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LWT 41 (2008) 391–400
LWT
www.elsevier.com/locate/lwt

Chemical composition and *in vitro* antioxidative activity of a lemon balm (*Melissa officinalis* L.) extract

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Received 12 December 2006; received in revised form 1 March 2007; accepted 6 March 2007

Abstract

The leaf material of lemon balm (*Melissa officinalis* L.) was extracted with 450 ml/l aqueous ethanol by medium pressure liquid–solid extraction. The total phenolic content of the extract was estimated as gallic acid equivalents by Folin–Ciocalteu reagent method and a qualitative–quantitative compositional analysis was carried out using high performance liquid chromatography coupled with photodiode array detection. The lemon balm extract contained hydroxycinnamic acid derivatives and flavonoids with caffeic acid, *m*-coumaric acid, eriodictyol-7-*O*-glucoside, naringin, hesperidin, rosmarinic acid, naringenin, hesperetin being identified based on their chromatographic behaviour and spectral characteristics. The extract was also investigated for potential *in vitro* antioxidant properties in iron(III) reduction, iron(II) chelation, 1,1-diphenyl-2-picrylhydrazyl, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate), superoxide anion and nitric oxide free-radical scavenging, and inhibition of β-carotene–linoleic acid bleaching assays. The extract demonstrated antioxidant activity in all the assays. However, it was not as potent as the positive controls except in the β-carotene–linoleic acid bleaching assay, where its activity was superior to that of gallic and caffeic acids and statistically indistinguishable from quercetin and BHA. The exceptionally high antioxidant activity and the fact that this assay is of biological relevance warrants further investigation of lemon balm extract in *ex vivo* and *in vivo* models of oxidative stress.

Keywords: Lemon balm; *Melissa officinalis*; Antioxidants; High performance liquid chromatography; Free radicals

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism and at low-to-moderate concentrations they are known to possess various physiological roles ranging from cellular signal transduction to defence against pathogens (Valko, Leibfritz, Moncol, & Cronin, 2007). However, during oxidative stress there is an overproduction of ROS and RNS on one side and a deficiency of enzymatic and non-enzymatic antioxidant defence system on the other, with the result that the excess reactive species produced bring about degradation of cellular components, viz., DNA, carbohydrates, proteins and lipids. This will eventually lead to cellular dysfunction and ultimately cell death. That is why oxidative stress has been proposed to play a cardinal role in the pathogenesis of many diseases as well as in the ageing process (Halliwell & Gutteridge, 1989a; Valko et al., 2007).

Recently there has been a growing interest in the search for natural antioxidants for three principal reasons (Dastmalchi, Dorman, Koşar, & Hiltunen, 2007): (i) numerous clinical and epidemiological studies have demonstrated that consumption of fruits and vegetables is associated with reduced risks of developing chronic diseases such as cancer, cardiovascular disorders and diabetes; (ii) safety consideration regarding the potential

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0023-6438/$30.00
doi:10.1016/j.lwt.2007.03.007

harmful effects of the chronic consumption of synthetic antioxidants in foods and beverages; and (iii) the public's perception that natural and dietary antioxidant are safer than synthetic analogues. The result has been an increased interest in spices, aromatic and medicinal plants as sources of natural antioxidants.

Lemon balm (*Melissa officinalis* L.) belongs to the family Lamiaceae and grows widely in central and southern Europe and in Asia minor (Zargari, 1990); however, it is cultivated throughout the world because of its culinary properties. In Iran, this plant is known locally by the names Badranjbooye, Varangboo and Faranjmoshk and is found in the north, north-west and western parts of the country (Anon, 2002). It is used in the Iranian traditional system of medicine for the treatment of headaches, flatulence, indigestion, colic, nausea, nervousness, anaemia, vertigo, syncope, malaise, asthma, bronchitis, amenorrhea, cardiac failure, arrhythmias, insomnia, epilepsy, depression, psychosis, hysteria, ulcers and wounds (Anon, 2002; Zargari, 1990).

Although lemon balm antioxidant studies have been carried out, studies reporting upon the antioxidant activity of polar extracts of lemon balm are limited (Hohman, Zupko, Redei, & Csanyi, 1999; Ivanova, Gerova, Chervenkov, & Yankova, 2005; Triantaphyllou, Blekas, & Boskou, 2001; Venkutonis, Gruzdiene, Trizite, & Trizite, 2005). As natural antioxidants can exert their effect via a variety of different mechanisms, it is important to assess fully their different modes of action and this is not possible by conducting few assays (Part, 1999). Therefore, as a part of our on going antioxidant research on spices and herbs, an aqueous ethanolic extract of the plant was screened for its antioxidant properties in a battery of *in vitro* assays. Furthermore, because it is important to determine the constituents present in the extract which may be contributing to the activity, a compositional fingerprint analysis was carried out using high performance liquid chromatography coupled to photodiode array detector (HPLC–PDA).

2. Materials and methods

2.1. Chemicals

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonate) diammonium salt (ABTS), ascorbic acid, bovine brain extract (type VII), β-carotene, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 2-deoxy-D-ribose, 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$), disodium salt of ethylenediamine tetraacetic acid (EDTA), ferrozine, Folin–Ciocalteu reagent, gallic acid, hypoxanthine, iron(III) chloride, iron(II) chloride, linoleic acid, nitroblue tetrazolium (NBT), phosphate-buffered saline (PBS) tablets, potassium peroxosulfate, trichloroacetic acid (TCA), Trizma base and Trolox were from Sigma Chemical Company (Germany). Potassium hydroxide, dibasic potassium phosphate, sodium carbonate and dibasic sodium phosphate were from Merck (Germany). Potassium hexacyanoferrate, sodium hydroxide, trifluoroacetic acid and Tween 20 were from Fluka BioChemika (Germany). The chromatographic standards were obtained from Extrasynthase (France). All the solvents were of HPLC grade and were purchased from Rathburn chemicals (UK). Ultrapure water was prepared using a Millipore Milli-RO 12 plus system (Millipore Crop., Bedford, MA, USA).

2.2. Plant material

Lemon balm (Specimen No. GeLM175) was obtained from the Institute of Medicinal Plants, Iranian Academic Centre for Education, Culture and Research (Halejerd, Iran). The taxonomic identity of the plants was confirmed by a botanist from the Department of Cultivation and Development of the Institute of Medicinal Plant (Tehran, Iran).

2.3. Extraction

The air-dried leaves of lemon balm obtained from the Institute of Medicinal Plants were extracted with 450 ml/l aqueous ethanol using medium pressure liquid–solid extraction. The column used was manufactured by Büchi laboratoriums-Tecnik Ag. (Flawil, Switzerland) and the ratio of material to the extractant was 1:5.4 (w/v). The incubation period with the extractant and the interval between the runs was of 3 h and the time taken for each run was 45 min at a flow rate of 3 ml/min. The plant material was extracted in two batches of 200 and 250 g and the number of extraction runs were 8 and 10, respectively. Finally extracts from all the runs were combined, filtered, reduced in vacuo (45 °C), freeze-dried and stored at 4 °C.

2.4. Determination of total phenol content

Total phenol content was estimated as gallic acid equivalents (GAE; mg gallic acid/g extract) as described by Singleton, Orthofer, and Lamuela-Raventós (1999). In brief, a 100 µl aliquot of dissolved extract was transferred to a 10 ml volumetric flask, containing ca. 6.0 ml H_2O, to which was subsequently added 500 µl Folin–Ciocalteu reagent. After 1 min, 1.5 ml 200 g/l Na_2CO_3 was added and the volume was made up to 10 ml with H_2O. After 2 h of incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve.

2.5. Qualitative–quantitative HPLC analysis

The HPLC system (Waters 600) consisted of an in-line degasser pump and controller coupled to a 2996 PDA detector equipped with a 717 autosampler (20 µl injection volume) interfaced to a PC running Millennium32 software (Waters Corporation, Milford, MA, USA). Separations were performed on a 250 × 4.6 mm i.d., 5 µm reverse-

phase Hypersil BDS-C18 analytical column (Agilent Technologies, Santa Clara, CA, USA) operating at room temperature with a flow rate of 1 ml/min. Detection was carried out with a sensitivity of 0.1 aufs between the wavelengths of 200 and 550 nm. Elution was done by using a binary non-linear gradient of the solvent mixture 0.2 g/l aqueous trifluoroacetic acid (solvent A) and MeOH (solvent B). The composition of the mobile phase 80:20 (A/B), remained unchanged for 5 min, and then it was changed to 70:30 (A/B) in 5 min, changed to 55:45 (A/B) in 10 min, held for 5 min, changed to 20:80 (A/B) in 20 min, held for 10 min, and then returned to initial condition in 5 min. A 20 min equilibrium time was allowed between the injections. Components were identified by comparison of their retention times to those of authentic standards under identical analysis conditions and by comparison of their UV spectra with an in-house PDA library.

Stock solutions of the extract and standards were prepared in 450 ml/l aqueous ethanol and 700 ml/l aqueous methanol to final concentration of 10 and 1 mg/ml, respectively. The concentration range used for calibration of the standard compounds was 0.001–0.10 mg/ml. The standards and samples were injected in duplicate.

2.6. Iron(III) to iron(II) reduction

The iron(III) reductive capacity of the extract was assessed spectrophotometrically according to the method of Oyaizu (1986). In brief, 1 ml of dissolved extract was mixed with 2.5 ml phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of a 10 g/l potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. After 30 min at 50 °C, 2.5 ml of a 100 g/l aqueous TCA solution was added and the mixture was centrifuged for 10 min (1800 rpm). Finally, a 2.5 ml aliquot was mixed with 2.5 ml ultra-pure water and 0.5 ml of a 1 g/l $FeCl_3$ solution and the absorbance was recorded at 700 nm. The data are presented as ascorbic acid equivalents (AscAE; mmol ascorbic acid/g sample).

2.7. Iron(II) chelation

The ability of the extract to chelate iron(II) was assessed by the method of Carter (1971). In brief, to a 200 μl of dissolved extract was added 100 μl (2.0 mmol/l) $FeCl_2 \cdot 4H_2O$ and 900 μl methanol. After a 5-min incubation, the reaction was initiated by the addition of 400 μl (5.0 mmol/l) ferrozine. After a further 10 min incubation period, the absorbance at 562 nm was recorded. The percentage chelating activity was calculated using Eq. (1) and the concentration at which the extract exerts 50% of its effect (EC_{50}) values were estimated by a linear regression algorithm.

$$\text{Percentage inhibition} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100. \quad (1)$$

2.8. DPPH$^\bullet$ free-radical scavenging

The ability of the extract to scavenge DPPH$^\bullet$ radicals was determined as described by Gyamfi, Yonamine, and Aniya (1999). In brief, a 50 μl aliquot of dissolved extract was mixed with 450 μl Tris–HCl buffer (50 mmol/l, pH 7.4) and 1.0 ml (0.1 mmol/l) DPPH$^\bullet$ dissolved in methanol. After a 30-min reaction period, the resultant absorbance was recorded at 517 nm. The percentage inhibition was calculated using Eq. (1) and the concentration of the extract at which it exhibits 50% inhibition (IC_{50}) value was estimated using a non-linear regression algorithm.

2.9. ABTS$^{\bullet+}$ free-radical scavenging

The ability of the extract to scavenge ABTS$^{\bullet+}$ radical scavenging was determined by the method of Re et al. (1999). The ABTS$^{\bullet+}$ radical was generated by reacting an (7 mmol/l) ABTS aqueous solution with $K_2S_2O_8$ (2.45 mmol/l, final concentration) in the dark for 12–16 h, at ambient temperature, and adjusting the $\text{Abs}_{734\ \text{nm}}$ to 0.700 (± 0.020) with ethanol. The samples were diluted, such that a 15 μl sample when added to 1.485 ml ABTS$^{\bullet+}$ resulted in a 20–80% inhibition of the blank absorbance. After 1.485 ml ABTS$^{\bullet+}$ solution was added to 15 μl sample, the absorbance at 734 nm was recorded 1 min after initial mixing and subsequently at 5 min intervals (40 min, in toto). The results are expressed as the Trolox equivalent antioxidant capacity (TEAC, mmol/l Trolox) at different time intervals.

2.10. β-Carotene–linoleic acid bleaching inhibition

The ability of the extract to inhibit the bleaching of the β-carotene–linoleic acid emulsion was determined using a modification of the method described by Koleva, van Beek, Linssen, de Groot, and Evstatieva (2002). In brief, 0.2 mg β-carotene dissolved in 1 ml chloroform, 20 mg of linoleic acid and 200 mg of Tween 20 were transferred into a round-bottom flask. Once the chloroform had been removed under nitrogen, 50 ml distilled H_2O was added and the resulting mixture was vigorous stirred for 30 min. Thereafter, 5 ml aliquots of this emulsion were transferred to tubes containing either 200 μl of dissolved extract (1 mg/ml) or 200 μl of positive controls (1 mg/ml). After mixing, the absorbance (Abs_0) at 470 nm was recorded. The remaining samples were placed in a water bath at 50 °C for a period of 2 h. Thereafter, the absorbance of each sample was remeasured at 470 nm (Abs_{120}). The data ($n = 2$) are presented as antioxidant activity % (AA%) values, calculated using

$$\text{AA\%} = [1 - (\text{Abs}_0\text{sample} - \text{Abs}_{120}\text{sample})/(\text{Abs}_0\text{control} - \text{Abs}_{120}\text{control})] \times 100. \quad (2)$$

2.11. Superoxide anion radical scavenging

The ability of the extract to scavenge superoxide anion radicals was determined by the method described by Lee, Kim, Kim, and Jang (2002). In brief, to a 100 μl aliquot of dissolved extract the following was added: 100 μl (30 mmol/l) Na_2EDTA, 100 μl (3 mmol/l) hypoxanthine in 50 mmol/l NaOH and 200 μl (1.42 mmol/l) NBT in NaH_2PO_4–NaOH (50 mmol/l, pH 7.4). After a 3-min incubation period at room temperature, 100 μl (0.5 U/ml) xanthine oxidase in the NaH_2PO_4–NaOH buffer was added followed 2.4 ml NaH_2PO_4–NaOH buffer. The resulting solution was incubated at room temperature for 20 min and the absorbance at 560 nm was measured. The absorbance was also measured at 293 nm to detect if the extract inhibited uric acid generation. Once it was confirmed that uric acid formation is not inhibited, then the percentage inhibition at 560 nm was calculated using Eq. (1) and IC_{50} values were estimated using a non-linear regression algorithm.

2.12. Nitric oxide radical scavenging

The ability of the extract to scavenge nitric oxide free radicals was determined using a modification of the method described by Marcocci, Maguire, Droy-Lefaix, and Packer (1994). In brief, a 0.5 ml aliquot of extract (1 mg/ml) or positive control (1 mg/ml) dissolved in KH_2PO_4–KOH (50 mmol/l, pH 7.4) was mixed with 0.5 ml of (10 mmol/l) sodium nitroprusside solution. The mixture was incubated at 37 °C for 2.5 h under normal light condition. After incubation the sample was placed in dark for 20 min. Thereafter, 1 ml of Griess reagent (1 g/l *N*-(1-naphtyl)ethylenediamine and 10 g/l sulphanilamide dissolved in 20 ml/l aqueous H_3PO_4) was added and the absorbance was taken after 40 min at 546 nm. The percentage inhibition was calculated using Eq. (1).

2.13. Statistical analysis

Data are presented as mean values ±95% confidence interval. Analysis of variance was performed using ANOVA procedures. Significant differences between means were determined by Tukey's pairwise comparison test at a level of $P<0.05$. IC_{50} values were estimated using a non-linear regression algorithm unless otherwise stated.

3. Results and discussion

3.1. Extract yield and total phenol content

The extract yield was 308.7 mg/g (dry wt.) plant material, with a total phenol content estimated as 268.9 ± 21.3 mg gallic acid/g (dry wt.) extract (Table 1).

Table 1
Extract yield, total phenol content and HPLC–PDA qualitative–quantitative analysis of the lemon balm extract

Sample	EY[c]	TP[d]	Identified components[a]							Unidentified components[b]		
			CA[e] (16.0)[f]	Erio-glu (23.5)	m-CA (25.0)	Nar-rham (26.7)	Hesp-rutin (27.5)	RA (28.9)	Naring (36.7)	Hesp (29.3)	∑HCA	∑Fl	∑
L. balm	308.7	268.9 ± 21.3	4.05 ± 0.00	2.26 ± 0.05	0.23 ± 0.01	0.60 ± 0.01	9.31 ± 0.14	96.45 ± 0.13	1.14 ± 0.01	7.46 ± 0.04	70.14 ± 0.25	8.26 ± 0.26	199.89 ± 0.41

[a] Values (mg/g dry extract) are mean values ± standard deviation ($n = 2$).
[b] ∑HCA, sum of hydroxycinnamic acid derivatives quantified using caffeic acid, ∑Fl, sum of flavonoids quantified using naringin.
[c] EY, extract yield expressed as mg extract/g (dry wt.) plant material.
[d] TP, total phenol content expressed as mg gallic acid/g (dry wt.) extract ($n = 2$).
[e] CA, caffeic acid; Erio-glu, eriodictyol-7-*O*-glucoside; *m*-CA, *m*-coumaric acid; Nar-rham, naringenin-7-*O*-rhamnoglucoside (Naringin); Hesp-rutin, hesperetin-7-*O*-rutinoside (Hesperidin); RA, rosmarinic acid; Naring, Naringenin; Hesp, hesperetin.
[f] Retention time (min).

3.2. Qualitative–quantitative HPLC analysis

The data from the qualitative–quantitative analysis of the lemon balm extract made using HPLC coupled with photodiode array detection, is presented in Table 1, while the chromatogram with detector responses at 280 and 330 nm overlaid are presented in Fig. 1. The components caffeic acid, eriodictyol-7-*O*-glucoside, *m*-coumaric acid, naringin, hespiridin, rosmarinic acid, naringenin, hesperetin (Fig. 2) were identified by comparisons to the retention times and UV spectra of authentic standards analysed under identical analytical conditions, while the quantitative data was calculated from their respective calibration curves. The major component present in the plant extract was identified as rosmarinic acid (96.45 ± 0.13 mg/g), which is in accordance with the previous lemon balm studies and other taxa of the Lamiaceae family (Carnat, Carnat, & Lamaison, 1998; Dorman, Bachmayer, Koşar, & Hiltunen, 2003; Zgórka & Glowniak, 2006; Ziaková & Brandšteterova, 2002, 2003). The least abundant compound present in the extract was *m*-coumaric acid (0.23 ± 0.01 mg/g). The flavonoids identified ranged from naringin (0.60 ± 0.01 mg/g) to that of hespiridin (9.31 ± 0.13 mg/g). There were compounds which could not be identified; however, based on their chromatographic behaviour and UV spectra their chemical class was determined as hydroxycinnamic acid derivatives and flavonoids.

From the compounds identified, rosmarinic and caffeic acids have already been reported to be present in the plant by other researchers (Carnat et al., 1998; Zgórka & Glowniak, 2006; Ziaková & Brandšteterova, 2002, 2003). Although the remaining phenolic components have not been reported previously, their presence in other species of the Lamiaceae family has been confirmed (Dastmalchi et al., 2007; Dorman, Bachmayer et al., 2003; Dorman, Koşar, Kahlos, Holm, & Hiltunen, 2003). In a number of compositional studies reported on this plant, benzoic acid derivatives, luteolin-7-*O*-glucoside and other glycosides of luteolin have been detected. The absence in this sample may be due to a variety of reasons ranging from climate and geography to difference in the specificity of the extraction procedures used (Carnat et al., 1998; Heitz, Carnat, Fraisse, & Carnat, 2000; Zgórka & Glowniak, 2006; Ziaková & Brandšteterova, 2002, 2003).

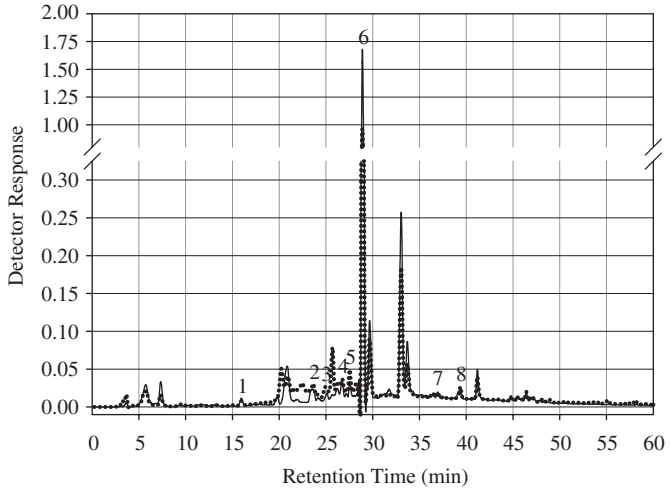

Fig. 1. HPLC–PDA analysis of the extract of lemon balm with detector responses at 280 (dotted line) and 330 nm (straight line) overlaid. 1, caffeic acid; 2, eriodictyol-7-*O*-glucoside; 3, *m*-coumaric acid; 4, naringin; 5, hesperidin; 6, rosmarinic acid; 7, naringenin and 8, hesperetin.

The amount of total phenolics calculated from the data obtained by compositional analysis (199.89 ± 0.41 mg/g) was less than that estimated by Folin–Ciocalteu method (268.9 ± 21.3 mg/g), which can be explained due to the limitation of the later assay i.e. the fact that other substances in addition to phenolic substance can participate in the assay reaction (Singleton et al., 1999).

3.3. Iron(III) to iron(II) reduction

Antioxidative activity has been proposed to be related to reducing power (Duh, 1998). Therefore, in order to assess the electron-donating power of lemon balm extract, its ability to reduce iron(III) was investigated. The results of iron(III) reduction are presented as AscE values, the higher the AscE value the greater the electron-donating power of the sample (Fig. 3A). The lemon balm extract was capable of reducing iron(III) ions and did so in a linear concentration-dependent manner (data not shown). On the basis of AscE values, the hierarchy of the activity was gallic acid (8.26 ± 0.30 mmol ascorbic acid/g) > ascorbic acid (5.68 ± 0.10 mmol ascorbic acid/g) > quercetin (4.92 ± 0.13 mmol ascorbic acid/g) > BHA (2.26 ± 0.05 mmol ascorbic acid/g) > lemon balm (1.47 ± 0.05 mmol ascorbic acid/g).

The results suggest that the plant extract has the ability to donate electrons, thus it can scavenge free radicals. However, this activity was less compared to the positive controls, which very much follows the trend observed in case of extracts of Lamiaceae plants (Dastmalchi et al., 2007; Dorman, Koşar et al., 2003). The reason is believed to be that the extracts contain antioxidant compounds at a much lower concentration than when they are used as positive controls.

3.4. Iron(II) chelation

One of the mechanisms of antioxidative action is chelation of transition metals, thus preventing catalysis of hydroperoxide decomposition and Fenton-type reactions (Gordon, 1990). Therefore, the ability of lemon balm extract to chelate iron(II) was investigated. As can be seen from the data presented in Fig. 3B, the extract was capable of chelating iron(II) and did so in a concentration-dependent manner (data not shown). The extract was a better chelator of iron(II) compared to ascorbic acid, gallic acid and BHA. The EC_{50} values for these substances could not be calculated due to the fact that they were not able to exert more than 50% chelating activity. The chelating

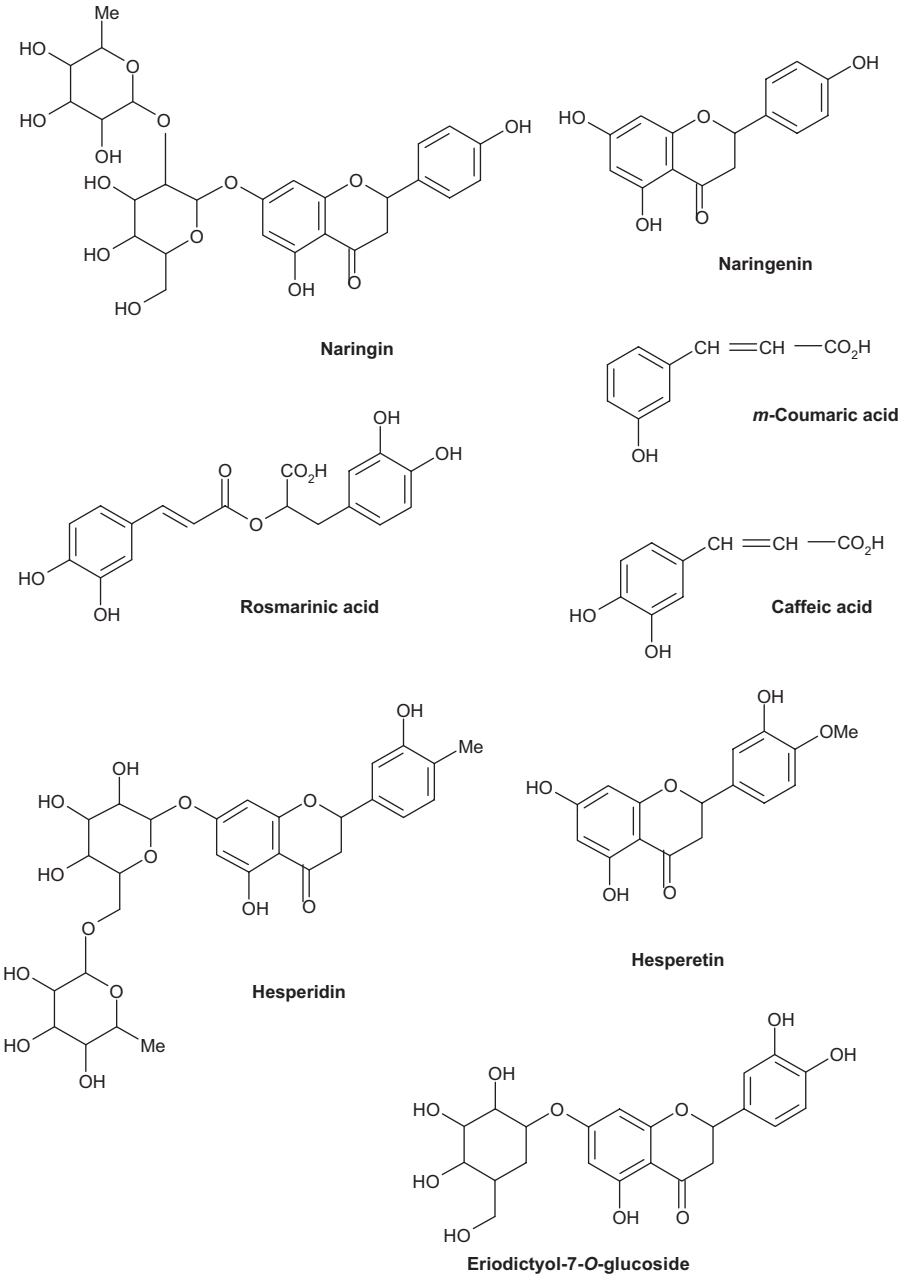

Fig. 2. Structural formulas of the compounds identified.

activity of EDTA (184.71 ± 2.01 μg/ml) was significantly higher ($P < 0.05$) than that of the plant extract (7562.85 ± 177.99 μg/ml). Therefore it can be said that the extract has mild chelating activity.

The relatively mild iron(II) chelating activity of the plant extract is of great significance, because it has been proposed that the transition metal ions contribute to the oxidative damage in neurodegenerative disorders, like Alzheimer's and Parkinson's diseases and one of the lines of treatments currently under investigation is selective low-affinity binding of transition metals (Bush, 2003; Vardarajan, Yatin, Aksenova, & Butterfield, 2000). Therefore, if the plant extract will also display mild chelating activity in *in vivo* studies then it can be of therapeutic potential in the treatment of diseases mentioned above; however, *in vivo* studies would be required to confer this.

The chelating activity of lemon balm extract can be of potential interest in the food industry where transition metal ions, by catalysing the initiation and decomposition of hydroperoxides, contribute to lipid oxidation which is the main source of degradation of food products (Antolovich, Prenzler, Pastalides, & Donald, 2002).

3.5. $DPPH^{\bullet}$ free-radical scavenging

Oxidative damage within the cellular systems is a multistep process involving free-radical chain initiation and propagation steps (Halliwell & Gutteridge, 1989b;

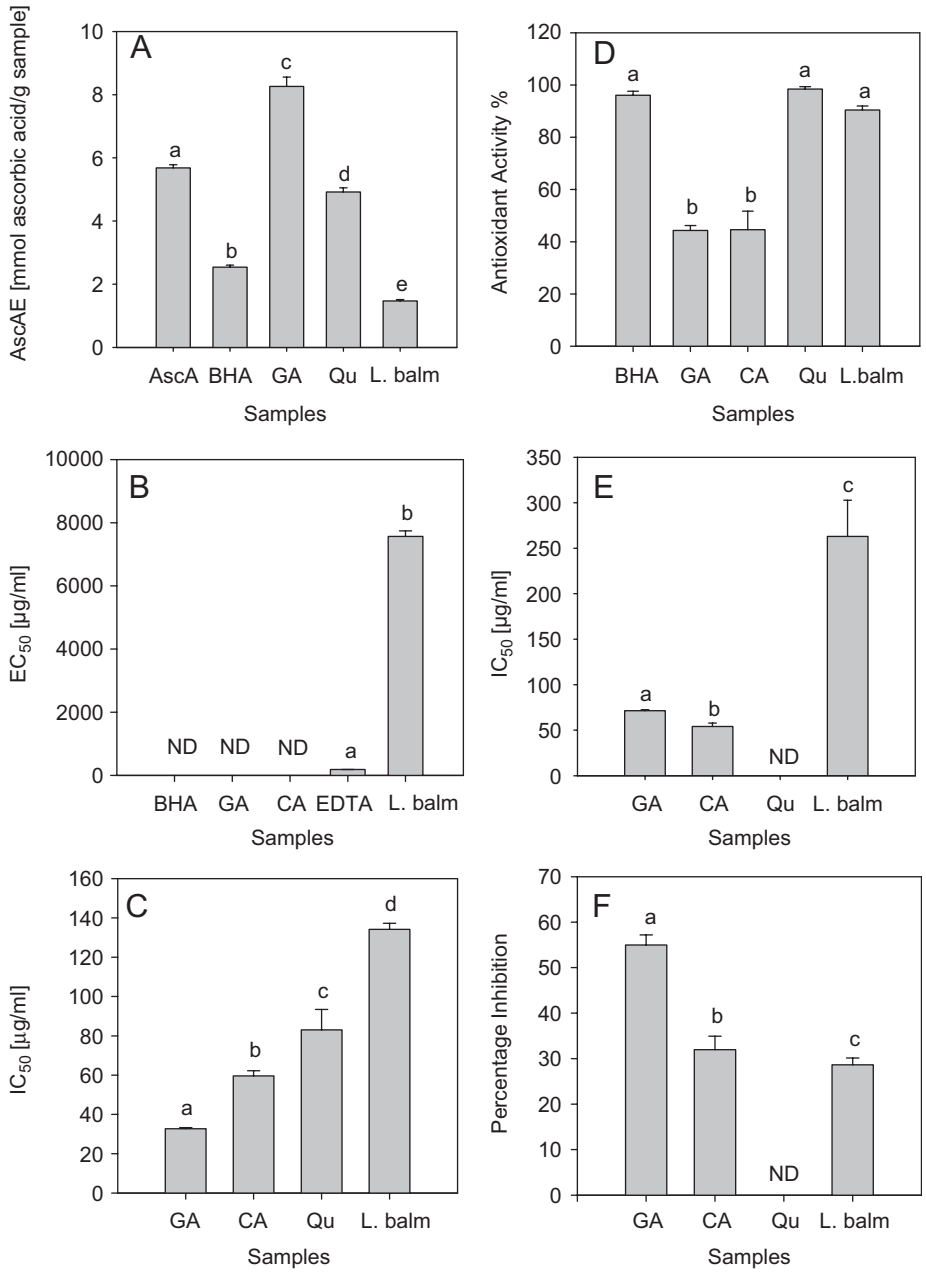

Fig. 3. The effect of the plant extract and positive controls upon (A) iron(III) reduction, (B) iron(II) chelation, (C) DPPH$^{\bullet}$ radical scavenging, (D) β-carotene–linoleic acid bleaching, (E) superoxide anion radical scavenging and (F) nitric oxide scavenging. AscA, Ascorbic acid; BHA, butylated hydroxy anisole; GA, gallic acid; CA, caffeic acid; Qu, quercetin; l. balm, Lemon balm. Values are presented as means $\pm$95% confidence intervals ($n = 4$, except for β-carotene–linoleic acid bleaching data, where $n = 2$). Bars with the same lower case letters (a-e) are not statistically ($P > 0.05$) different. ND, not determined.

Valko et al., 2007). One of the mechanisms by which antioxidants bring about their action is by scavenging free radicals (Dastmalchi et al., 2007). Hence, it is important to assess the scavenging ability of the lemon balm extract. This was estimated using nitrogen-centered free radical (DPPH$^{\bullet}$). This free radical serves both as an oxidising substrate and as the reaction indicator molecule.

The plant extract was capable of scavenging DPPH$^{\bullet}$ free radicals and did so in a concentration-dependent manner (data not shown). Based on the calculated IC_{50} values presented in Fig. 3C, the order of the activity is as follows: gallic acid (32.72 ± 0.6 µg/ml) > caffeic acid (59.6 ± 2.6 µg/ml) > quercetin (83.0 ± 10.5 µg/ml) > lemon balm (134.16 ± 3.09 µg/ml).

The DPPH$^{\bullet}$ scavenging data suggests that the extract is capable of scavenging free radicals at physiological pH; thus preventing the initiation and propagation of free-radical-mediated chain reactions which contribute to the oxidative stress. This can be of beneficial interest in preservation of foodstuffs, drug products and cosmetics, where free-radical-mediated chain reactions result in lipid oxidation and subsequent deterioration of these products.

The plant extract may also prove to have therapeutic potential, as free radicals are believed to be involved in the pathogenic cascade of events in many diseases.

3.6. $ABTS^{\bullet+}$ free-radical scavenging

Perhaps $DPPH^{\bullet}$ scavenging is the most commonly used method for assessment of free-radical scavenging activity of natural products; however, due to certain shortcomings with this method, an alternative assay based on scavenging of $ABTS^{\bullet+}$, a moderately stable nitrogen center radical, was used (Koleva et al., 2002). The $ABTS^{\bullet+}$ free-radical scavenging overcomes the limitations of the former method such as solubility, problems of spectral interference. The $ABTS^{\bullet+}$ model is more versatile as both the polar and non-polar samples can be assessed for their scavenging activity and the spectral interference is minimised as the absorption maximum used is 760 nm a wavelength not normally encountered by natural products Re et al. (1999). In addition with the help of this method is possible to monitor the activity of the sample over a specified period of time. In this way samples having different rates of antioxidant activity can be investigated.

The antioxidant activity is presented in the form of TEAC values presented in Table 2. From the presented data, it can be seen that the hierarchy of activity at 1 min was as gallic acid > BHA > caffeic acid > quercetin > lemon balm. However, the order of activity changed after 10 min to gallic acid > BHA > caffeic acid, quercetin (not significantly different, $P > 0.05$) > lemon balm.

As it can be seen from the TEAC data in Table 2, the scavenging activity of the extract increased up to 30 min after which there was no significant change in the activity ($P > 0.05$). The TEAC values of caffeic acid decreased up to 10 min, after which they did not change significantly ($P > 0.05$). However, in case of quercetin there was an opposite trend, an initial increase up to 10 min and then there was no significant change ($P > 0.05$). Looking at the TEAC data it is apparent there was no significant change in the scavenging activity of BHA and gallic acid throughout the assay ($P > 0.05$).

It can be deduced from the result that the plant extract unlike the positive controls is a long acting free-radical scavenger. However, throughout the period of the assay (45 min) the scavenging activity is less than that of the positive controls, which is an indication that lemon balm has a mild scavenging activity. The TEAC value calculated for lemon balm is different from that recently reported by Ivanova et al. (2005). The reason for the difference in the TEAC values can lie in the different extraction procedures of the plant. The higher TEAC value reported for the water infusion of the plant can be due to the higher content of phenolic substances, which have been extracted.

3.7. β-Carotene–linoleic acid bleaching inhibition

Although the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ free-radical scavenging assays are an indicator of potential antioxidant activity, neither assays use a biologically relevant matrix. Therefore, in order to simulate oxidation of membrane lipid oxidation, the authors decided that it would be of use to utilise the β-carotene–linoleic bleaching inhibition assay which is considered to be a good model for membrane-based lipid peroxidation (Ferreria, Proença, Serralheiro, & Araújo, 2006). In this oil-water emulsion-based system, linoleic acid undergoes thermally induced oxidation, thereby producing free radicals that attack the β-carotene's chromophore resulting in a bleaching effect (Koleva et al., 2002). An extract that inhibits β-carotene bleaching can be described as a free-radical scavenger and a primary antioxidant (Liyana-Pathirana & Shahidi, 2006).

As can be seen from the data presented in Fig. 3D, the lemon balm extract demonstrated an ability to inhibit the bleaching of β-carotene by scavenging linoleate-derived free radicals. It is important to note that the antioxidant activity of the extract (90.43 ± 1.55 μg/ml) was statistically superior ($P > 0.05$) to that of gallic and caffeic acids ($44.29 \pm 1.92\%$ and $44.59 \pm 7.10\%$ respectively) and was statistically indistinguishable ($P > 0.05$) from that of quercetin ($98.46 \pm 0.89\%$) and BHA ($96.08 \pm 1.58\%$).

The strong antioxidant effect of the less polar compounds, quercetin and BHA, and the plant extract, which is

Table 2
Effect of the lemon balm extract and positive controls on $ABTS^{\bullet+}$ radical scavenging

Sample	TEAC[a] (mmol/l Trolox)				
	1 min	10 min	20 min	30 min	40 min
BHA	5.83 ± 0.36 a,A	6.08 ± 0.39 a,A	6.20 ± 0.38 a,A	6.23 ± 0.38 a,A	6.27 ± 0.38 a,A
Gallic acid	11.99 ± 2.33 b,A	12.94 ± 2.18 b,A	13.22 ± 2.13 b,A	13.37 ± 2.11 b,A	13.51 ± 2.06 b,A
Caffeic acid	4.87 ± 0.05 c,A	4.64 ± 0.01 c,B	4.63 ± 0.01 c,B	4.61 ± 0.01 c,B	4.61 ± 0.01 c,B
Quercetin	3.95 ± 0.15 d,A	4.61 ± 0.03 c,B	4.63 ± 0.01 c,B	4.61 ± 0.01 c,B	4.62 ± 0.01 c,B
L. balm	1.40 ± 0.08 e,A	1.60 ± 0.09 d,A,B,C	1.72 ± 0.10 d,B,C	1.79 ± 0.10 d,C	1.84 ± 0.11 d,C

Data are presented as mean values ± 95% confidence limits ($n = 4$). Analysis of variance was performed by ANOVA procedures, with significant differences between means determined by Tukey's pairwise comparisons. Values with the same lower case letters (a–e) within each column and upper case letters (A–C) within each row are not significantly ($P > 0.05$) different.

[a]Trolox equivalent antioxidant capacity (TEAC) is defined as the concentration of Trolox in mmol/l having the $ABTS^{\bullet+}$ scavenging activity equal to a 1.0 mg/ml sample solution.

of heterogeneous nature, may be explained by the 'polar paradox' phenomenon as described by Frankel, Huang, Kanner, and German (1994). It has been proposed that apolar antioxidants exhibit stronger antioxidant properties in oil in water emulsions, because they get concentrated at the oil–water interface, thus protecting the lipids from oxidation. On the other hand, the polar antioxidants remain diluted in the bulk (aqueous) phase and thus are less effective in protecting the lipids.

The high antioxidant activity exhibited by the lemon balm extract in the current assay suggests that it has a potential for use in foods containing emulsified oils. The antioxidant effect of the extract may also be of biological relevance as it may prevent oxidation of lipid components within cell membranes. Therefore, the plant extract may prove to be of potential health benefit.

3.8. Superoxide anion radical scavenging

The superoxide anion radical ($O_2^{\bullet-}$) is the most common ROS formed *in vivo*. Although it is a relatively unreactive per se, during oxidative stress it forms more reactive species either directly by interacting with other ROS and RNS or indirectly through enzyme or metal-catalysed processes (Liu & Ng, 2000). Therefore, the $O_2^{\bullet-}$ scavenging activity of lemon balm extract was investigated. The plant extract exhibited $O_2^{\bullet-}$ scavenging activity and did so in a concentration-dependent manner (data not shown). As it can be seen from Fig. 3E, the IC_{50} value calculated for the plant extract ($262.87 \pm 39.90\,\mu g/ml$) was less than that of gallic acid ($71.54 \pm 1.06\,\mu g/ml$) and caffeic acid ($54.17 \pm 3.67\,\mu g/ml$). Quercetin had a poor scavenging activity and its IC_{50} could not be detected.

The results show that lemon balm extract has $O_2^{\bullet-}$ scavenging activity which can be of potential health interest as it may be effective in reducing the level of $O_2^{\bullet-}$ which is elevated during oxidative stress in the body. $O_2^{\bullet-}$-mediated oxidative stress is believed to be involved in the pathogenesis of cardiovascular disorders, diabetes mellitus, acute respiratory distress syndrome, neurodegenerative disorders like Alzheimer's and Parkinson's diseases.

The ability of the samples to inhibit uric acid generation was also investigated. None of the samples inhibited uric acid formation (data not shown), which indicates the samples did not prevent $O_2^{\bullet-}$ generation by inhibiting the enzyme xanthine oxidase.

3.9. Nitric oxide ($NO^{\bullet}$) radical scavenging

The nitric oxide radical ($NO^{\bullet}$), which is produced *in vivo* by variety of cell types, is an important bioregulatory molecule with a number of physiological functions (Law, Gauthier, & Quirion, 2001; Valko et al., 2007). However, under oxidative stress this RNS reacts with other reactive species to produce more toxic RNS and ROS (Valko et al., 2007). Therefore, the scavenging effect of lemon balm extract was assessed against this radical. It can be seen from the data presented in Fig. 3F, that the extract of lemon balm was capable of scavenging $NO^{\bullet}$. Based on the percentage inhibition values, the extract ($28.64 \pm 1.53\%$) was less active than the positive controls, gallic acid ($54.99 \pm 2.18\%$) and caffeic acid ($31.96 \pm 2.98\%$). Quercetin did not exhibit any scavenging activity.

The $NO^{\bullet}$ scavenging activity of the extract is of potential health interest as it has been proposed that $NO^{\bullet}$ plays an important role in the progression of many diseases and pathological conditions such as septic shock, atherosclerosis, ischaemia reperfusion, neurodegenerative disorders like Alzheimer's and Parkinson's diseases, cancer and diabetes (Law et al., 2001; Moncada, Palmer, & Higgs, 1991; Tamir & Tannenbaum, 1996; Valko et al., 2007; Vardarajan et al., 2000).

4. Conclusions

The antioxidative data presented in this study clearly demonstrate that the lemon balm extract is capable of scavenging a wide range of synthetic and naturally occurring free radicals. This is of significant importance as it indicates that the extract may have the potential to prevent oxidative damage *in vivo* by preventing free-radical-mediated oxidative stress. Another important finding was the iron(II) chelating activity of the extract, thus increasing its antioxidant potential. This activity is of significant value in the pharmacological investigation for the treatment of Alzheimer's and Parkinson's diseases.

In the β-carotene–linoleic acid bleaching assay, which simulates biologically relevant medium, lemon balm extract showed exceptionally high antioxidant activity. Therefore, it is suggested that the efficacy of the plant extract to be assessed in *ex vivo* and *in vivo* models of diseases, where oxidative stress is believed to play an important part in their pathogenesis.

The compositional finger print analysis reveals that the phenolic substances present in the plant are in the form of flavonoids and hydroxycinnamic acid derivatives. These compounds have proven to possess antioxidant activity; therefore, it is likely that they are contributing to the antioxidant properties of the extract. However, there is a need for activity-guided fractionation in order to isolate the compounds which are responsible for the antioxidant activity of the extract.

Acknowledgements

The authors gratefully acknowledge the Institute of Medicinal Plant, Iranian Academic Centre for Education, Culture and Research, Tehran, Iran for providing the plant material. Also we thank Mar Santamaria and Elisenda Sabate for their technical assistance. KD acknowledges the financial support of the Helsinki Research Fundation while PPO acknowledges the Academy of Finland (grant for Center of Excellence on Microbial Resources; Project no.

53305) and the Finnish Cultural Foundation for financial support.

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