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

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ABSTRACT

In this work enzyme-assisted extraction (EAE) and pressurized liquid extraction (PLE) are applied for extraction of natural compounds from lemon balm (*Melissa officinalis*). Cellulase, endo- β -1,4 xylanase and pectinase were studied in order to degrade cell wall of lemon balm leaves and to release phenolic compounds. On the other hand, in order to compare the performance obtained with EAE, PLE using water and ethanol was employed maintaining 150° C as extraction temperature.

The obtained extracts were characterized in terms of antioxidant capacity by using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging and trolox equivalent antioxidant capacity (TEAC) *in-vitro* assays, whereas the Folin-Ciocalteu procedure was employed to estimate the total phenols content. On the other hand, extracts were chemically characterized by liquid chromatography tandem mass spectrometry (LC-MS/MS). The results showed that EAE enhanced the total phenolic content and the antioxidant capacity compared to a non-enzymatic control. PLE extracts presented higher amount of phenols and antioxidant capacity than enzyme-assisted extracts, reaching the highest values on water extracts (193.18 mg gallic acid/g extract and $EC_{50} = 6.81 \mu\text{g/mL}$). Among the bioactive phenolic compounds identified in lemon balm, rosmarinic acid was the main component, although other important compounds were also identified, such as caffeic acid derivatives (salvianolic acids, lithospermic acid) and rosmarinic acid derivatives (rosmarinic acid hexoside, sagerinic acid, sulphated rosmarinic acid).

The present study confirms that EAE and PLE can be considered alternative methods for the extraction of natural compounds with biological activity from natural sources.

42 **Keywords:** rosmarinic acid; caffeic acid derivatives; enzyme-assisted extraction; lemon balm;
43 polyphenols; pressurized liquid extraction.

44

1. INTRODUCTION

In the last decades an increasing interest in functional foods has been observed, these products being able to provide with additional health benefits compared to traditional foods. Functional foods are especially designed containing functional ingredients with different bioactivities, among which, antioxidant activity is pointed out.

Antioxidants such as phenolic acids, flavonoids, tannins, coumarins, xanthenes, lignans and terpenoids are widely distributed in all parts of higher plants (bark, flowers, fruits, leaves, pods, seeds, stems and wood) and have been broadly investigated due to their natural origin as well as for their ability to act as free radical scavengers [1]. The therapeutic potential of natural antioxidants from plant materials has been studied for the prevention of certain chronic diseases such as some types of cancer [2], cardiovascular diseases [3] or neurodegenerative diseases [4], which are directly related to oxidative stress.

Lemon balm (*Melissa officinalis*), belonging to the Lamiaceae family, is a plant that grows extensively in central and Mediterranean Europe [5]; however, it is cultivated throughout the world because of its culinary properties. Several investigations have shown that lemon balm may possess many beneficial effects such as spasmolytic [6], antimicrobial [7], sedative [8], antitumoral and antioxidant [9] effects. Besides, this plant could also help reducing anxiety, depression, sleep disturbance [10] and gastrointestinal disorders [11]. Several studies have been carried out to determine the major constituents of lemon balm [12-14] and their role in the mentioned activities, and have shown that phenolic compounds such as protocatechuic acid, caffeic acid and rosmarinic acid are the most representative in this plant. Moreover, other phenolic compounds have been isolated from lemon balm, such as flavonoids [15] and, more

recently, dimers (salvianolic acid F), trimers (lithospermic acid A, salvianolic acid A) and tetramers (salvianolic acid B isomer) of caffeic acid [16].

Nowadays, effective extraction methods are required to use plant extracts as natural sources of functional ingredients with potential applications in the food industry. Advanced extraction methods are faster and more efficient than traditional ones and use lower volumes of organic solvents. Among these techniques, supercritical carbon dioxide extraction (scCO₂), pressurized liquid extraction (PLE), ultrasound-assisted (UAE) and microwave-assisted extraction (MAE), among others, are highlighted [17]. PLE is based on the application of high temperatures and pressures in order to perform solvent extractions with significantly improved mass transfer [18]. Although this technique has been already employed to extract bioactive compounds from different vegetable matrices, its application to lemon balm has been just marginally explored [19].

Enzyme-assisted extraction (EAE) is also gaining a lot of attention as an efficient procedure to enhance the release and recovery of bioactive compounds from plants and algae, among others [20-22]. Enzymes can effectively catalyze the degradation of vegetable cell walls, favoring the release of bioactive components contained inside the cells. The primary cell wall of plants is mainly composed of cellulose, hemicelluloses (xyloglucans), pectin and proteins [23]. Besides, phenolic compounds have been reported to be linked to cell wall polysaccharides by hydrophobic interactions and hydrogen bonds. Therefore, cellulases, hemicellulases, pectinases as well as other enzymes can be used to catalyze the hydrolysis of 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 [24, 25].

Consequently, the aim of this 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 lemon balm leaves purchased in a local herbal store (Madrid, Spain). Before extraction, cryogenic grinding of the sample was performed with dry ice (Grindomix GM200, Retsch, Haan, Germany). The samples were stored protected from light at 4 °C until their use.

1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH, 95% purity), ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), gallic acid, potassium persulfate and citric acid were obtained from Sigma-Aldrich (Madrid, Spain), ethanol from VWR BDH Prolabo (Madrid, Spain) and methanol from Panreac Quimica (Barcelona, Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and formic acid were provided by Fluka Chemie AG (Buchs, Switzerland). Folin-Ciocalteu phenol reagent, sodium carbonate (Na_2CO_3), and sodium phosphate, dibasic (Na_2HPO_4) were acquired from Merck (Darmstadt, Germany).

Acetonitrile (ACN) and propan-2-ol were obtained from LabScan (Dublin, Ireland) whereas the water used was Milli-Q Water (Millipore, Billerica, MA, USA).

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*) ≥ 1 U/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, with continuous stirring at 300 rpm. Extractions were carried out at 50° C during 2 h. Extraction solvent was phosphate-citrate buffer at pH 5. Temperature and pH conditions were selected as a compromise taking into consideration the manufacturers' instructions. In fact, those conditions were within the optimum pH and temperature ranges for the three tested enzymes. Commercial enzymes were applied directly on each dry and ground sample, prior to solvent incorporation, in a 5% (w/w) respect to dry matter. The following proportions 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). These ratios of enzymes were established based on simplex-centroid design (Figure 1). Non-enzymatic control extractions were also performed using phosphate/citrate buffer at pH 5 as solvent.

The enzymatic treatment and the phenolic compounds extraction were done altogether. After the 2 h treatment, each sample was centrifuged (3500 rpm, 4 °C, 10 min), and the resulting supernatant was collected and filtered with vacuum through a Whatman No. 40 filter paper. All

supernatants were lyophilized using a freeze-dryer (Labconco Corporation, Missouri, USA). 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), equipped with a solvent controller unit. Ultrapure water and ethanol were used as solvents. At the beginning of the day, the solvents were sonicated for 10 min. Extractions were carried out at 150 °C whereas the static extraction time was 20 min. An instrumentally-preset warming-up time of 7 min was also accomplished before the static extraction period. 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 [26].

Once extractions were finished, the solvents were removed; for the evaporation of the ethanol, a Rotavapor R-210 (from Buchi Labortechnik AG, Flawil, Switzerland) was used, whereas water extracts were lyophilized using a freeze-dryer (Labconco Corporation, Missouri, USA).

2.4. Determination of total phenols content (TPC)

The TPC of EAE 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 [27]. The total volume of reaction mixture was miniaturized to 1 mL. Briefly, 10 µl aliquot of extract solution (concentration range from 5 to 10 mg/mL) and 600 µL ultrapure water were mixed, to which 50 µL undiluted Folin-Ciocalteu reagent was subsequently added. After 1 min, 150 µL of 20% (w/v) Na₂CO₃ were added and the volume was made up to 1.0 mL with water. The samples were

incubated for 2 h at 25 °C in the darkness. Later on, 300 µL of each reaction mixture were transferred to a 96-well microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader Powerwave XS (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 [28]. Briefly, a solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Different concentrations of extracts (5 different concentrations for each extract) were tested. Twenty five microliters of these solutions were added to 975 µL of DPPH diluted solution to complete the final reaction medium (1 mL). After 4 h at room temperature in absence of light, 300 µL of each reaction mixture were transferred into a 96-well microplate where the absorbance was measured at 516 nm in a Powerwave XS microplate spectrophotometer reader (Bio Tek). DPPH-methanol solution was used as a control sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve (1.5 – 9 mM DPPH). 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 EC₅₀ value. Consequently, the lower the EC₅₀ obtained, the higher the antioxidant activity. 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. [29] with some modifications. ABTS^{•+} radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature during 16 h before use. The aqueous ABTS^{•+} solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.7 ($\pm$ 0.02) at 734 nm. Ten microliters of sample (5 different concentrations ranging from 0.062 to 2 mg/mL) and 1 mL of ABTS^{•+} solution were mixed in an eppendorf vial and 300 μ L of the mixture were transferred into a 96-well microplate. The absorbance was measured at 734 nm every 5 min during 45 min in a Powerwave XS 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) equipped with a diode array detector (DAD) and an autosampler. A Hypersil C₁₈-AR (150 mm $\times$ 4.6 mm, d.p. 3 μ m) (Thermo Scientific) column thermostated at 30 °C was used. The mobile phases used 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 were 10 μ L used as injection volume. The DAD recorded the spectra from 190 to 550 nm.

The instrument was also coupled to a TSQ Quantum triple quadrupole MS (Thermo) analyzer via an electrospray interface, operated under negative ionization mode. 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 °C; 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 primary cell wall of dicotyledonous plants is mainly composed by cellulose; the structure of cellulose is formed by β -(1,4)-linked D-glucoses, which are tightly linked by hydrogen bonds to forms microfibrils. Besides cellulose, all plant cell walls have a similar structure that consists of pectins (also known as pectic-polysaccharides) and hemicelluloses, among which xylans are the most abundant class [23]. Polyphenols may be linked to cell wall polysaccharides, and, therefore, their release could contribute to an enhanced antioxidant capacity [30]. Considering this typical composition of plant cell walls, cellulase, β -xylanase and pectinase were selected for enzymatic extraction of polyphenols from lemon balm. Additionally, to compare with the obtained extracts, another advanced extraction technique, PLE, that has already showed its potential for bioactive

extractions from diverse matrices [18] was also employed. To the best of our knowledge, this approach has not been used to extract potential bioactive phenolics from lemon balm so far. Extracts obtained by EAE and PLE were characterized in terms of their functional activity and chemical composition, in order to be able to correlate the antioxidant capacity observed to a particular chemical composition.

3.1. EAE and PLE of lemon balm and functional characterization

To more effectively use enzymes for enhancing the recovery of polyphenols and other antioxidant compounds it is important to understand their catalytic activity, mode of action and to adjust influencing factors to find the optimal reaction conditions. In this sense, reaction/extraction time, process temperature, concentration of the enzymes and pH of the reaction medium are the most important parameters that influence the extraction efficiency of polyphenols. Following enzymes suppliers' instructions, the extraction temperature and pH were selected in the middle of optimal range for the employed enzymes, 50 °C and pH 5, respectively. The pH was adjusted by using a phosphate/citrate buffer solution. Based on previous experience with natural matrices [24] which shows that total phenols diffused rapidly into the solvent, the extraction time was set at 2 h, considering that is sufficient to ensure the complete release of polyphenols. After selection of the suitable enzymes, the proportion of substrate to enzyme or the enzyme concentration are the most influencing parameters in the extraction process. Therefore, according to several previous studies [31, 32] which show that the use of higher concentration of enzymes improved the recovery of polyphenols, the enzyme concentration was selected at 5% respect to dry matter.

248 In order to find the optimal enzyme mixtures that provide the best extraction yield, a simplex
249 centroid experimental design for mixtures with three components was performed. As can be seen
250 in Figure 1, experiments 1, 2, 3 were vertex points of the design corresponding to pure enzymes.
251 Points 4, 5, 6 on the sides were 1:1 (w/w) binary mixtures (mix-1, mix-2, mix-3) while points 7,
252 8, 9 were ternary mixture (mix-4) with equal parts of all three enzymes 1:1:1 (w/w/w). As
253 mentioned, PLE was also employed in this study as a comparison method. Two different
254 solvents, ethanol and water, at 150 °C, were selected for PLE of lemon balm, to cover different
255 polarities. Those conditions were selected based on previous works in which these solvent-
256 temperature combinations showed to be useful for the extraction of phenolic antioxidants from
257 other matrices [18,33]. Figure 2 shows the extraction yields obtained at the different tested
258 conditions. As it can be observed, all the enzymes or enzyme mixtures involved in this study,
259 except endo 1,4 β -xylanase, show an increased extraction yield with respect to non-enzymatic
260 control. The highest yield was obtained when a ternary enzyme mixture (mix-4) was used,
261 indicating an eventual additive effect. Thereby, the mix-4 has proved its efficiency in increasing
262 extraction yield from 56.2% (non-enzymatic control) to 65.2%. These results suggest that when
263 the enzymes are used altogether a more complete breakdown of cell walls was attained, higher
264 than the hydrolysis degree that could be reached when the enzymes acted separately.

265 Concerning the results obtained by PLE, as can be seen in Figure 2, the extraction method and
266 the extraction solvent directly influenced the obtained yield for all the studied samples. Thus, in
267 the case of PLE extracts the extraction yield was lower than when treated with enzymes, being
268 60.5% and 12.8% for water and ethanol, respectively. This observation suggests that PLE is a
269 more selective extraction technique, above all depending on the solvent used.

270 The next step consisted on the functional analysis of the obtained extracts. As mentioned, the
271 antioxidant capacity of lemon balm extracts was measured by using two different *in vitro* assays
272 (DPPH and TEAC), whereas their total phenols content was also determined (Folin method). The
273 analysis of the extracts obtained by EAE and PLE revealed the presence of high amounts of
274 phenolic compounds in all of them (Table 1). The higher phenols content corresponded to the
275 extraction carried out by PLE with water at 150 °C (193.18 mg gallic acid/g extract; see Table
276 1). The lowest percentage of phenolic content was found in extract obtained by non-enzymatic
277 control and when using binary enzyme mixtures (mix-1, mix-2 and mix-3) suggesting that lignin-
278 carbohydrate bonds are more sensitive to temperature than acidity or enzymatic treatment.
279 Nevertheless, not very significant differences were observed for the extracts obtained using one
280 or two enzymes and the non-enzymatic control, as can be observed from the statistical groups
281 shown in Table 1. However, a statistically significant increase on total phenols amount was
282 observed when using the ternary enzymes mixture (mix-4) and when using PLE for the
283 extraction. Although this latter technique produced extracts with higher amounts of total phenols
284 independently of the solvent employed, water was shown to be more appropriate for the
285 extraction of phenols than ethanol, at the tested temperature. These results suggest that most of
286 the compounds present in lemon balm had a relatively high polarity and were preferentially
287 extracted with water, this behavior being previously reported with other plants [33].
288 As it has been already mentioned, the enzymatic treatment with mix-4 had a small but significant
289 effect on increasing the phenolic content in extract. This effect may be due to the presence of
290 lignin on the cell walls that is able to absorb enzyme and may retard the action of
291 polysaccharides' enzymes [25].

The results corresponding to the antioxidant activity of the obtained extracts are also summarized in Table 1. The methods employed provided the antioxidant capacities of extracts against two different radicals, and therefore, they may provide a deeper insight on the chemical constituents of the extracts. 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 EC₅₀) followed by the ethanol PLE extract. In fact, a good agreement was found between the two antioxidant capacity assays employed. 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. Comparing the results from the three *in vitro* methods employed, a good positive correlation between the total phenols amount and the antioxidant capacity shown by those extracts can be inferred.

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

A new RP-LC-DAD-MS/MS method was optimized to characterize the obtained lemon balm extracts. In order to improve the phenolic compounds separation and to attain a higher resolution of the complex profile of the different extracts, a quite slow gradient was employed. 0.1% Formic acid was added to the mobile phase to improve resolution and peak shapes. In fact, many caffeic derivatives in plants have isomers and are difficult to separate due to their very similar structures. Figure 3 shows the chromatograms (280 nm) corresponding to the enzymatic and non-enzymatic extracts whereas Figure 4 shows the chromatograms corresponding to PLE water and ethanolic extracts. As it can be observed, a relatively good separation of the complex extracts was achieved after a careful optimization of the chromatographic conditions. Besides, Table 2

summarizes the MS and UV-Vis information collected for the separated components present on the different extracts. This combined information, as well as their elution order, permitted the assignment of most of the peaks present in the extracts. Besides, these data was compared to those provided by commercial standards when available.

3.2.1. Lemon balm EAE extracts composition

As can be observed in Figure 3, both lemon balm EAE 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 the reference standard were protocatechuic acid (peak 2), p-hydroxybenzoic acid (peak 4) and rosmarinic acid (peak 15). The lemon balm extracts were very rich in caffeic acid derivatives, such as salvianolic acid H/I (peak 7), a caffeic acid trimer, and salvianolic acid B, E, L and L-isomer (peaks 10, 11, 22 and 23, respectively), caffeic acid tetramers. Figure 5 shows the chemical structure of the most important phenolic compounds detected in lemon balm extracts.

Rosmarinic acid was identified according to its molecular ion $[M-H]^-$ at m/z 359 and characteristic fragment ions at m/z 197, 179 and 161 [16]. The ESI-MS spectra of the $[M-H]^-$ ions of protocatechuic acid, caffeic acid and salvianolic acid H/I (peak 7) exhibited $[M-H-44]^-$ product ions, which are attributed to the loss of CO_2 , confirming the presence of the carboxyl group in these compounds. These fragments are typically produced from those compounds, thus, helping to confirm their identification.

Salvianolic acid H/I was identified based on its molecular ion ($[M-H]^-$) at m/z 537 and its corresponding fragment ions (at m/z 493, 359, 339, 295).

Four peaks (10, 11, 22, 23) presented the same molecular ion $[M-H]^-$ at m/z 717. Also the MS spectra of these four compounds showed similar product ions at m/z 519, 359 and 339 suggesting that these peaks could be assigned to salvianolic acid B (peak 10), salvianolic acid E (peak 11), salvianolic acid L (peak 22) and salvianolic acid L isomer (peak 23). The UV-vis spectra of these four components were quite similar with maxima at ca. 280 and 321 nm, which was in agreement with their tentative identification. These caffeic acid derivatives are characteristic of other Lamiaceae plants, especially *Salvia miltiorrhiza*, commonly known as “Danshen”. Their elution order was, therefore, elucidated based on the available literature for this plant [34-38], although very recently, these compounds have been also identified for the first time in lemon balm [16]. Mainly due to their polyphenolic structure, salvianolic acids have been found to be associated to several functional properties, such as prevention of cardiovascular diseases [39], or anti-inflammatory [40] and antioxidant activities [41].

A dimer of rosmarinic acid was identified as sagerinic acid, based of molecular ion $[M-H]^-$ at m/z 719, yielding a fragment at m/z 359. The dimerization had occurred by a $[2 + 2]$ union of the olefinic moieties (cyclobutane structure) [42].

Besides caffeic derivatives, that were found in abundance on these extracts, a flavonoid (peak 17) 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. The presence of flavonoids in lemon balm has been also reported by other researchers [43, 44].

3.2.2. Lemon balm PLE extracts

360 To the best of our knowledge, the possibility of extracting phenolic compounds using PLE from
361 lemon balm has been just marginally explored [19]. In the present study, water and ethanol have
362 been selected for the extraction of phenolic compounds from lemon balm; their chromatographic
363 profiles are shown in Figure 4.

364 The main phenolic compound found in the PLE extracts was rosmarinic acid. Three derivatives
365 of rosmarinic acid were also tentatively assigned, i.e., rosmarinic acid hexoside (peak 9),
366 sulphated rosmarinic acid (peak 13) and sulphated rosmarinic acid isomer (peak 14). The
367 tentative identification of these components was possible due to the loss of fragments of 162 Da
368 (hexoside moiety) and 80 Da (sulphated moiety) from the molecular ions at m/z 521 and 439,
369 respectively, and the detection of a fragment at m/z 359, corresponding to rosmarinic acid. Other
370 compounds present in the EAE extract were also identified on the PLE water extract, such as
371 peaks 1-5, 7, 10-15, 17, 20, 22, 23.

372 Other compounds tentatively identified on PLE water extract were lithospermic acid (peak 18),
373 lithospermic acid isomer (peak 21), rosmarinic acid derivative (peak 24), salvianolic acid C
374 derivative (peak 25), rosmarinic acid derivative (peak 27). The abundance of the carboxyl
375 fragment ion $[M-44-H]^-$ at m/z 493 in MS spectrum of peaks 18 and 21 together with the
376 molecular ion $[M-H]^-$ at m/z 537 suggested that these peaks could be tentatively assigned to
377 lithospermic acid and lithospermic acid isomer. It has been previously shown that these
378 compounds could be differentiated from other components possessing the same molecular
379 weight, such as salvianolic acid H/I, thanks to the lack of a fragment at m/z 339 [35] that would
380 be generated from the salvianolic acid H/I structure. Salvianolic acid C derivative present a
381 fragment at m/z 535 corresponding to the yielded of caffeic acid $[M-H-180]^-$ from the molecular

ion $[M-H]^-$ at 715. This peak presented also a fragment at m/z 491 (salvianolic acid C) corresponding to the loss of an additional carboxyl group (44 Da) from the fragment at m/z 535. Concerning the ethanol extract, its chromatographic profile was characterized by the presence of compounds with relatively lower polarity such as rosmarinic acid derivatives (peaks 24, 25, 27-29, 31-33, see Figure 4B). These peaks could be assigned as rosmarinic acid derivatives based on the fragments at m/z 359 detected and their similar UV-Vis spectra. Nevertheless, as in water PLE extract, the main compound in the ethanol extract was by far rosmarinic acid. Other peaks found in the different extracts could not be completely identified; their UV-Vis and MS spectral information is also summarized in Table 2.

3.3. Quantification of phenolic antioxidants

Once the tentative identification of the most important compounds present on the extracts was performed, their quantification by LC-DAD was carried out. For quantitative analysis, calibration curves were constructed by injecting known concentrations of the different available standard compounds diluted in methanol, at least, using 6 different concentration points (see Table 3). Due to the lack of some commercial reference compounds, the amounts of caffeic acid derivatives in the extracts (peaks 7, 10, 11, 18, 20-23, 25, 30) were estimated as caffeic acid equivalents, rosmarinic acid derivatives (peaks 9, 13, 14, 24, 27-29, 31-33) were calculated as rosmarinic acid equivalents, while 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. The results (expressed in $\mu\text{g}/\text{mg}$ extract) as well as linear ranges, regression equations, correlation coefficients, LODs and LOQs are summarized in Tables 3 and 4.

Despite that similar chromatographic profiles were observed for both enzymatic extract and non-enzymatic control, the quantity of each phenolic compound was higher in the enzymatic extract, as it can be observed in Table 4. This increase after the enzymatic treatment could 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 (3.94 $\mu\text{g}/\text{mg}$ on enzymatic extract and 3.44 $\mu\text{g}/\text{mg}$ on non-enzymatic control) followed by rosmarinic acid (3.48 $\mu\text{g}/\text{mg}$ and 2.96 $\mu\text{g}/\text{mg}$, respectively). Besides a strong antioxidant capacity of rosmarinic acid [45], other functional properties have also been reported, such as antiviral activity against herpes simplex virus [46], antiallergenic [11] and anti-inflammatory activities [47].

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 polarity compounds were determined. In both extracts, rosmarinic acid was, by far, the most abundant compound. PLE ethanol extract contained 90.53 $\mu\text{g}/\text{mg}$ rosmarinic acid whereas the PLE water extract possessed 45.73 $\mu\text{g}/\text{mg}$ extract. However, even if its contribution should be very important, other compounds found in the extracts would be greatly responsible for the total antioxidant capacity determined. This statement can be clearly appreciated by the fact that even if the PLE ethanol extract contained almost twice rosmarinic acid compared to the PLE water extract, the latter presented higher total antioxidant capacity. Thus, possible synergistic effects among the phenolic compounds present should not be neglected in the case of water extracts.

4. CONCLUSIONS

Enzyme assisted extraction (EAE) and pressurized liquid extraction (PLE) have been proposed as environmentally friendly extraction techniques to efficiently recover bioactive compounds from lemon balm. Different enzymes have been employed with the aim to find the best conditions to improve the quality of extracts. 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 walls of lemon balm leaves and a better mass transfer of the compounds into the solvent. On the other hand, even if the use of enzymes had a positive effect on the extraction process, 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, suggesting that PLE processes with ethanol as solvent are more selective for the extraction of phenolic compounds from natural matrices. 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 μ g/mL). The chemical characterization of the extracts by LC-DAD-MS/MS allowed the tentative identification of a wide variety of phenolic compounds that would be most probably related to the antioxidant capacity observed. This novel extraction approach provided extracts from Lemon balm which 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.

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FIGURE LEGENDS.

Figure 1. Ternary diagram illustrating the level combinations of cellulase, endo-1,4- β -xylanase, and pectinase in simplex centroid experimental design. Numbers shown refer to extraction conditions employed.

Figure 2. Extraction yield (%) produced after enzymatic treatment and PLE at the indicated conditions.

Figure 3. LC-DAD-MS chromatograms (280 nm) of different extracts (A – non-enzymatic control and B – enzymatic treatment with mix-4) obtained from lemon balm. For peak identification, see Table 2.

Figure 4. LC-DAD-MS chromatograms (280 nm) of different lemon balm extracts obtained using PLE water (A) and ethanol (B). For peak identification, see Table 2.

Figure 5. Chemical structures of the main phenolic compounds extracted and tentatively identified in lemon balm.

Table 1. Values of total phenols (as mg GAE (gallic acid equivalent)/g extract), EC₅₀ (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	Antioxidant capacity		
		mg GAE/g extract	DPPH EC ₅₀ (µg/mL)	TEAC (mmol/g)
1.	Cellulase	71.58 ± 3.31 ^{a,b,c}	28.80 ± 0.19 ^{a,b}	0.853 ± 0.010 ^a
2.	Xylanase	73.79 ± 2.17 ^{b,c}	28.63 ± 0.52 ^{a,b,c}	0.863 ± 0.011 ^a
3.	Pectinase	72.62 ± 2.43 ^{a,b,c}	28.16 ± 0.32 ^c	0.863 ± 0.021 ^a
4.	mix-1	65.39 ± 0.27 ^{a,d}	29.14 ± 0.1 ^b	0.849 ± 0.006 ^a
5.	mix-2	67.90 ± 1.72 ^{a,b,d}	28.24 ± 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 ± 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

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	18.5	Unknown	278, 326	683.5	221.5, 359.3, 485.3, 665.1
7	18.7	Salvianolic acid H/I	278, 325sh	537.3	493.4, 295.3, 359.6, 339.1
8	20.0	Unknown	275, 324sh	685.3	268.8, 553.4
9	20.6	Rosmarinic acid hexoside	288sh, 322	521.3	359.3
10	21.3	Salvianolic acid B	280, 325sh	717.1	519.3, 359.4, 339
11	21.5	Salvianolic acid E	285, 325	717.3	519.3, 359.4, 339
12	21.9	Sagerinic acid	282, 325sh	719.3	359.3
13	22.7	Sulphated rosmarinic acid	285, 328	439.2	213.9, 258, 229, 359.2, 132.72
14	22.9	Sulphated rosmarinic acid isomer	280, 326sh	439.2	213.9, 258, 229, 359.2, 132.72
15	24.2	Rosmarinic acid*	289sh, 328	358.7	160.7, 178.7, 197.3
16	25.1	Unknown	280, 322sh	745.5	583.7
17	26.2	Luteolin 7-O-glucuronide	269, 289sh, 338	461.1	284.4
18	26.5	Lithospermic acid	289, 327	537.2	493.4, 359.7
19	27.3	Unknown	260, 300sh	711.6	503.8, 665.5
20	28.1	Salvianolic acid C derivative	290, 325	829.3	535.3, 667.3, 491.6, 311.2
21	28.6	Lithospermic acid isomer	290, 328	537.3	359.7, 493.4
22	29.8	Salvianolic acid L	278, 325sh	717.3	519.3, 359.2, 339
23	31.5	Salvianolic acid L isomer	280, 325sh	717.3	519.3, 359.2, 339
24	32.8	Rosmarinic acid derivative	250sh, 350	535.8	359.1, 174.8, 158.7
25	33.6	Salvianolic acid C derivative	295sh, 326	715.3	535.3, 491.6, 311.2
26	35.9	Unknown	285, 327	693.4	517.3, 651.3, 457.3, 190.9
27	37.2	Rosmarinic acid derivative	280, 327	495.1	359.2, 158.7, 333.6, 313.1
28	38.0	Rosmarinic acid derivative	290sh, 327	565.3	359.4, 519.3
29	39.5	Rosmarinic acid derivative	286, 326	495.9	359.6, 196, 268, 406.4, 450.1
30	39.7	Salvianolic acid A isomer	292, 326	493.2	312.8, 179.1, 160.8
31	40.8	Rosmarinic acid derivative	300sh, 330	565.2	158.9, 359.3, 519.3
32	42.2	Rosmarinic acid derivative	290sh, 327sh	565.3	158.9, 359.7, 519.5
33	42.6	Rosmarinic acid derivative	288, 347	565.3	359.4, 519.6

Table 3. Calibration curves, concentration ranges employed for the quantification of the antioxidants, limits of detection (LOD) and limits of quantification (LOQ) reached using the optimized LC-DAD method.

Compound	Retention time (min) ± RSD (%)	Concentration range (µg/mL)	Slope ($n = 6$) ± sd	Intercept ($n = 6$) ± sd	R ²	LOD (ng/mL)	LOQ (ng/mL)
Protocatechuic acid	13.9 ± 0.6	0.1-20	(307.2 ± 6.3) 10 ⁵	(-17.7 ± 5.9) 10 ³	0.9932	113.06	376.88
p-hydroxybenzoic acid	16.2 ± 0.4	0.1-30	(111.2 ± 2.5) 10 ⁶	(-84.3 ± 36.6) 10 ³	0.9923	10.26	34.20
Caffeic acid	17.4 ± 0.3	5-160	(65.2 ± 1.4) 10 ⁶	(-53.7 ± 10.7) 10 ⁴	0.9924	110.90	369.67
Luteolin 7-O glucoside	20.5 ± 0.4	6.25-200	(235.1 ± 2.3) 10 ⁵	(-74.2 ± 21.8) 10 ³	0.9985	757.57	2525.25
Rosmarinic acid	24.2 ± 0.6	5-1000	(305.0 ± 3.4) 10 ⁵	(-73.9 ± 15.0) 10 ⁴	0.9978	154.66	515.56

Table 4. Quantification of phenolic antioxidants found in the lemon balm extracts. Concentration indicated as $\mu\text{g}/\text{mg}$ extract $\pm$ sd. NF: not found.

Peak no.	Retention time (min)	Compound identified	Concentration ($\mu\text{g}/\text{mg}$ extract)			
			Mix-4	Non-enzymatic control	Water 150 °C	Ethanol 150 °C
1	11.9	dimer R(+)- β -(3,4-dihydroxyphenyl) lactic acid	0.94 ± 0.05	0.97 ± 0.01	5.04 ± 0.44	1.38 ± 0.14
2	13.9	Protocatechuic acid	0.10 ± 0.00	0.08 ± 0.00	0.38 ± 0.03	NF
3	15.0	Caftaric acid	1.25 ± 0.04	1.16 ± 0.00	2.73 ± 0.36	NF
4	16.1	p-hydroxybenzoic acid	0.30 ± 0.01	0.20 ± 0.03	0.40 ± 0.03	1.03 ± 0.04
5	17.4	Caffeic acid	1.81 ± 0.17	1.52 ± 0.15	2.26 ± 0.02	3.79 ± 0.10
7	18.7	Salvianolic acid H/I	2.64 ± 0.10	2.10 ± 0.12	9.04 ± 0.46	1.29 ± 0.08
9	20.6	Rosmarinic acid hexoside	NF	NF	5.58 ± 0.35	5.80 ± 0.28
10	21.3	Salvianolic acid B	1.09 ± 0.01	1.07 ± 0.01	1.23 ± 0.03	NF
11	21.5	Salvianolic acid E	1.27 ± 0.04	1.10 ± 0.04	1.44 ± 0.11	NF
12	21.9	Sagerinic acid	3.94 ± 0.16	3.44 ± 0.16	6.97 ± 0.36	3.10 ± 0.11
13	22.7	Sulphated rosmarinic acid	3.01 ± 0.06	2.78 ± 0.01	3.31 ± 0.08	NF
14	22.9	Sulphated rosmarinic acid isomer	3.15 ± 0.06	2.78 ± 0.05	<LOQ	NF
15	24.2	Rosmarinic acid	3.48 ± 0.20	2.96 ± 0.02	45.73 ± 2.34	90.53 ± 4.74
17	26.2	Luteolin 7-O-glucuronide	2.00 ± 0.26	1.01 ± 0.04	5.62 ± 0.21	2.80 ± 0.01
18	26.5	Lithospermic acid	NF	NF	2.17 ± 0.03	NF
20	28.1	Salvianolic acid C derivative	0.90 ± 0.01	0.88 ± 0.00	1.51 ± 0.02	NF
21	28.6	Lithospermic acid isomer	NF	NF	3.80 ± 0.28	1.38 ± 0.03
22	29.8	Salvianolic acid L	1.09 ± 0.02	1.02 ± 0.03	NF	NF
23	31.5	Salvianolic acid L isomer	<LOQ	<LOQ	<LOQ	NF
24	32.8	Rosmarinic acid derivative	NF	NF	3.68 ± 0.16	3.73 ± 0.09
25	33.6	Salvianolic acid C derivative	NF	NF	1.69 ± 0.02	4.56 ± 0.32
27	37.2	Rosmarinic acid derivative	NF	NF	1.31 ± 0.03	1.12 ± 0.07
28	38.0	Rosmarinic derivative	NF	NF	NF	5.99 ± 0.31
29	39.5	Rosmarinic derivative	NF	NF	NF	2.76 ± 0.07
30	39.7	Salvianolic acid A isomer	NF	NF	NF	0.93 ± 0.01
31	40.8	Rosmarinic derivative	NF	NF	NF	13.31 ± 0.46
32	42.2	Rosmarinic acid derivative	NF	NF	NF	<LOQ
32	42.6	Rosmarinic acid derivative	NF	NF	NF	4.65 ± 0.04