Phenolic profiles of cultivated, *in-vitro* cultured and commercial samples of *Melissa officinalis* L. infusions

LILLIAN BARROS,[a,b] MONTSERRAT DUEÑAS,[b] MARIA INÊS DIAS,[a] MARIA JOÃO SOUSA,[a] CELESTINO SANTOS-BUELGA,[b,*] ISABEL C.F.R. FERREIRA[a,*]

[a]*CIMO/Escola Superior Agrária, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal.*

[b]*Grupo de Investigación en Polifenoles (GIP-USAL), Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain.*

*Authors to whom correspondence should be addressed (e-mail: iferreira@ipb.pt, telephone +351273303219, fax +351273325405; e-mail: csb@usal.es; telephone +34 923 294537; fax +34 923 294515).

Abstract

Melissa officinalis L. (lemon balm) is normally consumed as infusions and presents therapeutic properties such as sedative, carminative and antispasmodic, being also included in some pharmaceutical preparations. The phenolic profiles of different samples of lemon balm prepared as infusions were evaluated by HPLC-DAD-ESI/MS. The profiles were compared in order to understand the differences between cultivated, *in-vitro* cultured and commercial (bags and granulated) samples. All the samples showed a similar phenolic profile, presenting differences only in the quantities found of each compound. Rosmarinic acid was the most abundant compound, being higher in commercial samples, especially in tea bag sample (55.68 mg/g of infusion) and lower in *in-vitro* cultured sample (15.46 mg/g). Moreover, dimers, trimers and tetramers of caffeic acid were identified and quantified for the first time in lemon balm. Only one flavonoid, luteolin-3'-*O*-glucuronide was found in all the samples, ranging from 8.43 mg/g in commercial granulate sample to 1.22 mg/g in *in-vitro* cultured sample. Overall, cultivated and *in-vitro* cultured samples presented the lowest amounts of phenolic compounds (59.59 and 30.21 mg/g, respectively); otherwise, commercial samples showed the highest contents (109.24 mg/g for tea bag and 101.03 mg/g for granulate sample). The present study shows that infusion of lemon balm can be a source of phenolic compounds, known for their bioactive effects.

Keywords: *Melissa officinalis*; *In-vitro* culture; Phenolic acids; Flavonoids; HPLC-DAD-ESI/MS

1. Introduction

The interest in natural compounds has been increasing in the last decades. Phenolic compounds are a group of secondary metabolites, which includes flavonoids (e.g. anthocyanins, flavones, isoflavones) and phenolic acids, with antioxidant and redox and metal chelation properties, acting as reducing agents, hydrogen donators or singlet oxygen quenchers (Proestos, Chorianopoulos, Nychas & Komaitis, 2005). Furthermore, they present antibacterial, antiviral, anti-fungal, anti-inflammatory, anti-diarrheal and antiulcer properties, among other pharmacological activities (Hussain et al., 2011). Phenolic compounds can also act as phytoalexin protective agents against UV light, providing also an important role in plant growth and reproduction (Barros et al., 2012).

The Lamiaceae are a promising source of natural antioxidants due to the large amount of phenolic acids found in many species of this family (Ziaková & Brandsteterová, 2003). *Melissa officinalis* L., commonly known as lemon balm, is a perennial herb belonging to Lamiaceae family. Traditionally administered in infusion form, it has therapeutic properties such as sedative, carminative and antispasmodic but also it is used for treatment of headache, rheumatism, indigestion and hypersensitivities (Petersen & Simmonds, 2003; Weitzel & Peterson, 2011). All of these properties of lemon balm have been related to the high levels of phenolic acids found in this species, mainly hydroxycinnamic acid derivatives such as rosmarinic acid (Fecka & Turek, 2007).

Some studies already reported other phenolic compounds in lemon balm. Heitz, Carnat, Fraisse, Carnat, & Lamaison (2000) isolated luteolin 3'-*O*-glucuronide as the major flavonoid presented in *Melissa officinalis* from France. In 2002, Patora & Klimek (2002) isolated and determined the structure of six major flavonoids (apigenin and luteolin derivatives) in lemon balm from Poland on the basis of spectral data. There are also reports on validation of analytical techniques of phenolic acids in Lamiaceae species from USA and Slovakia including lemon balm (Lee, 2010; Ziaková, Brandsteterová, & Blahová, 2003). Nevertheless, as far as we know, there are no reports comparing the phenolic profile of garden cultivated, *in-vitro* cultured and commercial samples of *Melissa officinalis*.

In-vitro culture of plant cells is a technique with an increasing interest in academic and industrial areas, to explore new pharmaceutical and medicinal potential of plants, due to the ability of producing, in a sterile environment, numerous copies of the same plant tissue, through micropropagation, and consequently it is a mean to produce some active metabolites, such as phenolic compounds, alkaloids, triterpens or quinones (Dias, Barros, Sousa, & Ferreira, 2011; Oksman-Caldentey & Inzé, 2004).

The aim of the present work was to establish the individual phenolic profiles of garden cultivated, *in-vitro* cultured and commercial (bags and granulated) samples of *Melissa officinalis* (lemon balm), prepared as infusions.

2. Material and methods

2.1. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid and trifluoroacetic acid (TFA) were purchased from Prolabo (VWR International, France). The phenolic standards (caffeic acid, luteolin-7-*O*-glucoside and rosmarinic acid) were from Extrasynthese (Genay, France). All other chemicals were of analytical grade and purchased from chemical suppliers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples

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

Cultivated lemon balm was obtained from a local garden (Bragança, Portugal) and identified according to Flora Ibérica (2010). 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 distilled 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 & Skoog, 1962): macronutrients, 1 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 (benzylaminopurine). 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 ºC.

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

2.3. 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 ºC and further lyophilized. Afterwards, 10 mg were diluted in 2 mL of methanol:water 80:20 (*v/v*). The solution was filtered through a 0.22-µm disposable LC filter disk for High Performance Liquid Chromatographic (HPLC) analysis.

2.4. Analysis of phenolic compounds by HPLC-DAD-ESI/MS

The samples were analysed using a Hewlett-Packard 1100 chromatograph (Agilent Technologies) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 µm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15-25% B over 5 min, 25-35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed to perform a series of two consecutive scan modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to obtain full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1000. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) of the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V.

The phenolic compounds present in the samples were characterised according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, a calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standards compounds: caffeic acid (y=611.9x-4.5733; R^2=0.9998); luteolin-7-*O*-glucoside (y=80.829x-21.291; R^2=0.9999); and rosmarinic acid (y=336.03x+170.39; R^2=0.9998). The results were expressed in mg per g of lyophilized infusion.

2.6. Statistic analysis

For each sample, three infusions were prepared and the analysis of phenolic compounds was carried out in duplicate. The results are expressed as mean values and standard deviation. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with α = 0.05. This treatment was carried out using SPSS v. 18.0 program.

3. Results and Discussion

3.1. Identification of the phenolic compounds

Figure 2 shows the phenolic compounds profile of cultivated lemon balm sample. Data of the retention time, λ_{max} in the visible region, molecular ion, main fragment ions in MS^2, and tentative identification obtained by HPLC-DAD-MS analysis are presented in **Table 1**. All the lemon balm samples studied herein presented a similar profile, with rosmarinic acid (caffeic acid dimer) as the main compound. The samples also presented other caffeic acid derivatives, such as caffeic acid trimers (lithospermic acid, salvianolic acid A, C and F and yunnaneic acid F) and tetramers- in fact they are dimers of rosmarinic acid- (salvianolic acid B and sagerinic acid) (**Figure 3**). This identification was based on the literature available for these compounds (Chen, Zhang, Wang, Yang, & Wang, 2011; Lu & Foo, 2002; Neuengchamnog, Krittasilp, & Ingkaninan, 2011; Ruan, Li, Li, Luo, & Kong, 2012; Zeng, Xiao, Liu, & Liang, 2006; Zhu et al., 2007), comparing their UV spectra and fragmentation pattern. All these derivatives were calculated based on rosmarinic acid calibration curve.

Peak 1, was identified as 3-(3,4-dihydroxyphenyl)-lactic acid **(1)**, based on the fragmentation pattern found in the literature (Zeng et al., 2006). The 2*R* isomer of this compound is known as salvianic acid A or "Danshensu", usually reported in roots and rhizomes preparations of *Salvia miltiorrhiza* (Danshen in Chinese) used in Traditional Chinese Medicine (Chen et al., 2011).

Peak 2 showed a pseudo molecular ion $[M-H]^-$ at *m/z* 597, yielding prominent ions at *m/z* 359, 295 and 179, respectively. Similar characteristics were reported by Chen et al. (2011) for the compound yunnaneic acid F **(10)** identified in *Salvia miltiorrhiza.* Peaks 3, 7 and 9 presented similar UV spectra with λ_{max} at 320-330 nm. Peak 9 was identified as rosmarinic acid **(3)** according to its mass spectrum, showing a pseudo molecular ion $[M-H]^-$ at *m/z* 359, and characteristic fragment ions at *m/z* 197, 179 and 161 (**Figure 4A**) (Chen et al., 2011); its identity was confirmed by comparison with a commercial standard. Peaks 3 (*m/z* at 439) and 7 (*m/z* at 521) yielded a fragment at *m/z* 359 (rosmarinic acid) from the loss of 80 mu (sulphate moiety) and 162 mu (hexoside moiety), respectively, as well as other fragments identical to those observed for peak 9, which allowed their tentative identification as respective sulphated and hexoside derivatives of rosmarinic acid.

Peak 5 showed a UV spectrum with λ_{max} at 330 nm similar to caffeic acid, and a pseudo molecular ion $[M-H]^-$ at *m/z* 473, yielding product ions at *m/z* 311 ($[M-H-162]^-$ loss of a hexosyl moiety), *m/z* 149 (base peak, tartaric acid) and *m/z* 179 (caffeic acid), suggesting that it was a hexoside derivative of caffeoyltartaric acid (caftaric acid).

Peaks 4 and 13 presented the same pseudo molecular ion $[M-H]^-$ at *m/z* 537 and similar UV spectrum and fragmentation pattern (**Figure 4B)**, consistent with the caffeic acid trimer lithospermic acid A (Chen et al., 2011; Zeng et al., 2006). This compound can easily lose 8"-carboxyl group (-44 mu) releasing the peak at *m/z* 493 that further breaks down to form the fragment ion at *m/z* 313 and 295. Salvianolic acids H/I, with the same molecular weight as lithospermic acid A **(4)**, were discarded as possible identities for peaks 4 and 13 due to they have quite a different fragmentation pattern (Ruan et al., 2012; Zeng et al., 2006). Thus, peak 13 was assigned to lithospermic acid A that was expected to elute later than rosmarinic acid (Chen et al., 2011; Zeng et al., 2006; Zhu et al., 2007), whereas peak 4 could correspond to a lithospermic acid A isomer. Lithospermic acid and its mono- and dimethyl esters are known to be bioactive components able to inhibit adenylate cyclase (Lu & Foo, 2002).

Peak 12 presented a pseudo molecular ion $[M-H]^-$ at *m/z* 493, which together with the characteristic fragment ions at *m/z* 313, 295 and 197 (Chen et al., 2011; Ruan et al., 2012) and UV spectrum allowed assigning it as salvianolic acid A **(6)**. This compound is a stilbenoid caffeic acid trimer reported as having a strong oxygen free radical scavenging activity (Lu & Foo, 2002; Zhu et al., 2007), which may be the basis for some of its biological activities. Salvianolic acid A is apparently not very stable and it is likely converted to salvianolic acid C **(7)**, a benzofuran compound from its cyclization (Lu & Foo, 2002). Although salvianolic acid C ($[M-H]^-$ at *m/z* 491) was not found in our samples, other three detected compounds (peaks 11, 14 and 15) had MS^2 spectra showing fragments at *m/z* 491, 311, 293 and 197 characteristic of salvianolic acid C (Chen et al., 2011; Zeng et al., 2006), pointing to they could be derived from this compound. Peaks 11, 14 and 15 presented in their mass spectra other signals resulting from the loss of known fragments. Thus, the losses of 162 mu and 146 mu from the ions *m/z* 829 (peak 11) and 813 (peak 14) to yield the fragment at *m/z* 667 could correspond to hexosyl and rhamnosyl residues, respectively, and further loss from this latter fragment of 132 mu to yield the ion at *m/z* 535 may correspond to the loss of a tartaric acid or a pentose moiety. The same MS^2 fragment at *m/z* 535 was observed for peak 15, which might be attributed to the loss of caffeic acid (-180 mu) from the pseudomolecular ion (*m/z* at 715). Further loss of 44 mu (carboxyl group moiety) from the ion at *m/z* 535 would release the fragment at *m/z* 491 (salvianolic acid C). Despite these observations, no definite structure could be assigned to peaks 11, 14 and 15 that remain unknown.

Peak 6 presented a pseudo molecular ion $[M-H]^-$ at *m/z* 717 and a fragmentation pattern in which successive losses of danshensu (198 mu) or caffeic acid (180 mu) units are observed (**Figure 4C**). Similar mass spectra were reported for salvianolic acid B (also known as lithospermic acid B) and other related isomers (salvianolic acids E/L and isosalvianolic acid B) by different authors (Chen et al., 2011; Nuengchamnong et al., 2011; Ruan et al., 2012; Zeng et al., 2006). Those compounds derive from the condensation of two rosmarinic acid molecules via an oxidative cyclization leading to the formation of a 1,2-dihydronaphthalene ring structure **(8)**. The data here obtained did not allow deciding about the precise identity of the compound. According to the indicated authors all those compounds should elute after the peak of rosmarinic acid, which was not found in our case. For that reason, the compound was just tentatively assigned as a salvianolic acid B isomer.

Peak 8 showed a pseudo molecular ion $[M-H]^-$ at *m/z* 719 and a base peak at *m/z* 359 corresponding to $[M-2H]^{2-}$. These characteristics coincide with those reported by Nuengchamnong et al. (2011) for sagerinic acid, a rosmarinic acid dimer (caffeic acid tetramer), where dimerization occurs by a [2+2] union of the olefinic moieties leading to a cyclobutane structure **(9)** (Lu & Foo, 1999). Peak 16 gave a $[M-H]^-$ ion at *m/z* 313, yielding majority fragment ions at *m/z* 269 and 161 (respective losses of CO_2 and dihydroxy benzene units). Other fragments found were *m/z* 179 that resulted in a loss of 134 mu (also observed in peak 12), corresponding to caffeoyl moiety and *m/z* 135 loss of 44 mu (carboxyl group moiety of caffeic acid). These characteristics were coherent with the identity of salvianolic acid F **(5)** reported by Chen et al. (2011), although that compound was expected to elute earlier than rosmarinic acid (Chen et al., 2011; Zhu et al., 2007), which raises some doubts about its actual identity.

Finally, peak 10 was identified as a flavone, the only flavonoid found in this sample. This peak presented a UV spectra with λ_{max} at 350 nm, and a pseudomolecular ion $[M-H]^-$ at *m/z* 461, releasing a MS^2 fragment at *m/z* 285 ($[M-H-176]^-$, loss of a glucuronyl moiety), corresponding to luteolin. This compound was tentatively identified as luteolin-3'-*O*-glucuronide, described as the major flavonoid in *Melissa officinalis* by Heitz et al. (2000).

3.2. Quantification of the phenolic compounds

Despite the similar profile observed in all the studied lemon balm samples, the quantities found of each compound was different (**Table 2**). Cultivated and *in-vitro* cultured samples presented the lowest quantities of phenolic compounds (59.59 and 30.21 mg/g of infusion, respectively), whereas commercial samples showed the highest contents (109.24 mg/g for tea bag and 101.03 mg/g for granulate sample). In general, the antioxidants content of fresh plant materials is higher than that of dried plant materials due to their degradation during drying. However, some recent studies have shown that dried plant materials (including air-drying) contain higher antioxidants, such as polyphenolics, and antioxidant activity as compared to fresh plant materials (Suvarnakuta, Chaweerungrat, & Devahastin, 2011). This was also observed in the present study where commercial samples (submitted to drying procedures before being processed to the final preparations, bag or granulate) gave higher phenolic compounds probably due to a higher stress associated to their growth conditions. Otherwise, cultivated and, mostly, *in-vitro* cultured seem to be under less stress producing lower amounts of phenolic compounds (secondary metabolites). In fact, *in-vitro* cultured samples grown with controlled light, temperature and nutrients (Matkowski, 2008) optimized to achieve the best conditions (lower stress).

There are a few studies reporting a full characterization of lemon balm (Caniova & Brandsteterova, 2001; Fecka & Turek, 2007; Heitz et al., 2000; Lee, 2010; Patora & Klimek, 2002; Proestos et al., 2005; Ziaková & Brandsteterova, 2003). Some of those studies report the existence of rosmarinic acid as being the most abundant phenolic in this species (Caniova & Brandsteterova, 2001; Fecka & Turek, 2007; Lee, 2010; Ziaková & Brandsteterova, 2003). In all the samples studied herein, this phenolic acid was also the most abundant compound found, being higher in commercial samples, especially in tea bag sample (55.68 mg/g of infusion) and lower in *in-vitro* cultured sample (15.46 mg/g). These contents are slightly higher than those reported by Fecka & Turek (2007) that presented values of rosmarinic acid in *M. officinalis*, ranging from 32.6 to 5.1 mg/g of infusion. Only one flavonoid, luteolin-3'-*O*-glucuronide was found in all the samples, ranging from 8.43 mg/g in commercial granulate sample and 1.22

mg/g in *in-vitro* cultured sample. This compound was also reported by Heitz et al. (2000) as being the major flavonoid in *M. officinalis*. Other authors also described the existence of other luteolin and apigenin derivatives (Patora & Klimek, 2002; Fecka & Turek, 2007) namely luteolin-7-*O*-glucoside (Fecka & Turek, 2007), but they have not been detected in our samples.

This is the first time that caffeic acid derivatives (dimers, trimers and tetramers) were described in *M. officinalis* (lemon balm), although these compounds have been found in other Lamiaceae, especially in *Salvia* spp. Furthermore, the comparison of the phenolic compound profiles of cultivated, *in-vitro* cultured and commercial (bags and granulated) samples is novel. The results obtained highlight the importance of *in-vitro* culture for the production of phenolic compounds such as rosmarinic acid, making this compound always available, independently of environmental conditions that can influence *in-vivo* grown plants.

Information about phenolic compound bioactive forms *in-vivo* and the mechanisms by which they may contribute toward antioxidant effects is still necessary. Therefore, absorption, distribution, metabolism or excretion, or the plasma/tissue levels of the metabolites of phenolic compounds that might occur, should be considered on their bioactivity (Rechner et al., 2002)." Nevertheless, the *in-vitro* antioxidant activity of the main phenolic compounds identified in lemon balm, have been extensively studied. They can trap free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzyme. The antioxidant properties of rosmarinic acid, caffeic acid and luteolin-3'-*O*-glucuronide is well-established. According to Erkan, Ayranci, & Ayranci (2008), rosmarinic acid revealed a DPPH scavenging activity EC_{50} value of 26.03 μg/mL (72.3 μM), a value guaranteed by the levels of rosmarinic acid found in all the studied samples herein (69.6, 99.6, 102.7 and 134.2 μg/mL for *in-vitro* cultured, granulated, cultivated and bag samples, respectively; values calculated from data in **Table 2**). Moreover, this phenolic acid has been reported as potent antioxidant (Lu & Foo, 2001). Caffeic acid has the ability to inhibit LDL oxidation, but also quench radicals and singlet oxygen; at the concentration of 10 μg/mL, caffeic acid showed 68.2% inhibition on lipid peroxidation of linoleic acid emulsion, and proved to be an effective DPPH scavenging, superoxide anion radical scavenging, total reducing power and metal chelating on ferrous ions activities (EC_{50} values lower than 5 μg/mL; Gulçin, 2006). Finally, luteolin-3'-O-glucuronide was reported as having low antioxidant potential (Lu & Foo, 2001)".

Overall, the present study shows that infusion of lemon balm can be a source of phenolic compounds, known by their bioactive effects. The amounts of these compounds found in the different samples are in agreement with the antioxidant potential reported for these samples in a previous study (Dias, Barros, Sousa, & Ferreira, 2012) that were in the following order: commercial tea bag > commercial granulate > cultivated > *in-vitro* cultured lemon balm. Moreover, some of the individual compounds identified in the samples (mainly rosmarinic acid which is the most antioxidant according to literature) could be responsible for the antioxidant activity of lemon balm. Nevertheless, possible synergistic interaction among the antioxidants present in the samples should not be neglected.

Acknowledgements

The authors are grateful to strategic project PEst-OE/AGR/UI0690/2011 for financial support to CIMO. L. Barros thanks to FCT, POPH-QREN and FSE for her grant (SFRH/BPD/4609/2008). The GIP-USAL is financially supported by the Consolider-

Ingenio 2010 Programme (FUN-C-FOOD, CSD2007-00063). M. Dueñas thanks the Spanish "Ramón y Cajal" Programme for a contract.

References

Barros, L., Dueñas, M., Dias, M.I., Sousa, M.J., Santos-Buelga, C., & Ferreira, I.C.F.R. (2012). Phenolic profiles of *in vivo* and *in-vitro* grown *Coriandrum sativum* L. *Food Chemistry*, *132*, 841-848.

Caniova, A., & Brandsteterova, E. (2001). HPLC analysis of phenolic acids in *Melissa officinalis*. *Journal of Liquid Chromatography & Related Technologies*, *24*, 2647-2659.

Chen, H., Zhang, Q., Wang, X., Yang, J., & Wang, Q. (2011). Qualitative analysis and simultaneous quantification of phenolic compounds in the aerial Parts of *Salvia miltiorrhiza* by HPLCDAD and ESI/MSn. *Phytochemical Analysis*, *22*, 247–257.

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 for Human Nutrition*, *66*, 181-186.

Dias, M.I., Barros, L., Sousa, M. J., & Ferreira, I.C.F.R. (2012). Systematic comparison of nutraceuticals and antioxidant potential of cultivated, *in-vitro* cultured and commercial *Melissa officinalis* samples. *Food and Chemical Toxicology*, *50*, 1866-1873.

Erkan, N., Ayranci, G., & Ayranci, E. (2008). Antioxidant activities of rosemary (*Rosmarinus officinalis* L.) extract, blackseed (*Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. *Food Chemistry*, *110*, 76-82.

Fecka, I., & Turek, S. (2007). Determination of water-soluble polyphenolic compounds in commercial herbal teas from Lamiaceae: Peppermint, Melissa, and Sage. *Journal of Agricultural and Food Chemistry*, *55*, 10908-10917.

Flora Ibérica: Plantas Vasculares de la Península Ibérica e Islas baleares. Volume XII. Real Jardín Botánico, CSIC, Madrid 2010, pp. 482-483.

Gulçin, I. (2006). Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). *Toxicology*, *217*, 213-220.

Heitz, A., Carnat, A., Fraisse, D., Carnat, A.P., & Lamaison, J.L. (2000). Luteolin 3'-glucuronide, the major flavonoid from *Melissa officinalis* subsp. *officinalis*. *Fitoterapia*, *71*, 201-202.

Hussain, A.I., Anwar, F., Nigam, P.S., Sarker, S.D., Moore, J.E., Rao, J.R., Mazumdar, A. (2011). Antibacterial activity of some Lamiaceae essential oils using resazurin as an indicator of cell growth. *LWT - Food Science and Technology*, 44, 1199–1206.

Lee, J. (2010). Caffeic acid derivates in dried Lamiaceae and *Echinacea purpurea* products. *Journal of functional Foods*, *2*, 158-162.

Lu, Y., & Foo L.Y. (1999). Rosmarinic acid derivatives from *Salvia officinalis*. *Phytochemistry*, *40*, 91-94.

Lu, Y., & Foo L.Y. (2001). Antioxidant activities of polyphenols from sage (*Salvia officinalis*) *Food Chemistry*, *75*, 197–202.

Lu, Y., & Foo L.Y. (2002). Polyphenolics of Salvia—a review. *Phytochemistry*, 59,117–140.

Matkowski, A. (2008) Plant *in vitro* culture for the production of antioxidants - A review. *Biotechnological Advances*, *26*, 548-560.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473-497.

Nuengchamnong, N., Krittasilp, K., & Ingkaninan, K. (2011). Characterisation of phenolic antioxidants in aqueous extract of *Orthosiphon grandiflorus* tea by LC–ESI-MS/MS coupled to DPPH assay. *Food Chemistry*, *127*, 1287–1293.

Oksman-Caldentey, K. M., & Inzé, D. (2004). Plant cell factories in the post-genomic era: New ways to produce designer secondary metabolites. *Trends in Plant Sciences*, *9*, 433–440.

Patora, J., & Klimek. B. (2002). Flavonoids from lemon balm (*Melissa officinalis* L., Lamiaceae). *Polish Pharmaceutical Society*, *59*, 139-143.

Petersen, M., & Simmonds, M.S.J. (2003). Molecules of interest: rosmarinic acid. *Phytochemistry*, 62, 121–125.

Proestos, C., Chorianopoulos, N., Nychas, G.J.E., & Komaitis, M. (2005). RP-HPLC analysis of the phenolic compounds of plant extracts. Investigation of their antioxidant capacity and antimicrobial activity. *Journal of Agricultural and Food Chemistry*, *53*, 1190-1195.

Rechner A.R., Kuhnle G., Bremner P., Hubbard G.P., Moore K.P., Rice-Evans C.A. (2002). The metabolic fate of dietary polyphenols in humans. Free Radical Biology and Medicine, 33, 220-235.

Ruan, M., Li, Y., Li, X., Luo, J., & Kong, L. (2012). Qualitative and quantitative analysis of the major constituents in Chinese medicinal preparation Guan-Xin-

Ning injection by HPLC-DAD-ESI-MS^n. *Journal of Pharmaceutical and Biomedical Analysis*, 59, 184-189.

Suvarnakuta, P., Chaweerungrat, C., & Devahastin, S. (2011). Effects of drying methods on assay and antioxidant activity of xanthones in mangosteen rind. *Food Chemistry*, *125*, 240-247.

Weitzel, C., & Petersen. M. (2011). Cloning and characterisation of rosmarinic acid synthase from *Melissa officinalis* L. *Phytochemistry*, *72*, 572-578.

Zeng, G., Xiao, H., Liu, J., & Liang, X. (2006). Identification of phenolic constituents in Radix Salvia miltiorrhizae by liquid chromatography/electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry*, 20, 499–506.

Zhu, Z., Zhang, H., Zhao, L., Dong, X., Li, X., Chai, Y., & Zhang. G. (2007). Rapid separation and identification of phenolic and diterpenoid constituents from Radix Salvia miltiorrhizae by high-performance liquid chromatography diode-array detection, electrospray ionization time-of-flight mass spectrometry and electrospray ionization quadrupole ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry*, *21*, 1855–1865.

Ziaková, A., & Brandsteterová. E. (2003). Validation of HPLC determination of phenolic acids present in some *Lamiaceae* family plants. *Journal of Liquid Chromatography & Related Technologies*, *26*, 443-453.

Ziaková, A., Brandsteterová. E., & Blahová. E. (2003). Matrix solid-phase dispersion for the liquid chromatographic determination of phenolic acids in *Melissa officinalis*. *Journal of Chromatography A*, *983*, 271-275.

Figure 1. A. Garden cultivated lemon balm; B. *In-vitro* cultured lemon balm; C. Commercial sample of lemon balm: bag; D. Commercial sample of lemon balm: granulate.

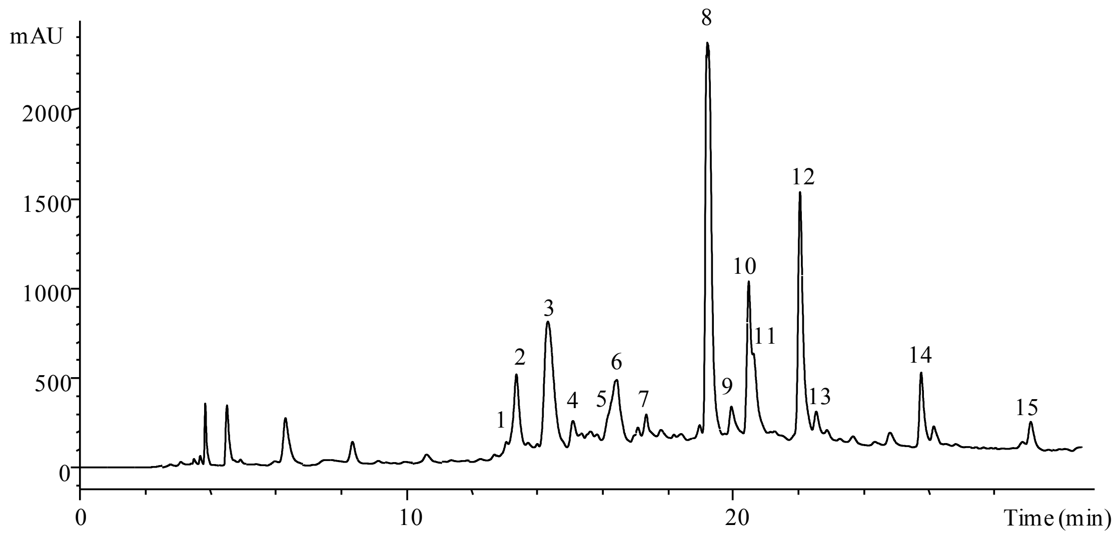
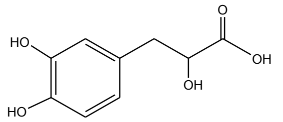
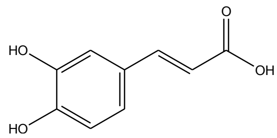


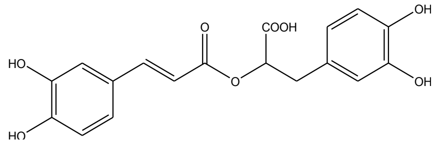
Figure 2. Individual chromatogram of cultivated lemon balm sample, recorded at 280 nm.



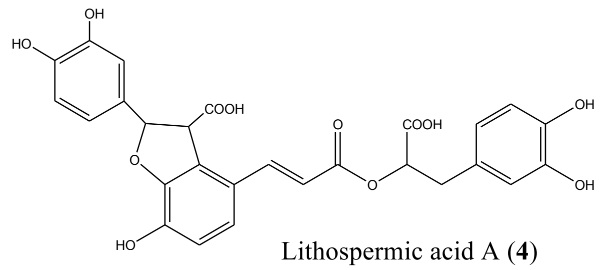
3-(3,4-dihydroxyphenyl)-lactic acid (**1**)



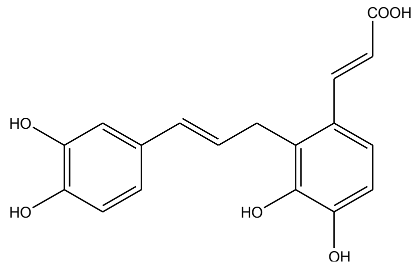
Caffeic acid A (**2**)



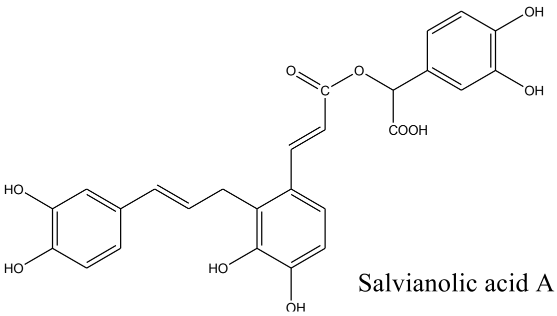
Rosmarinic acid (**3**)



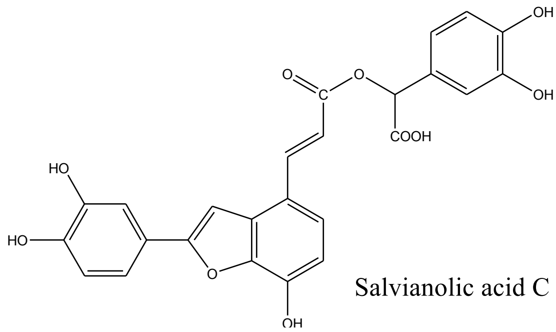
Lithospermic acid A (**4**)



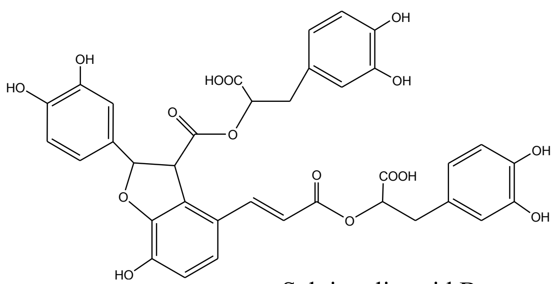
Salvianolic acid F (**5**)



Salvianolic acid A (**6**)

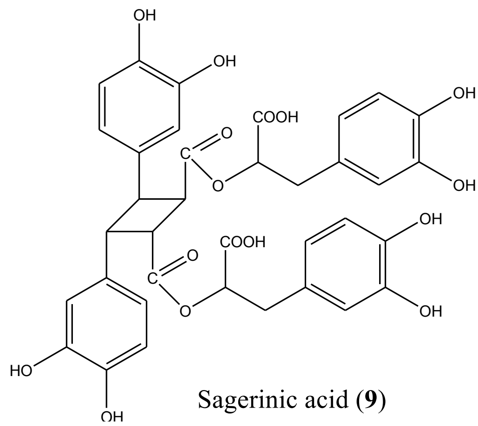


Salvianolic acid C (**7**)

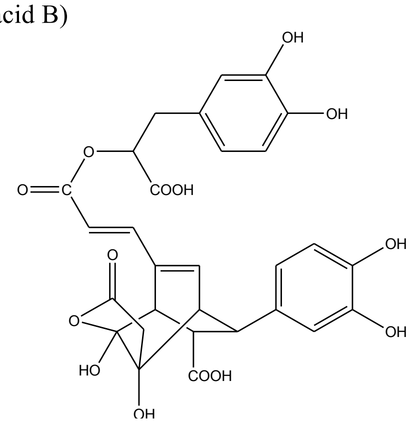


Salvianolic acid B (**8**)

(Lithospermic acid B)

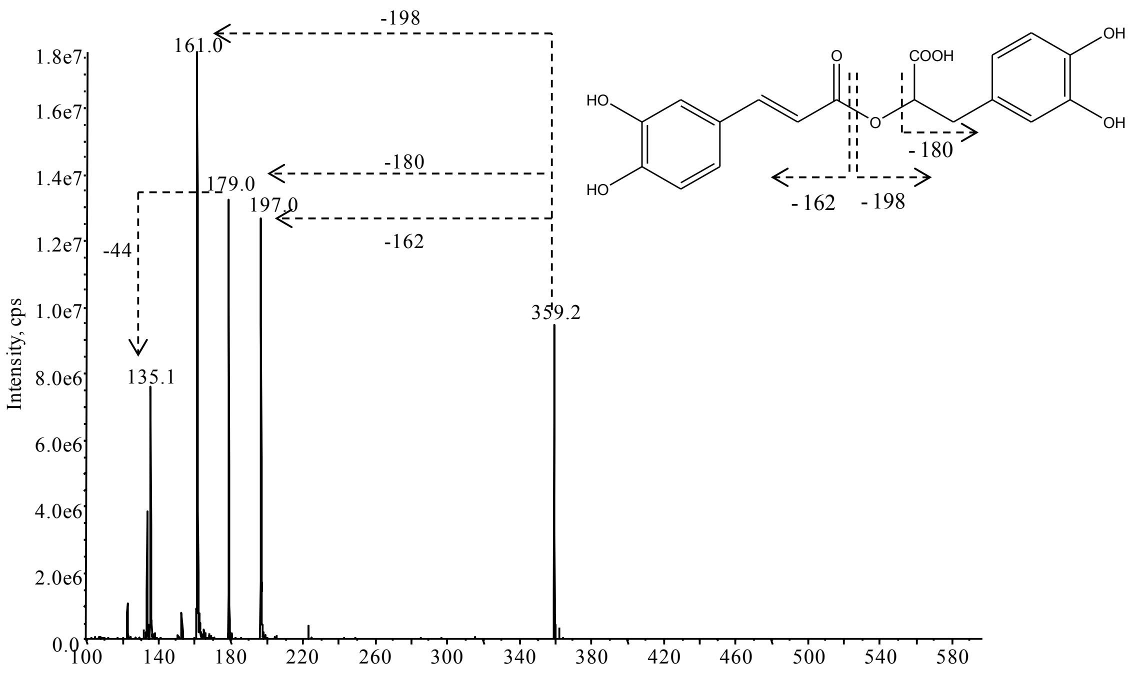


Sagerinic acid (**9**)



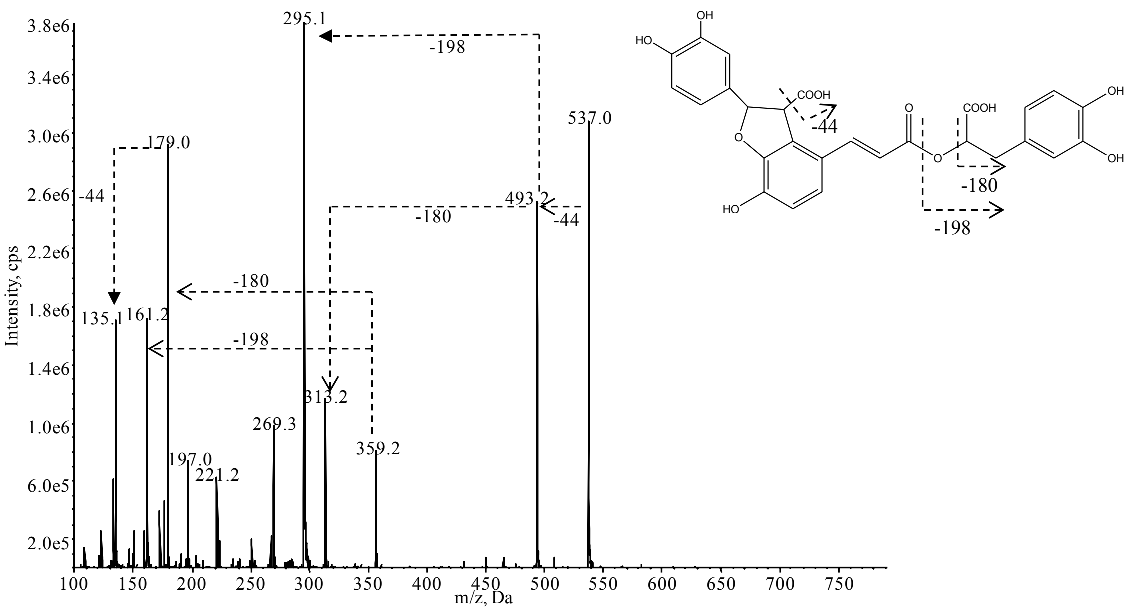
Yunnaneic acid F (**10**)

Figure 3. Chemical strutures of caffeic acid derivatives.



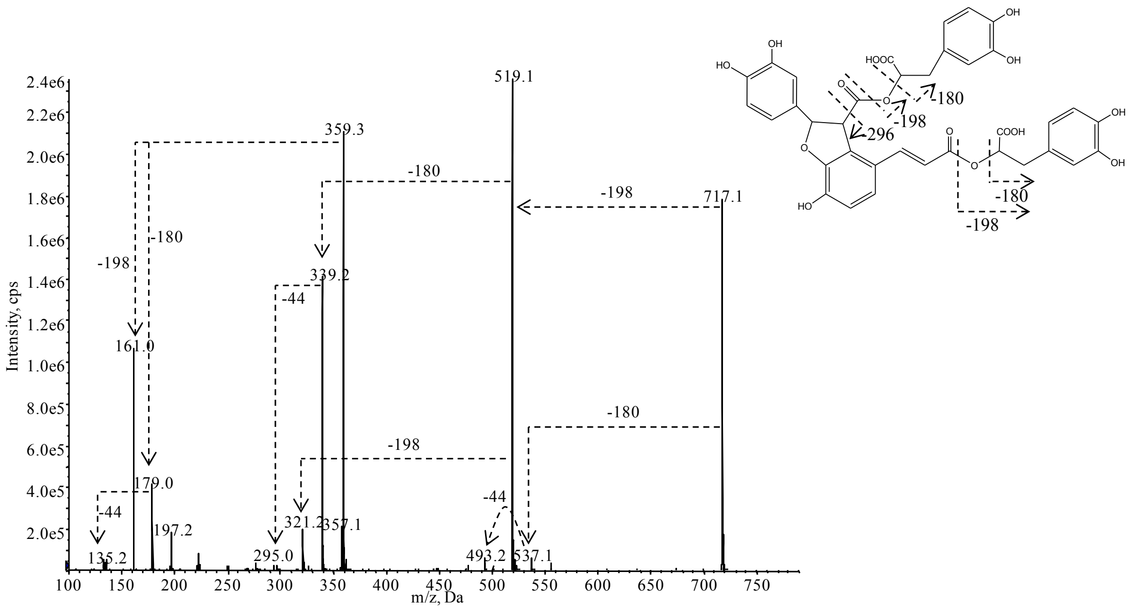
-198
161.0
-180
179.0
197.0
-162
-44
359.2
135.1
Intensity, cps
HO
HO
O
O
COOH
OH
OH
- 180
- 162
- 198

(A)



295.1
-198
537.0
179.0
-44
493.2
-180
-44
-180
-198
135.1
161.2
313.2
269.3
359.2
197.0
221.2
Intensity, cps
m/z, Da
HO
OH
COOH
O
HO
OH
-180
-198

(B)



(C)

Figure 4. Main fragment ions in MS^2 of compounds found in lemon balm: (A) Rosmarinic acid, precursor ion *m/z* 359; (B) lithospermic acid A, precursor ion *m/z* 537; (C) salvianolic acid B isomer, precursor ion *m/z* 717.

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and concentration of phenolic acids and flavonoids in lemon balm.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	6.22	280	197	179(30), 135(100)	3-(3,4-dihydroxyphenyl)-lactic acid
2	13.06	297,334(sh)	597	579(58), 359(8), 295(100), 179(25)	Yunnaneic acid F
3	13.36	330	439	439(100), 359(26), 179(25), 161(48), 135(49)	Sulphated rosmarinic acid
4	14.29	278,324(sh)	537	493(67), 359(17), 313(31), 295(100), 269(26), 197(20), 179(77)	Lithospermic acid A isomer
5	15.09	330	473	311(21), 293(15), 267*, 179(82), 149(100), 135(30)	Caftaric acid hexoside
6	16.22	286,324(sh)	717	537(4), 519(100), 493(7), 359(98), 339(84), 321(5), 295(12), 197(9), 179(16)	Salvianolic acid B isomer
7	16.44	320	521	359(100), 197(24), 179(38), 161(90), 135(24)	Rosmarinic acid hexoside
8	17.34	282,326(sh)	719	539(12), 521(9), 359(100), 197(9), 179(12), 161(51), 135(5)	Sagerinic acid
9	19.19	330	359	197(98), 179(94), 161(100), 135(58)	Rosmarinic acid
10	19.94	350	461	285(100)	Luteolin-3'-*O*-glucuronide
11	20.47	326	829	667(84), 535(100), 491(25),311(55), 293(5), 197(4), 179(9)	Salvianolic acid C derivative
12	20.62	276,338(sh)	493	359(100), 313(12), 295(67), 269(7), 197(21), 179(53)	Salvianolic acid A
13	22.07	288,326(sh)	537	493(47), 359(100), 313(3), 295(20), 269*, 197(42), 179(57)	Lithospermic acid A
14	22.55	330	813	667(12),535(100),491(32),311(70), 293*, 179*	Salvianolic acid C derivative
15	25.78	320	715	535(100), 491(37), 311(92), 293(4), 179*	Salvianolic acid C derivative
16	29.12	286,324(sh)	313	269(59), 203(29), 179*,161(100),135(6)	Salvianolic acid F isomer

Figures in brackets after MS^2 fragment ions refer to their relative abundances. *Relative abundance < 2%

Table 2. Phenolic compounds quantification (mg/g infusion) in different samples of lemon balm.

Peak	Cultivated	*In-vitro* cultured	Commercial bag	Commercial granulate
1	nq	nq	nq	nq
2	0.12 ± 0.01	0.01 ± 0.00	0.09 ± 0.00	0.35 ± 0.05
3	3.63 ± 0.11	2.45 ± 0.10	3.87 ± 0.18	6.34 ± 0.19
4	8.79 ± 0.14	0.57 ± 0.06	7.09 ± 0.46	11.64 ± 0.30
5	0.52 ± 0.02	0.63 ± 0.00	nd	0.44 ± 0.01
6	1.43 ± 0.02	nd	1.12 ± 0.00	1.77 ± 0.01
7	1.98 ± 0.22	2.01 ± 0.01	3.49 ± 0.07	2.90 ± 0.07
8	0.51 ± 0.03	0.36 ± 0.04	2.17 ± 0.06	1.59 ± 0.01
9	20.96 ± 0.16	15.46 ± 0.08	55.68 ± 0.20	39.86 ± 0.26
10	4.11 ± 0.78	1.22 ± 0.06	6.63 ± 0.02	8.43 ± 0.19
11	4.61 ± 0.16	2.12 ± 0.05	2.23 ± 0.37	1.06 ± 0.27
12	2.28 ± 0.35	1.29 ± 0.09	4.08 ± 0.04	3.22 ± 0.10
13	6.84 ± 0.51	3.08 ± 0.31	17.97 ± 1.64	13.68 ± 1.39
14	0.71 ± 0.06	0.68 ± 0.07	0.28 ± 0.06	1.25 ± 0.02
15	2.35 ± 0.17	0.10 ± 0.01	6.55 ± 0.27	8.50 ± 0.15
16	0.77 ± 0.04	0.23 ± 0.00	nd	nd
TPA	**55.48 ± 0.10 c**	**28.99 ± 0.23 d**	**102.61 ± 1.00 a**	**92.60 ± 0.85 b**
TF	**4.11 ± 0.78 c**	**1.22 ± 0.06 d**	**6.63 ± 0.02 b**	**8.43 ± 0.85 a**
TPC	**59.59 ± 0.88 c**	**30.21 ± 0.17 d**	**109.24 ± 0.98 a**	**101.03 ± 0.66 b**

In each row different letters mean significant differences ($p<0.05$). nd- not detected; nq-not quantified; TPA-total phenolic acids; TF- Total flavonoids; TP- Total phenolic compounds.