SUPPLEMENTARY MATERIAL

Antioxidant activity and cholinesterase inhibition studies of four flavouring herbs from Alentejo

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Abstract

Essential oils (EOs) and aqueous extracts of aerial parts of four aromatic species, *Calamintha nepeta*, *Foeniculum vulgare*, *Mentha spicata* and *Thymus mastichina*, from southwest of Portugal were characterized chemically and analysed in order to evaluate their antioxidant potential and cholinesterase inhibitory activities. The main components of EOs were oxygenated monoterpenes, and aqueous extracts were rich in phenol and flavonoid compounds. EOs and aqueous extracts presented a high antioxidant potential, with ability to protect the lipid substrate, free radical scavenging and iron reducing power. Furthermore, EOs and extracts showed AChE and BChE inhibitory activities higher than rivastigmine, the standard drug. Results suggested the potential use of EOs and aqueous extracts of these flavouring herbs as nutraceutical or pharmaceutical preparations to minimize the oxidative stress and the progression of degenerative diseases.

Keywords

Essential oils, Aqueous extracts, Aromatic herbs, Antioxidant, Cholinesterase inhibition, Alzheimer's Disease

1. Experimental

1.1. Chemicals

Analytical standards for chromatography (> 99%) were purchase from Sigma-Aldrich (St. Louis, USA) and Extrasynthese (Genay, France). Quercetin (> 99%), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (95%), β-carotene (95%), linoleic acid (99%), acetylcholinesterase (AChE) type VI-S-electric eel, 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI) and equine serum butyrylcholinesterase (BChE) were purchased from Sigma-Aldrich (St. Loius, MO, USA). All other chemicals and solvents were high purity grade and purchase from Sigma-Aldrich (St. Loius, MO, USA) and Merck (Darmstadt, Germany).

1.2. Plant material

Wild grown *F. vulgare (leaves)*, *M. spicata* (leaves), *T. mastichina* (leaves and flowers) and *C. nepeta* (leaves and flowers) were collected in Évora region and deposited at the herbarium of Aromatic Plants of the University of Évora, with the accession numbers of HPAMT_UE 000003, HPAMT_UE 000001, HPAMT_UE 000010 and HPAMT_UE 000006, respectively. Voucher specimens were identified by Professor Marízia Menezes (University of Évora).

1.3. Extraction of essential oils and aqueous extracts

Essential oils were obtained by hydrodistillation of aerial part of the plants during 3 h, using a Clevenger-type apparatus, according to the European Pharmacopoeia (COE 2007). The refractive index of EOs was measured using a refractometer (Leica Abbe Mark II, Model 10481). Aqueous extracts were obtained from decoction waters.

1.4. Chemical characterisation of essential oils

The characterisation of EOs was performed by gas chromatography with a flame ionization detector (GC-FID) on a HP-5890 SERIES II equipped with a 30 m x 0.25 mm i.d., film thickness 0.25 µm Supelcowax™10 fused-silica polar capillary column (Supelco, Milford, USA) and a ChemStation HP software, version A.04.02. GC-MS analyses were performed with a GC-MS-QP2010 Series (Shimadzu) gas chromatograph, fitted with a DB-WAX column 30 m × 0.25 mm i.d., film thickness 0.25 µm, interfaced with a detector model Polaris Q (E. I. quadrupole).

Experiments were conducted under the conditions described by Arantes et al. (2016). Compounds were identified based on their retention indices and their mass spectra of NIST08 (*National Institute of Standards and Technology*) library. Retention indices were determined by interpolation relative to the retention time of C8–C22 *n*-alkanes and they were compared with those of authentic standards and literature data (Cavaleiro et al. 2004, Mottram 2007). Quantitative data of individual components of EO were determined using relative percentage abundance and a normalization method without the use of

response factors for flame ionisation detection. Percentage values are the mean of peak areas of three injections per sample.

1.5. Chemical analysis of aqueous extracts

1.5.1. Phytochemical screening

Phytochemical tests to detect the presence of heterosides, saponins, tannins, flavonoids, steroids, triterpenes, coumarins, quinones, organic acids and alkaloids were performed according to the method described by Anyasor et al. (2010).

1.5.2. Total phenol content

Quantitative determination of total phenols content of aqueous extracts was performed using the Folin-Ciocalteau method, based on the reaction of the reagent with the functional hydroxyl groups of phenols. Aliquots of 5 µL of extract (17–133 µg/mL) were diluted with 235 µL of water and added with 15 µL of Folin-Ciocalteu's phenol reagent. After 5 min incubation, 45 µL of saturated NaCO3 solution was added and samples were incubated for 30 min at 40 °C. The absorbance was measured at 630 nm versus a blank sample, in a microplate spectrophotometer (Thermo Scientific, Finland).mixed. Gallic acid (0.2-9.0 µg/mL) was used as standard.

1.5.3. Total flavonoid content

Total flavonoid content of aqueous extracts was determined according to the colorimetric method of Deng et al. (2012), with some modifications. Briefly, 100 µL of diluted extracts (500–4000 µg/mL) were added to equal volumes of $AlCl_3.H_2O$ solution 2% (w/v). The solution was shaken and incubated at room temperature for 10 min. A blank assay was prepared adding distilled water to samples. The absorbance was measured at 490 nm using a microplate spectrophotometer. Quercetin was used as standard in a concentration range of 25-500 µg/mL.

1.6. Antioxidant activity

1.6.1. DPPH radical scavenging assay

Free radical scavenging activity was determined by measuring the bleaching of a purple-coloured solution of the stable radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH•). This method evaluates the ability of EO to act as donor of hydrogen atoms or electrons in transformation of DPPH• into its reduced form DPPH-H. Antioxidant activity of EOs (0.4–32.6 mg/mL), ascorbic acid (0.25–65.0 µg/mL) and quercetin (0.42–13.04 µg/mL) was performed according to by Tepe et al. (2007), with some modifications. In 96 wells microplates, 30 µL of each EO ethanol solution was added to 200 µL of DPPH ethanol solution 0.1 mM. A control sample using solvent was prepared. After incubation at room temperature, in the dark, absorbance at 517 nm was measured periodically during 180 min, using a

microplate spectrophotometer (Thermo Scientific, Finland) (Arantes et al. 2016). Inhibition of free radical by DPPH in percentage (I%) was calculated using the equation:

I (%) = [(AC-AS)/AC] x 100, where AC is the absorbance of the control and AS is the absorbance of the sample.

1.6.2. β-Carotene/linoleic acid bleaching method

Bleaching β-carotene/linoleic acid antioxidant capacity is determined measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Tepe et al. 2007). Antioxidant activity of EO (0.04–2.50 mg/mL), ascorbic acid (0.06-4.00 mg/mL) and quercetin (0.8–24.56 µg/mL) was determined measuring the absorbance at 490 nm at minute zero and after 2 h incubation in a water bath at 50°C, using a microplate spectrophotometer (Thermo Scientific).

Lipid peroxidation (LPO) inhibition was calculated using the following equation:

LPO (%) = $[(\Delta A_C - \Delta A_S) / \Delta A_C] \times 100$,

where ΔA_C is the difference between absorbance at 0 h and 2 h for the control and ΔA_S is the difference between absorbance at 0 h and 2 h for samples or standards.

1.6.3. Reducing power assay

Reducing power of EOs was determined according Ferreira et al. 2007, with some modifications. In microtubes, 200 µL of EOs ethanol solutions (0.4–60.0 mg/mL) were added to 50 µL of sample solvent, 200 µL of phosphate buffer (0.2M, pH 6.6) and 200 µL of potassium ferrocyanide (1%). Solutions were mixed and incubated at 50°C for 20 min. 200 µL of trichloroacetic acid (10%) was added, solutions were incubated during 2 min. and centrifuged (3000 rpm, 10 min). 50 µL of the supernatant was added with 50 µL distilled water and 100 µL $FeCl_3$ (0.1%). The absorbance was measured at 630 nm and compared with standards, quercetin and ascorbic acid.

1.7. In vitro *inhibition studies for AChE and BChE*

AChE and BChE inhibitory activities were determined using a modified Ellman's microassay method described by Ingkaninan et al. (2003), with modifications. In a total volume of 250 µL, 75 µL of samples (ethanol for EOs or buffer solution for extracts) in Tris-HCl buffer 0.1 M (pH 8) with different concentrations were added to 25 µL of 15 mM ATCI/ BTCI solutions, 125 µL of 3 mM DTNB in Tris-HCl buffer 50 mM, and 25 µL of enzyme solution containing 0.3 U/mL. Rivastigmine and galanthamine were used as a standard. The reactions were monitored for 20 min at 405 nm in a microplate spectrophotometer. Tests were carried out in triplicate. Sample concentrations providing 50% inhibition (IC50) were calculated from graphs plotted with I% values of the inhibition of AChE/ BChE versus the analysed concentrations, by nonlinear fit, using Origin 8.0 software (Microsoft Corporation).

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Captions

Table S1. Chemical composition of EOs of *C. nepeta*, *F. vulgare*, *M. spicata* and *T. mastichina*

Figure S1. Chemical characterization of aqueous extracts. Total phenol content (a); total flavonoid content (b). GAE – gallic acid equivalents. QE – quercetin equivalents.

Table S1. Chemical composition of EOs of *C. nepeta*, *F. vulgare*, *M. spicata* and *T. mastichina*

Compound	RI $_{exp}$ [a]	RI $_{ref}$	% Area [g]				Id [h]
			C. nepeta	*F. vulgare*	*M. spicata*	*T. mastichina*	
α - pinene	1028	1030 [d]	2.0	2.2	0.6	-	AS, RI
Camphene	1071	1073 [d]	2.1	0.3	0.7	1.5	AS, RI
Δ^3-carene	1159	1151[e]	-	-	0.6	-	AS, RI
β – pinene	1116	1116 [d]	1.3	0.8	0.9	3.3	AS, RI
α-terpinene	1190	1189 [d]	-	-	5.1	-	AS, RI
α- phellandrene	1165	1168 [d]	1.2	4.9	-	-	AS, RI, NIST
Myrcene	1169	1162 [d]	-	5.1	-	-	AS, RI, NIST
Limonene	1209	1205 [d]	-	6.0	1.1	-	AS, RI, NIST
1,8-cineole	1217	1215 [d]	18.3	1.2	-	72.0	AS, RI, NIST
β-o-cimene	1234	1235 [d]	0.2	-	2.5	-	AS, RI, NIST
ϒ-terpinene	1253	1251 [d]	0.1	0.2	1.5	0.6	AS, RI, NIST
Sabinene hydrate	1464	1464 [b]	1.5	-	-	-	AS, RI
ρ-Cymene	1275	1273[d]	-	1.7	-	-	AS, RI
α-Terpinolene	1290	1288 [b]	-	0.4	-	-	AS, RI
Fenchone	1400	1400 [f]	-	20.2	-	-	AS, RI
Menthone	1465	1461 [b]	1.6	-	-	-	AS, RI, NIST
Isomenthone	1490	1491 [b]	0.6	-	4.0	-	AS, RI, NIST
Linalool	1542	1542 [d]	-	-	-	2.0	AS, RI
Isopulegol	1562	1557 [c]	22.6	-	-	-	AS, RI, NIST
Isopulegone	1575	1570 [b]	20.5	-	-	-	AS, RI, NIST
β- cariophylene	1600	1594 [b]	3.6	0.1	2.9	-	AS, RI, NIST
4-terpineol	1606	1599 [b]	6.7	-	-	1.4	AS, RI, NIST
Menthol	1645	1635 [b]	5.1	-	-	-	AS, RI, NIST
trans-pinocarveol	1659	1649 [d]	2.1	-	-	-	AS, RI
Pulegone	1643	1640 [b]	8.6	-	-	-	AS, RI
iso-borneol	1670	1665 [d]	-	-	-	2.7	AS, RI
Estragol	1688	-	0.7	6.2	7.5	-	AS, NIST
Terpenyl acetate	1700	1692 [d]	0.3	-	1.5	-	AS, RI
α-terpineol	1703	1692 [d]	0.4	-	21.5	9.0	AS, RI
Carvone	1729	1727 [d]	-	-	45.0	0.8	AS, RI, NIST
Anethole	1847	-	-	46.2	-	-	AS, NIST
Geranyl acetate	1766	-	-	-	1.1	-	AS
α-humelene	1704	1662 [d]	-	-	2.4	-	AS, RI
Caryophyllene oxide	2016	1969 [d]	-	0.5	-	-	AS, RI, NIST
Carvacrol	2207	2206 [e]	-	-	-	2.8	AS, RI
Total identified			99.5	96.0	98.9	96.1	
Hydrocarbon monoterpenes			6.9	21.6	13.0	5.4	
Oxygenated monoterpenes			89.0	73.8	80.6	90.7	
Hydrocarbon sesquiterpenes			-	-	2.4	-	
Oxygenated sesquiterpenes			-	0.5	-	-	

Values are mean ± standard deviation of triplicate analyses.

[a] Retention indices relative to C8 - C22 n-alkanes on the Supelcowax™10 column.

[b] Retention indices reported by Gonçalves et al. (2007).

[c] Retention indices reported by Marongiu et al. (2010).

[d] Retention indices reported by Cavaleiro et al. (2004).

[e] Retention indices reported by Machado et al. (2010).

[f] Retention indices reported by Zuzarte et al. (2009).

[g] Relative quantitative data by GC-FID analysis.

[h] Id - Identification method: AS - Identification by adding standard, RI - Identification by the retention indices, NIST - Identification by mass spectrometry library (NIST).

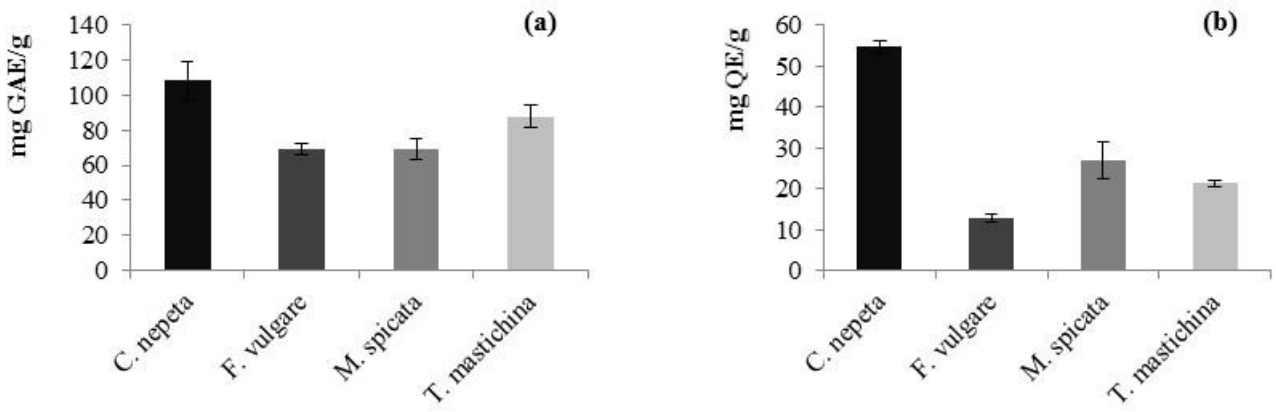

Figure S1. Chemical characterization of aqueous extracts. Total phenol content (a), total flavonoid content (b). GAE – gallic acid equivalents. QE – quercetin equivalents.