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Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices

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Abstract

In the present work, we report the results of a study aimed at evaluating the antiradical activity, the antioxidant activity and the acetylcholinesterase (E.C. 3.1.1.7.) inhibitory capacity of essential oils, ethanol and boiling water extracts from five aromatic herbs growing wild in Portugal and used in traditional food preparations: fennel (*Foeniculum vulgare*), mint (*Mentha spicata*), pennyroyal (*Mentha pulegium*), rosemary (*Rosmarinus officinalis*) and wild thyme (*Thymus serpyllum*). The water extracts of *M. spicata* and *M. pulegium* showed the highest radical-scavenging activity (DPPH test) values ($IC_{50} = 5.7 \pm 0.4$ and 8.9 ± 0.2 μg ml^{-1} respectively). This activity was higher than that found with the standard antioxidant BHT. The ethanol extracts of *M. spicata*, *T. serpyllum* and *F. vulgare* showed the highest antioxidant activities measured by the β-carotene/linoleic acid assay, $IC_{50} = 36.9 \pm 0.1$, 41.2 ± 0.1 and 68.7 ± 0.1 μg ml^{-1}, respectively. The inhibition of AChE was higher in the essential oil fraction. The highest activity was found for *R. officinalis* with an $IC_{50} = 69.8 \pm 0.1$ μg ml^{-1}.

Keywords: Essential oils; Aromatic herbs; GC–MS; Antioxidants; Antiacetylcholinesterase activity

1. Introduction

Many plants contain natural antioxidants that act in metabolic response to the endogenous production of free radicals and other oxidant species. These responses are due to ecological stress or are promoted by toxins produced by pathogenic fungi and bacteria (Grassmann, Hippeli, & Elstner, 2002). Recently, interest has increased in naturally-occurring antioxidants that can be used to protect human beings from oxidative stress damage (Scalbert, Manach, Morand, & Remesy, 2005).

Over production of reactive oxygen species (ROS) in human beings, by endogenous or external sources, e.g. tobacco smoke, certain pollutants, organic solvents or pesticides, leads to oxidative stress (Gulcin, Oktay, Kireçci, & Kufrevioglu, 2003). ROS are chemical entities that include oxygen free radicals, such as superoxide anion radicals ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), nitric oxide (NO), peroxinitrite and also non-radical species, such as H_2O_2 and singlet oxygen ($^1O_2$). In living organisms, different metabolic ways can give rise to various endogenous ROS from normal aerobic respiration (Gulcin et al., 2003). In healthy cells, there is an equilibrium between the production of these highly reactive species and the different defence systems, either enzymatic or non-enzymatic. When this equilibrium is disrupted, oxidative damage due to free radical accumulation, defined as oxidative stress, occurs and, as a consequence, many diseases, e.g. cancer,

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arteriosclerosis and other cardiovascular problems and diabetes and even ageing are promoted (Aruoma, 1998). The brain is an organ particularly vulnerable to oxidative stress, and the hypothesis that this process is involved in neurodegenerative events and neuronal cell death has emerged (Behl, 1997). Alzheimer's disease (AD), the most common cause of dementia in aged population, whose symptoms are cognitive decline and mental deterioration, is the result of massive and progressive loss of neurons from several different regions of the brain. It is still controversial but some studies suggest that dietary supplements with antioxidants and free radical-scavengers (including vitamin E) may display some benefits in slowing the mild cognitive impairment of AD (Morris et al., 2002; Stuchbury & Munch, 2005). Until now, the only treatment for this disease is based on the "cholinergic hypothesis" which means that the drugs approved for the Alzheimer therapy must act by counteracting the acetylcholine deficit, enhancing its level in the brain (Heinrich & Teoh, 2004). Acetylcholine is involved in the signal transfer in the synapses and, after being delivered in the synapses, is usually hydrolyzed, giving choline and acetate in a reaction catalyzed by the enzyme acetylcholinesterase. The molecular basis of the drugs used up to now is their action as acetylcholinesterase inhibitors (Ingkaninan, Temkitthawon, Chuenchon, Yuyaem, & Thongnoi, 2003). This enzyme is associated with the extra-cellular membrane surface and it plays an important role as a safeguard of the brain cells (Shen, 2004). Recently it was shown that the senile plaques seem to induce inflammatory processes in which radical oxygen species are liberated (Stuchbury & Munch, 2005; Vina, Lloret, Orti, & Alonso, 2004).

Aromatic herbs and spices have been used for a long time in Mediterranean cuisine, not only to improve or modify the flavour of foods, but also to avoid its deterioration. In Portugal, people who live in the countryside appreciate and continue to gather and consume wild aromatic herbs. In the present work we report the results of a study aimed at evaluating the antiradical activity, the antioxidant activity and the acetylcholinesterase (E.C. 3.1.1.7.) inhibitory capacity of essential oils, ethanol and boiling water extracts, of five aromatic herbs growing wild in Portugal and commonly used as spices in traditional food preparations. The studied plants were mint (*Mentha spicata* L., Lamiaceae, *port.* hortelã-comum) used to flavour chicken soup and rice, pennyroyal (*Mentha pulegium* L., Lamiaceae, *port.* poejo) used to prepare a liquor called "licor de poejo", added to fish stew made with river fish and also in a bread-soup very popular in the region of Alentejo, fennel (*Foeniculum vulgare*, Miller, Umbelliferae, *port.* funcho or erva doce) used in liquors as well, in the boiling of chestnuts and to aromatize many cakes, rosemary (*Rosmarinus officinalis* L., Lamiaceae, *port.* alecrim) and wild thyme (*Thymus serpyllum* L., Lamiaceae, *port.* serpão), both used in many recipes of meat dishes. Moreover, all these herbs have ethnobotanical uses, mainly as stimulant and anti-asthenia agents (Proença da Cunha, Pereira da Silva, & Roque, 2003).

2. Materials and methods

2.1. Plant material

F. vulgare, (fennel) (LISU 204108), *R. officinalis* (rosemary) (LISU 204109), and *T. serpyllum* (wild thyme) (LISU 204113), were collected in Beira Interior (east of Portugal), during the summer of 2004. *M. pulegium* (pennyroyal) and *M. spicata* (mint) were bought in a traditional market in the same summer, the latter in a southern region, Ribatejo-Portugal. All plant material was authenticated by Dr Ana Margarida Francisco (research team of Professor Lia Ascenção, Centro de Biotecnologia Vegetal, DBV), Faculty of Science University of Lisbon, where voucher specimens of collected plants were deposited.

2.2. Preparation of the extracts

Plant material was dried in the dark and ground to a powder to obtain essential oils and ethanol extracts.

Essential oils were obtained by hydrodistillation of the plant material until 250 ml of the water–oil layer was obtained. The aqueous layer was extracted three times with *n*-pentane and the organic solvent was removed by vacuum distillation at room temperature.

Ethanol extracts were obtained by extracting the plant material three times at room temperature and removal of the solvent by vacuum distillation at 50 °C. Aqueous extracts were obtained by boiling 5 g of dried plant material, broken into small pieces, in 100 ml of distilled water for 20 min, followed by filtration. For the enzymatic tests, aliquots of 1 ml were used immediately, or frozen and used when necessary. An aliquot of each water extract was evaporated to dryness to obtain the equivalent dry weight (dry wt).

2.3. Chemicals

All chemicals were of analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, β-carotene, 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT), acetylcholinesterase (AChE) type VI-S, from electric eel 349 U/mg solid, 411 U/mg protein, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), acetylthiocholine iodide (AChI), tris[hydroxymethyl]aminomethane (tris buffer), dimethylsulfoxide (DMSO) and Tween 40, were supplied from Sigma.

2.4. Analysis of the essential oils

Capillary GC–MS analyses were performed on an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 *N* mass selective detector (Agilent Technologies, Little Falls, DE, USA). A vaporization injector in

the split mode (1:100) at 250 °C, with a fused silica capillary column, 30 m × 0.25 mm ID × 0.25 μm d_f (TRB-5MS; 5% diphenyl 95% dimethyl polydimethylsiloxane, Teknokroma – Spain) were used. The oven temperature was programmed from 45 °C and then increased at 5 °C min^{-1} to 240 °C, followed by 10 °C min^{-1} to 300 °C and held isothermally for 5 min. High purity helium was used as carrier gas at 30 cm s^{-1}.

Electron ionisation mass spectra in the range 40–400 Da were recorded at 70 eV. The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 °C, respectively. A solvent delay of 6 min and a turbo molecular pump (10^{-5} torr) were used. All data were recorded using a MS ChemStation (G1701CA; Rev C.00.00; Agilent Technologies).

The identity of each compound was determined by comparison of its retention index (RI) relative to C_7–C_{27} *n*-alkanes (Adams, 2001), as well as of its spectral data with the Wiley library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies). For semi-quantification purposes, of the samples analysed (1 μl), the normalised peak area of each compound was used without any correction factors to establish abundances.

2.5. Antioxidant activity

2.5.1. General

The antioxidant activity was measured by the β-carotene/linoleic acid assay, as described by Tepe, Daferera, Sokmen, Sokmen, and Polissiou (2005) and by the DPPH assay described by Kirby and Schmidt (1997) and modified by Tepe et al. (2005).

2.5.2. β-Carotene/linoleic acid assay

The method described by Tepe et al. (2005) was used with a slight modification. A stock solution of β-carotene and linoleic acid was prepared by dissolving 0.5 mg of β-carotene in 1 ml of chloroform and 25 μl of linoleic acid, together; 200 mg of Tween 40 were added. The chloroform was evaporated under vacuum. One hundred ml of aerated water were then added to the residue. To 2.5 ml of this mixture, 300 μl of a solution of each extract were added. The ethanolic extract, aqueous extract or essential oil fraction were dissolved in ethanol, water or DMSO, respectively. The test tubes were incubated in hot water (50 °C) for 2 h, together with two blanks, one containing the antioxidant BHT as a positive control and the other with the same volume of distilled water instead of the extracts. In the test tube with BHT, the yellow colour is maintained during the incubation period. The absorbance was measured at 470 nm. Antioxidant capacities (AA) of the solutions tested were calculated using the following equation (Shon, Kim, & Sung, 2003):

$$\text{AA}\ (\%) = (\beta\text{-carotene content after 2 h assay} / \text{initial } \beta\text{-carotene content}) \times 100.$$

Tests were carried out in triplicate. Extract concentration providing 50% inhibition (IC_{50}) was obtained plotting inhibition percentage *versus* extract solution concentrations.

2.5.3. DPPH assay

The method described by Tepe et al. (2005) was used. To 5 ml of a methanol solution of 2,2′-diphenylpicrylhydrazyl (DPPH) 0.002% in methanol, 50 μl of essential oil or extract solutions were added and the mixture was incubated at room temperature for 30 min. The absorbance was measured at 517 nm against a corresponding blank. Inhibition percentage of free radical DPPH (I%) was calculated in the following way:

$$I\ (\%) = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the control reaction (a reaction with all the reagents except the test extract), and A_{sample} is the absorbance of the test extract. Tests were carried out in triplicate and the extract concentration providing 50% inhibition (IC_{50}) was obtained by plotting extract solution concentration *versus* inhibition percentage.

2.6. Determination of total phenolic compounds

The total phenolic compound contents in the ethanol and water extracts were determined by colorimetric assays, using the Folin-Ciocalteu reagent (Oktay, Gulcin, & Kufrevioglu, 2003) and pyrogallol as a standard. Briefly, 1 ml of the ethanol or aqueous solution containing 1 mg of extract was mixed with 45 ml of distilled water. One millilitre of Folin-Ciocalteu reagent was added and the contents of the flask mixed thoroughly. After 3 min, 3 ml of a 2% solution of sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm:

$$\text{Absorbance} = 1.475\ \text{pyrogallol} - 0.0731.$$

The concentration of total phenolic compounds, in the both ethanol and water extracts, was determined as microgrammes of pyrogallol equivalents by using an equation that was obtained from the standard pyrogallol plot. Results were means of three assays expressed as pyrogallol equivalents in mg per g of extract.

2.7. Acetylcholinesterase inhibition

The enzymatic activity was measured, using an adaptation of the method described by Ingkaninan et al. (2003). 325 μl of Tris–HCl buffer (pH 8), 100 μl of a buffer solution of extract (in DMSO for essential oils, ethanol for ethanol extracts or water for aqueous extracts) with different concentrations and 25 μl of an enzyme solution containing 0.28 U/ml were incubated during 15 min. Subsequently, 75 μl of a solution of AChI 0.023 mg ml^{-1} and 475 μl of 3 mM DTNB were added and the final mixture incubated for another 30 min at room temperature. Absorbance of the mixture was measured at 405 nm. A control mixture

was prepared, using 100 μl of a solution, similar to the sample mixture but with the respective solvent instead of extract. Inhibition, in %, was calculated in the following way:

$$I\ (\%) = 100 - (A_{\text{sample}}/A_{\text{control}}) \times 100$$

where A_{sample} is the absorbance of the extract containing reaction and A_{control} the absorbance of the reaction control. Tests were carried out in triplicate. Extract concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against extract solution concentrations.

3. Results and discussion

3.1. General

In a first approach, the extracts obtained relatively to 100 g (dry wt) of each plant were determined and the results are depicted in Table 1. The yields obtained presented the following increasing order of occurrence: essential oils < ethanol extract < water extract. The values obtained for the last extract were above 25%. The essential oils obtained by hydrodistillation presented the lowest yields (<1%) for all plants under study.

The essential oils analysis of each plant by GC–MS was carried out and their compositions determined. The β-carotene/linoleic acid and the DPPH free radical-scavenging tests were used to evaluate the potential antioxidant activity of the essential oils and both extracts obtained. Finally, the capacity of all the extracts to inhibit the enzyme acetylcholinesterase was also determined.

3.2. Essential oils compositions

The essential oils were shown to be complex mixtures of several components, characterised by a high percentage of the monoterpene fraction, dominated by oxygenated monoterpenes. The identified constituents represented 94.8%, 81.7%, 62.5%, 67.1% and 64.5% of the total oils from *F. vulgare* (fennel), *M. spicata* (mint), *M. pulegium* (pennyroyal), *R. officinalis* (rosemary) and *T. serpyllum* (wild thyme) samples, respectively. Table 2 shows the identified

Table 1
Yield of the essential oils, ethanol and water extracts obtained for each plant studied

g/100 g dry plant	*Foeniculum vulgare*	*Mentha spicata*	*Mentha pulegium*	*Rosmarinus officinalis*	*Thymus serpyllum*
Essential oil	0.1	0.9	0.7	0.3	0.7
Ethanol extract	6.9	7.9	8.2	29.2	23.6
Water extract	26.5	75.8	49.8	51.1	36.8

Table 2
Composition of the identified components of the essential oils from the plants studied, obtained by GC–MS

Components	% Composition[a]					
	RI[b]	*Foeniculum vulgare*	*Mentha spicata*	*Mentha pulegium*	*Rosmarinus officinalis*	*Thymus serpyllum*
1,8-Cineole	969				3.1	
cis-Sabinene hydrate	1018		2.7			
Linalool	1060				1.9	
α-Thujone	1070	6.0				
Camphor	1086				5.5	
Borneol	1121				2.1	0.9
4-Terpineol	1136		1.8		3.9	
α-Terpineol	1151				7.2	4.9
Verbenone	1163				35.4	
Pulegone	1194			35.1		
Carvone	1204		75.9			
(*Z*)-Anethole	1205	18.6				
Carvacrol	1271					56.0
(*E*)-Anethole	1297	70.2				
Piperitenone	1325			27.4		
β-Caryophyllene	1387		0.5		2.3	
α-Humulene	1423				1.2	
Germacrene D	1452		0.5			
Caryophyllene oxide	1550		0.3		2.4	
Veridiflorol	1559					2.7
α-Bisabolool	1648				2.1	
Total identified		94.8	81.7	62.5	67.1	64.5

[a] Normalised peak areas without using the correction factors.
[b] Relative to C_7–C_{27} *n*-alkanes on the TRB-5MS capillary column.

compounds and their percentages obtained by GC–MS, as well as the retention indices listed in order of their elution from the TRB-5MS capillary column.

The main compounds in fennel (*F. vulgare*) were (*Z*) and (*E*)-anethole, amounting to 18.6% and 70.2%, respectively. α-Thujone was also identified but in a lower percentage (6.0%). (*E*)-Anethole was also reported to be the major component (72–74%) of the essential oil obtained from the seeds of fennel from a Mediterranean origin where (*Z*)-anethole was present in a small percentage (traces) (Diaz-Maroto, Hidalgo, Sanchez Palomo, & Perez-Coello, 2005; Mimica-Dukié, Kujundzié, Soković, & Coulandis, 2003). The higher values found for this compound relatively to the values reported in the cited literature may be the result of the extraction of the whole aerial part of the plant and not only the seeds. The essential oil of pennyroyal (*M. pullegium*) is rich in pulegone (35.1%) and piperitenone (27.4%). However, none of these compounds were present in the essential oil of the other *Mentha* species, i.e. mint, whose main component is carvone (75.9%). Pulegone (48%) and menthone (41%) are described in essential oil of the *M. pullegium* species (Miyazawa, Watanabe, Kazuyas, & Kameoka, 1998).

The essential oil of rosemary contains several compounds in small proportions, ranging from 1.2% (α-humulene) to 7.2% (α-terpineol), and verbenone was the major component (35.4%). The amount achieved for verbenone is, however, greater than those previously reported for the Sardinian ecotype (21%) (Sachetti et al., 2005), the Spanish southern one (2.2–5.8%) (Salido, Altarejos, Nogueras, Sanchez, & Luque, 2003), as well as for the essential oil obtained from a Portuguese southern region species (0.7–1.5%) (Serrano, Palma, Tinoco, Venâncio, & Martins, 2002). Besides several minor compounds, such as borneol (0.9%), α-terpineol (4.9%) and veridiflorol (2.7%), the major component identified from wild thyme was carvacrol (56.0%), which is in good agreement with the high content usually found in other European *Thymus species* (Horvath, Szabo, Lemberkovics, Botz, & Kocsis, 2004; Juliano, Mattana, & Usai, 2000).

3.3. Inhibitory effect on lipid peroxidation

The inhibitory effect on lipid peroxidation was determined by the β-carotene/linoleic acid bleaching test. This test simulates the oxidation of the membrane lipid components in the presence of antioxidants inside the cells. It also measures the capacity to inhibit the formation of conjugated diene hydroperoxide arising from linoleic acid oxidation (Tepe et al., 2005). Essential oils, ethanol and water extracts were tested and results (IC_{50} μg ml^{-1}) are present in Table 3.

All extracts exhibited antioxidant activity, the best results being obtained with the ethanol extracts of *M. spicata* ($IC_{50} = 36.9 \pm 0.1$ μg ml^{-1}). According to a literature report (Oktay et al., 2003), the water extracts obtained from fennel (*F. vulgare*) seeds have high antioxidant activity. In this study, the antioxidant activity of the water extract was moderate compared with the antioxidant activity of ethanol extract. However, the whole plant was extracted, which may explain the opposite results here presented.

Essential oils are composed mostly of terpenoids (Table 2), and some of them, e.g. γ-terpinene, terpinolene and geraniol, have high radical-scavenging activity (Choi, Song, Ukeda, & Sawamura, 2000; Ruberto & Baratta, 2000). Antioxidant activity of essential oils followed the order: fennel (*F. vulgare*) > wild thyme (*T. serpyllum*) > rosemary (*R. officinalis*) > mint (*M. spicata*) > pennyroyal (*M. pullegium*). The antioxidant activity of numerous terpenes has already been evaluated (Ruberto & Baratta, 2000). Carvacrol and anethole, the terpenes found in wild thyme and fennel as major components (Table 2), may explain the low IC_{50} obtained with these oils, 96.9 ± 0.7 and 86.9 ± 0.1 μg ml^{-1}, respectively. The result obtained with the essential oil of rosemary was worse than that obtained from an Italian source (Sachetti et al., 2005). These results may be due to the low content of 1,8-cineole (3.1%) and higher content of verbenone (35.4%) found with the Portuguese species. 1,8-Cineole has a higher antioxidant activity than verbenone (Ruberto & Baratta, 2000).

Table 3
Antioxidant activity, represented by IC_{50} (μg ml^{-1})[a], of water, ethanol extracts and essential oils of each plant, measured by the DPPH test and by the inhibitory effect on lipid peroxidation

Plant	β-Carotene/linoleic acid test IC_{50} (μg ml^{-1})			DPPH test IC_{50} (μg ml^{-1})		
	Water extract	Ethanol extract	Essential oil	Water extract	Ethanol extract	Essential oil
Foeniculum vulgare	407 ± 0.7	68.7 ± 0.1	86.9 ± 0.1	48.0 ± 0.1	12.0 ± 0.1	n.a.
Mentha spicata	91.7 ± 0.7	36.9 ± 0.1	554.02 ± 0.5	5.7 ± 0.4	65.2 ± 0.1	n.a.
Mentha pulegium	167 ± 0.2	165 ± 7	1485 ± 0.3	8.9 ± 0.2	24.9 ± 0.2	n.a.
Rosmarinus officinalis	774 ± 0.5	246 ± 0.1	233 ± 0.5	37.3 ± 0.7	36.0 ± 0.1	n.a.
Thymus serpyllum	261 ± 0.2	41.2 ± 0.1	96.9 ± 0.7	31.6 ± 0.8	13.2 ± 0.3	n.a.
BHT		12.0 ± 0.7			15.7 ± 0.2	

The values reported are relative to μg of dried extract *per* ml of tested solution.
n.a., not available.
[a] Averages ± SD were obtained from three different experiments.

3.4. Free radical-scavenging activity

The free radical-scavenging activity was determined by the DPPH test. This test aims to measure the capacity of the extracts to scavenge the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$) formed in solution by donation of hydrogen atom or an electron (Tepe et al., 2005). If the extracts have the capacity to scavenge the DPPH free radical the initial blue/purple solution will change to a yellow colour due to the formation of diphenylpicrylhydrazine. This reaction is used as a measure of the ability of the extracts, or any other antioxidant, such as BHT, to scavenge any free radical. The results from the radical-scavenger assays for ethanol and water extracts are presented in Table 3.

Both ethanol and water extracts exhibit very good radical-scavenging activities. The best results were obtained with water extracts of the two *Mentha* species, pennyroyal ($IC_{50} = 8.9 \pm 0.2$ μg ml^{-1}) and mint ($IC_{50} = 5.7 \pm 0.4$ μg ml^{-1}). These values are lower than those found with the antioxidant standard, BHT. Water extracts of pennyroyal and mint were more active than the ethanol ones. Ethanol extracts of wild thyme ($IC_{50} = 13.2 \pm 0.3$ μg ml^{-1}) and fennel ($IC_{50} = 12.0 \pm 0.1$ μg ml^{-1}) were more active than were the corresponding water extracts ($IC_{50} = 31.6 \pm 0.8$ μg ml^{-1} and 48.0 ± 0.1 μg ml^{-1} respectively) and water and ethanol extracts of rosemary ($IC_{50} = 37.3 \pm 0.7$ μg ml^{-1} and $IC_{50} = 36.0 \pm 0.1$μg ml^{-1}, respectively) had the same antioxidant activity. Antioxidant studies with the fennel seed ethanol and water extracts have already been reported (Oktay et al., 2003). The authors obtained an antioxidant activity of about 47% for a solution containing 250 μg in 3 ml of water extract. In the present work, the whole aerial part was extracted, which may explain the better antioxidant properties found.

Rosmarinic and carnosic acids are two strong antioxidants present in rosemary (Shahidi, 2000). The activity of polar extracts of rosemary may be related to the concentration of the latter compound (Wellwood & Cole, 2004) and the results obtained suggest that these antioxidants are more concentrated in the ethanol extract. The antioxidant activity of the polar extracts may be related to their contents of phenolic compounds; therefore, the total phenolic contents of these extracts were determined and the results are shown in the next section.

The DPPH test is not applicable to essential oils, due to the low solubility of these oils under test conditions. An attempt to study these oils, at concentrations identical to those that gave 50% inhibition in the β-carotene/linoleic acid assay, gave inhibition values below 10%: pennyroyal (0.6%), mint (4.5%), fennel (4.7%), rosemary (7.1%) and wild thyme (8.0%).

Antioxidant activity is a complex process that can occur through several mechanisms. Due to its complexity more than one test must be carried out when evaluating the antioxidant activity of pure compounds or extracts (Aruoma, 2003). In this work, two classical antioxidant tests were carried out, the DPPH and β-carotene/linoleic acid test. The first gives information about the ability of the tested compounds to scavenge free radicals and the second about the ability of tested extracts to delay lipid peroxidation by reacting with chain-propagating peroxyl radicals faster than these radicals can react with proteins or fatty acid side-chains. Plant extracts are complex mixtures and reports of antioxidant activities evaluated by different tests are not always concordant (Sachetti et al., 2005; Tepe et al., 2005; Trouilla et al., 2003).

3.5. Determination of total phenolic compounds

The total phenolic compound contents in the ethanol and water extracts were determined by a colorimetric assay, using the Folin-Ciocalteu reagent (Oktay et al., 2003). Results, expressed as pyrogallol equivalents, are presented in Table 4. These results demonstrate the presence of natural antioxidant phenolic compounds in all these extracts. The value obtained for the ethanol extracts of rosemary 73.5 ± 6.5 mg g^{-1} is similar to that reported (79.4 mg g^{-1}, Trouilla et al., 2003). The value found for the ethanol extract of fennel, 63.1 ± 3.0 mg g^{-1}, is slightly below that reported in the literature, (90.0 mg g^{-1}, Oktay et al., 2003), although for the water extract, a much higher value was obtained, 63.8 ± 7.3 mg g^{-1} *versus* 21.25 mg g^{-1} (Oktay et al., 2003).

Pennyroyal (*M. pullegium*) and mint (*M. spicata*) contain flavonoids that may account for the high antioxidant activity observed for the polar extracts of these aromatic herbs (Justesen & Knuthsen, 2001; Zaidi, Voirin, Jay, & Viricel, 1998). Thyme (*T. vulgaris*) contains phenolic compounds that are responsible for the antioxidant activity of polar extracts (Dapkevicius et al., 2002; Wang, Li, Ho, Peng, & Ho, 1998) and, although the authors did not find any reference describing the presence of this type of chemical compound in wild thyme (*T. serpyllum*), it can be assumed that phenolics are responsible for the activity observed.

An attempt to establish a linear correlation between the total phenolic compound contents of the extracts and the

Table 4
Total phenolic compounds, determined as pyrogallol equivalents, in mg per g of extract by the Folin-Ciocalteu method[a]

	Foeniculum vulgare	*Mentha spicata*	*Mentha pulegium*	*Rosmarinus officinalis*	*Thymus serpyllum*
Ethanol extract	63.1 ± 3.0	81.2 ± 7.6	71.7 ± 2.1	73.5 ± 6.5	113.0 ± 6.2
Water extract	63.8 ± 7.3	64.5 ± 6.7	57.9 ± 1.6	58.4 ± 5.9	74.9 ± 3.3

[a] Averages ± SD were obtained from three different experiments.

Table 5
Acetylcholinesterase inhibition capacity represented by IC_{50} ($\mu g\ ml^{-1}$)[a], of essential oils, ethanol, water extracts and standard compounds (verbenone, carvacrol and *E*-anethole[b])

	Foeniculum vulgare	*Mentha spicata*	*Mentha pulegium*	*Rosmarinus officinalis*	*Thymus serpyllum*
Ethanol extract	308 ± 0.1	–	534 ± 0.1	219 ± 0.1	252 ± 0.1
Water extract	1490 ± 0.3	721 ± 0.1	1581 ± 53.6	769 ± 3.9	348 ± 0.1
Essential oil	252 ± 0.1	357 ± 0.1	324 ± 0.1	69.8 ± 0.1	190 ± 0.1
Verbenone	163 ± 0.2				
Carvacrol	115 ± 0.1				
(*E*)-Anethole	$5.9 \pm 0.1\%$ ($100\ \mu g\ ml^{-1}$)[b]				

The values reported are relative to μg of dried extract *per* ml of tested solution.
[a] Averages ± SD were obtained from three different experiments.
[b] % inhibitory activity.

free radical-scavenging or the lipid peroxidation inhibition activities was unsuccessful, corroborating results also found in the literature. One can assume that other compounds, without a phenolic structure, may be responsible for the antioxidant activity. The co-extraction in the polar fraction of terpenoids usually present in the essential oil, may explain, in part, the antioxidant activity found with these plants.

3.6. Determination of acetylcholinesterase inhibitory activity

The acetylcholinesterase (AChE) (E.C. 3.1.1.7.) inhibition was determined using an adaptation of the method described in the literature (Ingkaninan et al., 2003).

Essential oils, ethanol and water extracts of the aromatic herbs were tested to determine their ability as acetylcholinesterase inhibitors and results are depicted in Table 5.

For each plant, the inhibition capacity shows the following order: essential oils > ethanol extracts > water extracts. The best inhibitory activity was exhibited by the essential oil of rosemary where an $IC_{50} = 69.8 \pm 0.1\ \mu g\ ml^{-1}$ was obtained. Rosemary is used to prepare an aromatic "tea" and also in aroma-therapy. Its influence on mood and cognition was recently investigated and it was reported that its essential oil produced a significant enhancement of performance and overall quality of memory in healthy adult volunteers (Moss, Cook, Wesnes, & Duckett, 2003). GC–MS analysis of essential oils (Table 2), identified 1,8-cineole, camphor and 4-terpineol. All these compounds inhibit AChE, the first one being a potent inhibitor (Savelev, Okello, Perry, Wilkins, & Perry, 2003). However these compounds are present in small amounts. Verbenone, the major component of this oil (35.4%), was tested alone and the value of $IC_{50} = 163 \pm 0.2\ \mu g\ ml^{-1}$ obtained suggests that the activity exhibited by the essential oil is probably the summation of the activities of several active components.

Carvacrol, the major essential oil component of wild thyme has, alone, an $IC_{50} = 115 \pm 0.1\ \mu g\ ml^{-1}$, this compound being present only at 56%, does not explain the activity found for the oil fraction. The other components present in this oil probably contribute, to a small extent, to the activity exhibited.

(*E*)-Anethole, the major component of the essential oil of fennel (70.2%), was also tested and the values obtained were $5.9 \pm 0.1\%$ at $100\ \mu g\ ml^{-1}$, and $10.7 \pm 0.1\%$ at $200\ \mu g\ ml^{-1}$. The highest concentration that could be tested, due to precipitation under test conditions, indicated that this compound was not responsible for the activity, which may be due to the other isomer present, (*Z*)-anethole. This isomer is present in a higher amount (18.6%) than that usually found in the fennel essential oil (Diaz-Maroto et al., 2005; Mimica-Dukié et al., 2003). When the ethanol extract of this plant was tested at a concentration of $100\ \mu g\ ml^{-1}$, the inhibition obtained, 16%, was above that reported for *F. vulgare* of Greek origin, ⩽10% (The Local Food-Nutraceuticals Consortium, 2005).

The essential oils of the two *Mentha* species studied, pennyroyal and mint, exhibited only moderate AChE inhibition with $IC_{50} = 324 \pm 0.1\ \mu g\ ml^{-1}$ and $IC_{50} = 357 \pm 0.1\ \mu g\ ml^{-1}$, respectively. The main components of these oils, pulegone and carvone, are moderate inhibitors of AChE (Miyazawa et al., 1998), which is in accordance with the results obtained.

It was not possible to test the ethanol extract of mint, due to its intense green colour. The worst results, $IC_{50} = 1490 \pm 0.3\ \mu g\ ml^{-1}$ and $IC_{50} = 1581 \pm 53.6\ \mu g\ ml^{-1}$ were obtained with the water extract of fennel and pennyroyal, respectively.

4. Conclusions

Wild-gathered plants from Portugal, widely used in the country as aromatic herbs in cooking, and also for their medicinal properties in folk medicine, may act as important supplements of antioxidants in the diet, especially in the cooking of some dishes of poor nutritional value.

Plants studied showed activity at a concentration level that is in accordance with their use in culinary preparations. The ethanol and water extracts studied are, in general, as effective as a known standard, BHT, in scavenging free radicals.

All plants exhibited moderate to good antiacetylcholinesterase activity, at least in one of the extract tested, essential oil of rosemary being the more active.

Plants showing simultaneous antioxidant activity and AChE inhibitory capacity could be considered as foods, having some function besides their traditional value, which

makes them promising candidates for more detailed *in vitro* and *in vivo studies.*

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