities between the results presented by the mentioned authors and those obtained in the present study (Figueiredo et al., 2008b).

Matos et al. (2009) studied the essential oil of *Lavandula stoechas* subsp. *lusitanica* (Chaytor) Rozeira [=*Lavandula pedunculata* subsp. *lusitanica* (Chaytor) Franco] collected in the south Portugal (Algarve region). As observed in this study, they also found the predominance of oxygen-containing monoterpenes, although with some quantitative and qualitative differences.

The large chemical polymorphism of *T. lotocephalus* essential oil makes difficult to obtain a general characterisation of its composition (Figueiredo et al., 2008b). Figueiredo et al. (1993) identified the essential oil constituents isolated from different parts of *T. lotocephalus* (i.e. flowers and leaves). It was observed that the essential oils isolated from the leaves and flowers were quite different and dependent of the developmental stage, as well as of the quantity of flowers present in the plant material used. The composition of the essential oil from flowers was dominated by linalyl acetate while 1,8-cineole was the major compound in the essential oil from leaves. In addition, Salgueiro et al. (2000) compared the chemical compositions of the essential oils of four *T. lotocephalus* populations from relatively close

localities (Ludo, Amendoeira, Quinta de Marim and Malhão-Amendoeira). Linalool, geranyl acetate and 1,8-cineole were the major constituents identified. They reported a high intraspecific variability within the essential oils from plants collected at the same developmental stage, meaning that the chemical polymorphism of the essential oil of *T. lotocephalus* might be due, at least in part, to genetic factors. The heterogeneity of Portuguese environmental conditions, including humidity degree, thermic amplitude and soil type, can contribute to this genetic diversity (Figueiredo et al., 2008b).

The high-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis of the water, water:ethanol, ethanol and methanol extracts, and infusions from wild plants of *L. viridis*, *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus* allowed the identification of phenolic acids (i.e. 3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, caffeic and rosmarinic acids) and flavonoids (i.e. luteolin, apigenin and pinocembrin). Rosmarinic acid was the major compound identified in the infusions, water:ethanol and ethanol extracts of both *Lavandula* spp., and in the methanol extract of *L. viridis*. On the other hand, 4-*O*-caffeoylquinic acid was present in higher amounts in the water extracts of both *Lavandula* spp. Rosmarinic acid was also the major compound found in all the extracts of *T. lotocephalus*. The presence of this phenolic acid as the major compound is not surprising since it is abundant in the Lamiaceae family (Shekarchi et al., 2012). The water:ethanol mixture was the most efficient solvent for the recovery of phenolic compounds from the three aromatic species, while pure water was the one with the poorest performance. These results strongly highlight the influence of the solvent choice on the efficiency of extraction. In fact, aqueous organic solvents generally allow higher yields of phenolics than the corresponding absolute organic solvents (Sultana et al., 2009).

Many species are threatened due to overharvesting for medicinal, culinary or other purposes, and also due to other anthropogenic and natural factors. For this reason, the development of more sustainable techniques to produce natural compounds is required (Lubbe and Verpoorte, 2011). Plant tissue cultures have significant potential for the production of valuable phytochemicals allowing their large-scale production without a negative impact on natural habitats (Matkowski, 2008; Karuppusamy, 2009). This last point is particularly important for species critically endangered and legally protected, such as *T. lotocephalus*, meaning that wild plants cannot be exhaustively exploited as a source of bioactive compounds. Moreover, through *in vitro* propagation methods it is possible to obtain a selective, rapid, reliable and

predictable production of secondary metabolites with no seasonal constraints and independent of geographical and soil conditions (Matkowski, 2008; Karuppusamy, 2009).

In this work the phenolic profiles of extracts from *in vitro* cultures of *L. viridis* and *T. lotocephalus* were studied and compared with wild plants (Chapter 3). *In vitro* cultures of *T. lotocephalus* and *L. viridis* were used for the production of metabolites because *T. lotocephalus*, as mentioned above, is a critically endangered endemic species that is confined to a few populations in the Algarve region, and therefore alternatives for the production of plant compounds are mandatory. Cultures of *L. viridis* were already established in the Plant Biotechnology Laboratory and previous studies proved their capacity to produce high-value compounds, such as terpenes. The largest differences between the extraction yields of wild plants and *in vitro* cultures were observed by the water:ethanol extracts, with *in vitro* cultures presenting the highest values. As observed in wild plants, water:ethanol was the most suitable solvent for the recovery of phenolic compounds from both *in vitro* cultures, while water was the less efficient solvent.

Most extracts from *in vitro* cultures of *L. viridis* and *T. lotocephalus* were dominated by rosmarinic acid and the production of this compound was higher in *in vitro* cultures than in wild plants. Some quantitative differences found between the identified phenolics in wild plants and *in vitro* cultures can be explained by the respective *in vivo* and *in vitro* growth conditions (e.g. the composition of the culture medium and the controlled environmental conditions). For instance, it was observed that reduced light intensity of the *in vitro* environment can influence the production of secondary metabolites (García-Pérez et al., 2012). After characterising essential oils and extracts, and understood the effect of technique and solvent choice on their extraction yields and chemical compositions, their biological potential (anti-ChE and antioxidant activities) was evaluated.

7.1.2. Biological activities

7.1.2.1. *In vitro* cholinesterase inhibition

The aging of the population is a worldwide phenomenon and it is well-established that increasing age is a risk factor for dementia. Alzheimer's disease (AD) is, by far, the most prevalent form of dementia, accounting to 60-70% of cases (WHO, 2011). AD is caused by progressive and irreversible neurodegeneration. Nowadays, the most efficient therapeutic

approach is focused on enhancing cholinergic function by restoring the level of acetylcholine neurotransmitter through inhibition of ChEs (Park, 2010). The drugs currently used in the treatment of AD present some side effects and are not able to completely interrupt the progression of the disease (Park, 2010). Therefore, there is still a great interest in finding more effective ChE inhibitors.

In this research, the Ellman's method was applied to predict the potential of the studied species as ChE inhibitors. Excepting the SFE extracts from the second separator, all the essential oils and polar extracts were found to be active against both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes (Chapters 2 and 3). Albeit the SFE extracts obtained in the second separator have more compounds in common with the essential oils, particularly those obtained at 12 MPa, the quantitative differences can explain the observed absence of inhibition. Since most of the main compounds are absent, the activity exhibited by the first separator extracts could reflect the importance of the minor components for the overall bioactivity. In addition, other factors have to be considered such as the possible occurrence of interactions between components, including unidentified compounds.

Ellman's method has been widely used to identify new ChE inhibitors. This is a colorimetric method, where the enzyme hydrolyses the substrate generating thiocholine, which reacts with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, also called Ellman's reagent) to produce 2-nitrobenzoic-5-mercaptothiocholine (thiocholine-thionitrobenzoate disulphide) and 5-thio-2-nitrobenzoic acid (thionitrobenzoate) which can be detected at 405 nm (Rhee et al., 2003). A drawback of this method is that it does not distinguish between enzymatic inhibition and loss of activity caused by the reaction between thiocholine and DTNB. To elucidate this, in this work false-positive effects were qualitatively determined by thin layer chromatography. The first separator extracts of both species generated many white bands on the false-positive detection plates possibly reflecting the co-extraction of undesirable compounds during SFE; this suggest that the activity of these extracts can be overestimated. On the other hand only few false-positive effects were detected in the essential oils. In general, *L. viridis* was the most active species in inhibiting ChE activities; with the exception of SFE extracts (second separator), aqueous extracts and infusion, the remaining extracts from this species exhibited capacity to inhibit the activity of both enzymes (at least, 50% at the highest concentration tested). The methanol extract from this species, efficiently inhibited ChEs *in vitro* and also *in vivo* using mouse brain homogenates as the source of the enzyme after intraperitoneal administration of the extract (50 mg/kg). *L. pedunculata* subsp. *lusitanica* compounds were

the less potent; only the essential oil and ethanol extract reached around 50% inhibition of AChE and BChE activities, respectively. The anti-ChE activity of *in vitro* cultures was also evaluated and the results noticed that the ethanol extracts, from *in vitro* cultures of both *L. viridis* and *T. lotocephalus*, were more effective AChE and BChE inhibitors than those from wild plants.

Overall, the essential oils displayed the most promising results as compared with the extracts. Previous studies reported the AChE and BChE inhibitory activity of some terpenes also identified in *Lavandula* and *Thymus* essential oils, including borneol, bornyl acetate, camphor, caryophyllene oxide, 1,8-cineole, eugenol, linalool, myrtenal, terpinen-4-ol, α-terpineol, α-pinene, β-pinene, verbenone and viridiflorol (Miyazawa et al., 1998; Savelev et al., 2003; Savelev et al., 2004; Miyazawa and Yamafuji, 2005; Dohi et al., 2009; Aazza et al., 2011; Kaufmann et al., 2011). 1,8-Cineole, α-pinene and myrtenal, known as potent AChE inhibitors, are present in higher contents in the *L. viridis* essential oil than in the remaining essential oils which probably can explain its highest anti-ChE activity.

In this study, the contribution of some individual components to the inhibitory activity was evaluated based on their natural concentrations in the most active essential oils, namely those from *L. viridis* and *T. lotocephalus.* Camphor, linalool, 1,8-cineole, caryophyllene oxide and α-pinene were used for the analysis of ChEs inhibition for two main reasons; firstly, because some of them (i.e. camphor and linalool) represent the main compounds in the *L. viridis* and *T. lotocephalus* essential oils, respectively; and secondly, due to their well-known anti-ChE activity. The inhibition of AChE activity displayed by both essential oils seems to be mainly due to 1,8-cineole. This monoterpenoid accounted for ~36% and 20% of the inhibitory activity of the whole essential oil of *L. viridis* and *T. lotocephalus*, respectively, whereas the remaining compounds demonstrated moderate activity. In contrast, camphor and caryophyllene oxide were the most active against BChE accounting for ~19% and ~17% of the inhibitory effect of *L. viridis* and *T. lotocephalus* essential oil, respectively. Despite the tendency to attribute the biological potential of the most abundant components present in the essential oils, one should not neglect the contribution of the minor compounds as well as probable occurrence of synergistic and antagonistic effects among all components (Savelev et al., 2003). The results obtained for α-pinene and linalool confirm that α-pinene, representing only 0.2% and 0.3% of the essential oils of *T. lotocephalus* and *L. viridis*, respectively, had noticeable inhibitory effect on both AChE and BChE activities. In contrast, linalool, which is the major component found in the *T. lotocephalus* essential oil, showed a weak contribution to

the biological effect. Therefore, other minority constituents in the essential oils (some of them with recognized anti-ChE capacity) can also account to these inhibitions including borneol, bornyl acetate, eugenol, myrtenal, β-pinene, α-terpineol, terpinen-4-ol, verbenone and viridiflorol. The data obtained suggest that inhibitory activity of essential oils was caused not only by one strong inhibitor but rather by the collective activities of the different constituents.

7.1.2.2. Antioxidant activity

As mentioned throughout the different chapters, oxidative stress is recognized as a common factor in many neurodegenerative diseases and a proposed mechanism for age-related degenerative processes (Kelsey et al., 2010). The continuing growth of the antioxidants market reflects the expectation of consumers to prevent or treat diseases caused or promoted by oxidative stress (Pinchuk et al., 2012). Antioxidants have also a pivotal role in the food industry to preserve or prevent the oxidation processes. Nowadays, almost all processed foods have synthetic antioxidants incorporated, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *tert*-butylhydroquinone (TBHQ). However, their potential toxicologic effects have stimulated the demand for natural alternatives (Karre et al., 2013). Plants are a huge source of these powerful metabolites with phenolic acids and flavonoids among the most important, thus attracting considerable scientific interest. Recently, the European Union authorized the use of extracts from rosemary (Lamiaceae family) as new food additives for use in foodstuffs under Directive 95/2/EC and assigned E392 as its E number (European Union directives 2010/67/EU and 2010/69/EU). This approval strongly stimulates the research for new and more efficient natural antioxidants (Karre et al., 2013). To evaluate the antioxidant health-protecting effects of natural compounds, a single *in vitro* chemical method is not enough to predict their performance *in vivo*. Therefore, in the present study, different *in vitro* assays were employed in order to determine and compare the antioxidant properties of Lamiaceae essential oils and extracts, namely, oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Fe^{2+} chelation and thiobarbituric acid reactive substances (TBARS) assays.

7.1.2.2.1. Free radical scavenging activity

All the essential oils demonstrated acceptable antioxidant activity as assessed by the ORAC and DPPH assays, acting preferentially *via* a hydrogen atom transfer mechanism (Chapter 2). The essential oil from *L. viridis* showed the highest capacity to scavenge both peroxyl and DPPH radicals followed by *T. lotocephalus*. For *L. viridis* and *T. lotocephalus,* the antiradical activity was more pronounced in the essential oils in comparison with the SFE extracts. The activity observed probably reflects the high content of monoterpenes which are known to have free radical scavenging capacity. Camphor and linalool, the main compounds identified in the *Lavandula* and *Thymus* essential oils, respectively, have been previously demonstrated to have radical scavenging abilities (Çelik and Ozkaya, 2002; Zaouali et al., 2010). Again, the contribution of other components, including minor and unidentified, as well as interactions among them cannot be discarded. The SFE extraction conditions influenced the antioxidant activity of the SFE extracts of *L. viridis* and *T. lotocephalus*. Overall, the SFE extracts, of both Lamiaceae species collected in the first separator and at the highest pressure, were more effective as radical scavengers.

The polar extracts were also tested for their antioxidant activities assessed by the ORAC and TEAC assays (Chapters 2 and 3). All the extracts tested displayed antioxidant activity. It was found that natural antioxidants from the extracts also interact with free radicals preferentially by donating a hydrogen atom. Among all the extracts, the water:ethanol was the most efficient solvent for the extraction of free radical scavenging compounds from all the studied species, which is in agreement with previous studies showing that the addition of water improves extraction efficiency and increases the antioxidant activity of extracts (Sultana et al., 2009; Trabelsi et al., 2010; Chew et al., 2011). Infusions showed similar results to those obtained by the water:ethanol extracts. All the extracts from *in vitro* cultures displayed free radical scavenging activity. The ethanol extract from *in vitro* cultures of *L. viridis* showed higher radical scavenging activity than that from wild plants. Qualitative and quantitative differences between both materials, supported by HPLC analysis, can explain these divergences in terms of activity.

It is difficult to indicate exactly which compounds are responsible for the observed antioxidant activity but it is possible to speculate the contribution of some of them, based on previous reports. Rosmarinic (Huang and Zheng, 2006), caffeic (Wang and Yang, 2012) and 5-*O*-caffeoylquinic acids and its isomers (Xu et al., 2012), and luteolin (Özgen et al., 2011)

are strong free radical scavengers. Phenolic hydroxyl groups are excellent hydrogen atom and electron donors to free radicals (Dai and Mumper, 2010). The benzoic ring, which contains three double bonds, decreases very much the bond strength between hydrogen and oxygen in the linked hydroxyl group, turning it to a very active antioxidant (Erlank et al., 2011). In addition, the arrangement of the hydroxyl groups around the phenolic molecule also influences antioxidant reactions (Ndhlala et al., 2010). For instance, the presence of 3-OH and 4-OH groups in the B ring enhances the antioxidant activity when compared with compounds with one hydroxyl group (Fig. 7.1a) (Rice-Evans et al., 1996; Miguel, 2010). The 3-OH and 5-OH groups with 4-oxo function A and C rings are required for maximum radical scavenging potential (Fig. 7.1b) (Rice-Evans et al., 1996). In addition, the 3-OH group in the C ring in association with the C2-C3 double bond conjugated with the 4-oxo group forms an extremely active scavenger (Fig. 7.1c) (Rice-Evans et al., 1996; Miguel, 2010). However, synergistic and antagonistic interactions between extract components could also account to the observed antioxidant activity.

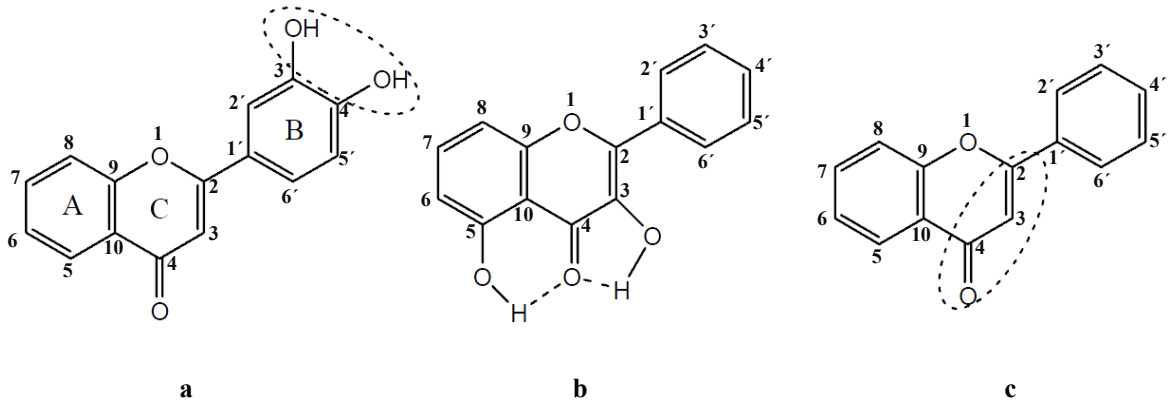

Figure 7.1. Structure-antioxidant activity relationship of flavonoids. Adapted from Yordi et al. (2012).

7.1.2.2.2. Fe^{2+} chelation

Metal chelation is another feature of natural phenolics which allows them to prevent the triggering of oxidative processes catalysed by biometals. Iron can promote lipid peroxidation by participating in the generation of initiating species and by accelerating peroxidation, decomposing lipid hydroperoxides into highly reactive lipid alkoxyl and peroxyl radicals, which perpetuate the chain of reaction of lipid peroxidation (Britton et al., 2002; Miguel, 2010). Therefore, the depletion of free iron by natural antioxidants can attenuate iron-

promoted radical production. To evaluate the ability of essential oils and extracts, from the studied species, to compete with ferrozine for iron ions in free solution different concentrations were tested. It was observed that only the polar extracts showed capacity to chelate Fe^{2+} and the highest results were obtained by the water extracts in a dose-dependent manner, followed by the water:ethanol extracts. The methanol extract of *L. viridis* showed similar results to the water extract. Excepting for *L. viridis*, the remaining infusions showed a capacity to chelate Fe^{2+} comparable to that exhibited by the water:ethanol extracts. Overall, the ethanol extracts were found to present the lowest efficiency to form complexes with Fe^{2+}. Regarding the *in vitro* cultures, all the extracts demonstrated Fe^{2+}-chelating activity. The ethanol extracts from both *in vitro* cultures showed the best capacity to chelate Fe^{2+} particularly that from *L. viridis*. These results highlight the influence of the solvent on the extraction of chelating agents. The high capacity of phenolics to chelate Fe^{2+} can be related with the presence of ortho-dihydroxyl groups, i.e., molecules bearing catechol or galloyl groups, 5-OH and/or 3-OH in conjunction with C4 keto group and a large number of hydroxyl groups which seem to be fundamental for Fe-binding (Khokhar and Apenten, 2003; Andjelković et al., 2006). However, the ethanol extracts showed little Fe^{2+}-chelating activity even possessing high content of catechol groups, suggesting that other organic substances, such as organic acids, amino acids and sugars, with capacity to chelate transition metal ions, may be also co-extracted and somehow contribute to the observed results.

7.1.2.2.3. Inhibition of lipid peroxidation

Lipid peroxidation is a marker of oxidative damage to membranes, affecting their permeability and fluidity and, consequently, their integrity. Lipid peroxidation is initiated by a radical attack towards fatty acids abstracting a hydrogen atom in the α-position, relative to the fatty acid chain double bond, generating the allyl radical (Miguel, 2010). Then, molecular oxygen adds to the carbon centered lipid radical forming the peroxyl radical (Repetto et al., 2012). These highly reactive species can, in turn, abstract hydrogen atoms from other molecules and propagate the chain reaction of lipid peroxidation (Carocho and Ferreira, 2013). The studied species were also tested for their capacity to inhibit Fe^{2+}-induced lipid peroxidation in mouse brain homogenates (Chapters 2 and 3). The extent of lipid peroxidation was assessed by measuring the production of malondialdehyde (MDA) which can be quantified colorimetrically following its controlled reaction with thiobarbituric acid (TBA).

The results showed that the polar extracts were more efficient than the essential oils and SFE extracts. These later ones were not able to inhibit MDA production at all the concentrations tested. Water:ethanol and water extracts contained the most efficient metabolites to protect mouse brain homogenates against lipid peroxidation induced by Fe^{2+}. Likewise, infusions from all the studied species and the methanol extract of *L. viridis* had a strong ability to prevent MDA production. These preventive effects could reflect the presence of rosmarinic (Fadel et al., 2011), 5-*O*-caffeoylquinic (Ohnishi et al., 1993) and caffeic (Khennouf et al., 2010) acids, and luteolin (Ashokkumar and Sudhandiran, 2008). In some cases, namely water extracts, the inhibition of MDA production was not related with their phenolic content. The presence of other active unidentified compounds in the extracts and the existence of synergistic and antagonistic effects cannot be excluded. In addition, it must also be considered the low specificity of the TBARS assay. Other substances, such as 4-hydroxyalkenals, 2,4-alkadienals and 2-alkenals, protein and sugar degradation products, amino acids, nucleic acids and anthocyanins are also able to react with TBA, forming a chromophore producing false-positive effects (Miguel, 2010).

The water:ethanol extracts, from *in vitro* cultures and wild plants of *L. viridis*, showed similar capacity to protect mouse brain homogenates against lipid peroxidation at the highest concentration tested. In addition, the ethanol extracts from *in vitro* cultures were more efficient than those from wild plants. The bioactive compounds, particularly found in the water:ethanol extracts, were efficient free radical scavengers and Fe^{2+} chelators, suggesting that both activities probably help to prevent Fe^{2+}-induced lipid peroxidation in mouse brain homogenates *in vitro*. Infusions from all the *in vitro* cultures demonstrated remarkable capacity to reduce MDA production.

7.1.3. Complexation of essential oils with cyclodextrins

Essential oils have distinctive flavour and fragrance properties and important biological activities. Due to their versatility, essential oils are applied in many industries. Although the food industry primarily uses them as flavouring agents, they represent an interesting source of natural antioxidants and antimicrobials for food preservation, reducing the oxidative reactions and microbial contaminations during food handling, processing and storage (Hyldgaard et al., 2012). Many essential oils have already been granted Generally Recognized As Safe (GRAS) status by the Food and Drug Administration (FDA) and, therefore, can be used in food

products (Hyldgaard et al., 2012). However, their limited water solubility, volatility and sensitivity to oxygen, light and heat can decrease their bioavailability and, consequently, restrict their applications. Cyclodextrins (CDs) have been widely applied to overcome these limitations due to their unique structural features and amphiphilic properties; the cavity is relatively hydrophobic while the external surfaces are hydrophilic. In this study, the ORAC assay was used to evaluate the antioxidant activity of all the essential oils and extracts. However, low ORAC values were obtained for the essential oils which can be related with their lipophilic character. This is a water-based method limited to hydrophilic compounds thus excluding lipophilic antioxidants which might be involved in important biological processes (Prior et al., 2005). Therefore, different CDs were used as solubilizing agents, namely *β*-CD and 2-hydroxypropyl-*β*-CD (HP-*β*-CD) (Chapter 4). HP-*β*-CD is a *β*-CD derivative with improved water solubility (above 60%); the introduction of hydroxypropyl moieties decreases the crystallinity thus enhancing solubility (Szente and Szejtli, 1999). In Chapter 4 the effect of *β*-CD and HP-*β*-CD on the antioxidant activity of the essential oils, from the three species, was evaluated by the ORAC assay. Since storage processes can induce chemical and biological modifications of volatiles (Marques, 2010) it was also investigated the storage stability of essential oils/CDs system at 4 ºC, over 30 days. Essential oils from *L. viridis*, *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus* were mixed with different concentrations of *β*-CD and HP-*β*-CD. Surprisingly, in the presence of *β*-CD, a remarkable antioxidant activity was noticed despite the use of a much less quantity of essential oils. The *L. viridis* and *T. lotocephalus* essential oils were found to be the most active radical scavengers in the presence of *β*-CD. Likewise, the activity of all the Lamiaceae essential oils was enhanced by the presence of HP-*β*-CD, being the effect more pronounced in the essential oil of *T. lotocephalus*, particularly at the highest concentration of HP-*β*-CD where the activity almost doubled. Conversely, no significant effect of the HP-*β*-CD concentration was detected in the activity of the *Lavandula* species.

The increase on the activity is probably related to the enhancement of solubility of the essential oils since in the absence of CDs, at the same low concentration, no effect was detected. Comparing both CDs, HP-*β*-CD was shown to have a more pronounced effect on the activity of the essential oils. It is believed that the process of complexation with HP-*β*-CD was facilitated in comparison with the native *β*-CD. The chemical structure of the hydroxypropyl moieties in HP-*β*-CD not only makes it more soluble than the *β*-CD but also changes the surface chemistry. Therefore, it is expected that less polar components, which have not been

trapped in the HP-*β*-CD cavity (and do not strongly interact with simple hydroxyl groups), can absorb at HP-*β*-CD external surface increasing their solubility thus contributing to the enhancement of the activity.

After 30 days, the essential oils of the studied species revealed storage stability in the presence of CDs. Generally, all formulations were more stable with HP-*β*-CD over time displaying a high activity after 30 days of storage. This reflects the higher solubility of HP-*β*-CD and favored interactions between the hydroxypropyl moieties and the essential oils constituents.

7.1.4. Neuroprotective effect

As mentioned before, oxidative stress is one of the mechanisms involved in neuronal damage. Hydrogen peroxide (H_2O_2) is among the main reactive species and is a potent inducer of oxidative damage causing mitochondrial dysfunction, calcium imbalance and apoptosis of neuronal cells (Halliwell et al., 2000; Giorgio et al., 2007). Natural antioxidants, namely phenolic compounds, are an important class of plant-derived secondary metabolites with recognized neuroprotective effects (Ebrahimi and Schluesener, 2012). The chemical analysis from *L. viridis* extracts showed high levels of phenolic compounds and, in addition, this species was the most consistent in terms of biological activities assessed by the chemical assays. Phytochemicals from *L. viridis* have capacity to act as free radical scavengers, metal chelators and lipid peroxidation inhibitors, allowing them to protect cells from the consequences of oxidative stress through different mechanisms of action. Therefore, for the neuroprotection and digestion assays only *L. viridis* was considered. The neuroprotective effect of *L. viridis* extracts (infusion, water:ethanol and methanol) and rosmarinic acid (the major compound present in the extracts), against oxidative damage induced by H_2O_2 in A172 human astrocyte cell line was investigated in Chapter 5. The possible neurotoxic effect of the *L. viridis* extracts and rosmarinic acid was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The viability of A172 human astrocyte cell line was not adversely affected by the *L. viridis* extracts and rosmarinic acid at the concentrations tested (i.e. 250 and 500 μg/ml); however, after 24 h of exposure to H_2O_2, the viability was reduced to ~35% compared to the untreated cells. Except for the lowest concentration of the methanol extract, all the remaining extracts and rosmarinic acid reduced the neurotoxicity induced by H_2O_2. Rosmarinic acid showed the greatest neuroprotective effect followed by the

water:ethanol and methanol extracts, particularly at the highest concentration tested (i.e. 500 μg/ml).

The ability of *L. viridis* extracts to reduce intracellular reactive oxygen species (ROS) production was estimated using a fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA), which can be deacetylated inside cells, where it can react with ROS, including H_2O_2, generating a fluorescent compound (Wang and Joseph, 1999; Gomes et al., 2005). The fluorescence intensity is believed to reflect the amount of ROS formed. The intracellular ROS accumulation resulting from H_2O_2 exposure was reduced (~20-30%) in the presence of water:ethanol, infusion and rosmarinic acid, compared with A172 cells treated only with H_2O_2. The methanol extract had no positive effect in the reduction of intracellular ROS production at the concentrations tested. The observed protective effects could be primarily due to their capacity to scavenge intracellular ROS production or to neutralize H_2O_2 itself. The protection mechanism of *L. viridis* extracts against ROS generation may be explained by their free radical scavenging activity (Chapters 2 and 3). Infusion was the most efficient at neutralizing the peroxyl and radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radicals, followed by the water:ethanol and methanol extracts (Chapters 2 and 3). The lower capacity of the methanol extract to attenuate intracellular ROS production may be a consequence of its lower free radical potential compared to the remaining extracts. In addition, the capacity of the extracts to chelate metal ions might prevent the *in vivo* toxicity of H_2O_2 by decreasing the occurrence of Fenton chemistry. Rosmarinic acid was highly active against H_2O_2-induced oxidative damage and able to reduce the intracellular ROS production, as already reported in previous studies (Gao et al., 2005; Qiao et al., 2005; Lee et al., 2008). Albeit, the protective effect of *L. viridis* extracts seems to be mainly due to rosmarinic acid, we cannot ignore the presence of other important phenolic compounds in the extracts which have also revealed neuroprotective effects, including 5-*O*-caffeoylquinic acid (Kim et al., 2012) and luteolin (Kang et al., 2004).

7.1.5. *In vitro* gastrointestinal metabolism

Among the wide range of active metabolites synthesised by plants, phenolics have been object of special interest, not only because they are potent and therapeutically useful bioactive substances but also because they are remarkably present in the human diet. It has been postulated that the consistent intake of phenolic compounds as exogenous defense systems,

provides health benefits associated with reduced risk of chronic diseases (Landete, 2013). However, their *in vivo* biological activity is dependent on their bioavailability which is affected by different factors including their release from the plant matrix, stability and absorption. It has been mentioned that phenolics have limited absorption and intense metabolism (Serra et al., 2012). During digestion processes, they may be structurally and chemically changed which, in turn, may not allow bioactive compounds to act at their full activity. For these reasons, the understanding of the active extracts behavior throughout the gastrointestinal tract is a key factor in assessing their implication in human health. Therefore, in Chapter 6, the influence of *in vitro* gastrointestinal tract conditions on the chemical profile and antioxidant and anti-ChE activities of the methanol and water:ethanol extracts from *L. viridis*, and rosmarinic acid is discussed. In addition, the effect of the plant matrix on the digestion of rosmarinic acid was compared. Pepsin and pancreatin were used to simulate gastric and pancreatic digestions, respectively, and Caco-2 cells to predict the absorption and metabolism of bioactive components. *L. viridis* extracts were studied at a concentration of 500 µg/ml and rosmarinic acid solutions were used at the same concentration found in each extract (i.e. 125 and 250 µg/ml, to the water:ethanol and methanol extracts, respectively). The results showed that the methanol extract matrix had no effect on the digestion of rosmarinic acid, unlike to the water:ethanol extract which probably contains metabolites that protect rosmarinic acid from the acid digestion. Unlike the water:ethanol extract, the antioxidant activity assessed by the TEAC assay of the methanol extract from *L. viridis* and rosmarinic acid was not affected by the simulated *in vitro* gastric juice. The pancreatic digestion induced an increase in the free radical scavenging activity of the methanol extract and rosmarinic acid. Cilla et al. (2011) found similar results; they referred that the presence of other compounds in the plant matrix (e.g. amino acids, sugars and uronic acids) can be released after *in vitro* digestion generating positive interfering effects in certain antioxidant capacity assays, such as TEAC. Other unknown interfering effects must be also considered.

The anti-ChE activity of the methanol and water:ethanol extracts was also investigated before and after *in vitro* digestion. Only the inhibition of BChE was affected; overall, methanol and water:ethanol extracts showed a moderate decrease after gastric and pancreatic digestions. Rosmarinic acid was not active at the concentrations tested, suggesting that the activity exhibited by the extracts was not primarily due to its presence. The viability of Caco-2 cell line was not affected by the extracts and pure solutions of rosmarinic acid. After 6 h incubation, the *L. viridis* extracts and pure solutions of rosmarinic acid were detected in the

medium and no changes were found on their chemical profiles. No phenolic compounds were found within the cells suggesting that the metabolites were not able to penetrate them. Other factors, such as dilutions of the extracts and rosmarinic acid, as well as interactions with other chemicals within the plant matrices could, in principle, affect the bioavailability of phenolic compounds present in the *L. viridis* extracts. For instance, rosmarinic acid has low intestinal absorption efficiency, occurring the transport mainly *via* paracellular diffusion as reported by Konishi and Kobayashi (2005). They also found that rosmarinic acid is unsusceptible to hydrolysis by mucosa esterase in Caco-2 cells.

7.2. GENERAL CONCLUSIONS

AD is the most prevalent dementia affecting an alarming number of people worldwide. Oxidative stress is a serious hallmark of AD and has been considered as therapeutic target for AD treatment. Plants and their active components play an important role in the prevention of chronic and neurodegenerative diseases. Lamiaceae family comprises important species, including *Lavandula* and *Thymus*, with a tremendous positive impact on the human health, producing valuable classes of phytochemicals, as terpenes and phenolic compounds. Therefore, this work traces natural products discovery, outlining the following aromatic plants from Portugal, collected in the Algarve region: *Lavandula viridis*, *Lavandula pedunculata* subsp. *lusitanica* and *Thymus lotocephalus*. This research also intends to provide scientific background to support the traditional usage of these species either as condiments or as therapeutics. Different techniques and extraction solvents were applied to assess the best approach for the recovery of bioactive metabolites from the target species. After prepared and chemically characterised, the extracts were subjected to biological screening in pharmacologically relevant assays. The main draw conclusions out from this research are presented next.

Regarding the volatile fraction, it was observed that the SFE alternative technique was the most suitable choice to obtain high extraction yields, whereas HD allowed the identification of the highest number of components (Chapter 2). The chemical analysis highlighted the abundance of oxygen-containing monoterpenes in all the essential oils and SFE extracts, excepted those from *T. lotocephalus* collected in the first separator at both pressures. It was found that the chemical composition of the essential oils and SFE extracts, collected in the second separator (volatiles), differ qualitatively and quantitatively. The results obtained confirm that the heaviest compounds were collected in the first separator, albeit some amount of volatile components was also retained.

The chemical analysis of the polar extracts allowed the identification of phenolic acids (3-*O*-caffeoylquinic, 4-*O*-caffeoylquinic, 5-*O*-caffeoylquinic, caffeic and rosmarinic acids) and flavonoids (luteolin, apigenin and pinocembrin), two important groups of secondary metabolites (Chapters 2 and 3). The water:ethanol extracts contained the highest amount of the identified compounds followed by the infusions. Most extracts, including those obtained from *in vitro* cultures, were dominated by rosmarinic acid. *In vitro* conditions were the most suitable for rosmarinic acid accumulation, suggesting *in vitro* cultures from *L. viridis* and *T. lotocephalus* as promising sources of valuable active metabolites. Therefore, in future studies, the manipulation of the chemical or physical environment can be performed to improve the production of these compounds.

In respect to the anti-ChE activity, excepting the infusions, all the remaining polar extracts were active against both AChE and BChE activities. However, the essential oils exhibited the best results as compared with the extracts, excepted for the methanol extract that showed similar results; the methanol extract inhibited ChEs both in *in vitro* and *in vivo* models. The activity of the SFE extracts was affected by the extraction pressure: the anti-ChE activities of *L. viridis* increased with the higher extraction pressure, whereas the SFE extracts of *T. lotocephalus* were more active at 12 MPa. False-positive effects were found in the SFE extracts (first separator), suggesting that the observed activity is possibly overestimated. The strong activity exhibited by the essential oils could reflect the presence of 1,8-cineole, α-pinene, myrtenal and viridiflorol, however the collective activities of other constituents, including minor components, must be considered.

Among the essential oils and extracts, the water:ethanol extract and infusions were generally the most efficient radical scavengers, acting preferentially by donating a hydrogen atom

(Chapters 2 and 3). In respect to the SFE extracts, the extraction pressure affected the capacity of the SFE extracts (first separator) to scavenge the peroxyl radical: the antioxidant activity increased with the higher extraction pressure. The highest capacity to chelate Fe^{2+} was obtained by the water extracts followed by the water:ethanol extracts and infusions. Both plant materials of *L. viridis* and *T. lotocephalus* were efficient Fe^{2+} chelating agents. In addition, the polar extracts revealed higher capacity to protect mouse brain homogenates against Fe^{2+}-induced lipid peroxidation than the essential oils and SFE extracts which were not active at the tested concentrations (Chapter 3). Water and water:ethanol extracts and infusions showed the highest protective effect, and the ethanol extracts, from *in vitro* cultures, were more efficient than those from wild plants. The remarkable antioxidant capacity can be due, at least in part, to the presence of 5-*O*-caffeoylquinic caffeic and rosmarinic acids, and luteolin. The results reflect the influence of the solvent choice on the recovery of target active metabolites, being water:ethanol mixture the most suitable choice for the studied species.

Summarizing Chapters 2 and 3, it can be concluded that both HD and SFE extraction techniques showed advantages for the recovery of valuable volatile compounds of the target species. SFE provides high extraction yields and selectivity allowing the extraction of targeting high-value compounds for the flavour and fragrance industries, as camphor and linalool. Biologically, the volatiles isolated from HD seem to be more promising. The water:ethanol mixture was the most efficient solvent for the extraction of phenolic compounds followed by infusion. The *in vitro* environment was favorable for the accumulation of high levels of rosmarinic acid. The extracts were the most efficient free radical scavengers, Fe^{2+} chelators and inhibitors of MDA production, while the essential oils were generally the most active against ChEs.

The effect of CDs on the antioxidant activity of Lamiaceae essential oils is described in Chapter 4 and an incredible positive effect on the activity of the essential oils in the presence of CDs was observed. This behavior is probably due to an enhancement in solubility of some less polar compounds of the essential oils. Comparing both CDs, HP-*β*-CD had a more pronounced effect on the free radical scavenging effect of essential oils, in particular for *T. lotocephalus*. These complexes not only increase the antioxidant activity but also show stability over 30 days, particularly in the presence of the modified *β*-CD. These outcomes point out these systems as interesting alternatives in food, pharmaceutical and cosmetic formulations as natural source of antioxidants.

L. viridis was the most consistent species in terms of biological activities. Bioactive compounds from *L. viridis* can protect cells from the consequences of oxidative stress through different mechanisms acting as free radical scavengers, metal chelators and lipid peroxidation inhibitors. Therefore, in the neuroprotection (Chapter 5) and in the *in vitro* gastrointestinal metabolism (Chapter 6) studies only *L. viridis* was tested. It was found that *L. viridis* extracts and its main identified compound, rosmarinic acid, were effective in protecting A172 cells from the damaging effects generated by H_2O_2. Except for the lowest concentration of the methanol extract, all the remaining extracts and rosmarinic acid attenuated the neurotoxicity induced by H_2O_2. In addition, the water:ethanol extract, infusion and rosmarinic acid reduced the intracellular ROS accumulation. The observed protection effect can be, at least partially, related to their free radical scavenging ability. However, it is important to understand how the extracts can exactly protect cells from the toxicity induced by the H_2O_2. Furthermore, a suitable neuroprotective agent has to be absorbed and cross the blood-brain barrier, in order to reach the target sites. For this reason, the transport through the blood-brain barrier of drug candidates should be determined in the drug discovery processes.

Concerning the behavior of *L. viridis* bioactive compounds under *in vitro* digestion conditions it was found that the methanol extract matrix does not influence the digestion of rosmarinic acid contrasting to the water:ethanol extract which protected rosmarinic acid from the acid digestion. These results reinforce the influence of the plant matrix on the bioaccessibility of natural compounds. The antioxidant activity of the methanol extract and pure rosmarinic acid was maintained after *in vitro* gastrointestinal processes. Regarding the anti-ChE activity, only the inhibition of BChE was affected after *in vitro* gastric and pancreatic digestions; however, it can be considered that the activity is assured. Based on these results, the *L. viridis* extracts have a high probability of going through the gastrointestinal tract and keeping their biological activity. In addition to the studied *in vitro* digestion conditions, there are other biological *in vitro* methods for predicting intestinal absorption and transport mechanisms which can also be applied. To obtain more accurate results, it is desired to perform *in vivo* methods since the results obtained through *in vitro* digestion models are often different to those found using *in vivo* models due to differences of physicochemical and physiological events occurring in animal and human gastrointestinal tracts.

This study highlights some extracts as promising sources of metabolites with therapeutic potential; in these cases, the plant extracts can be fractionated and the obtained fractions

subjected to bioassays to accurately identify which compounds are responsible for the observed activity.

The paradigm of *one-molecule-one-target* is not the most suitable approach to address all the complex pathways in AD. Multi-target therapeutics with diverse molecular targets becomes a promising alternative to reduce the incidence of this multifactorial disease and other neurodegenerative diseases. In this sense, terpenes and phenolic compounds appear as powerful molecules. The results from this thesis suggest *L. viridis*, *L. pedunculata* subsp. *lusitanica* and *T. lotocephalus*, highlighting *L. viridis*, as new sources of natural antioxidants and anti-ChEs auspicious for the prevention of neurodegenerative diseases acting by different intracellular mechanisms. Furthermore, this thesis provides a scientific support in terms of chemical composition and biological activity for these target species traditionally used. Based on these findings it seems that their characteristic benefits are associated to their high antioxidant potential.

7.3. References

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