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The content of heavy metal(oid)s, total phenols, total flavonoids, rosmarinic acid, and antioxidant activity of lemon balm leaves (*Melissa officinalis* L.)

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ABSTRACT

Lemon balm is a plant widely used across various industries worldwide, and it contains a significant number of phenolic compounds that can positively affect human health. Given its extensive use today, this study determined the total polyphenols and flavonoid contents, antioxidant activities, rosmarinic acid content, and heavy metal(oid)s levels in the leaves of the pot-grown lemon balm. The heavy metal(oid)s content was compared with the maximum permissible concentrations for medicinal plants, as the World Health Organization (WHO) recommended. The results showed that all analyzed elements (As, Cd, Co, Cu, Zn, Mn, Ni and Pb) concentrations were within the recommended limits. The phenolic compound content of lemon balm was compared with literature data for wild-grown lemon balm samples.

Keywords: lemon balm, phenolic compounds, flavonoids, rosmarinic acid, antioxidant activity, heavy metal(oid)s

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Introduction

A medicinal plant, lemon balm (*Melissa officinalis* L.) is cultivated worldwide today due to its various positive effects on human health and its applications in the food and cosmetic industries. It is known that lemon balm has antioxidant, anti-inflammatory, antimicrobial, antiviral, and sedative properties. Additionally, it has a pleasant taste and good aroma, which makes it a popular additive in various products to improve their quality (Turhan, 2006).

Considering the properties of the lemon balm and its application in various industrial products, there is a significant need for its analysis and quality control. This includes the assessment of heavy metal(oid)s content, which could pose health risks to consumers of lemon balm and its derivatives, as well as the determination of total phenols, total flavonoids, antioxidant activity, and rosmarinic acid content. It is known that rosmarinic acid has a positive effect on the *Herpes simplex* virus, contributing to the medicinal effects of lemon balm (Petersen and Simmonds, 2003).

As the products of secondary metabolism in plants, phenolic compounds play a crucial role in their defence from various stress factors such as low temperatures, pathogen infections, nutrient deficiencies, heavy metal-induced stress, and others. These stressors trigger the production of free radicals and other molecules that cause oxidative stress (Lattanzio, 2013). Phenolic compounds contents in plants are crucial for the efficacy of medicinal plant-based products because they are natural antioxidants.

Given that different factors can influence the phenolic content, such as the presence of heavy metal(oid)s (Márquez-García et al., 2012) and cultivation conditions (Nurzyńska-Wierdak, 2023), there is need to explore these effects. Since cultivation conditions can significantly impact phenolic content, one of the goals of this study is to examine the influence of growing lemon balm in pots on phenolic content and compare it with literature data on wild-grown lemon balm samples. This comparison will be used as a basis for further research, such as the impact of elevated concentrations of heavy metal(oid)s on the antioxidant activity and phenolic compound content of lemon balm grown in pots, as well as the uptake of other heavy metal(oid)s present, both essential and potentially toxic.

Rosmarinic acid, an ester of caffeic acid, was first isolated from rosemary (*Rosmarinus officinalis*) in 1958. It is found in numerous plants, such as *Ocimum tenuiflorum* L., *Thymus mastichina* L., and others from the *Lamiaceae* family. Rosmarinic acid is believed to exhibit antioxidant, anti-aging, anti-inflammatory, antibacterial, antiviral, anticancer, antidiabetic, cardioprotective, hepatoprotective, nephroprotective, antidepressant, antiallergic properties (Nadeem et al., 2019). Since rosmarinic acid has antioxidant and antimicrobial activities and positively affects human health, it is used in the cosmetic and food industry to extend the expiry date of products.

Heavy metal(oid)s are naturally present in plants and can be categorized into essential and non-essential elements. Essential elements are necessary for plant growth and development. However, excessive levels of some essential elements can also negatively influence plant growth. In contrast, non-essential elements are not required for plant growth, and their elevated presence leads to harmful effects, such as leaf chlorosis, reduced plant growth, and other symptoms (Asati et al.,

2016). Determining the content of heavy metal(oid)s in plants is of great importance, as excessive levels can negatively affect the health of people consuming these plants.

Experimental

Lemon balm leaf samples were obtained by growing the plant in pots under natural conditions from June until August 2023. The soil used for cultivation was collected near Niš, from an area where no agro-technical measures had been applied for many years, to minimize the influence of these factors on the parameters being investigated. The collected soil was first air-dried for two weeks, sieved, and then mixed with commercially available Hawita Professional substrate in a 1:1 volumetric ratio to improve soil fertility. One-month-old lemon balm seedlings, grown from seeds purchased from the Dr. Josif Pančić Institute, were planted in this prepared soil. The collected lemon balm samples were air-dried before further analysis.

The extraction of phenolic compounds from the lemon balm samples was performed as described in Adamczyk-Szabela et al. (2023). Briefly, approximately 0.5 g of dried plant material was accurately weighed and transferred to an Erlenmeyer flask. In the flask, 50 mL of 70% methanol was added, and the extraction was carried out by placing the flask on a shaker for 2 h at room temperature. After extraction, the liquid was separated from the solid by centrifugation at 3000 rpm for 10 min in plastic tubes. The resulting extract was then filtered using microfilters (0.45 µm) and stored in a refrigerator until further analysis.

Determination of heavy metal(oid)s content using ICP OES

To determine the heavy metal(oid)s content, dried lemon balm leaf samples were prepared using the microwave digestion (ETHOS EASYmicrowave digestion system, Milestone, Bergamo, Italy) method as follows: approximately 0.35 g of the sample was weighed, transferred to digestion vessels, and 3 mL of concentrated H_2O_2 and 6 mL of concentrated HNO_3 were added. The digestion program was as follows: a temperature of 180 °C was reached within 20 min and then maintained for 10 min. After the digestion process was completed, the samples were allowed to cool. The entire content from the vessels was then quantitatively transferred to volumetric flasks (25 mL) and filled to the mark with deionized water.

For the ICP OES analysis, an external calibration curve method was used, with the wavelength selection for reading the results based on the relative intensity of the emission lines, the correlation coefficient value, spectral interferences, and the matrix effect on emission. The following heavy metal(oid)s were determined in the lemon balm leaves: As, Cd, Co, Cu, Zn, Mn, Ni, and Pb. ICP analysis was performed using ICP OES, series iCAP 6000 (ThermoScientific, Cambridge, United Kingdom).

Determination of total polyphenolic compounds content

The total polyphenol content in the lemon balm samples was determined using the Folin-Ciocalteu method. 0.1 mL of the extract, obtained by the previously described method (Adamczyk-Szabela et al., 2023), was measured in a 10 mL volumetric flask. Then, 0.5 mL of Folin-Ciocalteu reagent was added, followed by 2 mL of a saturated Na_2CO_3 solution after 5 minutes. The volumetric flask was then filled with deionized water to a final volume of 10 mL, and left in the dark for 30 min, and the absorbance of the sample was measured at 760 nm, with deionized water as a blank using UV VIS Perkin Elmer Lambda 15 spectrometer. The calibration curve was constructed by measuring the absorbance of standard gallic acid solutions to which 0.5 mL of Folin-Ciocalteu reagent and 2 mL of saturated Na_2CO_3 solution were added, and the volume was adjusted with deionized water, resulting in final gallic acid concentrations ranging from 1 to 9 µg/mL. After 30 minutes in the dark, the absorbance of the standard solutions was measured, and the calibration curve was obtained: $A = 0.04385 + 0.10517c_{\text{gallic acid}}$, $r^2 = 0.99986$ (Singleton et al., 1999; Stratil et al., 2006; Huang et al., 2005). The results of the extract analysis were expressed as mg of gallic acid equivalents per g of dry lemon balm sample.

Determination of total flavonoid content

0.1 mL of the lemon balm extract was measured into a volumetric flask (10 mL), and 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 1.5 mL of $AlCl_3$ solution was added, and after another 5 min, 2 mL of 1M NaOH was added. The volumetric flask was then filled with deionized water to 10 mL. The absorbance was measured at 510 nm, with deionized water as a blank. A series of working solutions of catechin was prepared from a stock solution of catechin at a concentration of 0.5 mg/mL and used to create a calibration curve, which showed linearity in the concentration range from 1 to 10 mg/L. The calibration equation was $A = 0.03612 + 0.00491c_{\text{catechin}}$. Based on this equation, the total flavonoid content was calculated and expressed as mg of catechin equivalents per g of dry sample (mg CE/g).

Determination of antioxidant activity using the DPPH method

0.5 mL of the obtained extract was diluted to 50 mL with 70% (v/v) methanol. The DPPH method was used, as described by Brand-Williams et al. (1995), with minor modifications. A solution of 2,2-diphenyl-2-picrylhydrazyl (DPPH) with a concentration of $1 \cdot 10^{-4}$ mol/L in methanol was prepared. A 5.0 mL aliquot of this solution was placed in a volumetric flask (10 mL), along with 0.5 mL of the diluted sample. The flask was then filled to 10 mL with methanol. After 30 min, the colour change of the DPPH radical was determined spectrophotometrically at 520 nm. A calibration curve was created using Trolox solutions based on the decrease in absorbance ($\Delta A = A_{\text{blank}} - A$), which corresponded to the DPPH radical scavenging activity. The results were reported as µg of Trolox equivalents (TE) per g of dry lemon balm (µg TE/g).

Determination of rosmarinic acid

An Agilent 1200 model (Agilent Technologies, Santa Clara, California, USA) was employed for HPLC analysis. The analytical column used was a C18 Zorbax Eclipse XDBC18, 5 µm, 4.6×150 mm (Agilent Technologies, Santa Clara, California, USA). The mobile phase flow rate was set to 0.8 mL/min, and the analysis time was 40 min. Solvent A was 5% HCOOH in deionized water, and Solvent B was 5% HCOOH in 80% acetonitrile, with the following gradient program: 0% B during the first 10 min, 0–25% B from 10 to 20 min, 25–40% B from 20 to 30 min, 40–70% B from 30 to 35 min, and 70–80% B from 35 to 40 min.

Results and Discussion

The content of heavy metal(oid)s in the lemon balm leaves is provided in Table 1.

Table 1. Content of heavy metal(oid)s in lemon balm leaves and selected wavelengths for determination

Element/λ[nm]	Concentration [mg/kg]	Element/λ[nm]	Concentration [mg/kg]
As/189.0	0.5 ± 0.2	Zn/202.5	71.7 ± 0.2
Cd/214.4	0.08 ± 0.00	Mn/257.6	30.0 ± 0.2
Co/228.6	0.48 ± 0.02	Ni/221.6	3.24 ± 0.03
Cu/324.7	10.45 ± 0.06	Pb/220.3	0.84 ± 0.02

Among the analyzed elements, Zn, Mn, Cu, and Ni are the most prevalent, which can be explained by the fact that these are essential elements necessary for plant growth (Sarwar et al., 2017). In addition, Co is an essential metal, but its concentration is significantly lower than the previously mentioned elements. Pb, As, and Cd are less prevalent as they are non-essential elements that plants do not require. The content of the analyzed elements is shown in Figure 1.

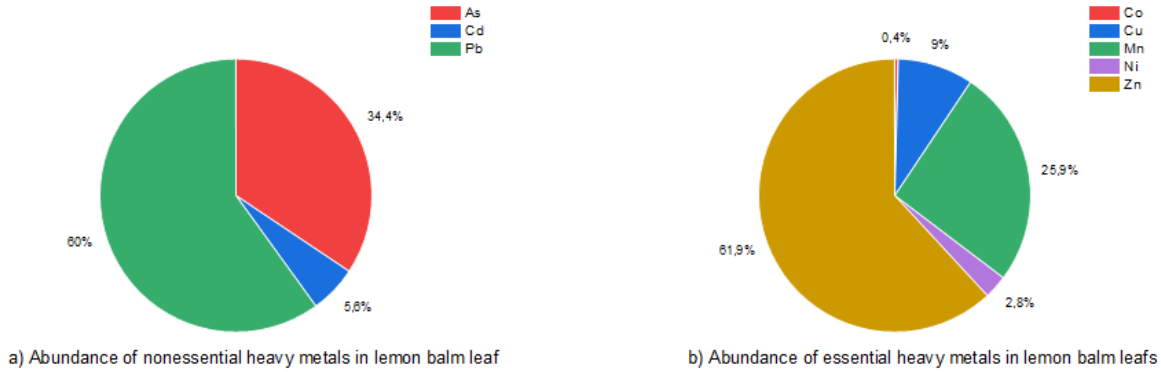

Figure 1. Distribution of heavy metal(oid)s in lemon balm leaves

As seen in Figure 1, the order of prevalence for essential elements is as follows: Zn > Mn > Cu > Ni > Co, and for non-essential elements, Pb > As > Cd. The concentrations of Cd and Pb are within the permissible limits for medicinal plants, based on the recommendations of the World Health Organization, as well as the values for Canada and China (WHO, 2007). The obtained concentrations of the analyzed elements in lemon balm are similar to the results obtained in the study by Sussa et al. (2022), where lemon balm was also grown in pots with the slightly lower concentration of Pb and Ni and somewhat higher the concentration of Zn.

The concentration of total polyphenols was 69 ± 3 mg/g gallic acid equivalent, expressed on a dry mass basis. In the study by Spiridon et al. (2011), the concentration of total phenols was 54.9 ± 2.14 mg/g, expressed on the dry extract mass of lemon balm. According to Boneza and Niemeyer (2018), the total phenol content ranged from 5.50 to 26.87 mg/g gallic acid equivalents, depending on the cultivar. In the works of Spiridon et al. (2011) and Boneza and Niemeyer (2018), samples from nature were used.

The concentration of total flavonoids, expressed on a dry mass basis of the plant, was 78 ± 2 mg/g catechin equivalents. According to Spiridon et al. (2011), the total flavonoid content in lemon balm was 25.8 ± 6.26 mg/g rutin equivalents, expressed on the dry extract mass, while for oregano (*Origanum vulgare*), the value for total flavonoids was 31.6 ± 4.25 mg/g rutin equivalents. Lin et al. (2012) determined total flavonoids in lemon balm leaves dried in two ways: warm air and freeze-drying. These authors extracted lemon balm leaves using ethanol and obtained results for the freeze-dried sample of 54.32 ± 4.13 mg/g catechin equivalents, and for the warm air-dried sample, 48.45 ± 3.24 mg/g catechin equivalents. Hassan et al. (2019) compared the impact of different solvents for extraction on the total flavonoid content in lemon balm extracts, obtaining the following results for the methanol extract: 72.38 mg/g dry extract of quercetin equivalents (QE), for methylene chloride extract: 59.76 mg/g QE eq., for ethyl acetate extract: 124.96 mg/g QE eq., and for butanol extract: 84.96 mg/g QE eq. The results of Hassan et al. (2019) clearly indicate that depending on the solvent used for extraction, the total flavonoid content varies, but the results obtained in this study, where a 70% (v/v) methanol extract was used, are similar to those obtained by Hassan et al. (2019) when methanol was used for extraction. All samples in the literature data include wild lemon balm.

The antioxidant activity of lemon balm leaves is one of its crucial properties, contributing to a wide range of applications. Thus, determining and controlling the antioxidant activity of lemon balm is very important. The antioxidant activity of lemon balm, determined by the DPPH method for the potted lemon balm leaves, is 379 ± 4 μmol/g Trolox equivalents, expressed on the dry mass of the lemon balm sample.

Rosmarinic acid is one of the most significant phenolic compounds in lemon balm. Based on the HPLC analysis, the content is 26.9 ± 0.1 mg/g of dried sample for potted lemon balm. Dastmalchi et al. (2008) reported a 96.45 ± 0.13 mg/g value for rosmarinic acid in their lemon balm samples. Arceusz and Wesolowski (2013), analyzing 19 lemon balm samples from Poland, found the concentration of rosmarinic acid to range from a minimum of 0.158 mg/g to a maximum of 48.608 mg/g, with an average of 27.05 mg/g, which is approximately consistent with the results obtained

in this study. This suggests no significant difference in rosmarinic acid content between potted lemon balm and wild lemon balm. Wang et al. (2004) found a value of 27.4 mg/g of rosmarinic acid in their lemon balm analysis, further supporting the earlier conclusion that there is no substantial difference between potted lemon balm and field-grown lemon balm regarding rosmarinic acid content.

Future studies should include research on the impact of elevated concentrations of some heavy metal(oid)s in soil on phenolic compounds content, antioxidant activity, and the content of heavy metal(oid)s (essential and potentially toxic) of lemon balm. Another focus of further research should be on improving the uptake of heavy metal(oid)s by lemon balm using different complexing agents, the prevention of heavy metal(oid)s uptake using zeolites, and the effect of different factors such as pH of soil, content of organic matter in soil, and the presence of some pesticides on heavy metal(oid)s uptake by lemon balm. This research will be conducted as pot experiments since concentrations of heavy metal(oid)s, phenolic compounds, flavonoids, and rosmarinic acid are similar in lemon balm grown in pots and naturally grown lemon balm.

Conclusion

In this study, the content of total polyphenols, total flavonoids, antioxidant activity, rosmarinic acid content, and the presence of eight heavy metal(oid)s (As, Cd, Co, Cu, Zn, Mn, Ni, and Pb) in lemon balm leaves was determined. The content of all heavy metal(oid)s was below the maximum allowed concentrations according to the World Health Organization (WHO) guidelines. Essential elements were present in higher quantities than non-essential. The total polyphenol content was 69 ± 3 mg/g gallic acid equivalents, total flavonoid content was 78 ± 2 mg/g catechin equivalents, antioxidant activity measured by the DPPH method was 379 ± 4 μmol/g Trolox equivalents, and the rosmarinic acid content was 26.9 ± 0.1 mg/g. All these values, as well as the values for heavy metal(oid)s content in lemon balm grown in pots in this study, are comparable to those found in the literature for wild lemon balm samples.

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Conflict-of-Interest Statement

The authors declare no conflict of interest.

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