Melissa officinalis extracts obtained using maceration, ultrasound- and microwave-assisted extractions: Chemical composition, antioxidant capacity, and physical characteristics

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Even though *Melissa officinalis* L. (lemon balm, fam. Lamiaceae) is a well-known medicinal, aromatic, and spicy plant, and its physicochemical profile and biological activity have been investigated, there is no detailed research regarding the influence of solvent nature (polarity) on the extraction of active compounds (total polyphenols and total flavonoids) and antioxidant activities of the obtained extracts. Therefore, this study aimed to evaluate the polyphenol and flavonoid contents, as well as the antioxidant potential of lemon balm extracts obtained by varying the polarity of the extraction solvents (methyl alcohol, acetone, ethyl acetate, and deionized water) and using three extraction techniques (maceration-MAC, ultrasound-UAE, and microwave-assisted extraction-MAE). Two *in vitro* tests (ABTS and FRAP assays) were used to determine the antioxidant activity of the extracts. Total tannin and total protein contents, extraction yield, and physical properties of the selected extracts were measured as well. The highest content of polyphenols was found for the methanolic and water extracts obtained by all three extraction techniques, while the highest flavonoid yield was detected only in the mentioned methanolic extracts. The microwave reactor provided methanolic, ethyl acetate, and water extracts with the highest ABTS radical scavenging activity, while in the case of UAE, it was methanolic and water extracts, and in the case of MAC only water extracts. On the other hand, among lemon balm extracts from MAC, water extract possessed the highest ferric reducing power, whereas in UAE and MAE, it was ethyl acetate extract. Total tannin content determined in selected water extracts was 2.55, 4.53, and 1.83 mg tannic acid equivalent (TAE)/mL, while total proteins amounted to 1.34, 1.50, and 1.31 mg/mL using MAC, UAE, and MAE, respectively. The content of total extractive substances in the form of the extraction yield was also determined for selected water extracts and amounted to 12.6 % for MAC, 17.2 % for UAE, and 36.8 % for MAE. Further, this research has included the investigation of some physical properties of lemon balm water extracts, such as conductivity (3.68-4.14 mS/cm), pH (5.99-6.43), density (0.854-0.901 g/mL), surface tension (26.0-31.7 mN/m), and viscosity (1.18-1.21 mPa·s). This research represents the base for the future encapsulation of lemon balm extracts, enriched in polyphenol content, in a novel type of biofunctional carrier that potentially can be applied in the pharmacy, chemical industry, and biotechnics.

Key words: extraction; extraction yield; lemon balm; total flavonoids; total polyphenols

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ABBREVIATIONS

ABTS - 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
FRAP - ferric reducing antioxidant power
GAE - gallic acid equivalent

MAC - maceration
MAE - microwave-assisted extraction
PCS - photon correlation spectroscopy
RSA - radical scavenging activity
TAE - tannic acid equivalent
TFC - total flavonoid content
TPC - total polyphenol content
UAE - ultrasound-assisted extraction
QE - quercetin equivalent

1. INTRODUCTION

Lemon balm (*Melissa officinalis* L.) is a perennial, herbaceous, bushy plant species belonging to the Lamiaceae family. Although it primarily originates from Southern Europe (Jastrzębska-Stojko et al., 2013), it is mostly cultivated in North America, Germany, France, Italy, Romania, and Bulgaria (Bagdat, 2006). Due to the essential oil content, its heart-shaped, hairy leaves emit a light, distinguishable lemony aroma which justifies their common use as a spice, natural seasoning, or flavoring. The pale-yellow flower buds develop into white or pale purple small flowers arranged in pseudo whorls. Early June to the middle of August is when the plants blossom and are ready for harvest.

Lemon balm is also utilized in conventional medicine, as well as in cosmetic products (Šalamon et al., 2019). Field-grown plants naturally release the antibacterial components which are involved in the defense against some pathogenic microorganisms (Szabo et al., 1999). The plant's infusion has been used as a moderate sedative throughout Europe to treat headaches, migraine, nervous tension, sleeplessness, as well as the common cold, fever, and cough (Stefanović and Čomić, 2012). In pharmaceutical uses, this plant has also been touted as having health benefits, including actions against gastrointestinal, cardiac, and thyroid disorders, rheumatism, nausea, vertigo, anemia, Graves' and Alzheimer's diseases, syncope, epilepsy, migraines, gynecological problems, nervousness, malaise, depression, insomnia, psychosis, hysteria, wounds, muscle tension, and obesity (Papoti et al., 2019; Świąder et al., 2019).

The bioactive compounds in lemon balm includes flavonoids, volatile compounds, triterpenes, tannins, and phenolic acids, which are responsible for its widespread use (Mencherini et al., 2007). Different phenolic acids that are usually found in lemon balm are those derived from benzoic acid, such as gallic acid, and those derived from cinnamic acid, such as caffeic and rosmarinic acids (D'Archivio et al., 2007). Rosmarinic acid, as a derivate of hydrocinnamic acid, is a particularly significant phenolic acid found in lemon balm, and the plant has to be standardized, i.e. to contain more than 4 % of hydrocinnamic acid for usage in pharmaceuticals (Fecka and Turek, 2008). All of lemon balm organs contain rosmarinic acid, which even accounts for roughly 6 % of the dry weight of the leaves (Petrisor et al., 2022). The phenolic components found in lemon balm extracts, including rosmarinic acid, tannins, and flavonoids mentioned above, are thought to be responsible for some of the health benefits (Petersen and Simmonds, 2003). Polyphenols, as natural antioxidants, are broadly distributed in most plant organs and have good reactive oxygen species scavenging abilities. The primary antioxidant of the Nepetoideae subfamily of the Lamiaceae family is precisely rosmarinic acid. Besides polyphenol types of chemical compounds, simple proteins, which are further separated into four categories (albumins, globulins, prolamins, and glutelins) are also present in all plant tissues.

The extractions that include a solid and a liquid phase have been divided into traditional and novel methods. The term traditional methods refer to maceration, leaching, Soxhlet extraction, etc. These types of extractions have been used for a long period, but they require a prolonged extraction time and a large amount of the extraction solvent (Jovanović et al., 2017). Whereas, newer methods do not only need a shorter period and less solvent but are more environmentally friendly, due to the pollution reduction. Additionally, chemical compounds can be extracted from solid plant matrixes quickly and effectively using modern extraction techniques like ultrasound-assisted extraction (Jovanović et al., 2017), microwave-assisted extraction (Jovanović et al., 2022), pressurized liquid extraction (Pagano et al., 2018), and supercritical fluid extraction (Rajapaksha and Shimizu, 2022). These methods can operate at high pressures and/or temperatures, considerably reducing the extraction time (Jovanović et al., 2017). Ultrasound- and microwave-assisted extractions have numerous advantages in comparison to maceration, among which are short extraction time, followed by a reduced amount of solvent, and higher extraction efficiency (Jovanović et al., 2022; 2017). On the other hand, the downsides are the degradation of heat-sensitive compounds, expensive equipment, the efficiency depending on the used solvent, etc. (Jovanović et al., 2017).

In the present study, different extracts of cultivated lemon balm obtained using maceration (MAC), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE), and four types of extraction medium (methyl alcohol, acetone, ethyl acetate, and deionized water), were characterized in terms of total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant potential (ABTS radical scavenging activity and ferric reducing antioxidant power, FRAP). Additionally, total tannin content (TTC), total proteins, extraction yield, pH, conductivity, density, surface tension, and viscosity of the selected lemon balm extracts were measured as well.

2. MATERIALS AND METHODS

2.1. Origin of plant material

Plant material used in this study derives from lemon balm plantation, which was established in 2021, on chernozem, at locality Bavanište (South Banat, Serbia), with nursery plants produced in the Institute for Medicinal Plant Research "Dr. Josif Pančić", Serbia. The plantation was in its first year of exploitation. The aerial plant portion (7 cm above the ground) was manually harvested at the beginning of flowering (June 8th, 2022), and left to air-dry in the shade at temperatures below 30 °C, prior to its further use for the preparation of the extracts. Before the extraction process, all of the plant samples were pulverized (to an average particle size of 0.5 mm, approximately) using a laboratory mill with water cooling (M20 Universal mill, IKA, Germany).

2.2. Reagents

The following reagents were used: methyl alcohol (Zorka-Farm, Republic of Serbia), acetone (Macron Fine Chemicals, Germany), sodium carbonate, and ethyl acetate (Fischer Chemicals, United Kingdom). Gallic acid, aluminium(III)-chloride, quercetin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium-hexacyanoferrate, sodium-hydroxide, hydrochloric acid (37 %), phosphoric acid (85 %), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) - ABTS, were purchased from Sigma Aldrich, Germany, while monosodium-phosphate monohydrate, disodium-phosphate dihydrate, albumin–protein standard, Coomassie® Brilliant blue G 250, and Folin-Ciocalteu reagent were purchased from Merck, Germany. Iron(III)-sulfate was purchased from Super-Lab Company, Belgrade, Serbia. Butyl alcohol 99 % was purchased from Zorka-Pharma, Serbia. Tannic acid powder (81 %)

was purchased from Kemika Group, Croatia. All reagents and standards were of practical grade and used without further purification. Filter paper CM (12-15 μm) was purchased from Chemlab Group, Spain.

2.3. Preparation of the extracts

Lemon balm extracts were prepared using MAC, UAE, and MAE; each extraction procedure is described below in the individual sub-chapters.

2.3.1. Maceration

The maceration was performed using a magnetic stirrer (Magnetic stirrer MM-530, Tehtnica Železniki, Yugoslavia) at 25±5 °C for 24 h. The plant material (1 g) was extracted with 20 mL of methyl alcohol, acetone, ethyl acetate, or water. Although ethanol is an non-expensive, non-toxic, and widely used extraction medium, it was not used in this study because there are already many studies that dealt with ethanol extraction from lemon balm. The samples were filtered using a laboratory filter paper. The collected analyte was stored in a dark bottle at 4 °C, until further analyses.

2.3.2. Ultrasound-assisted technique

The pulverized plant material (1 g) of lemon balm was extracted with 20 mL of the extraction medium (methyl alcohol, acetone, ethyl acetate, or water) for 60 min using an ultrasound bath at 60 °C (Sonorex, Bandelin, Germany). The extracts were collected, filtered through a laboratory filter paper, and stored at 4 °C, until analyses.

2.3.3. Microwave-assisted technique

The pulverized plant material (0.5 g) was extracted with 10 mL of the extraction solvent (methyl alcohol, acetone, ethyl acetate, or water) at 60 °C for 10 min using Microwave Synthesis Reactor (Monowave 300, Anton Paar, GmbH, Germany). The obtained raw extracts of lemon balm were collected and stored at 4 °C, until further analyses.

2.4. Determination of the active compounds

2.4.1. Total polyphenol content

The total polyphenol content (TPC) of the extracts was determined using the Folin-Ciocalteu assay (Singleton et al., 1999) with some modifications. Shortly, 20 μL of properly diluted extract, 100 μL of Folin-Ciocalteu reagent, and 1.5 mL of water were transferred into a 2 mL flask and mixed uniformly. After 5 min, 300 μL of sodium-carbonate (20 %, w/v) were added, followed by the addition of deionized water up to 2 mL. After 120 min of standing in the dark, the absorbance was measured at 765 nm using an 1800 UV/Vis spectrophotometer (Shimadzu, Japan). Gallic acid was used as an analytical standard (30-500 μg/mL) and the results of TPC were expressed as micrograms of gallic acid equivalent per milliliter of the extract (μg GAE/mL). All analyses were performed in triplicate and statistically processed.

2.4.2. Total flavonoid content

The total flavonoid content (TFC) determination of lemon balm extracts was performed using the modified method reported by Savi et al. (2017). In short, 250 μL of the properly diluted extract was mixed with 75 μL of sodium-nitrite (5 %, w/v) and 1250 μL of deionized water. Then, the mixture was left in dark for 5 min. After that time, 150 μL of aluminum (III)-chloride (10 %, w/v) and 500 μL of sodium-hydroxide (0.1 mol/L) were added to the mixture and topped up with deionized water to a final volume of 2000 μL. Finally, TFC was determined using a standard quercetin curve with a concentration in the range of 1 to 30 μg/mL. The results were expressed as micrograms of quercetin equivalent per milliliter of the extract (μg QE/mL). All tests were carried out in triplicate. The absorbance of the samples was measured at 425 nm.

2.4.3. Total tannin content

The total tannin content (TTC) in the selected extracts (the extracts with the highest TPC from all three extraction methods) was determined using the method described in the study of Makkar and Singh (1995). The extraction solution was prepared by mixing 50 mg of iron (III)-sulfate, 95 mL of butyl alcohol, and 5 mL of hydrochloric acid (35 %, w/v). For determining the tannin content (in a form of a condensed derivative), 10 mg of plant material in a glass test tube and 10 mL of extraction solution were added. Subsequently, the samples were exposed to the same conditions as in the case of all three used extraction procedures. The extraction solution was filtered through cellulose acetate filter paper (0.22 mm) and processed spectrophotometrically at 580 nm. The results of the analysis were expressed as milligrams of tannic acid equivalent per mL of raw extract (mg TAE/mL). The calibration curve was plotted by dissolving the standard of tannic acid in deionized water in a few different concentrations (1, 2, 4, 8, and 16 mg/mL). All assays were carried out in triplicate.

2.4.4. Total proteins

Total proteins of the selected lemon balm extracts (the extracts with the highest polyphenol concentration from all three extraction procedures) were analyzed by using a standard test for the determination of total proteins defined by Bradford (1976). The Bradford dye reagent was prepared according to the following protocol: 100 mg of Coomassie brilliant blue G-250 was dissolved in 50 mL of ethyl alcohol (97 %) and then 100 mL of phosphoric acid (85 %, v/v) was added. Lastly, the stock solution was diluted to 1000 mL with deionized water. The assay was performed using 50 μL of extract and 2500 μL of Bradford dye reagent in plastic microplate wells. Then, the blend was mixed with a plate shaker for 1 min at 25±5 °C and analyzed spectrophotometrically at 595 nm of wavelength. Albumin was used to calculate the standard curve and the results were expressed as mg/mL.

2.5. Determination of antioxidant capacity

2.5.1. ABTS assay

The $ABTS^{\bullet+}$ scavenging assay was based on the analytical protocol described by (Prior et al., 2005) with some modifications. In short, 200 μL of the extract was mixed with 2800 μL of $ABTS^{\bullet+}$ radical cation solution and incubated in dark for 30 min, at 25±5 °C. The $ABTS^{\bullet+}$ radical solution (7.8 mmol/L) was prepared by dissolving 20 mg ABTS(s) in 5 mL of deionized water and adding 88 μL of 2.45 mmol/L of potassium-persulfate(aq) (380 mg of potassium-persulfate(s) was dissolved into 10 mL of deionized water). Before use, the mixture of ABTS stock solution was incubated in a dark place at 4 °C, for 16-20 h to prepare (activate) radical cation solution ($ABTS^{\bullet+}$). After activation, $ABTS^{\bullet+}$ radical cation solution was diluted with ethyl alcohol to achieve an initial absorbance of 0.70±0.02, at 734 nm. The control solution was prepared by adding 200 μL of extraction solvent (instead of the extract) into 2800 μL of $ABTS^{\bullet+}$ radical cation solution. All measurements were performed in triplicate. These experiments monitored the decrease of absorption of $ABTS^{\bullet+}$ radical cation solution at 734 nm after the addition of antioxidant solution (extract solution) for the time of 30 min. $ABTS^{\bullet+}$ radical scavenging activity (RSA, %) was calculated by following the equation:

$$\text{RSA}\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\,,$$

where $A_{control}$ was the absorbance of the working solution and extraction medium, A_{sample} was the absorbance of the working solution and the extract.

2.5.2. Ferric reducing antioxidant power assay

FRAP assay was based on the ability of Fe^{2+}, present in the complex of potassium-hexacyanoferrate, to be reduced into the Fe^{3+} form. This method was described by Kammoun et al. (2013). Briefly, 100 μL of the extract was mixed with 1000 μL of 0.2 mol/L phosphate buffer (pH 6.6) and 1000 μL of potassium-hexacyanoferrate (1 %, w/v). The mixture was then incubated for 240 min at 50 °C. Then, 500 μL of that mixture was mixed with 250 μL of trichloroacetic acid (10 %, v/v) and centrifuged at 3000 rpm for 10 min. The supernatant was collected and united with a mixture of 750 μL of deionized water and 170 μL of iron (III)-chloride (0.1 %, w/v). The control was prepared under the same conditions (without the extract). The absorbance of the sample was measured at 700 nm. All measurements were performed in triplicate. Increased absorbance of the reaction mixture was an indication of an increase in reducing power. The serial dilutions of iron (II)-sulfate heptahydrate (10-1000 μmol Fe^{2+}/L) were made for the construction of the calibration curve. The results were expressed as μmol of iron ions per L of raw extract (μmol Fe^{2+}/L).

2.6. Determination of the extraction yield

Extraction yield (EY) of the selected lemon balm extracts was calculated as a relation between the weight of dry extract after evaporation and the weight of dry plant material, expressed in percentages.

2.7. pH and conductivity measurements

pH analysis of the selected lemon balm extracts was performed using a pH Meter (HI 2210, Hanna Instruments, Italy), while the conductivity was determined by photon correlation spectroscopy (PCS) on a Zetasizer Nano Series, Nano ZS (Malvern Instruments Ltd., United Kingdom). Each sample was measured three times at room temperature.

2.8. Density, surface tension, and viscosity measurements

The density and surface tension of the selected lemon balm extracts were measured using silicon crystal (as the immersion body) and Wilhelm plate, respectively, in Force Tensiometer K20 (Kruss, Germany). Each sample was examined three times at room temperature. Viscosity was determined using Rotavisc lo-vi device equipment with VOL-C-RTD chamber, VOLS-1 adapter, and spindle (IKA, Germany). Each sample was examined three times at room temperature.

2.9. Statistical analysis

The statistical analysis was performed by using the analysis of variance (one-way ANOVA) followed by Duncan's *post hoc* test within the statistical software, STATISTICA 7.0. The differences were considered statistically significant at $p<0.05$, n=3.

3. RESULTS AND DISCUSSION

Lemon balm extracts, obtained by MAC, UAE, and MAE using methanol, acetone, ethyl acetate, and water as an extraction medium, were analyzed *via* TPC, TFC, ABTS radical scavenging activity, and ferric reducing antioxidant power; the results are presented in Table 1 and Figures 1 and 2. The selected extracts (the samples with the highest polyphenol concentration from three extraction procedures) were additionally characterized in terms of TTC, total proteins, extraction yield, pH, conductivity, density, surface tension, and viscosity; the results are presented in Table 2.

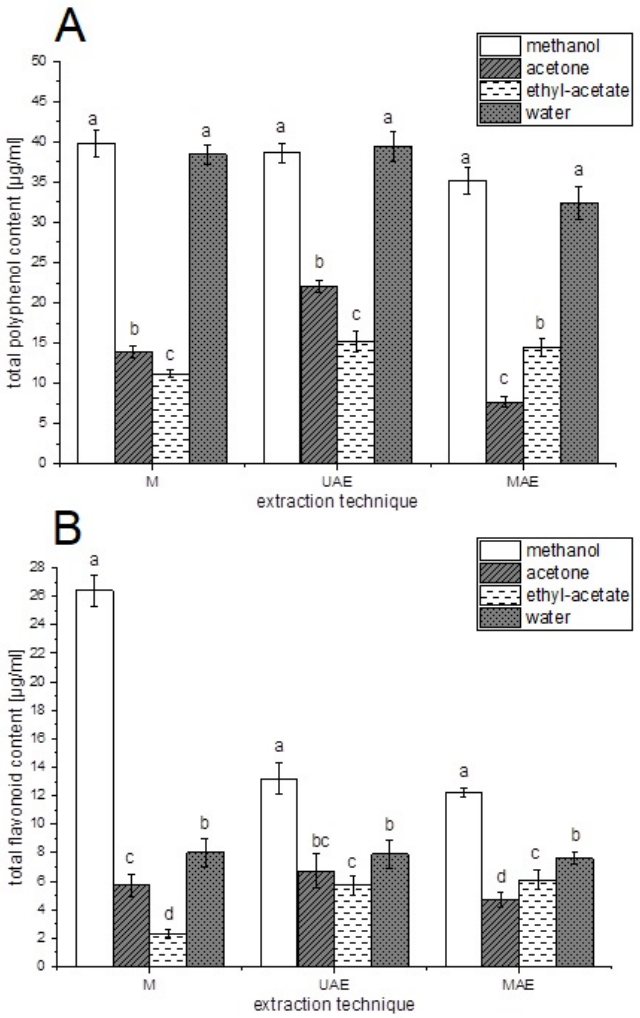

Fig. 1. Total polyphenol content (A) and total flavonoid content (B) of lemon balm (*Melissa officinalis* L.) extracts obtained in maceration (M), ultrasound- and microwave-assisted extractions (UAE and MAE, respectively); values with the same letter (a-d) in each group of the extraction technique showed no statistically significant differences ($p<0.05$; n=3; analysis of variance, Duncan's post-hoc test).

3.1. Chemical composition of the extracts

As can be seen from Table 1 and Figure 1A, the highest polyphenol concentration was determined in methanol and water extracts in all three extraction procedures. On the other hand, the lowest TPC was measured in ethyl acetate extracts in MAC and UAE, and in acetate extract in MAE. The content of complex polyphenol structures in the *Melissa species* (lemon balm, honey balm, etc.) can vary and it depends on the cultivation region (Shakeri et al., 2016). However, the influence of the extraction parameters and the methodology of the extraction process (extraction technique, time, polarity of the solvent, mixture of solvents, temperature mode, and temperature uniformity in the system, etc.) are not negligible. Duda et al. (2015) have reported that TPC of lemon balm extracts prepared using water and methyl alcohol mixture (70 %, v/v) generates higher amounts of TPC. Namely, Stanković (2011) determined that methanol and water effectively extract phenolic compounds (polyphenols and flavonoids) from the plant material in comparison to acetone and ethyl acetate. The solubility of phenolic compounds in the polar organic solvents

provides a high concentration of these compounds in the extracts obtained using the mentioned agents for the extraction process. Furthermore, the results from the research of Zhou and Yu (2004) showed that a mixture of highly polar solvents, methanol, and water, in an appropriate ratio, was more effective than only water to extract total phenolic compounds and other antioxidant components.

Further, the highest TFC was in methanol extracts from all three extraction techniques, followed by water extracts, in which TFC was statistically significantly lower (Table 1 and Figure 1B). These results of flavonoid content using methanol as a solvent correlated with the literature (Ince et al., 2013). Namely, Hassan et al. (2019) report that methanol effectively extracts TFC from lemon balm. In opposition to these findings, some studies suggest that ethyl acetate as a slightly polar solvent has the highest efficiency on the TFC from lemon balm plant in comparison to some conventional solvents (Pereira et al., 2014). Moreover, the literature data show that the acetate extracts of lemon balm derive from higher amounts of extracted flavonoid substances, such as quercetin, rutin, and epicatechin (Pereira et al., 2014).

Since that methanol and water lemon balm extracts possessed the highest TPC and ABTS radical scavenging potential, while water is a more favorable extraction medium compared to organic solvents in food, pharmaceutical, and cosmetic products, water extracts were selected for additional characterization analyses.

Total tannin content varied in all selected water extracts and reached the highest value in UAE (4.53±0.24 mg TAE/mL), followed by the statistically significantly lower value obtained by maceration (2.55±0.12 mg TAE/mL), while the lowest value was detected in the extract prepared by MAE (1.83±0.10 mg TAE/mL). Ultrasound-assisted extraction proved to be effective in increasing the content of phenolic compounds and tannins matters compared to conventional extraction procedures like maceration (Thomas et al., 2012). Namely, in the liquid system where the solid and liquid particles were vibrated and accelerated, the intramolecular forces are not able to hold the structure of the molecule in the intact state, so the formed bubbles can produce the mechanical effect which results in cell disruption of biological membranes. At the same time, the phenomenon of mass transfer of active compounds was the consequence of the impulsive penetration of the solvent into the structure of cell material followed by the direct release of extractable compounds. Results from our study suggest that microwave-assisted extraction of tannins matters was less effective compared to ultrasound-assisted. The explanation for this phenomenon could be that tannin matters were degraded by instantaneous heating of the residual moisture in the plant particle when it was exposed suddenly to microwave radiation. Consequently, when the solid content and solvent heat unequally, the formed high vapor pressure of the free aqueous molecules in the plant substrate causes the interruption of the cell wall and intensifies the release of the content into the surrounding extraction medium (Gupta et al., 2012). Further, Caleja et al. (2017) have established that ultrasound-assisted extraction effectively extracts tannin derivate than microwave-assisted and heat-assisted extraction. Finally, some studies report that the solid-to-liquid ratio can influence the results of the analysis. Namely, increased solid-to-liquid ratio in the process of ultrasound extraction showed negative content of tannins matters which probably reflects a tendency toward solvent saturation (Sousa et al., 2014). The level of extracted proteins was the highest in the water extract obtained in the ultrasound bath (1.50±0.01 mg/mL), whereas there was no statistically significant difference in total proteins between the extracts from maceration and MAE (1.34±0.02

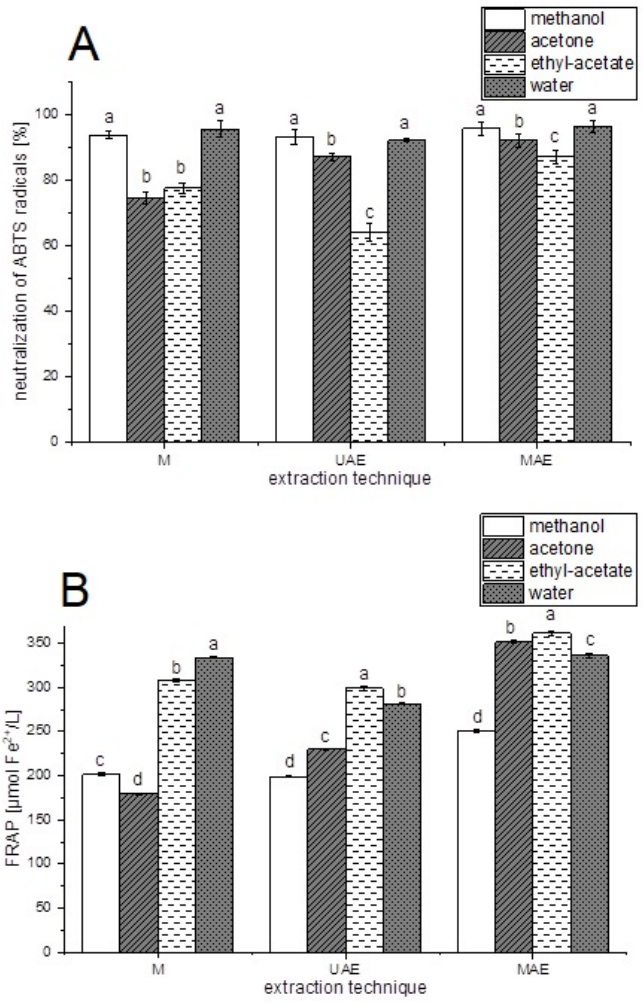

Fig. 2. ABTS radical scavenging activity (A) and ferric ion-reducing antioxidant potential (B) of lemon balm (*Melissa officinalis* L.) extracts obtained in maceration (M), ultrasound- and microwave-assisted extractions (UAE and MAE, respectively); values with the same letter (a-d) in each group of the extraction technique showed no statistically significant differences ($p<0.05$; n=3; analysis of variance, Duncan's *post-hoc* test).

and 1.31±0.01 mg/mL, respectively). The fresh plant of lemon balm has about 33 % of proteins, which classifies it as a nutritional valuable food. Doğan et al. (2021) reported that the content of proteins in the leaves of lemon balm was about 13.5±0.1 %. The content of proteins in the extract obtained by the microwave-assisted method of extraction was significantly lower compared to the ultrasound-assisted method of extraction and this occupation probably occurs as a consequence of the denaturation of plant proteins. The general conclusion could be that protein degradation was the consequence of changes in the internal structure of macromolecules and variation of space conformations which results in the cross-linking, formation of isopeptide bonds, and aggregates (Xiang et al., 2020). The formed aggregates can make difficult the measurements based on the colorimetric assay. Also, in the literature, the total protein content of lemon balm leaf extract was about 4.14 % (Silva et al., 2021). Moreover, that study revealed that the total protein content in extract was higher when 70 % ethyl alcohol was used as the extraction agent. Finally, the assumption is that degradation of proteins into extract caused by the ultrasound radiation might be explained in the same way as in the case of degradation of tannic substances. Ultrasound radiation generates acoustic cavitations consequently caused by the

Table 1. Total polyphenol content (TPC), total flavonoid content (TFC), and antioxidant potential (ABTS and FRAP assays) of *Mellisa officinalis* L. extracts obtained in maceration (MAC), ultrasound- and microwave-assisted extractions (UAE and MAE, respectively) and using methanol, acetone, ethyl acetate, and water as an extraction medium.

sample		TPC[a] [μg GAE/mL]	TFC [μg QE/mL]	ABTS [%]	FRAP [μmol Fe^{2+}/L]
MAC	methanol	39.8±1.6 a	26.4±1.1 a	93.7±1.4 a	201.2±1.8 c
	acetone	13.9±0.8 c	5.7±0.8 c	74.5±2.0 b	179.3±0.8 d
	ethyl acetate	11.2±0.5 d	2.3±0.3 d	77.5±1.6 b	307.7±1.4 b
	water	38.4±1.2 a	8.0±1.0 b	95.6±2.4 a	334.1±1.1 a
UAE	methanol	38.6±1.2 a	13.2±1.1 a	93.1±2.2 a	199.0±1.4d
	acetone	22.0±0.7 b	6.7±1.2 c	87.1±1.1 b	229.8±0.9 c
	ethyl acetate	15.2±1.3 c	5.7±0.7 c	64.1±2.6 c	298.9±2.0 a
	water	39.4±1.9 a	7.9±1.0 b	92.1±0.4 a	281.4±0.5 b
MAE	methanol	35.2±1.7 a	12.2±0.3 a	95.7±1.9 a	250.6±1.2d
	acetone	7.70±0.7 c	4.7±0.5 d	92.2±2.1 a	351.6±1.7 b
	ethyl acetate	14.4±1.1 b	6.1±0.7 c	87.2±2.1 b	361.5±2.5 a
	water	32.4±2.0 a	7.6±0.4 b	96.4±1.7 a	336.1±2.8 c

[a] Values with different letters (a-d) in each row showed statistically significant differences ($p<0.05$; n=3; analysis of variance, Duncan's post-hoc test); GAE, gallic acid equivalent, QE, quercetin equivalent.

instant increase of the temperature and pressure thereby rupturing the cell wall and extracting the protein matters through the rifts of the cells. In that way, the released intracellular protein fraction will be dissolved in a solvent environment. Certainly, it is important to point out that the frequency and intensity of applied waves, power, reached temperature, and time interval significantly influence the total protein content (Kumar et al., 2021).

3.2. Antioxidant capacity of the extracts

Lemon balm extracts' antioxidant capacity was examined *via* analyzing ABTS radical scavenging potential and ferric-reducing antioxidant power.

According to the results from the ABTS assay (Table 1 and Figure 2A), the highest antioxidant activity was measured in the methanol and water extracts in all used extraction procedures. Additionally, the extraction in a microwave reactor has provided the acetone and ethyl acetate extracts with higher antioxidant potential compared to their parallels from maceration and extraction in an ultrasound bath. Some studies report a high radical scavenging and antioxidant potential of polar lemon balm extracts (Miraj et al., 2017). These antioxidant activities have arisen from the content of some representatives of polyphenols, such as rutin, quercetin, chlorogenic acid, rosmarinic acid, and similar (Miraj et al., 2017). The highest values of antioxidant activity of acetone and ethyl acetate extracts obtained by microwave-assisted extraction revealed that the mentioned technique generates high extraction efficiency in the matter of antioxidant components in those extraction mediums. The capacity of the extraction medium to absorb microwave radiation affects the extraction efficiency. Namely, the physical interaction of microwaves with the molecules of extraction solvent causes an increase in internal temperature and internal pressure within the plant material, facilitating the breakage of the cell wall, and delivering bioactive substances in the solvent (Jovanović et al., 2022). Since most bioactive substances of lemon balm are polar molecules (free organic acids, alcohols, water-soluble vitamins, pigments, carbohydrates, and their derivates), methyl alcohol, ethyl alcohol, acetone, or water are suitable solvents for the process of extraction. Phenomenologically observed, microwave radiation assists polar solvents to penetrate the structure of plant cells causing the solubilization and hydrogen bond breaking. Consequently, in that environment, there is a gradual increase of extracted polyphenols which scavenge and neutralize the free radical species, superoxide anions, etc. (Radomir et al., 2019). On the other side, the mechanical vibrations caused by ultrasound radiation can provoke a sudden rise in temperature in the reaction mixture and intensify the process of bubbles forming. This phenomenon causes the asymmetric cavity to collapse and produce speed liquid jets which induce the destruction of cell walls with the simultaneous release of the cell content (Jovanović et al., 2017). Further, Šic Žlabur et al. (2016) claim that time as a parameter in the UAE is crucial for high extraction efficiency and content of polyphenols, the main carriers of antioxidant potential. Namely, in the time range of 20 min, the content of polyphenols increases by 6.5 times and it is partially provoked by an impulsive increase in the temperature of the reaction mixture (Šic Žlabur et al., 2016).

On the other hand, prepared lemon balm extracts have shown a slightly different ferric reducing antioxidant power compared to radical scavenging potential (Table 1 and Figure 2B). Namely, in maceration, the highest ferric reducing activity was possessed water extract, whereas, in UAE and MAE, it was ethyl acetate samples. Some papers indicate that ethyl acetate can help extract more phenolic compounds than water regardless of the higher polarity of water in comparison to ethyl acetate which is a less polar solvent (Boeing et al., 2014; Shafazila et al., 2010). Namely, some typical flavonoid molecules such as quercitrin, rhamnocitrin, and luteolin are insoluble in water and their solubility was determined by their structure and polarity. On the other side, the water has a higher capability to extract polar compounds (rosmarinic, gallic, caffeic, and phenolic acids) and some flavonoids such

Table 2. Physico-chemical properties of *Mellisa officinalis* L. water extracts obtained in maceration, ultrasound- and microwave-assisted extractions (MAC, UAE, and MAE, respectively).

Sample	Total tannins [mg TAE/mL]	Total proteins [mg/mL]	EY [%]	pH	G [mS/cm]	ρ [g/mL]	γ [mN/m]	η [mPa·s]
MAC	2.55±0.12b	1.34±0.02b	12.6±1.0c	6.43	4.14±0.43a	0.901±0.007a	26.0±0.7b	1.19±0.01a
UAE	4.53±0.24a	1.50±0.01a	17.2±1.5b	5.99	4.10±0.40a	0.854±0.005c	31.3±2.6a	1.18±0.02a
MAE	1.83±0.10c	1.31±0.01b	36.8±1.2a	6.08	3.68±0.35a	0.872±0.003b	31.7±0.4a	1.21±0.01a

[a] Values with different letters (a-c) in each row showed statistically significant differences ($p<0.05$; n=3; analysis of variance, Duncan's *post-hoc* test); EY, extraction yield; G - conductivity; ρ - density; γ - surface tension; η - viscosity; TAE - tannic acid equivalent.

as anthocyanins. Ethyl acetate cannot extract anthocyanins and some hydrophilic antioxidants in comparison to other highly polar solvents, like water and methanol (Shafazila et al., 2010). Probably, water and methanol can build strong hydrogen bonds with a molecule of antioxidants across their polar sites and cause their dissolution. Also, the assumption is those polar solvents like water, methanol, and similar, due to their value of dielectric constant and solvation energy, can efficiently extract phenolic compounds (Shafazila et al., 2010). Those findings are opposite to the results from this study where ethyl acetate, as a semipolar solvent, compared to other solvents, shows the highest antioxidative activity determined by FRAP assay. These contradictions can perhaps be explained by the fact that generated heat during the microwave and ultrasound treatment causes the partial degradation of some less stable water-soluble antioxidants. Consequently, the other highly stable flavonoid structures like cynaroside, apigenin, quercetin, and derivates of rutin remain in the extract solution and react with copper ions, neutralizing them, which positively reflects on the results of the FRAP test. Certainly, the presence of some interfering substances and organic molecules with a reduction potential lower than 0.77 V can intensify the reduction of copper and generates a significant change in values of absorbance (Boeing et al., 2014). Further, the samples obtained by maceration technique when the water was used as a solvent generates a higher reduction potential measured by FRAP assay compared to other used solvents (Figure 2B). This exception compared to other results from this study can be ascribed to the partial recovery of some polar antioxidants, in the residual forms, additionally conditioned by a long time of extraction. Sentkowska et al. (2015) have found that the water extract of lemon balm obtained by maceration/infusion technique gives the highest values of total polyphenols. Furthermore, FRAP assay is based on the ability of natural antioxidants, such as polyphenols, to reduce Fe^{3+} to Fe^{2+} in the presence of FRAP reagent, forming a blue chromophore that has a maximum absorption of 593 nm. Polyphenols present in the sample are the donors of electrons and can react with free radical species to stabilize it and block chain reactions. However, MAE has given the extracts with statistically significantly higher reducing potential in comparison to the other two extraction techniques at all solvent type levels, as in the case of ABTS scavenging activity. Generally, the period of harvesting, the genotype, the growing conditions, and the anatomical part of the plant can influence the content of phenols derivatives and antioxidant activity (Duda et al., 2015; Petrisor et al., 2022). Solvent, also, in a significant dose can influence the change of the antioxidant potential (Boeing et al., 2014). Jaimez et al. (2018) reported that the ferric reducing antioxidant potential of lemon balm water extract correlate with the total amount of phenolic compound when the extract was obtained by the extraction at a high temperature.

3.3. Physical characteristics of the selected extracts

Physical characteristics, including extraction yield (EY), pH, conductivity (G), density (ρ), surface tension (γ), and viscosity (η) of the selected lemon balm water extracts obtained in maceration, ultrasound- and microwave-assisted extractions (the extracts with the highest TPC) are presented in Table 2. The extraction yield was expressed as the dry matter content of lemon balm water extracts (%). As was expected, the highest extraction yield was obtained in the extract from MAE (36.8±1.2 %), due to the mechanism of microwave extraction. Namely, water in the plant cells absorbs the energy of microwaves, which causes interior superheating and degradation of cells, consequently enhancing the release of different compounds (polyphenols, sugars, lipids, and proteins) into the extraction medium, and the extraction yield (Jovanović et al., 2022). pH values of the selected lemon balm extracts varied in a range from 5.99 to 6.43 (Table 2) which are adequate values for the potential drying or encapsulation processes. Apart from the extraction yield and pH value, the conductivity of the selected extracts was determined as the third physical characteristic (Table 2). In specific, conductivity represents a measure of how easily an electrical current can pass through any medium and it also can be used as a predictor of the extract's antioxidant potential (Suliman et al., 2015). However, between selected extracts, there was no statistically significant difference in conductivity. According to Jurinjak Tušek et al. (2018), plant extracts that exerted a good antioxidant capacity also showed a higher conductivity. Suliman et al. (2015) have reported that conductivity is affected by the presence of extraneous ions, thus analysis of the antioxidant capacity of the extracts by using antioxidant methods is necessary. The obtaining of the experimental data on lemon balm extract rheological characteristics is an important applied task, because of its future application, drying, or encapsulation. Based on density measurements, the highest density was obtained for the extract prepared in MAE (0.872±0.003 g/mL), and the lowest for the extract from MAC (0.841±0.007 g/mL), which was correlated with extraction yields (Table 2). Namely, the density of herbal extracts depends on extraction conditions and techniques and correlates to the extraction yield (Mladenović et al., 2018). The obtained results are in agreement with the results reported by Mladenović et al. (2018), where the density of *Portulaca oleracea* extracts varied from 0.700 to 0.850 g/mL. According to the results of surface tension (Table 2), it can be noticed that the extracts obtained using UAE and MAE have shown the highest surface tension (~31 mN/m), whereas the extract from maceration possessed statistically significantly lower values of surface tension. Namely, due to lower surface tension, the adsorption of polyphenols by the extraction solvent is provided, as well as faster penetration of the extraction medium into the plant material that leads to the increase of the plant matrix-medium contact surface, and consequently to better polyphenol recovery. It can be the

reason for the absence of statistically significant differences between TPC in MAC and MAE extracts (Table 1), despite the potential of microwaves compared to only the shaking process in maceration.

The viscosity of herbal extracts, particularly high-yield samples, should be examined to predict potential difficulties, which can occur in the application of excessive viscous extracts in various food and pharmaceutical formulations. In all selected lemon balm extracts viscosity did not differ significantly and was in a range of 1.18-1.21 mP·s. The presented results are in agreement with the literature data, where *Hibiscus sabdariffa* L. water extract showed a similar viscosity, 1.5 mP·s (Hassan and Hobani, 1998).

CONCLUSION

These experiments were the first attempt to evaluate the influence of different solvent types on the extraction of polyphenol compounds from lemon balm herb using various extraction techniques (MAC, UAE, and MAE). Apart from polyphenol and flavonoid concentrations, this study revealed the antioxidant potential of different *M. officinalis* extracts, as well as total tannins, total proteins, and extraction yield of the most representative samples. The obtained results indicate that the polarity of the solvent has significantly affected the content of total polyphenols and flavonoids. In all three methods, methanol and water have caused the highest polyphenol release, as well as ABTS radical scavenging activity, while methanol was the most suitable extraction medium for the recovery of flavonoids. In MAC, the highest ferric-reducing activity was possessed water extract, while in UAE and MAE, it was ethyl acetate samples. Considering the total tannin content in lemon balm water extracts, there was a significant difference between the used extraction techniques (UAE$\gg$MAC$\gg$MAE), while according to the amount of total proteins, the trend was UAE$\gg$MAC$\sim$MAE. The content of total extractive substances in the form of extraction yield was determined for water extracts, and the trend was MAE$\gg$UAE$\gg$MAC. Conductivity and viscosity were the same for all investigated water extracts, while surface tension was the lowest for MAC extract. Further analyses will include the variation of other extraction parameters, such as temperature, particle size, solvent-to-solid ratio, pH, extraction time, the ratio of water and organic solvent in the extraction medium, etc. Finally, this research was an initial step in the production of the extracts with high biopotential aimed to be used for the formulation of different preparations applied in pharmacy, biomedicine, and agriculture. Also, further studies will be based on the investigation of different encapsulation processes for these types of lemon balm extracts, and their pharmacological activities.

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