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European pennyroyal (*Mentha pulegium*) from Portugal: Chemical composition of essential oil and antioxidant and antimicrobial properties of extracts and essential oil

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ABSTRACT

There is a growing interest of industry to replace synthetic chemicals by natural products with bioactive properties from plant origin. The aim of this study was to determine the chemical composition of European pennyroyal (*Mentha pulegium*) essential oil and to characterize the *in vitro* antioxidant and antimicrobial activities of its water (hot and cold) and ethanolic extracts and of the essential oil. The essential oil revealed menthone, pulegone and neo-menthol as the main constituents, comprising 35.9, 23.2 and 9.2% of the essential oil, respectively. The hot water extract exhibited the highest antioxidant activity and phenol content. In contrast, the extracts were not very effective to inhibit the growth of the seven foodborne spoilage and pathogenic bacteria tested, but the essential oil showed antibacterial activity against all bacterial strains. In conclusion, extracts and essential oil of *M. pulegium* from Mediterranean origin have huge potential as an alternative to chemical additives for the food industry.

1. Introduction

There has been growing interest in the investigation of natural products for the discovery of active compounds with antimicrobial and antioxidant properties that can be applied to the food industry. As consumers are avoiding the consumption of products with synthetic additives or preservatives, natural active compounds could be an alternative to the employ of synthetic chemicals. Such compounds can be used to prolong the storage stability of food, by inhibiting the growth of foodborne spoilage or pathogenic microorganisms and protecting food from oxidative stress damage. Several authors have tested essential oils of aromatic plants to prolong the shelf-life of food (Atrea et al., 2009; Kostaki et al., 2009; Mosqueda-Melgar et al., 2008), while others have focused on the antioxidant and antimicrobial properties of plant extracts and essential oils (Cao et al., 2009; Sökmen et al., 2004).

The European pennyroyal (*Mentha pulegium*) is an aromatic herb that belongs to the family Lamiaceae, is naturalized in America and thrives in Western, Southern and Central Europe, Asia, Iran, Arab countries and Ethiopia (Gruenwald et al., 2000). Its essential oil and dry parts have been traditionally used in medicine (digestive, liver and gallbladder disorders, amenorrhea, gout, colds, increased micturition, skin diseases and abortifacient), gastronomy (culinary herb), aromatherapy and cosmetics (Gruenwald et al., 2000; Agnihotri et al., 2005).

Most studies performed so far on *M. pulegium* were carried out with its essential oil in different regions of the world, including Iran (Aghel et al., 2004), Greece (Petrakis et al., 2009), Turkey (Müller-Riebau et al., 1997) and Portugal (Reis-Vasco et al., 1999), and focused mainly on its chemical composition.

Currently, there is a lack of information concerning the bioactive properties of *M. pulegium* extracts. Recently, Mata et al. (2007) characterized antioxidant properties of *M. pulegium* essential oil and extracts, yet no information is available on antimicrobial properties of this species. In this context, the aim of this study was to study the chemical composition of *M. pulegium* essential oil from Portuguese origin, and to evaluate the antioxidant

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activity, total phenol contents and antimicrobial potential of water (hot and cold) and ethanolic extracts and essential oil against food-borne spoilage and pathogenic bacteria.

2. Materials and methods

2.1. Preparation of extracts and essential oil

M. pulegium was collected in Santarém (Ribatejo, Portugal) (39° 21′ 37.44″ N, 8° 45′ 41.04″ W) in summer 2008. Voucher specimens were deposited in the Herbarium of the Portuguese National Institute of Biological Resources, I.P. The dried aerial parts were used to prepare three extracts (hot water, cold water and ethanolic) and the essential oil. The extracts were obtained by maceration of dry plant material (150 g) in: boiling water for 3 h (hot water extract); water for 3 days at room temperature (cold water extract); or ethanol for 3 days at room temperature (ethanolic extract). The macerates were filtered under vacuum through a Buckner funnel with filter paper (Whatman #4), whereas the ethanolic extract was dried in a rotary evaporator under vacuum (40 °C, 178 mbar). The extracts were freeze-dried at −50 °C (Heto – Powerdry, LL3000, Mukarov, Czech Republic). The essential oil was obtained from dry plant material (100 g) by hydrodistillation using a modified Clevenger system, for 3 h. Afterwards, the essential oil was dried through sodium sulphate anhydrous. The extracts and essential oil were stored at −20 °C and their final concentrations were 32.3 mg mL^{-1} (hot water extract), 23.1 mg mL^{-1} (cold water extract) and 927.4 mg mL^{-1} (essential oil), while the dry weight of the ethanolic extract was 8.2 g. Yields of the extractions were 21.5% (hot water extract), 12.3% (cold water extract), 5.4% (ethanolic extract) and 0.9% (essential oil).

2.2. Chemicals

Phosphate buffer, trichloroacetic acid, ferric chloride, ascorbic acid and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Fluka (Buchs, Germany); Folin-Ciocalteu reagent, gallic acid, α,α-diphenyl-β-picrylhydrazyl (DPPH), potassium hexacyanoferrate III and dimethylsulfoxide (DMSO) from Sigma–Aldrich (Steinheim, Germany); ferrous sulphate, sodium acetate, potato dextrose agar, tryptic soy agar and plate count agar from Merck (Darmstadt, Germany); sodium carbonate and butylated hydroxytoluene (BHT) from BDH (Poole, England); sodium sulphate anhydrous from Panreac Quimica S.A.U. (Barcelona, Spain); brain heart infusion broth from Oxoid (Basingstoke, Hampshire, UK); ethanol had a purity grade of 99% and the water used was Milli-Q purified and distilled.

2.3. GC–MS analysis

The *M. pulegium* essential oil was analyzed on an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N mass selective detector (Agilent Technologies, Palo Alto, USA). A vaporization injector operating in the split mode (1:50) at 250 °C was used, into which a fused silica capillary column (30 m length × 0.32 mm internal diameter × 0.25 μm film thickness; HP-5MS; 5% diphenyl, 95% dimethyl polydimethylsiloxane, Agilent Technologies) was installed. The oven temperature was programmed at 45 °C for 1 min, raised to 250 °C at 5 °C min^{-1} and maintained at 250 °C for 5 min. Helium was used as carrier gas at 30 cm s^{-1} and the injection volume was 1 μL. The transfer line, ion source and quadrupole analyzer temperatures were maintained at 280 °C, 230 °C and 150 °C, respectively, and a turbo molecular pump (10^{-5} Torr) was used. In the full-scan mode, electron ionization mass spectra in the range 40–400 Da were recorded at 70 eV electron energy. A solvent delay of 3 min was selected. The acquisition data and instrument control were performed by the MSD ChemStation Software (G1701CA; version C.00.00; Agilent Technologies, Santa Clara, CA, USA). The identity of each compound was assigned by comparison of their retention index, relative to a standard mixture of *n*-alkanes (Adams, 2001), as well as by comparison with the mass spectra characteristic features obtained with the Wiley's library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies, Santa Clara, CA, USA). For semi-quantification purposes the normalized peak area abundances without correction factors were used.

2.4. Antioxidant activity assays

2.4.1. Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay was based on the methodology of Benzie and Strain (1996) modified by Deighton et al. (2000). Briefly, FRAP reagent was prepared by combining 1 mM TPTZ with 2 mM ferric chloride in 0.25 M sodium acetate (pH 3.6). The sample (0.2 mL) was mixed with FRAP reagent (1.8 mL), stood for 4 min at room temperature and the absorbance was determined at 593 nm (ATI-UNICAN-UV2, Cambridge, UK). *M. pulegium* extracts were diluted in water and the essential oil was diluted in ethanol 50%. Negative controls with water (aqueous and ethanolic extracts) or ethanol 50% (essential oil) were also included. All determinations were performed in triplicate.

The difference between the absorbance of sample and the negative control was calculated and the reducing capacity of samples was compared with that of a reaction with a ferrous ion standard solution. The standard was prepared from ferrous sulphate that reacted with the TPTZ reagent, following the same procedure as with samples. The absorbance was plotted against ferrous ion concentration (0.125–3.500 μM Fe^{2+}), and FRAP values were expressed as μmol Fe^{2+} per g of sample.

2.4.2. Reducing power

The capacity of both *M. pulegium* extracts and essential oil to reduce iron (III) to iron (II) was determined according to the modified method of Oyaizu (1986). Briefly, the sample (1 mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium hexacyanoferrate III (1%, w/v). After 30 min of incubation at 50 °C in the dark, 2.5 mL trichloroacetic acid (10%) were added and the mixture kept at room temperature for 10 min. Afterwards, 2.5 mL of this mixture was added to 2.5 mL water and 0.5 mL ferric chloride (0.1%), vigorously mixed, and the absorbance measured at 700 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). Extracts and essential oil were diluted in ethanol. All determinations were performed in triplicate. Negative (ethanol) and positive (ascorbic acid in the 10–40 μg mL^{-1} range) control reactions were performed, in order to plot the absorbance of ascorbic acid against concentration. The results were expressed as μmol of ascorbic acid per g of sample.

2.4.3. Free radical scavenging

The scavenging effect of DPPH free radical was assessed by the modified method of Kondo et al. (2002). Briefly, 0.1 mL of each plant extract or essential oil at different concentrations (in 95% ethanol) was added to 2 mL DPPH (0.21 mM in 95% ethanol). The mixture was shaken, left for 60 min at room temperature in the dark, and the absorbance was measured at 517 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). The percentage of DPPH inhibition was calculated using the following equation:

$$\text{Percentage of inhibition} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where $\text{Abs}_{\text{control}}$ is the absorbance of the control reaction (blank with 0.1 mL of ethanol and DPPH) and $\text{Abs}_{\text{sample}}$ is the absorbance of the sample reaction (0.1 mL sample diluted in ethanol and DPPH). The sample concentration (in 1 mL reaction mixture) providing 50%

inhibition (EC_{50}) was estimated by plotting percentages of inhibition against concentrations of sample. All determinations were performed in triplicate. EC_{50} was also estimated for the synthetic antioxidant reagent BHT.

To standardize DPPH results, the antioxidant activity index (AAI), proposed by Scherer and Godoy (2009), was calculated as follows:

$$AAI = \frac{\text{DPPH concentration in reaction mixture}\,(\mu g\ mL^{-1})}{EC_{50}\,(\mu g\ mL^{-1})}$$

Samples were classified as showing poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when 0.5 < AAI < 1.0, strong antioxidant activity when 1.0 < AAI < 2.0, and very strong when AAI > 2.0 (Scherer and Godoy, 2009).

2.4.4. Phenol content

Total phenol content was measured using a modified Folin-Ciocalteu assay (Asami et al., 2003). Briefly, water (5 mL), sample (1–3 mL) and Folin-Ciocalteu Reagent (0.5 mL) were mixed, allowed to stand for 5–8 min at room temperature, followed by the addition of 1.5 mL sodium carbonate (20%, w/v) together with water to obtain a final volume of 10 mL. The solution was mixed, allowed to stand for 2 h and filtered (0.45 μm poly-tetrafluoroethylene filter, Whatman) prior to absorbance reading at 750 nm in a spectrophotometer (ATI-UNICAN-UV2, Cambridge, UK). Aqueous and ethanolic extracts were diluted in water, whereas essential oil was diluted in ethanol 50%. All determinations were performed in triplicate. Negative controls were performed with water (aqueous and ethanolic extracts) or ethanol 50% (essential oil). Total phenol content was quantified by comparison of samples absorbance values with those of gallic acid reaction. The calibration curve of gallic acid was prepared in the 5–25 mg L^{-1} range, and results were expressed as mg of gallic acid per g of sample.

2.5. Antibacterial activity

2.5.1. Strains and growth conditions

The antibacterial activity tests included foodborne spoilage and pathogenic bacteria purchased from American Type Culture Collection (ATCC) or Spanish Type Culture Collection (CECT): *Brochothrix thermosphacta* (CECT 847), *Escherichia coli* (ATCC 25922), *Listeria innocua* (CECT 910), *Listeria monocytogenes* (CECT 5873), *Pseudomonas putida* (CECT 7005), *Salmonella typhimurium* (ATCC 14028) and *Shewanella putrefaciens* (CECT 5346). These strains kept at −70 °C in a cryopreservative solution (Microbank, Pro-lab Diagnostics, Richmond Hill, ON, Canada) were inoculated in tryptic soy agar (TSA) and incubated overnight at 30 °C, except *L. monocytogenes* that was inoculated in plate count agar (PCA). Subsequently, one colony from these cultures was inoculated on brain heart infusion broth (BHI) and incubated at 30 °C for 18–24 h with shaking (75 rpm), in order to obtain freshly cultured microbial suspensions (10^8–10^9 cells mL^{-1}) for tests.

2.5.2. Paper disc diffusion method

The antibacterial activity of *M. pulegium* extracts and essential oil was determined using the paper disc diffusion method (NCCLS, 2002). Briefly, the bacterial suspensions were adjusted to 1×10^7 CFU mL^{-1} (equivalent to 0.5 McFarland) and spread in TSA or PCA (10–15 mL per 90 mm Ø Petri dishes) using a sterile cotton swab. Subsequently, filter paper discs (6 mm Ø; Whatman #1) were placed in the surface of Petri dishes and impregnated with 20 μL of the extracts or essential oil at different concentrations. Essential oils were diluted in DMSO, while ethanolic and aqueous extracts were diluted in ethanol and water, respectively. Negative controls were prepared using the same solvents employed to dissolve samples. After staying at 4 °C for 2 h, Petri dishes were incubated at 30 °C for 24 h, except *L. monocytogenes* that was incubated for 48 h. All determinations were performed in triplicate. Antibacterial activity was evaluated by measuring the radius of the inhibition zones to the nearest millimetre, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited growth of bacteria.

2.5.3. Effect of MIC on liquid medium

The MIC of plant extracts and essential oil was tested for antibacterial activity using liquid media. The bacterial suspensions were adjusted to 1×10^7 CFU mL^{-1} in BHI, and 180 μL were added to each well of 96-well plates. Subsequently, 20 μL of plant extract or essential oil were added, in order to obtain the MIC determined with the paper disc diffusion method. Water extracts were not tested with this assay because it was not possible to concentrate these extracts to the levels required for tests. Negative controls were prepared with the solvents used to dissolve extracts and essential oil, as well as positive controls with solvents and bacteria, but without the plant extract or essential oil. Plates were incubated at 4 °C (2 h) and 30 °C (24 h). The absorbance at 595 nm was measured using a micro plate reader (Bio-Rad model 680, Hercules, CA, USA) before and after incubation period, and the microbial concentration was determined by viable counts on TSA or PCA. All determinations were performed in triplicate.

2.6. Statistical analysis

Differences between extracts and essential oil were tested with analysis of variance (ANOVA). In order to satisfy ANOVA assumptions data were transformed, followed by multiple comparisons tests (Tukey's HSD) to identify differences between groups. If transformed data could not met ANOVA assumptions, non-parametric analysis of variance (Kruskall–Wallis) was performed, followed by non-parametric multiple comparisons test (Dunn). All statistical analyses were tested at a 0.05 level of probability with the software STATISTICA™ 6.1 (Statsoft, Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Chemical composition of essential oil

Volatiles of *M. pulegium* essential oil revealed 53 different compounds accounting for 86.3% of the essential oil composition that are identified in Table 1. The essential oil contained a complex mixture consisting mainly by oxygenated monoterpenes (76.8%), such as menthone (35.9%), pulegone (23.2%), neo-menthol (9.2%) and 8-hydroxy-δ-4(5)-*p*-menthen-3-one (2.1%). Menthone and pulegone have known antioxidant and antibacterial properties, whereas neo-menthol has also antibacterial activity against several bacteria (Ruberto and Baratta, 2000; Sivropoulou et al., 1995). Previous studies with the same plant harvested in Iran, India and Turkey reported menthone and pulegone as the major components of the essential oil, but in different proportions (Table 2; Agnihotri et al., 2005; Aghel et al., 2004; Müller-Riebau et al., 1997). Nonetheless, there is a great variability in the chemical composition of *M. pulegium* essential oil among the studies performed so far, being pulegone, menthone, piperitone, piperitenone and isomenthone among the major components (Table 2; e.g. Aghel et al., 2004; Sivropoulou et al., 1995; Mahboubi and Haghi, 2008). Such variability may be related with different vegetative phases of the plant, and also with environmental conditions (e.g. seasonal and geographical variations, soil composition (Müller-Riebau et al., 1997)).

Table 1
Chemical composition of the *Mentha pulegium* essential oil volatiles.

Compound	RI	%[a]
Monoterpene hydrocarbons		0.9
α-Thujene	853	tr
α-Pinene	861	0.2
Camphene	877	tr
Sabinene	909	tr
2-β-Pinene	912	0.2
n-Cymene	970	0.1
L-Limonene	975	0.2
Carene	1335	0.2
Sesquiterpenes hydrocarbons		0.1
β-Bourbonene	1426	0.1
Oxygenated monoterpenes		76.8
Eucalyptol	988	0.5
Linalool	1076	0.1
Menthone	1138	35.9
Neo-menthol	1164	9.2
trans-5-Methyl-2-(1-methylethenyl)-cyclohexanone	1173	1.1
Menthol	1179	0.1
3-*p*-Menthanol	1182	0.1
α-Terpineol	1189	0.1
Berbenone	1213	0.1
4-Hydroxy-δ-8-*p*-menthen-3-one	1221	0.9
(+)-trans-Carveol	1223	0.1
Pulegone	1262	23.2
(1RS,4SR)-8-hydroxy-*p*-menthan-3-one	1267	0.4
Piperitone	1271	0.4
3-Menthene	1295	1.6
8-Hydroxy-δ-4(5)-*p*-menthen-3-one	1315	2.1
1-Menthene	1317	0.2
Piperitenone	1374	0.4
Mint furanone 1	1555	0.2
Mint furanone 2	1590	0.1
Oxygenated sesquiterpenes		1.1
(−)-Allo-spathulenol	1643	0.1
Caryophyllene oxide	1649	0.5
Veridiflorol	1658	0.1
Muurolol	1708	0.1
α-Cadinol	1723	0.1
Palustrol	1794	0.2
Oxygenated diterpenes		0.1
Epimanoyl oxide	2040	0.1
Esters		0.2
3-Octyl acetate	1100	0.2
Others		7.1
1-Ethyl-3-methyl-2-(2-methylpropylidene) imidazolidine	1282	0.8
4,6-Diethyl-2-methoxypyrimidine	1309	0.3
3-Methyl-cyclopentanone	771	0.1
5-Methyl-3-heptanone	923	0.1
3-Octanol	935	0.7
1-Methyl-3,5-dimethoxy-1H-pyrazole	1394	0.6
2-Cyclohexen-1-ol	1404	1.1
Methyleugenol	1452	0.2
3-(2-Oxocyclopentyl) propanal ethylene ketal	1463	0.5
2-Amino-6-chloro-4-(2-ethenylamino) pyrimidine	1476	0.9
2,6-Dimethoxytoluene	1486	0.9
3-Methyl-hexanedioic acid	1491	0.2
2-tert-butyl-4-methylphenol	1552	0.1
Elemicin	1618	0.1
β-(3-Thienyl)acrylic acid	1628	0.4
Hexadecanoic acid	2005	0.1
Total identified		86.3

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

3.2. Antioxidant activity of extracts and essential oil

The antioxidant activity of *M. pulegium* measured by the FRAP method ranged between 4.7 and 10.3 μmol of Fe^{2+} per g of sample (Fig. 1). The hot water extract had statistically the highest antioxidant activity, followed by essential oil, ethanolic extract and cold water extract.

The ferric reducing power of *M. pulegium* revealed that the hot water extract had statistically the highest antioxidant activity, followed by the ethanolic extract, and the cold water extract (all varying between 116.6 and 154.2 μmol ascorbic acid per g of sample). Yet, the lowest value was observed for the essential oil (2.2 μmol ascorbic acid per g of sample). Previous studies with hot water extracts of *Mentha* species (*M. pulegium* not included) showed slightly higher ferric reducing power (ca. 100–250 mg ascorbic acid per g of extract; Dorman et al., 2003).

The DPPH assay also identified *M. pulegium* hot water extract ($EC_{50} = 16.3 \pm 0.4\ \mu g\ mL^{-1}$) as having statistically the highest free radical scavenging activity (i.e. highest antioxidant activity and lowest EC_{50}), followed by ethanolic extract, cold water extract and essential oil ($EC_{50} = 6.2 \pm 0.2\ mg\ mL^{-1}$; Fig. 1). Accordingly to the categories defined by Scherer and Godoy (2009), *M. pulegium* essential oil and cold water extract tested in the present study presented poor antioxidant activity, while ethanolic extract and hot water extract were classified as moderate and very strong antioxidants, respectively (AAI values: essential oil = 0.01; cold water extract = 0.45; ethanolic extract = 0.80; hot water extract = 4.83). Previous studies reported a wide variation in the antioxidant activity of *M. pulegium* extracts or essential oil. For example, the ethanolic extract had identical antioxidant activity (AAI = 0.8; Mata et al., 2007) or higher (AAI = 1.9; Nickavar et al., 2008), compared to the values obtained in the present study, the essential oil of *M. pulegium* exhibited higher antioxidant activity (AAI = 1.6; Hajlaoui et al., 2009), the opposite was also observed (AAI = 0.003; Kamkar et al., 2010), and variability also occurred with hot water extracts (Mata et al., 2007; Kamkar et al., 2010; Guimarães et al., 2011). The antioxidant activity of hot water extract was even higher than that found for BHT ($EC_{50} = 20.2 \pm 2.6\ \mu g\ mL^{-1}$; AAI = 3.95), and AAI value for BHT was within values obtained by previous authors, i.e. between 0.26 (Borneo et al., 2009) and 5.96 (Gourine et al., 2010).

Phenols are organic compounds that contain a hydroxyl group bound directly to the aromatic ring, and the H-atom of the hydroxyl group can trap peroxyl radicals, preventing other compounds to be oxidized (Nguyen et al., 2003). In this way, antioxidant activity may be partially due to the presence of phenols. In this study, total phenol contents varied between 0.7 and 13.3 mg gallic acid per g of sample (Fig. 1). The essential oil had statistically the lowest phenol content, followed by ethanol extract, cold water extract, and hot water extract. These values were rather low compared to those obtained with hot water extract of other *Mentha* species, where values varied between 128.1 to 230.8 mg gallic acid per g of sample (Dorman et al., 2003). In the present study, hot water was more efficient to extract phenol compounds from *M. pulegium* than the remaining extraction methods. In contrast, Mata et al. (2007) observed that total phenol contents of *M. pulegium* was higher in ethanolic extract (71.7 mg pyrogallol per g of sample) than in hot water extract (57.9 mg pyrogallol per g of sample). The differences found between the results obtained in this study and those reported by previous authors might be related with the duration of the extraction procedure, or with intrinsic characteristics of plants, like origin, not specified in the study of Mata et al. (2007), and season.

In this study, hot water extract of *M. pulegium* showed simultaneously the highest antioxidant activity and phenol content. The tendency was not so clear for the remaining extracts and essential oil, indicating that other compounds may also play an important role in their antioxidant activity.

3.3. Antibacterial activity

The antibacterial activity of *M. pulegium* extracts and essential oil suggested that almost all tested bacteria were resistant

Table 2
Major constituents of *Mentha pulegium* essential oils reported in different studies.

Origin	Sample period	Extraction method details	Compounds and compounds concentration in the essential oil	Reference
Bandar-Anzeli (Iran)	Summer 2000	4 h hydrodistillation (Clevenger apparatus)	Pulegone (37.8%); menthone (20.3%); piperitone (6.8%)	Aghel et al. (2004)
Jammu region and Kashmir valley (India)	July 2001 and 2003	3 h hydrodistillation (Clevenger apparatus)	Pulegone (65.9–83.1%); menthone (8.3–8.7%); isomenthone (3.8–4.0%)	Agnihotri et al. (2005)
Algeria	nd	Clevenger apparatus	Pulegone (4.4–87.3%); piperitenone (0.1–26.7%); isomenthone (trace to 22.6%); β-pinene (0.4–20.9%)	Beghidja et al. (2007)
Spanish market	nd	2 h simultaneous distillation-extraction	Pulegone (41.1–42.3%); piperitone oxide (14.9–16.9%); piperitenone (4.6–6.1%); piperitone (5.4–6.0%)	Díaz-Maroto et al. (2007)
Ksour Essef (Tunisia)	nd	3 h hydrodistillation (Clevenger apparatus)	Pulegone (61.1%); isomenthone (17.0%); menthone (5.9%)	Hajlaoui et al. (2009)
Gilan province (Iran)	Spring 2008	Hydrodistillation (Clevenger apparatus)	Pulegone (40.5%); menthone (35.4%); piperitone (5.2%)	Kamkar et al. (2010)
Kazeron (Iran)	August 2007	Hydrodistillation	Piperitone (38.0%); piperitenone (33.0%); α-terpineol (4.7%); menthone (3.0%); pulegone (2.3%)	Mahboubi and Haghi (2008)
Portuguese market	Summer 2004	Hydrodistillation	Pulegone (35.1%); piperitenone (27.4%)	Mata et al. (2007)
Monastir (Tunisia)	May, July	3 h hydrodistillation (Clevenger apparatus)	Menthol (40.6–51.6%); menthone (7.3–20.0%); 1,8-cineole (11.1–18.5%); pulegone (3.9–7.0%)	Marzouk et al. (2008)
Akzu (Turkey)	Summer 1993	3 h steam-distilled	Pulegone (205.5 mg mL^{-1}); 1,8–cineole (34.7 mg mL^{-1}); borneol (13.8 mg mL^{-1}); menthone (5.4 mg mL^{-1})	Müller-Riebau et al. (1995)
Antalya, Termessus, Aksu, Düden and Kalkan (Turkey)	April–August 1994	3 h steam-distilled (Clevenger apparatus)	Pulegone (39.6–419.6 mg mL^{-1}); menthone (12.2–166.0 mg mL^{-1}); borneol (16.7–47.6 mg mL^{-1}); 1,8 cineole (19.8–40.1 mg mL^{-1})	Müller-Riebau et al. (1997)
Tunisia	nd	12 h maceration in hexane	Pulegone (17.5–70.2%; carvone (trace to 55.7%); isomenthone (2.9–34.2%); menthol (0.1–21.2%); menthofuran (0.7–10.0%)	Mkaddem et al. (2007)
Samos, Argos, Evia, Samothraki and Kalamata (Greece)	Summer 2007, 2008	4 h hydrodistillation (Clevenger apparatus)	Pulegone (61.3–77.9%); iso-menthone (10.6–18.5%); menthone (0.6–8.3%); piperitone (0.3–3.2%); cis-isopulegone (0–1.7%)	Petrakis et al. (2009)
Island of Crete (Greece)	Summer 2007, 2008	4 h hydrodistillation (Clevenger apparatus)	Piperitone (69.3%); iso-menthone (24.8%); limonene (1.8%); menthone (1.6%)	Petrakis et al. (Petrakis et al., 2009)
Sintra (Portugal)	July	3 h hydrodistillation (Clevenger apparatus); supercritical CO_2	Pulegone (78.3–80.9%); menthone (8.5–9.2%)	Reis-Vasco et al. (1999)
Island of Paros and Mt. Vermio (Greece)	nd	2 h hydrodistillation	Pulegone (44.7–50.4%); piperitone (1.9–13.4%); isomenthone (0.2–4.5%); menthone (1.0–1.7%)	Sivropoulou et al. (1995)
Mt. Pangaio (Greece)	nd	2 h hydrodistillation	Isomenthone (77.5%); menthone (10.3%); pulegone (1.0%)	Sivropoulou et al. (1995)

nd, not described.

to hot and cold aqueous extracts (Table 3), except *S. putrefaciens* (MIC = 323 mg mL^{-1} hot water extract). The ethanolic extract did not expose inhibition against all Gram negative bacteria (*S. typhimurium*, *E. coli*, *S. putrefaciens* and *P. putida*), but all Gram positive bacteria (*L. innocua*, *L. monocytogenes* and *B. thermosphacta*) were sensitive to this extract. The resistance of Gram negative bacteria could be due to the complexity of their double layer cell membrane in comparison with the simpler cell membrane of Gram positive bacteria (Hogg, 2005).

The essential oil of *M. pulegium* was very effective to inhibit the growth of all bacteria tested, as shown by the low MIC (Table 3). The antibacterial activity of this essential oil might be due to the presence of pulegone, menthone and neo-menthol. Duru et al. (2004) have demonstrated the strong antimicrobial activity of pulegone against a set of bacteria, including *S. typhimurim* and *E. coli*. Cytotoxicity of this essential oil appears to include a bacterial membrane damage that occurs when the essential oil passes through the cell wall and cytoplasmic membrane, and disrupts the structure of their different layers of polysaccharides, fatty acids and phospholipids (Bakkali et al., 2008).

The lowest MIC was found for *P. putida*, while the highest values were found for *S. typhimurium* and *L. innocua*. Previous authors

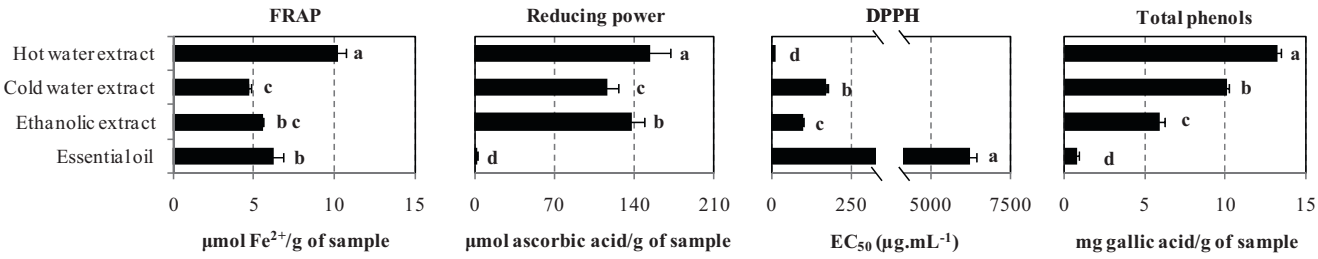

Fig. 1. Antioxidant activity (FRAP, reducing power and DPPH) and total phenols of *Mentha pulegium* extracts and essential oil. The bars represent the average, and the error bars represent the standard deviation. In each graphic, different letters denote significant differences ($p < 0.05$) between extracts and essential oil. *Abbreviations*: FRAP, ferric reducing antioxidant power; DPPH, α,α-diphenyl-β-picrylhydrazyl; EC_{50}, sample providing 50% inhibition.

Table 3
Antibacterial activity of *Mentha pulegium* extracts and essential oil against pathogenic and spoilage bacteria. The radius of paper disc (3 mm) was not included in the values of inhibition zone radius. The values of absorbance change with "+" and "−" indicate an increase and decrease in the absorbance values, respectively.

	Salmonella typhimurium	*Escherichia coli*	*Listeria innocua*	*Listeria monocytogenes*	*Shewanella putrefaciens*	*Brochothrix thermosphacta*	*Pseudomonas putida*
Disc diffusion method							
Inhibition zone radius (mm)							
Hot water extract (32.3 mg mL^{-1})	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Cold water extract (23.1 mg mL^{-1})	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Ethanolic extract (30.5 mg mL^{-1})	N.D.	N.D.	2	2	N.D.	2	N.D.
Essential oil (927.4 mg mL^{-1})	3	4	3	11	32	10	4
MIC (mg mL^{-1})							
Hot water extract	N.D.	N.D.	N.D.	N.D.	323	N.D.	N.D.
Cold water extract	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Ethanolic extract	N.D.	N.D.	15.3	15.3	N.D.	30.5	N.D.
Essential oil	9.3	2.3	9.3	2.3	2.3	4.6	1.2
Effect of MIC in liquid medium							
Logarithmic reductions							
Ethanolic extract	–	–	0	0	–	1.8	–
Essential oil	3.8	3.2	1.8	2.6	–	0.8	1.3
Absorbance change							
Ethanolic extract	–	–	+0.044	−0.067	–	−2.536	–
Essential oil	+0.015	+0.206	−0.445	−0.255	+0.037	+0.169	+0.080

MIC, minimum inhibitory concentration; N.D., not detected inhibition zone radius or MIC.

highlighted strong antimicrobial activity of *M. pulegium* essential oil against *L. monocytogenes* (Mahboubi and Haghi, 2008) and *E. coli* (Marzouk et al., 2008), but the opposite was observed in others' studies with no activity against *E. coli* and *S. typhimurium* (Mahboubi and Haghi, 2008). Such differences can be due to distinct plant origins and essential oil chemical composition (Tables 1 and 2).

The effect of MIC determined by the disc diffusion method on viable counts of bacteria was also tested in liquid medium (Table 3). The assay revealed different sensitivities depending on the bacterial strain, and generally viable cells were considerably reduced, except with the ethanolic extract tested against both *Listeria* species. The ethanolic extract caused only a 1.8 log reduction on viable cells of *B. thermosphacta*, whereas the essential oil reduced viable cells of almost all the bacteria tested, except for *S. putrefaciens*. This bacterial strain was very sensitive to the solvents used to dilute the essential oil and ethanolic extract (DMSO and ethanol), and consequently, it was impossible to determine their MIC effect. The highest inhibition was observed for *S. typhimurium* (3.8 log reductions), whereas the lowest value was obtained with *B. thermosphacta* (0.8 log reductions). These results indicate that the use of microdilution methods to determine MIC might reveal lower values, as the concentrations obtained with the disc diffusion method were able to reduce the bacterial population.

The relationship between the bacterial counts and the absorbance measured for the effect of MIC on viable cells was analyzed to evaluate the accuracy of using the absorbance and visual inspection in the determination of MIC of *M. pulegium* extract and essential oil. As shown in Table 3 no relationship was obtained between the variables, thus indicating that future studies with microdilution methods should focus on bacterial counts for MIC evaluation rather than absorbance or visual inspection.

4. Conclusions

This study allowed concluding that *M. pulegium* hot water extract exhibited the highest antioxidant activity and phenol content. In contrast, the essential oil showed strong antibacterial properties. In this way, *M. pulegium* extracts and essential oil have a huge potential as alternatives to synthetic preservatives in food industry. Further studies should evaluate the safety and toxicity of *M. pulegium* extracts and essential oil to human consumption before considering their use for food preservation or medicinal purposes.

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