Systematic comparison of nutraceuticals and antioxidant potential of cultivated, *in vitro* cultured and commercial *Melissa officinalis* samples

MARIA INÊS DIAS,[a,b] LILLIAN BARROS,[a,b] MARIA JOÃO SOUSA,[b]

ISABEL C.F.R. FERREIRA[a,b,*]

[a]Mountain Research Centre, CIMO-ESA, *Campus* de Santa Apolónia, Apartado 1172, 5301-854 Bragança, Portugal.

[b]School of Agriculture, Polytechnic Institute of Bragança, *Campus* de Santa Apolónia, Apartado 1172, 5301-854 Bragança, Portugal.

* Author to whom correspondence should be addressed (e-mail: iferreira@ipb.pt telephone +351-273-303219; fax +351-273-325405).

Abstract

Melissa officinalis (lemon balm) infusions are used worldwide for digestive, analgesic and other pharmaceutical applications. Herein, the nutraceuticals production and antioxidant potential in garden cultivated, *in vitro* cultured and two commercial samples (bags and granulated) of lemon balm was compared. The profile of *in vitro* cultured lemon balm is closer of garden cultivated sample than of both commercial samples (bag or granulate). It presented the highest levels of proteins and ash, and the lowest energetic value. The most favourable n6/n3 ration, as also the highest PUFA (mostly α-linolenic acid), tocopherols (including α-, γ- and δ-isoforms) and ascorbic acid contents were also observed in this sample. Nevertheless, it was the commercial bag lemon balm that gave the highest antioxidant activity and the highest levels of phenolics and flavonoids. As far as we kwon, this is the first comparison of nutraceuticals and antioxidant potential of cultivated, *in vitro* cultured and commercial lemon balm samples. Moreover, it proved that *in vitro* culture might be used to stimulate vitamins production.

Keywords *Melissa officinalis*; Infusions; *In vitro* culture; Nutraceuticals; Antioxidant potential

1. Introduction

Oxidative stress results from an increasing concentration of reactive oxygen species (ROS) like $O_2^{\bullet-}$, H_2O_2 and $ROO^{\bullet}$, that lead to cellular damage caused by the interaction of ROS with cellular constituents. To maintain a healthy biological system it is important to balance the presence of these species with antioxidant defenses (Halliwell, 1996; Valko et al, 2007). Nowadays, antioxidants like polyphenolics, among others are in the front line of investigation not only due to their natural origin but also to their ability to act as free radical scavengers, helping the endogenous antioxidant system (Katalinic et al., 2006; Ferreira et al, 2009). A whole range of plant-derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating disease are now being described as functional foods and nutraceutical sources (Bernal et al., 2011). Some examples of bioactive compounds are fatty acids, carotenoids, vitamins and polyphenols that could be find in *Melissa officinalis* L., commonly known as lemon balm. This plant belongs to Lamiaceae family being reported as one of the most interesting sources of antioxidant compounds (Ciriano et al., 2010). It is an edible plant used worldwide in the form of infusion, important to prevent some human diseases (Ferreira et al., 2006; Ivanova et al., 2006). Its digestive, analgesic, sedative, spasmolytic and hypotensive properties are well known, being one of the few aromatic plants used in pharmaceutical preparations (Marongiu et al., 2004). Lemon balm essential oil also revealed spasmolityc and antimicrobial activity being citral the main compound (Carnat et al., 1998).

There are some studies on the antioxidant activity of lemon balm in the form of infusions (Katalinic et al., 2006; Bouayed et al.., 2007; Ciriano et al., 2010), ethanolic extracts (Ferreira et al., 2006) and supercritical residues (Ribeiro et al., 2001; Marongiu

et al., 2004). Other experiments with lemon balm were also performed in terms of preventing lipid deterioration is sausages (Berasategi et al., 2011) and in cooked pork meat patties (Lahucky et al, 2010; Lara et al., 2011). Ciriano et al. (2010) studied the effect of lemon balm extract in the stabilization of long-chain fatty acids of algae oil. Furthermore, some antioxidants were identified in lemon balm, such as phenolic compounds, mainly rosmarinic acid (Ivanova et al., 2006; Dastmalchi et al., 2008; Spiridon et al., 2011), carotenoids and ascorbic acid (Capecka et al., 2005). Nevertheless, as far as we know, there are no scientific reports on *in vitro* cultured lemon balm, and *in vitro* technique can be very useful to explore potentialities of plants with industrial applications (Dias et al., 2011; Matkowski, 2008).

The main purpose of the present work was to compare the nutraceuticals and antioxidant potential of cultivated, *in vitro* cultured and commercial (available in two different forms) samples of lemon balm (*Melissa officinalis*).

2. Material and Methods

2.1. Samples

Four different samples of lemon balm (*Melissa officinalis* L.) were studied: a cultivated sample, a sample obtained by *in vitro* culture and two commercial samples available in bag and granulate forms (**Figure 1)**.

Cultivated lemon balm was obtained from a local garden (Bragança, Portugal). Commercial samples (bag and granulate lemon balm) were purchased in a local supermarket.

In vitro culture of lemon balm was achieved with commercial seeds. Using bleach and detergent, the seeds were sterilized for 7 min with agitation, washed with distillate water

and inoculated in a germination basic medium with water and agar (0.9%), and kept in dark until germination. The seedlings were then transferred from the germination medium to a modified culture medium (Murashige and Skoog, 1962): macronutrients, l mg/l thiamine, 1 mg/l nicotinic acid, 1 mg/l pyridoxine, 2% sucrose, 0.5 mg/l NAA (1-naphthaleneacetic acid) and 0.1 mg/l BAP (benzilaminopurine). The pH of the culture medium was adjusted to 5.7 before autoclaving. The culture conditions were T_{min}. [16-19] ºC, T_{max} [23-26] ºC, photoperiod of 16/8 h (light/dark) supplied by light-bulbs Silvana day light (Phillips, Amsterdam, Netherlands). They were kept in the same culture conditions and subculture occurred every two months; vegetative parts were stored at -20^{o}C.

Cultivated and *in vitro* cultured samples were lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas, USA) and reduced to a fine dried powder (20 mesh).

2.2. Standards and Reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Panreac (Spain). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U), other individual fatty acid isomers, L-ascorbic acid, tocopherols (α-, β-, γ-, and δ-isoforms), sugars (D(-)-fructose, D(+)-glucose anhydrous, D(+)-melezitose, D(+)-sucrose, D(+)-rafinose, D(+)trehalose), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and catechin standards were purchased from Sigma (St.Louis, MO, USA). Racemic tocol, 50 mg/ml, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Water was treated in a Milli-Q water purification system (TGI PureWater Systems, USA).

2.3. Nutritional and nutraceutical composition

2.3.1. Nutritional value

The samples were analysed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein content (N×6.25) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 ^{o}C. Total carbohydrates were calculated by difference. Energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein) + 3.75 × (g carbohydrate) + 9 × (g fat).

2.3.2. Sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC–RI) as described by Pinela et al. (2012), using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and a RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH2 column (4.6×250 mm, 5 mm, Knauer) operating at 30 ^{o}C (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 ml/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed

using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.3.3. Fatty acids

Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GC–FID)/capillary column as described previously by the authors (Pinela et al., 2012). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 ^{o}C) and a Macherey–Nagel column (30 m x 0.32 mm ID × 0.25 μm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ^{o}C, held for 2 min, then a 30 ^{o}C/min ramp to 125 ^{o}C, 5 ^{o}C/min ramp to 160 ^{o}C, 20 ^{o}C /min ramp to 180 ^{o}C, 3 ^{o}C/min ramp to 200 ^{o}C, 20 ^{o}C/min ramp to 220 ^{o}C and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 ml/min (0.61 bar), measured at 50 ^{o}C. Split injection (1:40) was carried out at 250 ^{o}C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW1.7 software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

2.3.4. Tocopherols

Tocopherols content was determined following a procedure previously described by Pinela et al. (2012) using tocol as IS. The analysis was carried out in the HPLC system described above connected to a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II normal-phase column (250×4.6 mm; YMC Waters)

operating at 30 °C. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 ml/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response, using the internal standard method, and tocopherols contents were further expressed in mg per 100 g of dry weight (dw).

2.3.5. Ascorbic acid

Ascorbic acid was determined following a procedure previously described by the authors (Pinela et al., 2011) with 2,6-dichloroindophenol, and measuring the absorbance at 515 nm (spectrophotometer Analytik Jena, Germany). Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.0125-0.1 mM, $y = 4.459x-0.0538$, $R^2 = 0.9955$), and the results were expressed as mg of ascorbic acid per 100 g of dry weight (dw).

2.3.6. Carotenoids

β-Carotene and lycopene were determined following a procedure previously described by the authors (Barros et al., 2010; Pinela et al., 2011), measuring the absorbance at 453, 505, 645, and 663 nm. Contents were calculated according to the following equations: β-carotene (mg/100 ml) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$; lycopene (mg/100 ml) = $-0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$, and further expressed in mg per 100 g of dry weight (dw).

2.4. Antioxidant potential in infusions

2.4.1. General

For infusions preparation, the samples (1 g) were mixed with 200 ml of boiling water, stand for 5 min, filtered through Whatman No. 4 paper, frozen at -20°C and further lyophilized. Successive dilutions of the lyophilized infusion in distilled water were prepared and submitted to *in vitro* assays already described by the authors (Pinela et al., 2011) to evaluate the antioxidant activity of the infusions. The concentrations of lyophilized infusion in distilled water, providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene/linoleate and TBARS assays) or absorbance at 690 nm (reducing power assay) against sample concentrations. Trolox was used as standard.

2.4.2. DPPH radical-scavenging activity

This methodology was performed using an ELX800 Microplate Reader (Bio-Tek). The reaction mixture in each one of the 96-wells consisted of one of the different concentrations of the lyophilized infusions (1-0.0625 mg/ml; 30 µl) and a methanolic solution (270 µl) containing DPPH radicals (6×10^{-5} mol/l). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Pinela et al., 2011). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution.

2.4.3. Reducing power

This methodology was performed using the Microplate Reader described above. The different concentrations of the lyophilized infusions (0.25-0.0625 mg/ml; 0.5 ml) were mixed with sodium phosphate buffer (200 mmol/l, pH 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The mixture was incubated at 50 ^{o}C for 20 min, and trichloroacetic acid (10% w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured in the 48-wells, as also deionised water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm.

2.4.4. Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving 2 mg in chloroform (10 ml). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 ^{o}C under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 ml) were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing different concentrations of the lyophilized infusions (0.5-0.0625 mg/ml; 0.2 ml). The tubes were shaken and incubated at 50 ^{o}C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm (Pinela et al., 2011). β-Carotene bleaching inhibition was calculated using the following equation: (Abs after 2 h of assay/initial Abs) × 100.

2.4.5. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Brains were obtained from Sus scrofa, dissected and homogenized with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue

homogenate which was centrifuged at 3000g (Centorion K24OR refrigerated centrifuge) for 10 min. An aliquot (0.1 ml) of the supernatant was incubated with the different concentrations of the lyophilized infusions (0.125-0.00156 mg/ml; 0.2 ml) in the presence of FeSO4 (10 µM; 0.1 ml) and ascorbic acid (0.1 mM; 0.1 ml) at 37 ^{o}C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 ml), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 ml), and the mixture was then heated at 80 ^{o}C for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm (Pinela et al., 2011). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = [(A - B)/A] × 100%, where A and B were the absorbance of the control and the compound solution, respectively.

2.4.6. Phenolics

Total phenolics were determined by adding a solution of the lyophilized infusion (0.125 mg/ml for cultivated sample, 0.25 mg/ml for *in vitro* cultured sample and 0.625 mg/ml for commercial samples) to a Folin:Ciocalteu solution (1:10 v/v; 2,5ml) and sodium carbonate (75g/l, 2 ml). The tubes were vortexed and incubated at 40 ^{o}C for 30 min. Absorbance was then measured at 765 nm. Gallic acid was used to calculate the standard curve (0.05–0.8 mM; y = 1.8392x + 0.0281; R^2 = 0.999), and the results were expressed as mg of gallic acid equivalents (GAEs) in the lyophilized infusion per ml of distilled water.

Total flavonoids were determined by mixing the infusion (0.5 ml; 0.25 mg/ml for cultivated sample, 0.5 mg/ml for *in vitro* cultured sample and 0.125 mg/ml for

commercial samples) with distillated water (2 ml) and a $NaNO_3$ solution (5%, 0.15 ml). The samples were vortexed and stand for 6 min; afterwards, a $AlCl_3$ solution (10%, 0.15 ml) was added, vortexed, and stand for another 6min. NaOH (4%, 2 ml) and distilled water (0.2 ml) were added and stand for 15 min. The intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.05-0.8 mM; $y = 1.5431x + 0.028491$; $R^2 = 0.9997$) and the results were expressed as mg of (+)-chatequin equivalents (CEs) in the lyophilized infusion per ml of distilled water.

2.6. Statistic analysis

For each one of the analytical procedure, three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 16.0 program.

3. Results and Discussion

Data on macronutrients, energetic value and individual sugars composition are shown in **Table 1**. Carbohydrates (that include fiber) were the major macronutrients in all the studied samples, followed by proteins. *In vitro* cultured lemon balm revealed the highest levels of proteins (~8 g/100 g dw) and ash (~12 g/100 g dw), while the highest levels of carbohydrates were found in granulate commercial sample (85 g/100 g dw). Bag commercial lemon balm gave the highest energetic value (377 kcal/100 dw) certainly due to its highest fat content (~3 g/100 g dw). Regarding composition in free sugars, fructose, glucose, sucrose, threhalose and raffinose were found in all the samples

(**Figure 2A**) and in similar amounts (**Table 1**). Total sugars content in cultivated, *in vitro* cultured or commercial lemon balm is considered low (~3 g/100 g dw).

The percentages of the fatty acids found in the different lemon balm samples, including total saturated fatty acids (SFA), monoansutareted fatty acids (MUFA) and polyunsuturated fatty acids (PUFA) levels are given in **Table 2**. Up to 22 fatty acids were detected and quantified, being α-linolenic acid (C18:3n3, PUFA) the main fatty acid found in all the samples. Nevertheless, the levels of this fatty acid in commercial samples (29-30%) were much lower than in garden cultivated or in *in vitro* cultured samples (56-57%). The second major fatty acid found was linoleic acid (C18:2n6c, PUFA) for garden cultivated and *in vitro* cultured samples, or palmitic acid (C16:0, SFA) in the case of both commercial samples (bag and granulate). This fact is responsible for the highest levels of of SFA found in the latter samples, mainly in the granulate form (42%). Otherwise, garden cultivated lemmon balm presented the highest levels of PUFA (78%), but the lowest SFA content (17%).

Fatty acids might be considered nutraceuticals such is the case of some long chain PUFA, especially the n-3 series that have many beneficial effects in human health in preventing some diseases like coronary heart diseases, but also inflammation and autoimune disorders (Bernal et al., 2011). Moreover, for "good nutritional quality", including health beneficial effects, PUFA/SFA ratios should be higher than 0.45, while n-6/n-3 fatty acids ratios should be lower than 4.0 (Guil et al., 1996), as it is observed in the present study for all the samples (**Table 2**). The best PUFA/SFA ratio was registered in garden cultivated (4.68), while the most convenient n-6/n-3 ratio was observed in *in vitro* cultured lemon balm (0.27).

Lemon balm composition in other important nutraceuticals such as tocopherols, ascorbic acid and carotenoids, was also evaluated and data are shown in **Table 3**. A significant higher total tocopherols content was observed in *in vitro* cultured sample (213 mg/100 g dw), due to the highest levels of α-tocopherol (180 mg/100 g dw), γ-tocopherol (33 mg/100 g dw) and δ-tocopherol (0.21 mg/100 g dw) found in this sample. The increase in tocopherols production by *in vitro* cultured samples was already described by us for other plants (e.g. coriander, Dias et al., 2011) as also for mushrooms (Reis et al., 2011). β-tocopherol vitamer was found only in the garden cultivated sample, but in low levels (0.55 mg/100 g dw, **Table 3**). Commercial samples showed low tocopherols contents and low diversity of isoforms: α-and γ-tocopherols in bag lemon balm, and only α-tocopherol in the granulate form (**Figure 2B**).

The same tendency was observed for ascorbic acid and lycopene, presenting the *in vitro* cultured sample the highest levels (94 mg/100 g dw and 0.16 mg/100 g dw, respectively; **Table 3**). Garden cultivated lemon balm gave the highest levels of β-carotene (3.2 mg/100 g dw), followed by the *in vitro* cultured sample (1.3 mg/100 g dw). The levels of ascorbic acid found in the presented were much higher than the ones reported by Capecka et al. (2005) in a *Melissa officinalis* sample from Poland (3.3 mg/100 dw), while the carotenoids content reported by those authors (21 mg/100 g dw) were much lower than the values found herein.

Data reported on nutritional composition of lemon balm (obtained by analyzing the whole plant) is important because nowadays lemon balm is also available in formulations to be taken directly (e.g. capsules) rather than as infusions.

The results of antioxidant activity, measured by different *in vitro* assays, of the four studied lemon balm samples are shown in **Figure 4**. The response of antioxidants to

different radical or oxidant sources may be different. Depending upon the experimental conditions, test samples can demonstrate either antioxidant or pro-oxidant activity. Therefore, no single assay accurately reflects the mechanism of action of all radical sources or all antioxidants in a complex system (Prior et al., 2005). Therefore, the antioxidant activity of the samples was assessed through four different methods.

It should be highlighted that infusions of all the samples were prepared to evaluate their antioxidant potential, since this is the most common form of lemon balm consume For an easier comparison of the obtained data, EC_{50} values (sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power) were calculated and presented in **Table 4**. In general, commercial samples revealed the highest antioxidant potential, mostly the bag form. It revealed the lowest EC_{50} values in all the assayed methods: DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS inhibition ($\leq$ 0.16 mg/ml). These results are coherent with the existence of higher levels of phenolics (960 mg GAE/ml) and flavonoids (428 mg CE/ml) in commercial bag lemon balm. Otherwise, *in vitro* cultured sample gave the lowest antioxidant potential with the highest EC_{50} values ($\geq$0.13 mg/ml) and lowest phenolic (293 mg GAE/ml) and flavonoid (118 mg CE/ml) contents. In fact, the antioxidant activity of lemon balm infusions seemed to be positively correlated with phenolics and flavonoids content (**Table 5**), mainly DPPH radical scavenging activity and reducing power (R^2>0.72).

Lemon balm infusions studied herein showed higher antioxidant potential than ethanolic extracts studied by Ferreira et al. (2006). Nevertheless, despite the existance of other studies with infusions of lemon balm from Croatia (Katalinic et al., 2006), Iran

(Bouayed et al.., 2007) and Spain (Ciriano et al., 2010), it was not possible to compare their antioxidant activity because the results are expressed in different forms.

Overall, nutrients and nutraceuticals profile of *in vitro* cultured lemon balm is closer of garden cultivated sample than of both commercial samples (bag or granulate). It presented the highest levels of proteins and ash, and the lowest energetic value. The most favourable n6/n3 ration, as also the highest PUFA (mostly α-linolenic acid), tocopherols (including α-, γ- and δ-isoforms) and ascorbic acid contents were also observed in this sample. Nonetheless, it was the commercial bag lemon balm that gave the highest antioxidant activity and the highest levels of phenolics and flavonoids. As far as we kwon, this is the first comparison of nutraceuticals and antioxidant potential of cultivated, *in vitro* cultured and commercial lemon balm samples. Moreover, it proved that *in vitro* culture might be used to stimulate vitamins (e.g. tocopherols and ascorbic acid) production.

Acknowledgements

The authors are grateful to Fundação para a Ciência e a Tecnologia (FCT, Portugal) and COMPETE/QREN/EU for financial support to this work (research project PTDC/AGR-ALI/110062/2009) and to CIMO (strategic project PEst-OE/AGR/UI0690/2011). L. Barros also thanks to FCT, POPH-QREN and FSE for her grant (SFRH/BPD/4609/2008).

References

AOAC, 1995. Official Methods of Analysis, 16. Association of Official Analytical Chemists, Arlington VA, USA.

Barros, L., Carvalho, A.M., Ferreira, I.C.F.R. 2010. Leaves, flowers, immature fruits and leafy flowered stems of Malva sylvestris: a comparative study of the nutraceutical potential and composition. Food Chem. Toxicol. 48, 1466–1472.

Berasategi, I., Legarra, S., Ciriano, M.G., Rehecho, S., Calvo, M.I., Cavero, R.Y., Navarro-Blasco, I., Ansorena, D., Astiasarán, I. 2011. "High in omega-3 fatty acids" bologna-type sausages stabilized with an aqueous-ethanol extract of *Melissa officinalis*. Meat Sci. 88, 705-711.

Bernal, J., Mendiola, J.A., Ibáñez, E., Cifuentes, A. 2011. Advanced analysis of nutraceuticals. J. Pharm. Biomed Anal. 55, 758-774.

Bouayed, J., Piri, K., Rammal, H., Dicko, A., Desor, F., Younos, C, Soulimani, R. 2007. Comparative evaluation of the antioxidant potential of some Iranian medicinal plants. Food Chem. 104, 364-368.

Capecka, E., Mareczek, A., Leja, M. 2005. Antioxidant activity of fresh and dry herbs of some *Lamiaceae* species. Food Chem. 93, 223-226.

Carnat, A.P., Carnat. A., Fraisse, D., Lamaison. J.L. 1998. The aromatic and polyphenolic composition of lemon balm (*Melissa officinalis* L. subsp. *officinalis*) tea. Pharm. Acta Helvet.72, 301-305.

Ciriano, M.KI., Rehecho, S., Calvo, M.I., Cavero, R.Y., Navarro, I., Astiasarán, I., Ansorena, D. 2010. Effect of lyophilized water extracts of *Melissa officinalis* on the stability of algae and linseed oil-in-water emulsion to be used as a functional ingredient in meat products. Meat Sci. 85, 373-377.

Dastmalchia, K., Dormana, H.J.D., Oinonen, P.P., Darwisd, Y., Laaksoa, I., Hiltunena, R. 2008. Chemical composition and in vitro antioxidative activity of a lemon balm (*Melissa officinalis* L.) extract. LWT 41, 391-400.

Dias, M.I., Barros, L., Sousa, M.J., Ferreira, I.C.F.R. 2011. Comparative study of lipophilic and hydrophilic antioxidants from *in vivo* and *in vitro* grown *Coriandrum sativum*. Plants Food Hum. Nutr. 66, 181-186.

Ferreira A., Proença C., Serralheiro M.L.M., Araújo M.E.M. 2006. The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J. Ethnopharm. Pharm. 108, 31-37.

Ferreira, I.C.F.R., Barros, L., Abreu, R.M.V. 2009. Antioxidants in wild mushrooms. Curr. Med. Chem. 16, 1543-1560.

Guil, J.L., Torija, M.E., Giménez, J.J., Rodriguez, I. (1996). Identification of fatty acids in edible wild plants by gas chromatography. J. Chromatog. A. 719, 229-235.

Halliwell, B. 1996. Antioxidants in human health and disease. Annu. Rev. Nutr. 16, 33-50.

Ivanova, D., Gerova, D., Chervenkov, T., Yankova, T. 2005. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. J. Ethnopharm. Pharm. 96, 145-150.

Katalinic, V., Milos, M., Kulisic, T., Jukic, M. 2006. Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. Food Chem. 94, 550-557.

Lahucky, R., Nuernberg, K., Kovac, L., Bucko, O., Nuernberg, G. 2010. Assessment of the antioxidant potential of selected plant extracts – *In vitro* and *in vivo* experiments on pork. Meat Sci. 85, 779-784.

Lara, M.S., Gutierrez, J.I., Timón, M., Andrés, A.I. 2011. Evaluation of two natural extracts (*Rosmarinus officinalis* L. and *Melissa officinalis* L.) as antioxidants in cooked pork patties packed in MAP. Meat Sci. 88, 481- 488.

Marongiu, B., Porcedda, S., Piras, A., Rosa, A., Deiana, M., Dessì, M.A. 2004. Antioxidant Activity of Supercritical Extract of *Melissa officinalis* Subsp. *officinalis* and *Melissa officinalis* Subsp. *Inodora*. Phytother. Res. 18, 789-792.

Matkowski, A. 2008. Plant in vitro culture, for the production of antioxidants - A review. Biotechnol. Adv. 26, 548-560.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473-497.

Ribeiro, M.A., Bernardo-Gil, M.G., Esquível, M.M. 2001. *Melissa officinalis*, L.: study of antioxidant activity in supercritical residues. J. SuperCrit. Fluids 21, 51-60.

Pinela, J., Barros, L., Carvalho, A.M., Ferreira, I.C.F.R. 2011. Influence of the drying method in the antioxidant potential of four shrubby flowering plants from the tribe Genisteae (Fabaceae). Food Chem. Toxicol. 49, 2983-2989.

Pinela, J., Barros, L., Carvalho, A.M., Ferreira, I.C.F.R. 2012. Nutritional composition and antioxidant activity of four tomato (*Lycopersicon esculentum* L.) farmer' varieties in Northeastern Portugal homegardens Food Chem. Toxicol. 50, 829-834.

Prior, R.L., Wu, X., Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J. Agric. Food Chem. 53, 4290-4302.

Reis, F.S., Ferreira, I.C.F.R., Barros, L., Martins, A. 2011. A comparative study of tocopherols composition and antioxidant properties of *in vivo* and *in vitro* ectomycorrhizal fungi. LWT 44, 820-824.

Spiridona I., Colcerub S., Anghela N., Teacaa C.A., Bodirlaua R., Armatub A., 2011. Antioxidant capacity and total phenolic contents of oregano (*Origanum vulgare*),

lavender (*Lavandula angustifolia*) and lemon balm (*Melissa officinalis*) from Romania. Nat. Prod. Res. 25, 1675-1661.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44-84.

Figure 1. A. *In vitro* grown lemon balm; Commercial samples of lemon balm: bag (B) and granulated (C).

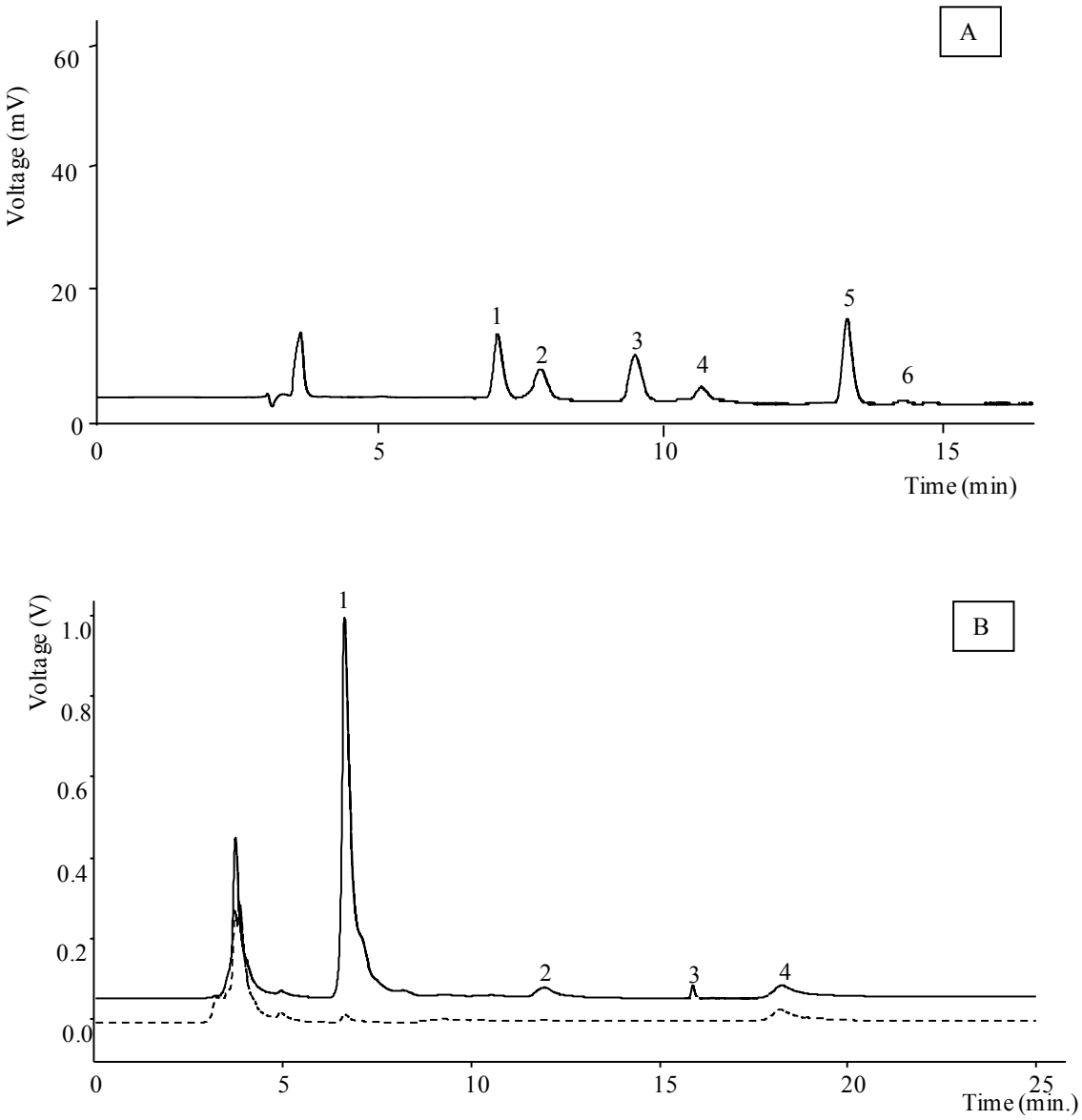
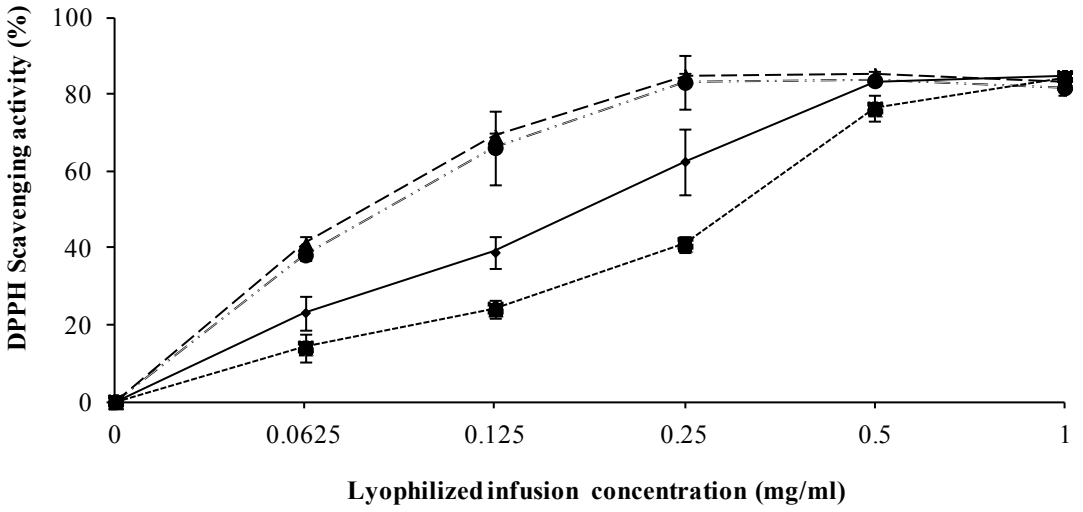
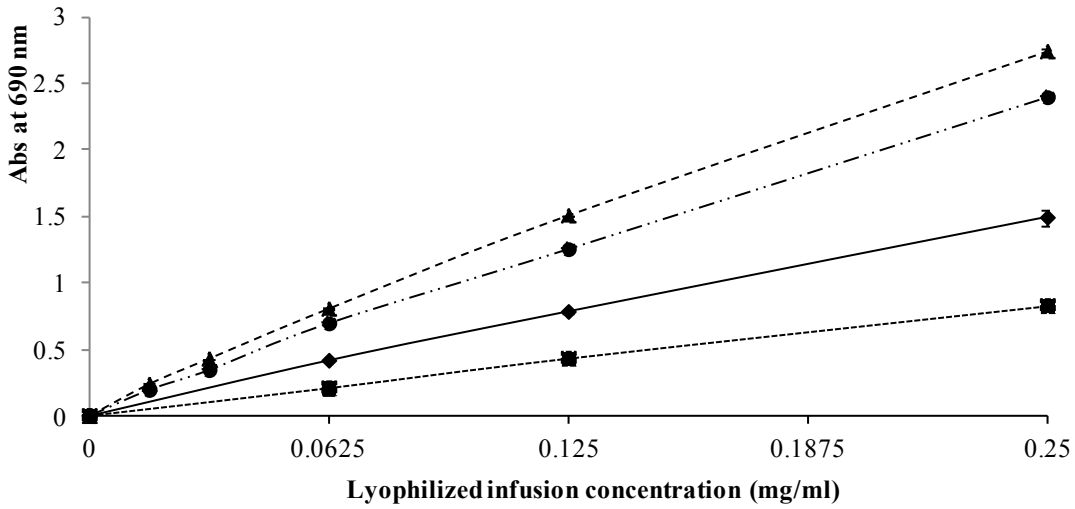
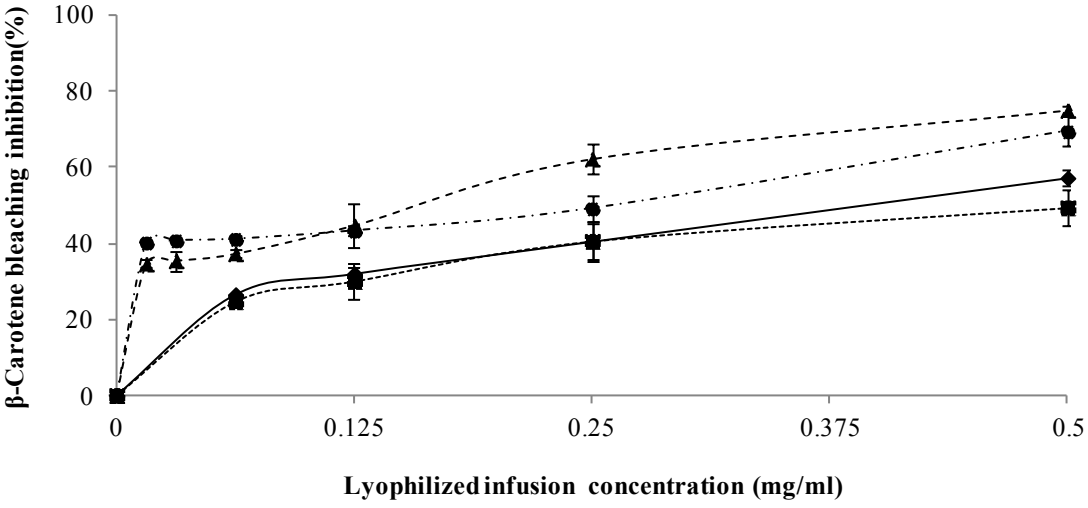


Figure 2. (A) Free sugars of lemon balm *in vitro* cultured sample: 1-frutose, 2- glucose, 3-sucrose, 4-trehalose, 5-melezitose(IS), 6-raffinose; **(B)** Tocopherols of *in vitro* cultured (—) and granulate commercial (---) samples: 1- α-tocopherol, 2- γ-tocopherol, 3- δ-tocopherol, 4- tocol (IS).


DPPH Scavenging activity (%)
100
80
60
40
20
0
0
0.0625
0.125
0.25
0.5
1
Lyophilized infusion concentration (mg/ml)


Abs at 690 nm
3
2.5
2
1.5
1
0.5
0
0
0.0625
0.125
0.1875
0.25
Lyophilized infusion concentration (mg/ml)


β-Carotene bleaching inhibition(%)
100
80
60
40
20
0
0
0.125
0.25
0.375
0.5
Lyophilized infusion concentration (mg/ml)

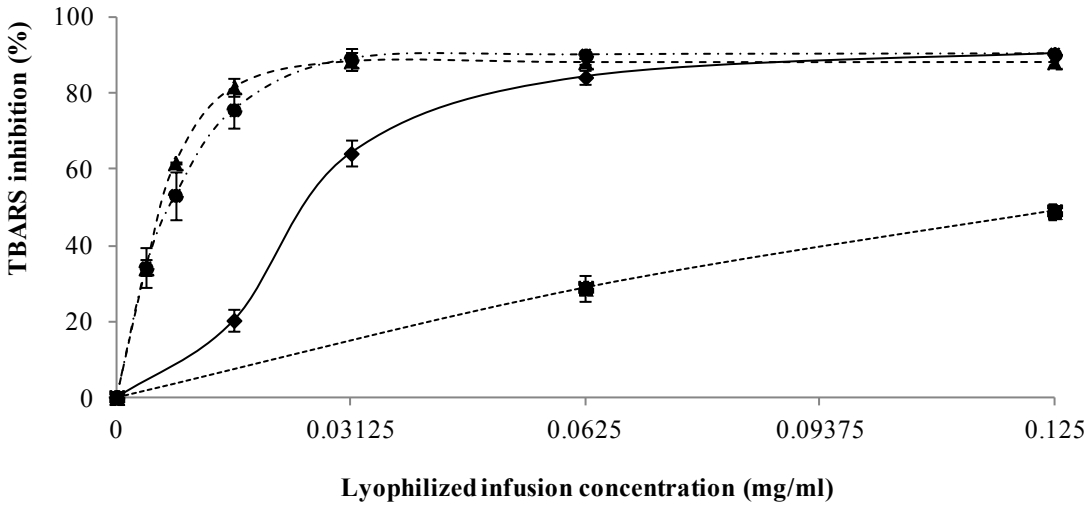


Figure 3. DPPH radical scavenging activity (%), Reducing power at 690 nm, β-Carotene bleaching inhibition (%) and TBARS formation inhibition (%) of garden cultivated (♦), *in vitro* culture (■), commercial bag (▲) and commercial granulated (●) lemon balm samples.

Table 1. Macronutrients, energetic value and individual sugars composition of different lemon balm samples.

	Garden cultivated	*In vitro* cultured	Commercial bag	Commercial granulate
Fat (g/100 g dw)	0.29 ± 0.06^{d}	1.41 ± 0.11^{c}	2.77 ± 0.12^{a}	1.84 ± 0.08^{b}
Proteins (g/100 g dw)	6.44 ± 0.36^{b}	7.74 ± 0.02^{a}	6.10 ± 0.26^{b}	4.14 ± 0.37^{c}
Ash (g/100 g dw)	10.13 ± 0.11^{b}	12.28 ± 0.00^{a}	9.31 ± 0.07^{c}	8.88 ± 0.13^{d}
Carbohydrates (g/100 g dw)	83.14 ± 0.31^{b}	78.57 ± 0.09^{d}	81.82 ± 0.36^{c}	85.14 ± 0.56^{a}
Energy (Kcal/100 g dw)	360.93 ± 0.15^{c}	357.93 ± 0.40^{d}	376.61 ± 0.17^{a}	373.69 ± 0.25^{b}
Fructose	1.09 ± 0.02^{a}	1.09 ± 0.02^{a}	1.11 ± 0.02^{a}	1.10 ± 0.03^{a}
Glucose	0.98 ± 0.01^{a}	0.97 ± 0.01^{a}	0.99 ± 0.01^{a}	0.98 ± 0.01^{a}
Sucrose	0.44 ± 0.02^{a}	0.44 ± 0.02^{a}	0.45 ± 0.02^{a}	0.45 ± 0.03^{a}
Trehalose	0.25 ± 0.03^{a}	0.24 ± 0.03^{a}	0.25 ± 0.02^{a}	0.25 ± 0.02^{a}
Raffinose	0.17 ± 0.02^{a}	0.17 ± 0.02^{a}	0.17 ± 0.02^{a}	0.17 ± 0.02^{a}
Total Sugars (g/100 g dw)	2.93 ± 0.03^{a}	2.91 ± 0.03^{a}	2.97 ± 0.03^{a}	2.95 ± 0.05^{a}

In each row, different letters mean significant differences ($p < 0.05$).

Table 2. Fatty acids composition (relative percentage of each fatty acid) of different lemon balm samples.

	Garden cultivated	*In vitro* cultured	Commercial bag	Commercial granulate
C6:0	0.05 ± 0.01	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.01
C8:0	0.06 ± 0.02	0.02 ± 0.00	0.04 ± 0.01	0.05 ± 0.00
C10:0	0.02 ± 0.00	0.04 ± 0.00	0.59 ± 0.08	0.05 ± 0.00
C12:0	0.05 ± 0.00	0.09 ± 0.00	0.17 ± 0.03	0.26 ± 0.05
C14:0	0.66 ± 0.05	0.48 ± 0.01	0.49 ± 0.05	0.54 ± 0.05
C14:1	0.26 ± 0.04	0.54 ± 0.01	1.34 ± 0.02	0.30 ± 0.04
C15:0	0.16 ± 0.03	0.23 ± 0.01	0.64 ± 0.07	1.01 ± 0.00
C16:0	10.54 ± 0.66	11.55 ± 0.22	19.94 ± 0.23	22.19 ± 0.59
C16:1	0.15 ± 0.04	0.42 ± 0.01	0.51 ± 0.01	0.23 ± 0.04
C17:0	0.25 ± 0.05	0.55 ± 0.02	0.62 ± 0.05	0.87 ± 0.01
C18:0	2.48 ± 0.24	2.77 ± 0.00	6.03 ± 0.06	5.61 ± 0.14
C18:1n9c	4.65 ± 0.35	4.03 ± 0.02	10.89 ± 0.20	8.88 ± 0.18
C18:2n6c	21.58 ± 1.34	15.60 ± 0.02	17.71 ± 0.03	17.66 ± 0.13
C18:3n3	56.35 ± 1.68	57.41 ± 0.04	29.12 ± 0.39	29.95 ± 0.76
C20:0	1.16 ± 0.03	2.38 ± 0.01	5.66 ± 0.20	4.41 ± 0.10
C20:1	0.07 ± 0.02	0.02 ± 0.01	0.07 ± 0.00	0.07 ± 0.00
C20:2	0.02 ± 0.00	0.02 ± 0.00	0.06 ± 0.01	0.03 ± 0.00
C20:3n3+C21:0	0.20 ±0.03	0.49 ± 0.03	0.550± 0.03	0.46 ± 0.08
C22:0	0.51 ± 0.07	1.93 ± 0.09	2.47 ± 0.34	2.48 ± 0.40
C22:2	nd	0.05 ± 0.01	0.20 ± 0.01	0.07 ± 0.00
C23:0	0.05 ± 0.01	0.17 ± 0.05	1.03 ± 0.01	2.53 ± 0.05
C24:0	0.74 ± 0.08	1.20 ± 0.13	1.86 ± 0.16	2.32 ± 0.12
SFA	16.72 ± 0.68^{d}	21.43 ± 0.05^{c}	39.55 ± 0.66^{b}	42.34 ± 1.16^{a}
MUFA	5.14 ± 0.36^{c}	5.02 ± 0.03^{c}	12.81 ± 0.24^{a}	9.48 ± 0.19^{b}
PUFA	78.15 ± 0.32^{a}	73.55 ± 0.02^{b}	47.64 ± 0.43^{c}	48.18 ± 0.98^{c}
PUFA/SFA	4.68 ± 0.21^{a}	3.43 ± 0.01^{b}	1.20 ± 0.03^{c}	1.14 ± 0.05^{c}
n6/n3	0.38 ± 0.03^{b}	0.27 ± 0.00^{c}	0.60 ± 0.01^{a}	0.58 ± 0.01^{a}

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9c); Linoleic acid (C18:2n6c); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); *cis*-11-Eicosenoic acid (C20:1); *cis*-11,14-Eicosadienoic acid (C20:2); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3+C21:0); Behenic acid (C22:0); *Cis*-13,16-Docosandienoic acid (C22:2); Tricosanoic acid (C23:0); Lignoceric acid (C24:0).
SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

Table 3. Nutraceuticals composition of different lemon balm samples.

	Garden cultivated	*In vitro* cultured	Commercial bag	Commercial granulate
α-tocopherol	24.39 ± 0.46^{b}	180.09 ± 21.8^{a}	1.28 ± 0.04^{c}	1.02 ± 0.17^{c}
β-tocopherol	0.55 ± 0.12^{a}	nd	nd	nd
γ-tocopherol	3.03 ± 0.02^{b}	33.04 ± 1.66^{a}	0.33 ± 0.00^{c}	nd
δ-tocopherol	0.04 ± 0.01^{b}	0.21 ± 0.05^{a}	nd	nd
Total tocopherols (mg/100 g dw)	28.01 ± 0.54^{b}	213.34 ± 20.09^{a}	1.61 ± 0.04^{c}	1.02 ± 0.17^{c}
Ascorbic acid (mg/100 g dw)	88.28 ± 0.33^{b}	93.72 ± 1.30^{a}	35.96 ± 0.29^{d}	52.84 ± 1.29^{c}
β-carotene (mg/100 g dw)	3.23 ± 0.04^{a}	1.27 ± 0.02^{b}	0.15 ± 0.00^{c}	0.02 ± 0.00^{d}
Lycopene (mg/100 g dw)	0.03 ± 0.02^{c}	0.16 ± 0.04^{a}	0.11 ± 0.00^{b}	0.05 ± 0.00^{c}

nd – not detected. In each row different letters mean significant differences ($p < 0.05$).

Table 4. Antioxidant potential of different lemon balm infusions.

	Garden cultivated	*In vitro* cultured	Commercial bag	Commercial granulate
DPPH scavenging activity (EC_{50}, mg/ml)	0.19 ± 0.01^{b}	0.31 ± 0.02^{a}	0.08 ± 0.00^{c}	0.09 ± 0.00^{c}
Reducing power (EC_{50}, mg/ml)	0.08 ± 0.00^{b}	0.15 ± 0.01^{a}	0.04 ± 0.00^{d}	0.05 ± 0.00^{c}
β-carotene bleaching inhibition (EC_{50}, mg/ml)	0.38 ± 0.05^{b}	0.61 ± 0.02^{a}	0.16 ± 0.03^{c}	0.26 ± 0.04^{cb}
TBARS inhibition (EC_{50}, mg/ml)	0.03 ± 0.00^{b}	0.13 ± 0.01^{a}	0.01 ± 0.00^{c}	0.01 ± 0.00^{c}
Phenolics (mg GAE/ml)	595.34 ± 10.39^{c}	293.32 ± 2.16^{d}	959.54 ± 10.02^{a}	657.06 ± 0.8^{b}
Flavonoids (mg CE/ml)	262.14 ± 7.87^{c}	118.09 ± 4.30^{d}	428.26 ± 6.97^{a}	263.69 ± 3.72^{b}

In each row different letters mean significant differences ($p < 0.05$). EC_{50} values are expressed in mg of lyophilized infusion per ml of distilled water; GAE/ml means gallic acid equivalents in the lyophilized infusion per ml of distilled water; CE/ml means catechin equivalents in the lyophilized infusion per ml of distilled water.

Table 5. Correlations established between total phenolics and flavonoids, and antioxidant activity EC_{50} values of different lemon balm infusions.

	DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		TBARS inhibition	
EC_{50} value (mg/ml)	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2
Phenolics (mg GAE/ml)	Y=-2248.8x+1003.8	0.8045	Y=-5011x+1007.2	0.8363	Y=-952.34x+962.53	0.6569	Y=-3979.9x+793.3	0.7370
Flavonoids (mg CE/ml)	Y=-990.42x+434.29	0.7229	Y=-2224.7x+437.16	0.7635	Y=-429.12x+419.55	0.6178	Y=-1778.1x+342.66	0.6814