ENRICHMENT OF ANTIOXIDANT COMPOUNDS FROM LEMON BALM (*Melissa officinalis*) BY PRESSURIZED LIQUID EXTRACTION AND ENZYME-ASSISTED EXTRACTION

Lucian T. Miron, Miguel Herrero, Elena Ibáñez*

Bioactivity and Food Analysis Department
Institute of Food Science Research (CIAL-CSIC)
Nicolás Cabrera 9, Campus Cantoblanco, 28049 – Madrid, Spain

Email: elena@ifi.csic.es

Abstract. The search of new natural sources of bioactive compounds to be employed in the food industry is a hot topic in the field of functional foods. Besides, the use of environmentally respectful technologies focused on the extraction of bioactives from natural matrices is of great importance nowadays. Pressurized liquid extraction (PLE) is a novel extraction method that allows obtaining higher extraction efficiencies while minimizing both, the amount of solvents and the time. On the other hand, enzyme assisted extraction (EAE) can be seen as a way to further improve the extraction mass transfer kinetics when dealing with plant-based materials. In fact, the use of enzymes to break down cells walls might offer new possibilities because the potential bioactive compounds found in cells will be more available to the extraction solvent. In these work, the two extraction mechanisms are studied and tested for the extraction of natural antioxidant compounds from lemon balm (*Melissa officinalis*). Different PLE extraction conditions are tested using ethanol or water as extracting solvents. Besides, the use of different enzymes, namely cellulose, xylanase and pectinase, alone or in combination, is also studied. Best results in terms of total antioxidant activity and extraction yield were obtained when using PLE using water as extracting solvent at 150 °C. Besides, the obtained extracts were chemically characterized using an HPLC-DAD-MS method. Thanks to the combination of these techniques, several interesting phenolic compounds could be identified in the obtained extracts, among which rosmarinic acid is highlighted.

Keywords:Lemon balm, rosmarinic acid, pressurized liquid extraction, enzyme-assisted extraction.

1. Introduction

Lemon balm (*Melissa officinalis*), belonging to the Lamiaceae family, is a plant that grows extensively in central and Mediterranean Europe [1]. Different investigations have shown that lemon balm may possess many beneficial properties such as spasmolytic [2], antimicrobial [3], sedative [4], antitumoral and antioxidant [5] effects. Several studies have been carried out to determine the major constituents of lemon balm [6-8] and have shown that phenolic compounds such as protocatechuic acid, caffeic acid and rosmarinic acid are the most representative in this plant.

Advanced extraction methods allow the attainment of the compounds of interest using low volumes of organic solvents while, at the same time, in a faster and more efficient (higher extraction yield) way. Supercritical carbon dioxide extraction ($scCO_2$), pressurized liquid extraction (PLE), ultrasound-assisted (UAE) and microwave-assisted extraction (MAE), among others, are highlighted [9] within this group.

Enzyme-assisted extraction (EAE) is also gaining attention as an advanced procedure to increase the recovery of bioactive compounds from natural matrices. Cellulases, hemicelullases, pectinases as well as other enzymes, can be used to catalyze the hydrolysis of the cell wall polysaccharides, thus, enabling a better release and a more efficient extraction of phenolic compounds, as it has been previously reported by several authors [10, 11].

Considering these important points, the aim of the present study was to increase polyphenols' recovery from lemon balm using two advanced extraction methods, namely, enzyme-assisted extraction (EAE) and pressurized liquid extraction (PLE). The obtained extracts were exhaustively characterized from a functional point of view by determining their corresponding total phenols concentration (Folin method) and their antioxidant capacities, measured using DPPH radical scavenging assay and trolox equivalents antioxidant capacity assay (TEAC). Besides, a method based on the use of LC-ESI-MS/MS was employed to chemically characterize the extracts as well as to quantify the phenolic compounds present.

2. Materials and methods

2.1 Samples and chemicals

Lemon balm (*Melissa officinalis*) samples consisted of dried leaves purchased in a local herbal store (Madrid, Spain). Before extraction, cryogenic grinding of the sample was performed with dry ice. The samples were stored protected from light at 4 ºC until their use.

Cellulase (from *Trichoderma viride*) 1,5 U/mg for biochemistry was purchased from Merck (Darmstadt, Germany) while endo-1,4-β-xylanase (from *Trichoderma longibrachiatum*) ≥1U/mg was obtained from Sigma-Aldrich (Steinheim, Germany) and lafase® HE Grand Cru (pectolytic enzyme) was supplied by Laffort (Bordeaux, France).

2.2 Enzyme treatment and extraction of phenolic compounds

Samples of 1.0 g of dried, ground lemon balm material were extracted in 50 ml polyethylene centrifuge tubes with a 1:20 solid/liquid ratio, using continuous stirring at 300 rpm. Extractions were carried out at 50º C during 2 h. Extraction solvent was phosphate-citrate buffer at pH 5. Commercial enzymes were applied in a 5% respect to dry matter. The following ratios of enzymes were used: 100% Cellulase (C), 100% endo-1,4-β-xylanase (X), 100 % pectinase (P), C:X (1:1 *w/w*, mix-1), C/P (1:1 *w/w*, mix-2), X/P (1:1 *w/w*, mix-3), C/X/P (1:1:1 *w/w/w*, mix-4). Non-enzymatic control extractions were also performed using phosphate/citrate buffer at pH 5 as solvent.

After the 2 h treatment, each sample was centrifuged (3500 rpm, 4 °C, 10 min), and the resulting supernatant was collected and filtered. All supernatants were lyophilized using a freeze-dryer. The dried extracts obtained were protected from light and stored at -20 °C until analysis.

2.3 Pressurized liquid extraction (PLE)

Extractions were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA). Ultrapure water and ethanol were used as solvents. Extractions were carried out at 150 °C whereas the static extraction time was 20 min. One gram of lemon balm material was packed into 11 mL stainless steel extraction cells after being mixed with 2 g of sea sand. Extraction method was performed according to a procedure previously described [12].

2.4 Determination of total phenols content (TPC)

The TPC of enzymatic and PLE extracts was estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g d.m. (dry matter) according to the Folin-Ciocalteu assay [13]. The absorbance was measured at 760 nm in a microplate spectrophotometer reader (Bio Tek Instruments, Winooski, VT). A standard curve with serial gallic acid solutions (0.031 – 2 mg/mL) was used for calibration. Data were presented as the average of triplicate analyses.

2.5 DPPH radical scavenging activity assay

The antioxidant activity of all the obtained extracts was estimated using the DPPH radical scavenging assay according to a widely-employed method [14]. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, that is, the EC_{50} value. Measurements were done, at least, by triplicate.

2.6 Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC was determined using the method described by Re *et al.* [15] with some modifications. The absorbance was measured at 734 nm every 5 min during 45 min in a microplate spectrophotometer reader (BioTek). Trolox was used as reference standard and results were expressed as TEAC values (mmol of trolox/g extract). These values were obtained from five different concentrations of each extract tested in the assay giving a linear response between 20 and 80% of the blank absorbance. All analyses were done in triplicate.

2.7. Analysis of phenolic compounds by LC-DAD-MS/MS

The samples were analyzed using an Accela liquid chromatograph (Thermo Scientific, San Jose, CA). A Hypersil C_{18}-AR (150 mm×4.6 mm, d.p. 3 µm) column thermostated at 30 °C was used. The mobile phases employed were (A) 0.1% formic acid in propan-2-ol/acetonitrile 30:70 (v/v) and (B) 0.1% formic acid in water, eluted according to the following gradient: 0 min, 95% B ; 6 min, 95% B; 12 min, 75% B; 30 min, 65% B; 40 min, 50% B; 45 min, 5% B; 55 min, 5% B; 60 min, 95% B. The employed flow rate was 0.4 mL/min, whereas 10 µL was the injection volume. The DAD recorded spectra from 190 to 550 nm.
The following parameters were selected for the correct ionization and detection (under full-scan mode) of the studied compounds: Q1 resolution of 0.7 Da FWHM; scan time, 0.351 s; spray voltage, 3000 V; sheath gas pressure, 35 arbitrary units; auxiliary gas pressure, 5 arbitrary units; capillary temperature, 350 ºC; mass range, m/z 90-1000.

2.8. Statistical analysis

IBM SPSS Statistics software v.19 was employed for data elaboration and statistical analysis using a level of significance set at 95 %. One-way analysis of variance (ANOVA), together with Student-Newman-Keuls test, was employed to group extracts based on statistically significant differences. Differences were considered statistically significant if $p < 0.05$.

3. Results and discussion

The main component of the primary plant cell wall is cellulose. This polysaccharide is formed β-(1,4)-linked D-glucoses, forming microfibrils. Besides cellulose, all plant cell walls have a similar structure that consists of pectins (also known as pectic-polysaccharides) and hemicelluloses, including [16]. It is well-known that phenolic compounds may be linked to cell wall polysaccharides. Consequently, their release from these interactions could contribute to an enhanced antioxidant capacity of the extracts derived from plant materials [17].

3.1. EAE and PLE of lemon balm and functional characterization

In this work, several enzymes have been employed, mainly cellulose, β-xylanase and pectinase in order to study the possible influence of an enzymatic treatment for the attainment of bioactive phenolic compounds from lemon balm. Besides, PLE has been also used in parallel with the aim to compare the capabilities of both techniques. To optimize the EAE process pure enzymes were employed, using 1:1 (*w/w*) binary mixtures (mix-1, mix-2, mix-3) and a ternary mixture (mix-4) with equal parts of all three studied enzymes 1:1:1 (*w/w/w*). Figure 1 shows the extraction yields obtained at the different tested conditions, including the use of EAE and PLE using water and ethanol as extracting solvents.

All the extracts resulting from the enzyme treatment, excepting endo 1,4-β-xylanase, showed an increased extraction yield with respect to non-enzymatic control. The highest yield was obtained when a ternary enzyme mixture (mix-4) was used, indicating an eventual additive effect. Thereby, mix-4 demonstrated its efficiency in significantly increasing extraction yield from 56.18% (non-enzymatic control) to 65.17%.

Concerning the results obtained by PLE, as can be seen in Figure 1, the extraction solvent directly influenced the obtained yield for all the studied samples. In fact, the extraction yield was lower than when treated with enzymes, being 60.48% and 12.82% for water and ethanol, respectively.

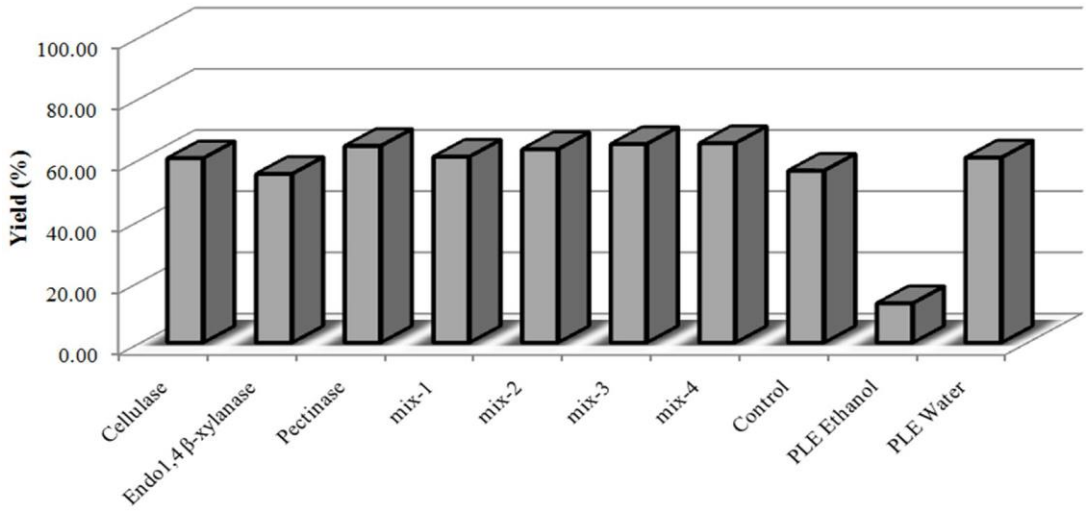

Figure 1. Extraction yield (%) produced after enzymatic treatment and PLE.

The obtained extracts were subsequently characterized in terms of total phenols content and antioxidant capacity (Table 1). The highest phenols content corresponded to the extraction carried out by PLE with water at 150 °C (193.18 mg gallic acid/g extract; see Table 1). On the other hand, the lowest phenolic content was found in the non-enzymatic control as well as when using binary enzyme mixtures (mix-1, mix-2 and mix-3). However, a statistically significant increase on total phenols amount was observed when using the ternary enzymes mixture (mix-4) and when using PLE for the extraction. Although this latter technique produced extracts with higher amounts of total phenols independently of the solvent employed, water was shown to be more appropriate for the extraction of phenols than ethanol, at the tested temperature.

All extracts were able to act against DPPH scavenger radical. As it can be observed in Table 1, extracts from lemon balm obtained by PLE using water as solvent showed significantly higher radical scavenging effect (higher TEAC and lower EC_{50}) followed by the ethanol PLE extract. Thus, both PLE extracts presented by far higher activities than the EAE extracts. On the other hand, the non-enzymatic control presented lower, although significant, antioxidant capacity than the EAE extracts, particularly when using the DPPH assay.

Table 1. Total phenols (as mg gallic acid/g extract), EC_{50} (µg/ml), TEAC (mmol/g) obtained for the different extracts attained at the indicated conditions. Different superscripts letters group statistically significant different samples ($p < 0.05$) for each variable.

No.	Reaction conditions	mg gallic acid/g extract	DPPH EC_{50} (µg/ml)	TEAC (mmol/g)
1.	Cellulase	$71.58 \pm 3.31^{a,b,c}$	$28.80 \pm 0.19^{a,b}$	0.853 ± 0.010^{a}
2.	Xylanase	$73.79 \pm 2.17^{b,c}$	$28.63 \pm 0.52^{a,b,c}$	0.863 ± 0.011^{a}
3.	Pectinase	$72.62 \pm 2.43^{a,b,c}$	28.16 ± 0.32^{c}	0.863 ± 0.021^{a}
4.	mix-1	$65.39 \pm 0.27^{a,d}$	29.14 ± 0.1^{b}	0.849 ± 0.006^{a}
5.	mix-2	$67.90 \pm 1.72^{a,b,d}$	$28.24 \pm 0.23^{a,c}$	0.853 ± 0.010^{a}
6.	mix-3	63.11 ± 0.96^{d}	29.87 ± 0.16^{d}	0.838 ± 0.011^{a}
7.	mix-4	78.55 ± 4.00^{c}	25.71 ± 0.14^{e}	1.017 ± 0.019^{b}
8.	Non-enzymatic control	$65.39 \pm 0.86^{a,d}$	33.14 ± 0.29^{f}	0.827 ± 0.004^{a}
9.	PLE Ethanol	167.19 ± 7.27^{e}	8.09 ± 0.29^{g}	2.527 ± 0.033^{c}
10.	PLE Water	193.18 ± 2.41^{f}	6.81 ± 0.28^{h}	2.999 ± 0.099^{d}

3.2 Chemical characterization of lemon balm extracts using LC-DAD-MS/MS

Figure 2 shows the chromatograms (280 nm) corresponding to the enzymatic, non-enzymatic extracts and to PLE water and ethanolic extracts. Besides, Table 2 summarizes the MS and UV-Vis information collected for the separated components present on the different extracts.

Lemon balm EAE extracts. As can be observed in Figure 2, both lemon balm extracts (control and treated) presented a similar profile, being caffeic acid (peak 5) the main phenolic compound detected. Other important compounds identified in these extracts by comparison with commercial standards were protocatechuic acid (peak 2), p-hydroxybenzoic acid (peak 4) and rosmarinic acid (peak 13). The lemon balm

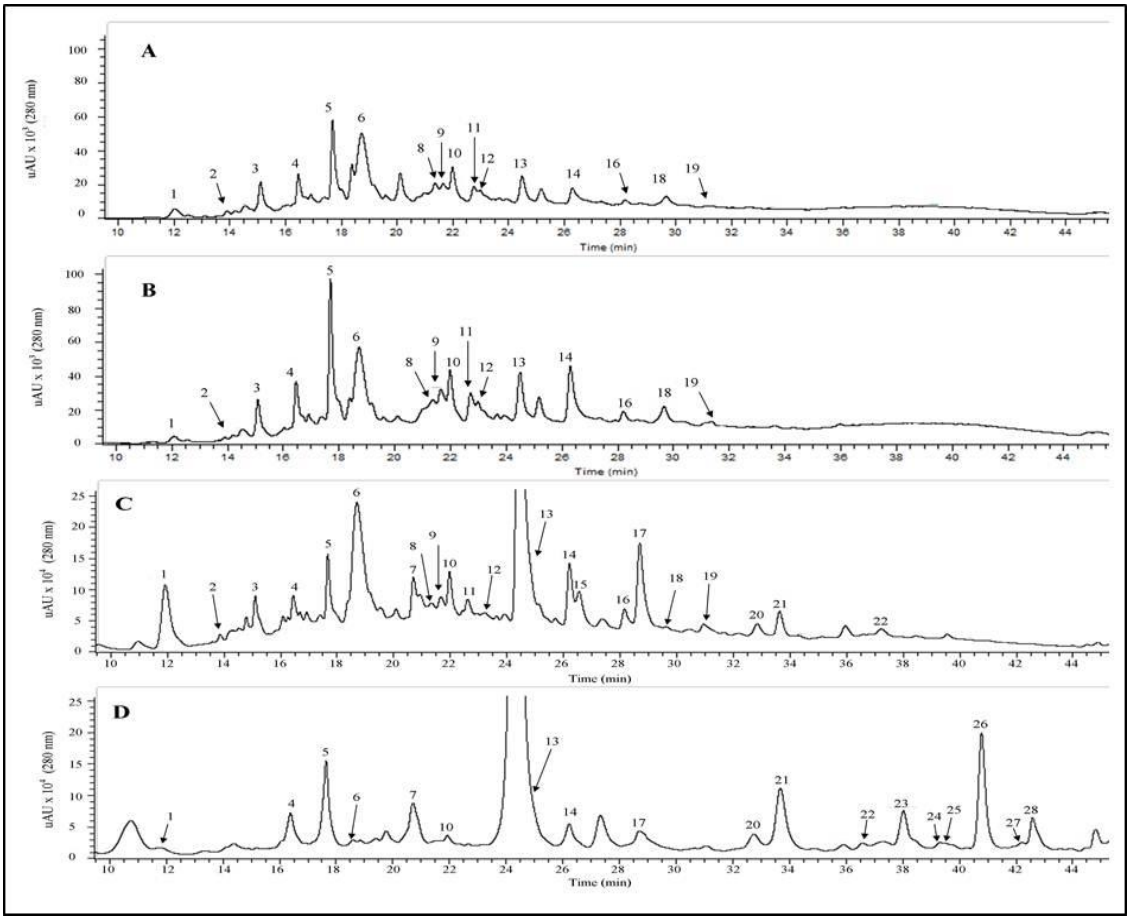

Figure 2. LC-DAD-MS chromatograms (280 nm) of different extracts obtained from lemon balm. A, non-enzymatic control; B, enzymatic treatment with mix-4; C, PLE using water; D, PLE using ethanol.

extracts were very rich in caffeic acid derivatives, such as salvianolic acid H/I (peak 6), a caffeic acid trimer, and salvianolic acid B, E, L and L-isomer (peaks 8, 9, 18 and 19, respectively), caffeic acid tetramers.

Besides caffeic derivatives, that were found in abundance on these extracts, a flavonoid (peak 14) was also tentatively identified; this peak possessed a molecular ion $[M-H]^-$ at m/z 461.1 that yielded a fragment at m/z 284.4 corresponding to luteolin. The UV spectrum presents a maximum absorbance at 338 nm, suggesting that this peak could be tentatively identified as luteolin 7-O-glucuronide.

Lemon balm PLE extracts. The main phenolic compound found in the PLE extracts was rosmarinic acid. Three derivatives of rosmarinic acid were also tentatively assigned, i.e., rosmarinic acid hexoside (peak 7), sulphated rosmarinic acid (peak 11) and sulphated rosmarinic acid isomer (peak 12). Other compounds present in the EAE extract were also identified on the PLE water extract, such as peaks 1-5, 6, 8-13, 14, 16, 18, 19.

Other compounds tentatively identified on PLE water extract were lithospermic acid (peak 15), lithospermic acid isomer (peak 17), rosmarinic acid derivative (peak 20), salvianolic acid C derivative (peak 21), rosmarinic acid derivative (peak 22).

Concerning the ethanol extract, the chromatographic profile showed that this extract was characterized by the presence of compounds with relatively lower polarity such as rosmarinic acid derivatives (peaks 20-24, 26-28).

Table 2. Compounds identified in the lemon balm extracts by LC-MS. Peaks which identification was confirmed using standards are marked with asterisk. Sh, spectral shoulder

Peak no.	Retention time (min)	Identification	UV-Vis maxima (nm)	$[M-H]^-$	Main fragments
1	11.9	dimer R(+)-β-(3,4-dihydroxyphenyl) lactic acid	280	395.3	196.7
2	13.9	Protocatechuic acid*	254, 290	153.2	109
3	15.0	Caftaric acid	295sh, 328	311.2	178.6
4	16.1	p-hydroxybenzoic acid*	280, 312	136.8	-
5	17.4	Caffeic acid*	295sh, 325	178.9	135.09
6	18.7	Salvianolic acid H/I	278, 325sh	537.3	493.4, 295.3, 359.6, 339.1
7	20.6	Rosmarinic acid hexoside	288sh, 322	521.3	359.3
8	21.3	Salvianolic acid B	280, 325sh	717.1	519.3, 359.4, 339
9	21.5	Salvianolic acid E	285, 325	717.3	519.3, 359.4, 339
10	21.9	Sagerinic acid	282, 325sh	719.3	359.3
11	22.7	Sulphated rosmarinic acid	285, 328	439.2	213.9, 258, 229, 359.2
12	22.9	Sulphated rosmarinic acid isomer	280, 326sh	439.2	213.9, 258, 229, 359.2
13	24.2	Rosmarinic acid*	289sh, 328	358.7	160.7, 178.7, 197.3
14	26.2	Luteolin 7-O-glucuronide	269, 289sh, 338	461.1	284.4
15	26.5	Lithospermic acid	289, 327	537.2	493.4, 359.7
16	28.1	Salvianolic acid C derivative	290, 325	829.3	535.3, 667.3, 491.6, 311.2
17	28.6	Lithospermic acid isomer	290, 328	537.3	359.7, 493.4
18	29.8	Salvianolic acid L	278, 325sh	717.3	519.3, 359.2, 339
19	31.5	Salvianolic acid L isomer	280, 325sh	717.3	519.3, 359.2, 339
20	32.8	Rosmarinic acid derivative	250sh, 350	535.8	359.1, 174.8, 158.7
21	33.6	Salvianolic acid C derivative	295sh, 326	715.3	535.3, 491.6, 311.2
22	37.2	Rosmarinic acid derivative	280, 327	495.1	359.2, 158.7, 333.6, 313.1
23	38.0	Rosmarinic acid derivative	290sh, 327	565.3	359.4, 519.3
24	39.5	Rosmarinic acid derivative	286, 326	495.9	359.6, 196, 268, 406.4, 450.1
25	39.7	Salvianolic acid A isomer	292, 326	493.2	312.8, 179.1, 160.8
26	40.8	Rosmarinic acid derivative	300sh, 330	565.2	158.9, 359.3, 519.3
27	42.2	Rosmarinic acid derivative	290sh, 327sh	565.3	158.9, 359.7, 519.5
28	42.6	Rosmarinic acid derivative	288, 347	565.3	359.4, 519.6

3.3. Quantification of phenolic antioxidants

For quantitative analysis, calibration curves were constructed by injecting known concentrations of the different available standard compounds diluted in methanol. Due to the lack of some commercial reference compound, the amounts of caffeic acid derivatives in the extracts (peaks 6, 8, 9, 15-19, 21, 25) were estimated as caffeic acid equivalents, rosmarinic acid derivatives (peaks 7, 11, 12, 20, 22-24, 26-28) were calculated as rosmarinic acid equivalents, while the luteolin 7-O-glucuronide was quantified using luteolin-7-O-glucoside standard. Dimer R (+)-β-(3,4-dihydroxyphenyl) lactic acid and caftaric acid were calculated as caffeic acid equivalents. Table 3 summarized the obtained results. The amount of each phenolic compound was higher in the enzymatic extract when compared to the control.

This increase after the enzymatic treatment would be the responsible for the higher antioxidant capacity observed in the EAE extracts, particularly in the mix-4 extract. Sagerinic acid was the compound found in highest quantity on these extracts (3.944 µg/mg on enzymatic extract and 3.442 µg/mg on non-enzymatic control) followed by rosmanic acid (3.482 µg/mg and 2.956 µg/mg, respectively).

Concerning the PLE extracts, content of each phenolic compound varied significantly. Thus, on the water extracts higher quantities of the more polar compounds were found, while on the ethanolic extracts higher quantities of low polar compounds were determined. In both extracts, rosmarinic acid was, by far, the most abundant compound. PLE ethanol extract contained 90.527 µg/mg rosmarinic acid whereas the PLE water extract possessed 45.725 µg/mg extract. Possible synergistic effects among the phenolic compounds present should not be neglected in the case of water extracts.

Table 3. Quantification of phenolic antioxidants found in the lemon balm extracts. Concentration indicated as µg/mg extract ± sd. NF: not found.

Peak no.	Compound identified	Mix-4	Non-enzymatic control	Water 150 °C	Ethanol 150 °C
1	dimer R(+)-β-(3,4-dihydroxyphenyl) lactic acid	0.943 ± 0.05	0.969 ± 0.012	5.041 ± 0.443	1.379 ± 0.140
2	Protocatechuic acid	0.096 ± 0.001	0.083 ± 0.001	0.383 ± 0.026	NF
3	Caftaric acid	1.250 ± 0.042	1.157 ± 0.001	2.733 ± 0.356	NF
4	p-hydroxybenzoic acid	0.296 ± 0.011	0.195 ± 0.026	0.395 ± 0.028	1.025 ± 0.039
5	Caffeic acid	1.812 ± 0.171	1.516 ± 0.147	2.255 ± 0.016	3.793 ± 0.104
6	Salvianolic acid H/I	2.636 ± 0.104	2.100 ± 0.123	9.040 ± 0.460	1.289 ± 0.079
7	Rosmarinic acid hexoside	NF	NF	5.582 ± 0.348	5.803 ± 0.284
8	Salvianolic acid B	1.088 ± 0.007	1.065 ± 0.011	1.225 ± 0.031	NF
9	Salvianolic acid E	1.269 ± 0.044	1.101 ± 0.040	1.435 ± 0.108	NF
10	Sagerinic acid	3.944 ± 0.159	3.442 ± 0.159	6.972 ± 0.360	3.095 ± 0.111
11	Sulphated rosmarinic acid	3.009 ± 0.058	2.778 ± 0.014	3.308 ± 0.084	NF
12	Sulphated rosmarinic acid isomer	3.146 ± 0.058	2.784 ± 0.045	<LOQ	NF
13	Rosmarinic acid	3.482 ± 0.203	2.956 ± 0.020	45.725 ± 2.336	90.527 ± 4.744
14	Luteolin 7-O-glucuronide	2.001 ± 0.257	1.012 ± 0.041	5.621 ± 0.208	2.801 ± 0.013
15	Lithospermic acid	NF	NF	2.174 ± 0.029	NF
16	Salvianolic acid C derivative	0.902 ± 0.014	0.882 ± 0.001	1.509 ± 0.023	NF
17	Lithospermic acid isomer	NF	NF	3.799 ± 0.280	1.375 ± 0.029
18	Salvianolic acid L	1.088 ± 0.019	1.021 ± 0.027	NF	NF
19	Salvianolic acid L isomer	<LOQ	<LOQ	<LOQ	NF
20	Rosmarinic acid derivative	NF	NF	3.678 ± 0.161	3.729 ± 0.091
21	Salvianolic acid C derivative	NF	NF	1.686 ± 0.016	4.555 ± 0.321
22	Rosmarinic acid derivative	NF	NF	1.312 ± 0.026	1.119 ± 0.065
23	Rosmarinic derivative	NF	NF	NF	5.991 ± 0.305
24	Rosmarinic derivative	NF	NF	NF	2.759 ± 0.065
25	Salvianolic acid A isomer	NF	NF	NF	0.926 ± 0.009
26	Rosmarinic derivative	NF	NF	NF	13.309 ± 0.457
27	Rosmarinic acid derivative	NF	NF	NF	<LOQ
28	Rosmarinic acid derivative	NF	NF	NF	4.651 ± 0.041

4. Conclusions

Results showed that a mixture of enzymes (cellulose, endo 1,4-β-xylanase and pectinase) presented higher extraction yield and antioxidant capacity compared to non-enzymatic control, suggesting a disruption of cell wall of lemon balm. PLE was able to provide water and ethanol extracts that presented significantly better antioxidant capacities, providing similar extraction yields in the case of water. The use of ethanol limited the extraction yield, although the amount of phenolic compounds was higher compared to EAE. Nevertheless, PLE water extract presented the highest amount of total phenols reaching 193.18 mg gallic acid/g extract, that corresponded also to the highest antioxidant capacity (EC_{50} = 6.81 µg/ml). Lemon balm extracts were very rich in caffeic acid derivatives and rosmarinic acid derivatives, some of them being identified for the first time in this plant, such as: salvianolic acid H/I, salvianolic acid E, salvianolic acid L and salvianolic acid L isomer. In conclusion, enzyme assisted extraction (EAE) and pressurized liquid extraction (PLE) have been shown as useful environmentally friendly extraction techniques to efficiently recover bioactive compounds from lemon balm.

Acknowledgements

This work was financed thanks to AGL2011-29857-C03-01 (Ministerio de Economía y Competitividad, MINECO) and ALIBIRD, S2009/AGR-1469 (Comunidad de Madrid) projects. MH would like to thank MICINN for a “Ramón y Cajal” research contract.

References

[1] K. Dastmalchi, H.J. Damien Dorman, P.P. Oinonen, Y. Darwis, I. Laakso, R. Hiltunen, Chemical composition and in vitro antioxidative activity of a lemon balm (Melissa officinalis L.) extract, LWT - Food Science and Technology, 41 (2008) 391-400.
[2] E. Yarnell, K. Abascal, Spasmolytic botanicals, Alternative and Complementary Therapies, 17 (2011) 169-174.
[3] N. Ceyhan, D. Keskin, A. Ugur, Antimicrobial activities of different extracts of eight plant species from four different family against some pathogenic microoorganisms, Journal of Food, Agriculture and Environment, 10 (2012) 193-197.
[4] M.E. Petenatti, E.M. Petenatti, L.A. del Vitto, M.R. Téves, N.O. Caffini, E.J. Marchevsky, R.G. Pellerano, Evaluation of macro and microminerals in crude drugs and infusions of five herbs widely used as sedatives, Brazilian Journal of Pharmacognosy, 21 (2011) 1144-1149.
[5] A.C. de Sousa, D.S. Alviano, A.F. Blank, P. Barreto Alves, C.S. Alviano, C.R. Gattass, Melissa officinalis L. essential oil: Antitumoral and antioxidant activities, Journal of Pharmacy and Pharmacology, 56 (2004) 677-681.
[6] S. Kim, E.J. Yun, J.S. Bak, H. Lee, S.J. Lee, C.T. Kim, J.H. Lee, K.H. Kim, Response surface optimised extraction and chromatographic purification of rosmarinic acid from Melissa officinalis leaves, Food Chemistry, 121 (2010) 521-526.
[7] J.T. Lin, Y.C. Chen, Y.C. Lee, C.W. Rolis Hou, F.L. Chen, D.J. Yang, Antioxidant, anti-proliferative and cyclooxygenase-2 inhibitory activities of ethanolic extracts from lemon balm (Melissa officinalis L.) leaves, LWT - Food Science and Technology, 49 (2012) 1-7.
[8] A. Caniova, E. Brandsteterova, HPLC analysis of phenolic acids in *Melissa officinalis*, Journal of Liquid Chromatography and Related Technologies, 24 (2001) 2647-2659.
[9] P. Garcia-Salas, A. Morales-Soto, A. Segura-Carretero, A. Fernández-Gutiérrez, Phenolic-compound-extraction systems for fruit and vegetable samples, Molecules, 15 (2010) 8813-8826.
[10] M. Pinelo, B. Zornoza, A.S. Meyer, Selective release of phenols from apple skin: Mass transfer kinetics during solvent and enzyme-assisted extraction, Separation and Purification Technology, 63 (2008) 620-627.
[11] M. Pinelo, A.S. Meyer, Enzyme-assisted extraction of antioxidants: Release of phenols from vegetal matrixes, Electronic Journal of Environmental, Agricultural and Food Chemistry, 7 (2008) 3217-3220.
[12] M. Herrero, E. Ibáñez, J. Señoráns, A. Cifuentes, Pressurized liquid extracts from Spirulina platensis microalga: Determination of their antioxidant activity and preliminary analysis by micellar electrokinetic chromatography, Journal of Chromatography A, 1047 (2004) 195-203.
[13] M. Koşar, H.J.D. Dorman, R. Hiltunen, Effect of an acid treatment on the phytochemical and antioxidant characteristics of extracts from selected Lamiaceae species, Food Chemistry, 91 (2005) 525-533.
[14] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, LWT - Food Science and Technology, 28 (1995) 25-30.
[15] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biology and Medicine, 26 (1999) 1231-1237.
[16] D.B. Jordan, M.J. Bowman, J.D. Braker, B.S. Dien, R.E. Hector, C.C. Lee, J.A. Mertens, K. Wagschal, Plant cell walls to ethanol, Biochemical Journal, 442 (2012) 241-252
[17] C.Y. Gan, A.A. Latiff, Extraction of antioxidant pectic-polysaccharide from mangosteen (Garcinia mangostana) rind: Optimization using response surface methodology, Carbohydrate Polymers, 83 (2011) 600-607.