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ORIGINAL ARTICLE

# Phenolic profile, antioxidant activity and enzyme inhibitory activities of extracts from aromatic plants used in Mediterranean diet

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**Abstract** The antioxidant and enzyme inhibitory properties of methanolic extracts from four aromatic plants used in traditional medicine and food [*Calamintha nepeta* (L.) Savi subsp. *nepeta*, *Helichrysum italicum* subsp. *picardii* Franco, *Mentha spicata* L. and *Origanum vulgare* subsp. *virens* (Hoffmanns. & Link) Bonnier & Layens] were evaluated. The extract from *O. vulgare* exhibited the strongest DPPH ($IC_{50}$ of 4.65 ± 0.12 μg/ml) and ABTS (1479.56 ± 12.29 $\mu mol_{TE}/g_{extract}$) scavenging capacities, as well as the largest ferric reducing ability (1746.76 ± 45.11 $\mu mol_{AAE}/g_{extract}$). This extract also showed the highest total phenolic content (1597.20 ± 24.10 $\mu mol_{GAE}/g_{extract}$) and although HPLC–DAD analysis revealed rosmarinic acid as the main compound of the extract, other compounds seem to be involved in the antioxidant activity. Furthermore, the extract from *H. italicum*, which was found to be rich in caffeoylquinic and dicaffeoylquinic acids and in pinocembrin, showed the highest inhibitory potential against acetylcholinesterase, tyrosinase and α-glucosidase. Overall, the results obtained validate the usefulness of the studied plants as valuable sources of natural agents beneficial for human health.

**Keywords** Antioxidants · Curry plant · Lesser calamint · Oregano · Phenolic compounds · Spearmint

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## Introduction

The demand for aromatic plants as sources of natural antioxidants has been increasing in the last years. Indeed, the use of aromatic plants is highly encouraged by health professionals nowadays in order to reduce the abusive addition of salt in the diet, or by presenting health benefits other than their antioxidant properties (Costa et al. 2015a). Furthermore, many aromatic plants are recognized as safe for human consumption (FDA 2014). Among bioactive compounds in aromatic plants, phenolics are one of the most important and probably the main candidates contributing to the claimed beneficial effects associated with these plants. They have been linked to a number of health benefits, including protection against cardiovascular diseases, cancer, diabetes mellitus (DM) and neurological diseases (Del Rio et al. 2013; Nikousaleh and Prakash 2016). With such a rich array of potential benefits, it is not surprising that phenolic compounds are being sought to replace synthetic antioxidants in the food supply (Fernandes et al. 2016). Natural antioxidants originating from plant sources became a wise option for the management of oxidative stress-related disorders as neurodegenerative diseases and DM (Maritim et al. 2003; Lee et al. 2010). Nowadays, there has been also much interest in the use of phytochemicals with enzyme inhibitory properties for the management of Alzheimer's disease (AD), Parkinson's disease (PD) and DM, because some drugs in use are reported to have side effects or low efficacy (Orhan et al. 2015; Zengin et al. 2015; Shanmugam et al. 2016).

Mediterranean diet includes the use of wild plants, such as herbs and aromatic species, harvested and eaten seasonally. The systematic investigation of such plants will help defining their precise pharmacological properties and to determine their value as functional foods or as a source

of nutraceutical and pharmaceutical compounds. The knowledge about the health properties of those plants will also contribute to increase their importance as a part of the Mediterranean diet. The aim of this study was to obtain homogeneous and quantitative data on the antioxidant activity and inhibitory capacity against enzymes linked to AD, PD and DM of four wild aromatic plants widely used in traditional medicine and Mediterranean diet (Table 1), namely *Calamintha nepeta* (L.) Savi subsp. *nepeta*, *Helichrysum italicum* subsp. *picardii* Franco, *Mentha spicata* L. and *Origanum vulgare* subsp. *virens* (Hoffmanns. & Link) Bonnier & Layens. Additionally, the phenolic compounds present in the extracts were identified and quantified by high-performance liquid chromatography with diode-array detection (HPLC–DAD), and their relationships coupled to the biological properties discussed.

## Materials and methods

### Chemicals and reagents

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tablets, 2,2-diphenyl-1-picrylhydrazyl (DPPH), methanol, formic acid, trichloroacetic acid (TCA), $K_2S_2O_8$, acetylthiocholine iodide (ATCI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), acetylcholinesterase (AChE) (Electric-eel, EC 3.1.1.7, Type VIS), mushroom tyrosinase (EC 1.14.18.1), 3,4-dihydroxy-L-phenylalanin (L-DOPA), porcine pancreatic α-amylase (EC 3.2.1.1), yeast α-glucosidase (EC 3.2.1.20), *p*-nitrophenyl-α-D-glucopyranoside, starch, galanthamine hydrobromide, kojic acid, acarbose, quercetin, pinocembrin, caffeic acid and 3,4-dihydroxybenzoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rosmarinic acid, quercetin-3-*O*-rutinoside, 5-*O*-caffeoylquinic acid, gallic acid, catechin, epicatechin, hesperetin-7-*O*-rutinoside and *p*-hydroxybenzoic acid were from Extrasynthèse (Genay, France). 3-*O*-Caffeoylquinic, 4-*O*-caffeoylquinic, 3,4-di-*O*-caffeoylquinic, 3,5-di-*O*-caffeoylquinic, 1,5-di-*O*-caffeoylquinic and 4,5-di-*O*-caffeoylquinic were purchased from Chengdu Biopurity Phytochemicals Ltd. (Schuan, China). Folin–Ciocalteu's phenol reagent (F–C reagent), gallic acid, $Na_2CO_3$, NaCl and $FeCl_3$ were acquired from VWR (Leuven, Belgium). Potassium ferricyanide, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and butylated hydroxytoluene (BHT) were purchased from Acros Organics (Geel, Germany). Methanol Lichrosolv was from Merck (Darmstadt, Germany).

### Plant material and extraction procedure

Randomly selected leaves of *C. nepeta* subsp. *nepeta*, *H. italicum* subsp. *picardii*, *M. spicata* and *O. vulgare* subsp. *virens* were collected from plants growing wild in the Algarve region (South Portugal). A representative sample of each plant was authenticated by JM Rosa Pinto from the herbarium of the University of Algarve (Faro, Portugal). The plant material was dried at 40 °C until constant weight (24–48 h) and powdered in a blender to achieve a mean particle size lower than 2 mm. Dried plant material was extracted twice by maceration with 80% methanol (1:20, w/v) during 24 h at room temperature and the resulting extracts were filtered through a 5–13 μm membrane. Finally, the extracts were concentrated to dryness in a rotary evaporator at 40 °C and under reduced pressure, and stored at −20 °C. The dried extract was later re-suspended in the appropriate solvent for each biological activity test, methanol in the case of DPPH assay and HPLC–DAD analyses, and the specific buffer (indicated below) used in each of the other assays.

### HPLC–DAD analysis

The extracts (50 mg/ml) were analyzed on an analytical HPLC unit (Gilson), using a Spherisorb ODS2 column

**Table 1** Local name, English common name, family, edible tissues and traditional medicinal uses of the four aromatic species studied

| Plant species | Local name | English common name | Family | Edible tissues | Traditional medicinal uses |
|---|---|---|---|---|---|
| *Calamintha nepeta* (L.) Savi subsp. *nepeta* | Erva-das-azeitonas | Lesser calamint | Lamiaceae | Leaves | As expectorant and tonic, and to alleviate digestive disorders |
| *Helichrysum italicum* subsp. *picardii* Franco | Perpétua-das-areias | Curry plant | Asteraceae | Leaves | To relieve inflammation and several skin diseases, including for wound healing |
| *Mentha spicata* L. | Hortelã-comum | Spearmint | Lamiaceae | Leaves | To treat numerous digestive and respiratory problems |
| *Origanum vulgare* subsp. *virens* (Hoffmanns. & Link) Bonnier & Layens | Oregão | Oregano | Lamiaceae | Aerial parts | As antitussive, expectorant, antiseptic and to alleviate digestive disorders |

(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 using a gradient of 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, 55% B at 47 min, 75% B at 56 min, 100% B at 60 min, at a solvent flow rate of 0.9 ml/min. Detection was achieved with a Gilson DAD. Spectral data from all peaks were accumulated in the range 200–400 nm. Chromatograms were recorded at 280 nm for hydroxybenzoic acids, flavan-3-ols and flavanones, at 320 nm for hydroxycinnamic acids and at 350 nm for the other flavonoids. 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 injected in the same conditions. Extracts were also spiked with the standards of the identified compounds in order to verify the identification. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, by using the following equation:

$$C(c) = \frac{A(c)}{A(st)} \times C(st)$$

where, C(c) and A(c) are the concentration and the area of the compound in the sample, respectively, and C(st) and A(st) are the concentration and the area of the standard, respectively.

## Determination of the total phenolic contents

The total phenolic content of each extract was measured using a modified F–C colorimetric method (Ainsworth and Gillespie 2007). Briefly, 200 µl of 10% (v/v) F–C reagent was mixed with 100 µl of each extract in phosphate buffer (75 mM, pH 7.0). Then 800 µl of 700 mM $Na_2CO_3$ were added and the reaction was incubated at room temperature for 2 h. Instead of the plant extracts, gallic acid was used as a positive control or phosphate buffer as a negative control. Two hundred microliters were transferred from each reaction to a clear 96-well microplate (NUNC, Rochester, New York, USA) and the absorbance was measured at 765 nm. A standard curve was calculated using several gallic acid concentrations and the results were expressed as gallic acid equivalents per gram of extract ($\mu mol_{GAE}/g_{extract}$).

## Antioxidant activity

### *DPPH free radical scavenging assay*

The ability of the extracts to scavenge DPPH radicals was determined according to the procedure described by Soler-Rivas et al. (2000) with slight modifications. One hundred microliters of 90 µM DPPH methanolic solution was added to 10 µl of sample solution at different concentrations, and the mixture was diluted with 190 µl of methanol in a clear 96-well microplate. The extract was replaced with solvent for the control. After 30 min, the reduction of DPPH radicals was measured at an absorbance of 515 nm. BHT was used as the reference antioxidant in this assay. The radical scavenging activity was expressed in terms of the amount of sample necessary to decrease the initial absorbance by 50% ($IC_{50}$).

### *$ABTS^{\bullet+}$ radical cation decoloration assay*

The ABTS free radical-scavenging activity of each sample was determined as described by Re et al. (1999) and the results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC). 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. The sample dilution diluted in phosphate buffer (75 mM, pH 7) that achieved 20–80% inhibition of the blank absorbance was used to calculate the TEAC values and the results were expressed as trolox equivalents per gram of extract ($\mu mol_{TE}/g_{extract}$).

### *Ferric reducing antioxidant power (FRAP)*

The reducing properties of the extracts were determined using $FeCl_3$ as described by Pulido et al. (2000) with some modifications. Briefly, 100 µl of plant extract was mixed with 250 µl sodium phosphate buffer (200 mM, pH 6.6) and 250 µl 1% $K_3[Fe(CN)_6]$ and was incubated at 50 °C for 20 min. After the addition of 250 µl 10% TCA the mixture was centrifuged at 650 rpm for 10 min. Then 100 µl of the supernatant were mixed with 100 µl of water and 20 µl 0.1% $FeCl_3$ in a 96-well microplate. Instead of the plant extracts, ascorbic acid was used as a positive control and phosphate buffer as a negative control. Reducing activity was measured by determining the absorbance at 700 nm and the results were expressed as ascorbic acid equivalents ($\mu mol_{AAE}/g_{extract}$).

## Enzyme inhibitory activities

### *Acetylcholinesterase inhibition*

The evaluation of AChE inhibitory activity was based on Ellman's method (Ellman et al. 1961), using a 96-well microplate reader. Firstly, 3 mM DTNB, 15 mM substrate (ATCI), 100 mM phosphate buffer (pH 8.0) and extract at 10 mg/ml, buffer or galanthamine at 5 µg/ml (standard inhibitor) were mixed. Finally, AChE (0.28 U/ml) was

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.

*Tyrosinase inhibition*

Inhibition of tyrosinase was determined using the modified dopachrome method as described by Masuda et al. (2005) Assays were conducted in 96-well microplates, an aliquot of the extracts at 10 mg/ml with 80 µl of phosphate buffer (pH 6.8), 40 µl of tyrosinase, and 40 µl of L-DOPA were mixed. The absorbance was measured at 475 nm and the tyrosinase inhibitory activity was expressed as percentage inhibition. Kojic acid (200 µg/ml) was used as the reference.

*α-Amylase inhibition*

α-Amylase inhibition assay was performed according to the procedure described by Kwon et al. (2008) with slight modifications. A total of 500 µl of plant extract at 10 mg/ml and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing porcine pancreatic α-amylase (0.5 mg/ml) were incubated at 25 °C for 10 min. After pre-incubation, 500 µl of a 1% starch solution in phosphate buffer was added to each tube. After incubation at 25 °C for 10 min the reaction was stopped with 1.0 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was diluted with distilled water and absorbance was measured at 540 nm. A control with buffer instead of plant extract was performed and the α-amylase inhibitory activity was expressed as percentage inhibition. Acarbose at 300 µg/ml was used as reference.

*α-Glucosidase inhibition*

α-Glucosidase inhibition assay was performed according to the procedure described by Kwon et al. (2008) with slight modifications. A volume of 50 µl of plant extract at 10 mg/ml or acarbose (1000 µg/ml) were mixed with 100 µl of 0.1 M phosphate buffer (pH 6.9) containing yeast α-glucosidase solution (1.0 U/ml) in 96-well plates and were incubated at 25 °C for 10 min. After pre-incubation, 50 µl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in phosphate buffer was added to each well. The reaction mixtures were incubated at 25 °C for 5 min and then absorbance readings were recorded at 405 nm and compared to a control which had 50 µl of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as percentage inhibition.

### Statistical analysis

All the experiments were carried out three times using triplicate samples. The data were presented as the mean ± standard error and were processed by one-way analysis of variance (ANOVA). Significant differences between means were identified using Duncan's New Multiple Range Test ($p < 0.05$), and correlations were calculated using Pearson's test. All statistical analysis was carried out using the SPSS statistical package for Windows (release 18.0; SPSS Inc., Chicago, IL, USA).

## Results and discussion

### Phenolic composition

The values of extraction yield obtained in this study, expressed as weight of extract relative to the weight of the initial plant material, were in the same range for the four species, 23.89, 24.86, 26.24 and 27.95% for *C. nepeta*, *H. italicum*, *M. spicata* and *O. vulgare*, respectively.

The phenolic composition of the extracts was analysed by HPLC–DAD and several phenolic acids and flavonoids were identified (Table 2; Fig. 1). *H. italicum* and *M. spicata* extracts contained the highest amount of the identified phenolic compounds (51.65 and 47.93 mg/g of dry extract, respectively). Rosmarinic acid was the major compound identified in *M. spicata* and *O. vulgare* extracts (35.63 and 23.53 mg/g of dry extract, respectively) similarly to the observed in other Lamiaceae species (Costa et al. 2013), which is not surprising as this is a taxonomic marker of this botanical family (Cantino and Sanders 1986; Janicsák et al. 1999). Substantial amounts of the flavanone hesperetin-7-*O*-rutinoside and the flavan-3-ol (−)-epicatechin were also found respectively in *M. spicata* and *O. vulgare* extracts (Table 2). The extract from *C. nepeta* (Lamiaceae) also contained rosmarinic acid but in much lower amount (1.44 mg/g of dry extract). This extract contained 4-*O*-caffeoylquinic acid (14.69 mg/g of dry extract) as the major compound as well as considerable amount of quercetin-3-*O*-rutinoside (Table 2). Pacifico et al. (2015) recently reported qualitative and quantitative variations in the phytochemical constitution of *C. nepeta* (L.) Savi during the life cycle. For rosmarinic acid the authors observed a strong variation in its content throughout the year. 3,5-di-*O*-Caffeoylquinic acid (14.48 mg/g of dry

**Table 2** Phenolic compounds identified in the extracts (mg/g of dry extract)

| Peak | Compound | RT (min) | λ (max) | *C. nepeta* | *H. italicum* | *M. spicata* | *O. vulgare* |
|---|---|---|---|---|---|---|---|
| 1 | Gallic acid | 6.48 | 270.7 | – | – | – | 1.19 ± 0.43 |
| 2 | 3,4-Dihydroxybenzoic acid | 9.47 | 259.3, 294.1 | – | – | – | 1.97 ± 0.33 |
| 3 | 3-*O*-Caffeoylquinic acid | 9.95 | 249.1, 296.4, 324.2 | 3.80 ± 0.13 | – | – | – |
| 4 | (+)-Catechin | 12.35 | 279.3 | – | – | – | 1.03 ± 0.10 |
| 5 | *p*-Hydroxybenzoic acid | 13.43 | 254.7 | – | 1.88 ± 0.09 | – | – |
| 6 | 4-*O*-Caffeoylquinic acid | 15.43 | 249.7, 296.9, 325.9 | 14.69 ± 0.28 | – | 1.52 ± 0.10 | – |
| 7 | 5-*O*-Caffeoylquinic acid | 17.38 | 248.7, 297.8, 325.7 | 2.76 ± 0.22 | 6.89 ± 0.86 | – | – |
| 8 | Caffeic acid | 17.84 | 249.1, 295.9, 323.3 | – | – | 1.00 ± 0.03 | 0.06 ± 0.002 |
| 9 | (−)-Epicatechin | 18.98 | 278.8 | – | – | – | 4.65 ± 2.20 |
| 10 | 3,4-di-*O*-Caffeoylquinic acid | 36.00 | 251.0, 296.9, 324.2 | – | 2.03 ± 0.44 | – | – |
| 11 | 3,5-di-*O*-Caffeoylquinic acid | 38.58 | 251.9, 298.3, 327.0 | – | 14.48 ± 2.84 | – | – |
| 12 | Hesperetin-7-*O*-rutinoside | 40.00 | 287.0, 337.7 | – | – | 9.78 ± 0.45 | – |
| 13 | 1,5-di-*O*-Caffeoylquinic acid | 40.78 | 250.5, 299.2, 328.4 | – | 3.53 ± 0.92 | – | – |
| 14 | Rosmarinic acid | 40.99 | 254.2, 289.0, 329.5 | 1.44 ± 0.08 | – | 35.63 ± 2.09 | 23.53 ± 0.47 |
| 15 | Quercetin-3-*O*-rutinoside | 42.85 | 257.4, 265.2, 299.1, 355.6 | 5.20 ± 0.60 | – | – | – |
| 16 | 4,5-di-*O*-Caffeoylquinic acid | 43.51 | 251.0, 299.7, 327.9 | – | 8.86 ± 1.60 | – | – |
| 17 | Pinocembrin | 55.08 | 290.4, 334.4 | – | 13.98 ± 0.88 | – | – |
| Σ | | | | 27.89 | 51.65 | 47.93 | 32.43 |

Values are expressed as mean ± SD ($n = 3$); –: not detected

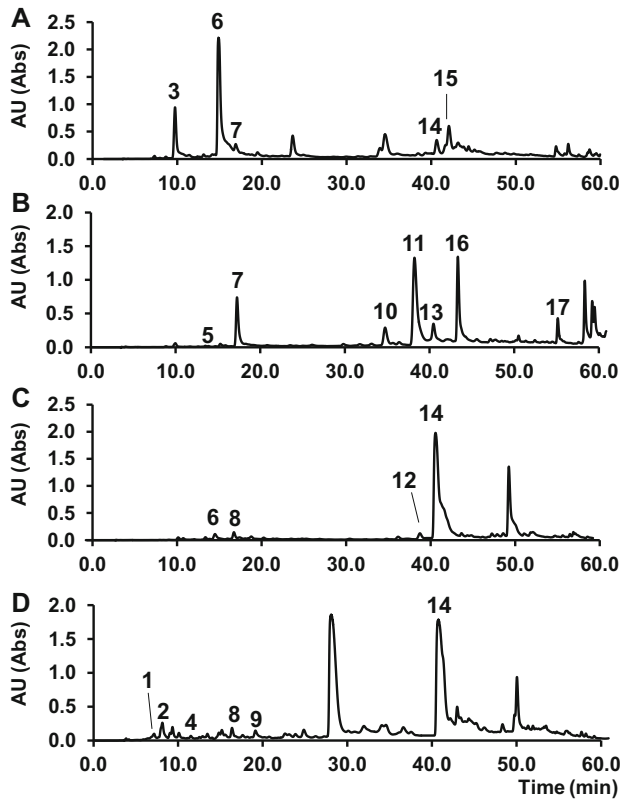


**Fig. 1** HPLC–DAD chromatograms of *C. nepeta* (**a**), *H. italicum* (**b**), *M. spicata* (**c**) and *O. vulgare* (**d**). Detection at 320 nm except in D (280 nm). Identity of compounds as in Table 2

extract) was the predominant phenolic compound in *H. italicum* extract followed by pinocembrin (13.98 mg/g of dry extract). In addition, another caffeoylquinic acid (5-*O*-caffeoylquinic acid) and three dicaffeoylquinic acids (3,4-, 1,5- and 4,5-di-*O*-caffeoylquinic acids) were identified in this extract (Table 2). Some caffeoylquinic and dicaffeoylquinic acids were also identified in other species of the same genus (Barroso et al. 2014). Furthermore, Scognamiglio et al. (2015) also identified 5-*O*-caffeoylquinic acid (chlorogenic acid) and dicaffeoylquinic acids in *Helichrysum italicum* G. Don. Studies reporting the phenolic composition of extracts from the subspecies investigated in this study, *C. nepeta* subsp. *nepeta*, *H. italicum* subsp. *picardii* and *O. vulgare* subsp. *virens*, are scarce although there are some studies describing the chemical composition of the essential oils or volatile extracts (Vale-Silva et al. 2012; Costa et al. 2015b). Conversely, there are reports describing the phenolic compounds of *M. spicata* extracts from different origins (Dorman et al. 2003; Fatiha et al. 2015). Similarly to what was observed in this study, rosmarinic acid was one of the main compounds identified in the extracts analyzed by most authors but in variable amounts, possibly reflecting the effect of edafoclimatic factors, and the application of distinct extraction and analytical techniques. Indeed, *Mentha* species have been considered promising sources of rosmarinic acid, which has attracted a great deal of attention due to its reported health benefits (Fatiha et al. 2015).

### Total phenolic contents and antioxidant activity

Plant-based products capable of protecting humans against oxidative damage have recently attracted interest worldwide. Phenolic compounds, the main class of plant antioxidants, can reduce the oxidative stress in the body maintaining a balance between oxidants and antioxidants due to their reducing, free radical scavenging or metal chelating properties (Aguilera et al. 2016). The total phenolic contents of the extracts from the four aromatic species were determined by F–C assay and the results ranged from 549.27 ± 23.86 to 1597.20 ± 24.10 $\mu mol_{GAE}/g_{extract}$. The highest phenolic level was observed in the extract of *O. vulgare,* whereas *C. nepeta* extract contained relatively low levels of phenolic compounds.

The free radical-scavenging capacity of the four extracts was investigated by two methods, DPPH and ABTS (Table 3). Furthermore, the reducing capacity of the extracts was evaluated by determining their ability to reduce ferricyanide/$Fe^{3+}$ complex to the corresponding $Fe^{2+}$ type (Table 3). The results of DPPH assay were expressed as $IC_{50}$ values and ranged from 4.65 ± 0.12 to 13.27 ± 0.46 μg/ml. The results of ABTS assay, expressed as trolox equivalents, varied between 877.93 ± 22.38 and 1479.56 ± 12.29 $\mu mol_{TE}/g_{extract}$. Among the four extracts *O. vulgare* was the most efficient at neutralizing both $ABTS^{\bullet+}$ and $DPPH^{\bullet+}$ radicals. This extract also had the highest ferric reducing ability expressed as ascorbic acid equivalents, 1746.76 ± 45.11 $\mu mol_{AAE}/g_{extract}$. There are some reports describing the antioxidant activity of phenolic extracts from *M. spicata* and *O. vulgare* subsp. *virens* but the comparison between results from different investigations is not always possible because of the different assay formats, experimental conditions and data presentation strategies. The DPPH assay is recurrently used and overall the $IC_{50}$ values obtained in this work were lower for both species (Barros et al. 2010; Fatiha et al. 2015). Studies describing the antioxidant activity from the subspecies of *H. italicum* and *C. nepeta* studied in this work are limited and mainly focused on essential oils and volatile extracts (Conforti et al. 2012; Costa et al. 2015b).

The correlation between antioxidant activity and the total phenolic content was evaluated by calculating Pearson's correlation coefficients and, in accordance with a wide range of reports, high correlation coefficients were found for all the used assays (FC-DPPH: 0.909; FC-ABTS: 0.809; FC-FRAP: 0.955). The outstanding antioxidant activity of *O. vulgare* extract could be partially attributed to the higher content in rosmarinic acid, the main phenolic compound found in this extract, which has been described as a potent antioxidant compound (Kim et al. 2015). However, other active compounds, as (+)-catechin and (−)-epicatechin, which are only present in this extract, certainly play an important role for the antioxidant activity of the extract. This is supported by the fact that *M. spicata* extract, while containing the highest amount of rosmarinic acid, did not show the greatest antioxidant activity.

Although phenolic compounds are the most abundant and potent antioxidants in plants, there is also a pool of non-phenolic compounds that contribute to the overall antioxidant activity of the extracts. Different phenolic compounds also vary in their antioxidant activities, and may work cooperatively, antagonistically or synergistically with other compounds present in crude extracts. Additionally, it is important to note that results of F–C assay should be interpreted carefully as it is nonspecific to phenolic compounds and other substances, including nonphenolic organic substances and some inorganic substances, could be oxidised by the F–C reagent (Huang et al. 2005).

### Enzyme inhibitory activities

The cholinergic neurotransmitter acetylcholine is reduced in AD patients and cholinesterase inhibitors have been shown to augment the activity of surviving cholinergic neurons in patients with the disease, resulting in

**Table 3** Total phenolic contents determined by Folin–Ciocalteu assay and antioxidant activity evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays, of extracts from the four aromatic species studied

| Plant species | Total phenolics | Antioxidant activity | | |
|---|---|---|---|---|
| | F–C ($\mu mol_{GAE}/g_{extract}$) | DPPH ($IC_{50}$, μg/ml) | ABTS ($\mu mol_{TE}/g_{extract}$) | FRAP ($\mu mol_{AAE}/g_{extract}$) |
| *C. nepeta* | 549.27 ± 23.86 c | 13.27 ± 0.46 c | 1050.31 ± 81.45 b | 582.83 ± 6.59 c |
| *H. italicum* | 814.23 ± 35.14 b | 13.27 ± 0.89 c | 947.52 ± 8.68 bc | 638.28 ± 22.04 c |
| *M. spicata* | 870.62 ± 45.69 b | 8.93 ± 0.27 b | 877.93 ± 22.38 c | 996.88 ± 13.08 b |
| *O. vulgare* | 1597.20 ± 24.10 a | 4.65 ± 0.12 a | 1479.56 ± 12.29 a | 1746.76 ± 45.11 a |

Values are expressed as mean ± SE ($n = 3$). In each column values followed by different letters are significantly different at $p < 0.05$. Reference in DPPH assay, BHT: $IC_{50} = 462.24 \pm 0.03$ μg/ml

**Table 4** Inhibitory activity (%) of methanol extracts (at 10 mg/ml) from the four aromatic species studied against acetylcholinesterase (AChE), tyrosinase, α-amylase and α-glucosidase

| | AChE | Tyrosinase | α-Amylase | α-Glucosidase |
|---|---|---|---|---|
| *C. nepeta* | 48.78 ± 5.21 b | 38.45 ± 4.06 c | 16.45 ± 0.94 a | 66.62 ± 1.61 c |
| *H. italicum* | 78.29 ± 2.44 a | 74.13 ± 3.79 a | 10.03 ± 1.87 b | 96.65 ± 1.34 a |
| *M. spicata* | 72.33 ± 2.65 a | 57.90 ± 1.44 b | 6.81 ± 0.65 b | 79.64 ± 0.69 b |
| *O. vulgare* | 20.11 ± 3.56 c | 69.65 ± 1.45 ab | 6.79 ± 0.57 b | 58.41 ± 1.97 d |

Values are expressed as mean ± SE ($n = 3$). In each column values followed by different letters are significantly different at $p < 0.05$. References: α-amylase, acarbose (300 μg/ml) = 72.47%; α-glucosidase, acarbose (1000 μg/ml) = 80.71%; AChE, galanthamine (5 μg/ml) = 67.78%; tyrosinase, kojic acid (200 μg/ml) = 94.46%

improvement of memory and cognition (Massoud and Gauthier 2010). Phytochemical substances have demonstrated AChE inhibitory activity and thus could be beneficial in the treatment of neurodegenerative disorders, such as AD. Evaluation of the effects of the extracts (at 10 mg/ml) studied in this work on AChE enzyme are listed in Table 4. *H. italicum* and *M. spicata* extracts showed the highest inhibitory percentages (78.29 and 72.33%, respectively). As far as our literature survey could ascertain the AChE inhibitory activity of *H. italicum* is reported for the first time. The HPLC–DAD analysis showed that *H. italicum* extract is rich in pinocembrin and results from Liu et al. (2014) indicated that this compound is inactive against AChE although it improves cognition and protects the neurovascular unit in Alzheimer related deficits (Liu et al. 2014). Thus, *H. italicum* extract can be effective for the prevention and/or treatment of AD through different mechanisms.

Tyrosinase is a copper-containing polyphenol oxidase that converts L-tyrosine to L-DOPA and oxidizes L-DOPA to form dopachrome, which induces the production of melanin pigments (Seo et al. 2003). Melanin production is critical in preventing UV damage to skin, hair and eyes, however its overproduction is associated with pigment variations and also with neurodegenerative disorders namely PD (Khan 2012). Tyrosinase is also responsible for browning in some fruits and vegetables thus it is frequent the treatment of plant-based foods with tyrosinase inhibitors (Loizzo et al. 2012). Concerns over the toxicity and side effects of synthetic inhibitors have led to a search for new safe and effective tyrosinase inhibitors particularly from natural sources (Zengin et al. 2015). The extracts (at 10 mg/ml) of the four species studied in this work were tested against tyrosinase activity and, although all the extracts inhibit the enzyme, the highest inhibitory activity was again obtained with *H. italicum* extract (74.13%). According to our literature survey, tyrosinase inhibitory activity of this species has never been studied up to now. This activity might be associated to the high amount of hydroxycinnamic acids, particularly dicaffeoylquinic acids in this extract (Iwai et al. 2004) (Table 2). Additionally, the tyrosinase inhibitory capacity of plant extracts is also frequently associated to the presence of flavonoids (Fatiha et al. 2015; Orhan et al. 2015) that can act as cofactor and/or substrate of tyrosinase or by active site chelation. This may lead to the assumption that the tyrosinase inhibitory action of *H. italicum* extract might also be related to the presence of pinocembrin, the only flavonoid identified in the extract. *O. vulgare* and *M. spicata* extracts also considerably inhibit this enzyme and rosmarinic acid is probably involved in that effect (Tundis et al. 2015).

α-Amylase and α-glucosidase enzymes are responsible for carbohydrate digestion and have been identified to be the therapeutic targets for the management of postprandial hyperglycemia (Shobana et al. 2009). The inherently associated adverse side effects of the synthetic inhibitors necessitate further efforts to find natural and safer alternatives. In this study the methanolic extracts (at 10 mg/ml) of the four aromatic plants were evaluated for their α-amylase and α-glucosidase inhibitory capacity. All the extracts showed a weak activity against α-amylase (Table 4). On the other hand, all the extracts were considerably more effective against α-glucosidase, with inhibition percentages in the range of 58.41 to 96.65%. The selective inhibition of α-glucosidase is the preferred effect for plant extracts to control glucose uptake because simultaneous inhibition of both enzymes would result in abnormal bacterial fermentation in the colon due to the presence of undigested carbohydrates (Costamagna et al. 2016). Indeed, plant extracts from many plants, including plants traditionally used to control diabetes or hyperglycemia, were reported to exert strong inhibition of α-glucosidase and moderate or negligible effect on α-amylase activity (Pradeep and Sreerama 2015). The best α-glucosidase inhibition result was once more obtained with *H. italicum* extract (Table 4). Since caffeoylquinic acids possess strong inhibition potential against α-glucosidase (Xiao et al. 2013) these compounds are almost certainly implicated in the α-glucosidase inhibitory properties of *H. italicum* extract.

## Conclusion

The present study evaluated the phenolic profile, and the antioxidant and enzyme inhibitory properties of extracts from four aromatic species used in traditional medicine and Mediterranean diet. Phenolic acids and flavonoids are the main identified phenolic compounds in the extracts. The *O. vulgare* extract showed the highest antioxidant activity and the *H. italicum* extract exhibited the highest enzyme inhibitory potential. Overall, the results obtained demonstrate that the studied plants represent promising sources of food rich in natural antioxidants and of active ingredient agents for the pharmaceutic and cosmetic industries.

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