

CONTENT OF BIOACTIVE COMPOUNDS IN LEMON BALM (*Melissa officinalis* L.) SAMPLES GROWN IN BOSNIA AND HERZEGOVINA

Lejla Čengić^{*1}, Edna Pipić¹, Zilha Ašimović¹, Drena Gadžo¹, Nermina Đulančić¹

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Abstract

Lemon balm leaves are a potential source of biologically active compounds, especially phenolic compounds. These compounds contribute to its antioxidative activity, serving as the plant's natural response to stress. The aim of the research was to determine the content of bioactive compounds and antioxidative activity in lemon balm samples grown in different parts of Bosnia and Herzegovina. Total phenolics, total flavonoids, and antioxidative activity were analyzed using aqueous and ethanolic extracts with the Folin-Ciocalteu and method with AlCl_3 for phenolics and flavonoids, respectively. The pFRAP method was used for antioxidative activity. Research showed that geographic location had an impact on bioactive compound content and their antioxidative activity. It can also be concluded that the extraction solvent influenced the antioxidant activity of lemon balm leaves.

Keywords: *lemon balm, bioactive compounds, antioxidant activity, location, extraction solvent*

INTRODUCTION

Herbs are known to be used to enhance the sensory properties of foods, as natural preservatives, and for their health-promoting and nutritional characteristics. Nowadays, focus is on the development of new phytocomplexes from food and plant-derived compounds (Tsoukalas *et al.*, 2021). Plants with potent health benefits for human health were recognized among the *Melissa* genus (Burlec, 2020). *Melissa officinalis*, known as lemon balm, is indigenous to the Mediterranean region, but is known and cultivated worldwide (Verma *et al.*, 2015). It is called the “elixir of life” by Paracelsus (Sawicka *et al.*, 2020), who believed it could completely restore a human’s strength. Extracts of *Melissa officinalis* can be applied in pharmaceutical products, cosmetics, and biopesticides, contributing to the production of bioactive metabolites with potential therapeutic effects against various diseases (Sawicka *et al.*, 2020). Previous research confirmed that lemon balm, especially its ethanolic extracts, showed strong antidiabetic and antihyperlipidemic effects (Moacă *et al.*, 2018). Its health benefits are reflected in β -cell prevention, improved insulin sensitivity, and regulation of glucose and lipid metabolism (Chung *et al.*, 2010). Numerous studies indicated a significant role of lemon balm extracts in the treatment of diabetes type 2, dyslipidemia and metabolic syndrome

¹ Faculty of Agriculture and Food Sciences, Zmaja od Bosne 8, 71000 Sarajevo, Bosnia and Herzegovina

^{*}Correspondence: l.cengic@ppf.unsa.ba

(Changizi-Astiyani *et al.*, 2013; Weidner *et al.*, 2015). Due to increasing resistance to antibiotics, lemon balm (*Melissa officinalis* L.) stands out as a natural alternative with antibacterial properties, particularly effective against Gram-positive bacteria, attributed to its bioactive compounds (Mimica-Dukić *et al.*, 2004; Cimpeanu *et al.*, 2025).

Phenolic compounds, classified as secondary metabolites, represent the predominant group of phytochemicals. They are characterized by the presence of one or more aromatic rings bearing hydroxyl groups, with structural complexity ranging from simple monomeric phenolics to large, high-molecular-weight polymers (Nile and Park, 2014). With regard to these compounds, several studies of lemon balm showed high content of phenolic acids, especially rosmarinic acid (Awad *et al.*, 2009; Spiridon *et al.*, 2010; Barros *et al.*, 2013), caffeic and p-coumaric acid (Astani *et al.*, 2012; Miraj *et al.*, 2017), as well as tartaric, malic, citric, quinic, succinic, salicylic, and chicoric acids (Aubert *et al.*, 2019). Moreover, several glucosides were identified, including luteolin 3'-O- β -D-glucoside and ethyl caffeate (Aubert *et al.*, 2019). Polyphenolic acids, such as rosmarinic acid, promote antiviral activity, particularly against the HSV-1 virus (Sharifi-Rad *et al.*, 2021), due to their ability to inhibit virus binding to cell receptors, consequently limiting the spread of the infection (Astani *et al.*, 2012). Flavonoids, isolated from a wide range of vascular plants, are known to be phenolic substances that act as antioxidants, antimicrobials, and photoreceptors and exhibit biological activities with a major interest in antioxidant activity due to their ability to reduce free radical formation (Pietta, 2000). The form in which they circulate in vivo will influence their polarity, thus their localization and bioactivities (Rice-Evans, 2001).

Many authors reported that growing conditions, along with genotype, ripeness, storage time, and seasonal differences, had a major influence on the accumulation of phenolic compounds (Josuttis *et al.*, 2013; Raudone *et al.*, 2021).

The present study aimed to quantify the bioactive constituents, including total phenolics and flavonoids, and to assess the antioxidant activity of lemon balm (*Melissa officinalis* L.) samples collected across various regions of Bosnia and Herzegovina. Additionally, the study sought to investigate the influence of different extraction solvents on the levels of these bioactive components and their associated antioxidant properties.

MATERIALS AND METHODS

Materials

Approximately 1 kg of whole lemon balm was collected at different locations in B&H. The location's altitude varied from 900 m, being the highest, to 384 m, being the lowest. Samples were classified based on their location altitude from S1 (the highest altitude) to S5 (the lowest altitude). Samples were grown in an open field. Samples were prepared for analysis as follows: only green, healthy leaves with no signs of damage were subjected to the drying process prior to analysis; every damaged leaf was discarded, and undamaged leaves were kept in a dark place at ambient temperature for seven days. The purpose of drying was to decrease the content of water in order to avoid the growth of

the fungus. Once it was dry, the sample was crushed in a mortar with a pestle and used for further analysis.

Extraction

Bioactive compounds were extracted using two solvents: a 70% (v/v) ethanol solution and distilled water. Extractions were performed under controlled conditions to ensure reproducibility and maximal yield of target metabolites. 1 g of dried and crushed lemon balm sample was mixed with 70% ethanol, previously heated at 50°C, manually homogenized, transferred to a volumetric flask with a round bottom, and heated in an ultrasonic bath at 24°C for 30 minutes. After sonication, the sample was filtered through Whatman no. 1, and another portion (20 mL) of ethanol was added to the residue. The mixture was sonicated repeatedly in an ultrasonic bath three times. Samples were transferred to a volumetric flask and adjusted to the volume (100 mL). The extraction procedure was repeated with distilled water.

Total phenolic content determination (TPC)

The spectrophotometric method based on a color reaction between phenolics and the Folin-Ciocalteu reagent was used for total phenolics content determination (Ough and Amerine, 1988). Briefly, 1 mL of extract was mixed with 2.5 mL Folin-Ciocalteu reagent (1:10, v/v), and after 5 minutes, 6.5 mL of saturated sodium carbonate Na_2CO_3 was added. The mixture was placed in a water bath (50°C) for 20 minutes, and absorbance was read at 750 nm against a blank. Calibration was performed using gallic acid, and the content of phenolic compounds was reported as gallic acid equivalents per 100 g of lemon balm.

Total flavonoid content determination (TFC)

The spectrophotometric method based on a color reaction between flavonoids and AlCl_3 was used for the determination of total flavonoid content, and the intensity of the colored complex was measured at 510 nm (Zhinsen *et al.*, 1999). Briefly, 1 mL of extract was mixed with 4 mL of distilled water and 300 μL of 5% NaNO_2 , after 5 minutes, 300 μL of 10% AlCl_3 was added, and 1 minute after, 2 mL of 1M NaOH . A calibration curve (0-100 mg/L) was made with catechin and used for the calculation of flavonoid content. Results were expressed as catechin equivalents /100 mg of dry weight.

Antioxidant activity determination

The spectrophotometric method based on a color reaction of phenolic compounds with $\text{K}_3[\text{Fe}(\text{CN})_6]$ and FeCl_3 was used for the determination of antioxidant activity. The intensity of the blue colored complex was measured at 700 nm (Meng *et al.*, 2011). Briefly, 1 mL of extract was mixed with 1 mL of $\text{K}_3[\text{Fe}(\text{CN})_6]$, and after 5 minutes, 1 mL of FeCl_3 and 7 mL of water were added. Gallic acid was used as a standard for calibration (0-100 mg/L), and the results were expressed as gallic acid equivalents.

Statistical data analysis

The obtained data were statistically analyzed using program PAST (*ver* 5.1) (Hammer *et al.*, 2001). In order to examine the influence of location on the investigated parameters (content of bioactive compounds and antioxidative activity), one-way ANOVA with Tukey *post hoc* test was used. To examine the influence of extraction solvents on the investigated parameters the non-parametric Mann-Whitney (MW) test was selected. Differences with $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The lemon balm samples were collected during the summer of 2024 from various locations across Bosnia and Herzegovina, including the Nevesinje area in eastern Herzegovina, Butmir and Trnovo near Sarajevo, and the Vareš and Kakanj area in central Bosnia. The location sites and their corresponding coordinates are given in Table 1. Samples were classified based on their location altitude, ranging from S1 (the highest altitude) to S5 (the lowest altitude). Notably, samples S3 and S4 were collected in close proximity, resulting in nearly identical coordinates.

Table 1. Locations of lemon balm (*Melissa officinalis* L.) samples collected in Bosnia and Herzegovina

Sample No. Location of sample	Latitude	Longitude	Altitude (m)
S-1 (Nevesinje)	43.2575°	18.1124°	898
S-2 (Vareš)	44.1644°	18.3283°	845
S-3 (Trnovo)	43.6658°	18.4459°	817
S-4 (Butmir)	43.8191°	18.3257°	506
S-5 (Kakanj)	44.1272°	18.1181°	387

The total phenolic content (Table 2) of lemon balm samples ranged from 289.1 ± 6.60 to 512.99 ± 2.21 mg GAE/100 g of dry weight in ethanolic extracts and 162.60 ± 12.30 to 360.40 ± 21.20 mg GAE/100 g of dry weight in aqueous extracts (Fig. 1). Levels of phenolics varied in dependence on altitude, and for both types of extractions, it was found that the samples from locations of the highest altitude had the highest TPC. This is in accordance with previous research. It is well established that higher altitudes can affect the accumulation of phenolic compounds (Taamalli *et al.*, 2018). Bilgin and Sahin (2013), in their research on olive leaves, showed TPC decreased with decreasing geographical altitude. The content of bioactive compounds is highly dependent on geographical location due to variations in environmental factors such as soil composition, climate, altitude, and sun exposure (Bilgin *et al.*, 2013; Zhang *et al.*, 2022), all of which influence plant growth and biosynthesis of bioactive compounds. Zakraoui *et al.* (2023) showed significant variation in phenolic compounds in olive leaves across different altitudes, which can affect plant height and density, contributing to differences

in bioactive compound content. Urbonaviciene *et al.* (2022), in their study on wild bilberries reported total phenolics to be the highest in the most northern regions and the lowest in the most southern regions. The observed results align partially with previous studies, potentially reflecting the influence of environmental conditions, including sunlight, precipitation, and related factors. Other researchers (Klimiene *et al.*, 2021) found that TPC and TFC are affected by the chemical properties of the soil (pH, N, P₂O₅, Ca, Mg, and soil humus).

The high content of phenolic compounds observed in this study is consistent with the findings reported by Slowianek and Leszcynska (2016). This characteristic is typical of the Lamiaceae family, to which *Melissa officinalis* belongs. Consequently, plants belonging to this family are widely employed in traditional medicinal practices and are valued for their beneficial effects on health (Modnicki and Blacerek, 2009).

Statistical analysis showed no significant effect ($p > 0.05$) of sampling locations on the average values of total phenolic content (TPC). Sampling locations had a statistically significant effect ($p < 0.05$) on total flavonoids content (TFC) (Table 2). The highest TFC content was determined at sampling site S-5 and it was statistically significantly higher than the TFC content at sampling sites S-1 and S-3. Statistically significant differences in the content of the mentioned parameter were also found between sampling localities S-1 and S-3 (Table 2).

Statistical analysis showed no significant effect ($p > 0.05$) of the extraction solvent on the average values of TPC and TFC. Although certain differences in the examined parameters were observed among the extraction methods, but these differences were not statistically significant (Table 2).

One of the most commonly applied methods for isolating bioactive compounds from plant materials is solvent extraction. Nevertheless, the efficiency of extraction and the corresponding antioxidant potential of the obtained extracts are greatly influenced by the type of solvent used. The heterogeneous distribution of phenolic compounds across plant tissues may limit the extraction efficiency of antioxidant compounds (Antolovich *et al.*, 2000). Our research revealed higher TPC and TFC values in ethanolic extracts (ethanol:water, 70:30), which is in accordance with previous results, confirming that aqueous ethanol is more effective in recovering the highest amounts of bioactive compounds with a phenolic nature. The impact of extraction solvent on phenolic and flavonoid levels has been reported (Khatode and Deshmukh, 2022) and was reflected in our study, where 70% ethanol extracts contained higher amounts of these compounds than extracts obtained with water. Sultana *et al.* (2009) reported that aqueous ethanolic and methanolic solutions had the highest content of TPC among selected medicinal plants. Jovanović *et al.* (2022) found that the highest content of polyphenols was found in methanolic and aqueous extracts of lemon balm.

Binello *et al.* (2017) focused their study on green extraction of polyphenolics in lemon balm, suggesting ultrasound and microwave-assisted extraction protocols, with ethanol as the best solvent for this type of extraction. Previous studies (Kamdem *et al.*, 2013; Duda *et al.*, 2015) reported higher amounts of phenolic compounds in methanolic extracts. Methanolic and ethanolic extraction solvents can improve the solubility of phenolic compounds, which was the topic of the study conducted by Singapurwa *et al.* (2024). These authors studied the influence of polarity of extraction solvents on components with antioxidant properties and showed that solvents with high polarity (methanol, ethanol) can exhibit higher values for TPC of higher molecular weight.

Table 2. Total phenolic and flavonoids content in 70% ethanolic extracts and aqueous extracts

Parameters		TPC			TFC	
Extraction solvents	Ethanolic extracts	Aqueous extracts	Average	Ethanolic extracts	Aqueous extracts	Average
Location						
S-1	446.69	162.60	304.65 A	3.93	0.73	2.33 A
S-2	289.18	328.60	308.89 A	2.91	4.49	3.70 AB
S-3	340.7	282.67	311.69 A	3.70	5.40	4.55 B
S-4	496.17	165.89	331.03 A	3.54	2.06	2.80 AB
S-5	512.99	360.40	436.70 A	5.55	4.68	5.12 C
Average	417.15 a	260.03 a		3.93 a	3.47 a	

* capital letters indicate differences between sampling locations; lowercase letters indicate differences between extraction solvents

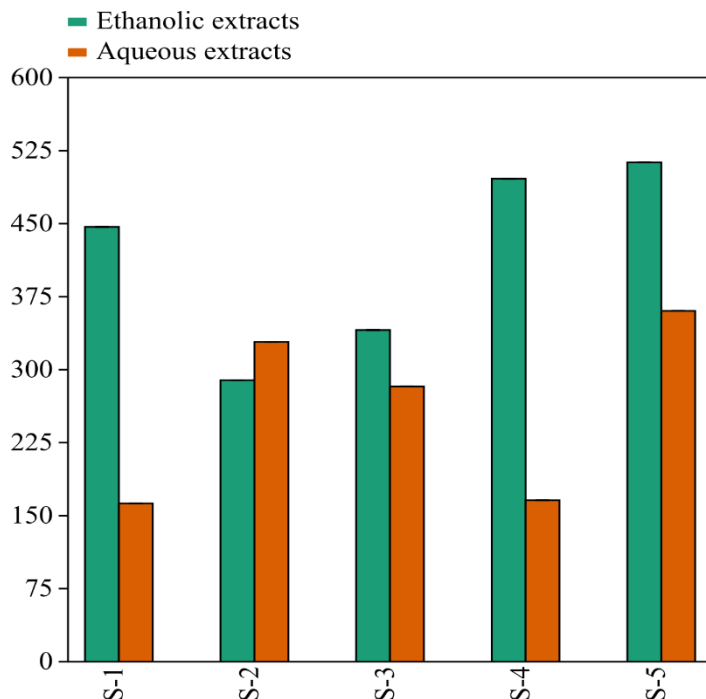


Figure 1. Total phenolic content (TPC) in lemon balm samples in mg of gallic acid equivalents

The content of phenolic compounds can also be influenced by various external and internal factors, such as temperature, extraction time, physical state of the sample (fresh or dehydrated), plant used for extraction, etc. Ordaz *et al.* (2018) used different temperature / time conditions for aqueous extraction of lemon balm samples, which resulted with a range of 5.58-49.16 mg GAE/g of sample and a strong influence of variable parameters. Interactions temperature/time showed a positive influence on the liberation of antioxidants (Ordaz *et al.*, 2018). Our results were slightly lower than reported, which can be explained by the extraction conditions and quantity of samples. Fernandes *et al.* (2016) showed that an increase in time of infusion favors the extraction, which can enhance solute solubility. Šić Žlabur *et al.* (2016) reported an increasing trend in the time of ultrasonic extraction for phenolic compounds. Tülek *et al.* (2020) showed optimum extraction conditions to be 100°C for temperature and 120 minutes for the time (6365 mg GAE/ 100g), although this type of extraction affected phenolic acids content, which was undesirable, knowing that rosmarinic acid is the most abundant phenolic acid in lemon balm (Silva *et al.*, 2023).

Table 3. Antioxidant activity measured by pFRAP method in 70% ethanolic and aqueous extracts

Parameter	Antioxidant activity		
	Ethanolic extracts	Aqueous extracts	Average
Extraction solvents			
Location			
S-1	2531.58	1548.63	2040.11 A
S-2	2579.77	2034.20	2306.99 A
S-3	2614.16	2093.13	2353.65 A
S-4	2562.58	1786.7	2174.64 A
S-5	2628.32	2173.27	2400.80 A
Average	2583.26 a	1927.19 b	

* capital letters indicate differences between sampling locations; lowercase letters indicate differences between extraction solvents

The pFRAP method was employed to evaluate the antioxidant potential of *Melissa officinalis* (lemon balm) samples, and the results are given in Table 3. As expected, the S5 samples exhibited the highest antioxidant capacity, indicating the greatest reducing power among the tested extracts. The results obtained from the pFRAP method, based on the reaction of components with antioxidant activity indicate a high Fe(II) - chelating capacity of both ethanolic and aqueous extracts of lemon balm, thereby enhancing their overall antioxidant potential.

Statistical analysis showed no significant effect ($p>0.05$) of sampling locations on the average values of *M. officinalis* antioxidant potential (Table 3).

Statistical analysis revealed significant differences in the antioxidant activity of lemon balm leaves between the extraction solvents used. On average, the antioxidant activity of the leaves was significantly higher in the ethanolic extract compared to the aqueous extract (Table 3). This can be explained by the presence of additional antioxidant compounds in lemon balm leaves that are more efficiently extracted with ethanol due to their lipophilic properties.

The antioxidant activity is largely attributed to phenolic constituents, including rutin, quercetin, kaempferol, and phenolic acids, known for their high radical-scavenging capacity (Omoba *et al.*, 2019). In general, higher concentrations of phytochemicals are associated with increased antioxidant activity, as these compounds can neutralize free radicals. Flavonoids, in particular, act as primary antioxidants by donating hydrogen atoms to stabilize reactive species (Speisky *et al.*, 2021). Furthermore, extracts of lemon

balm have demonstrated efficacy against both naturally occurring and synthetic free radicals (Dastmalchi *et al.*, 2008).

Antioxidant activity of lemon balm samples found to be in a positive correlation with total phenolic content (Table 4).

Table 4. Correlation

	TPC	TFC	pFRAP
TPC	1	0.978	0.964
TFC		1	0.973
pFRAP			1

The extracts with a high amount of phenolic components exhibit high antioxidant activity. The content of total phenolics and flavonoids is directly correlated with antioxidant activity, which is consistent with previous *in vitro* studies (Wong *et al.*, 2006). Li *et al.* (2008) also reported a strong positive correlations between phenolic compounds and antioxidant activity, confirming the significant role of phytochemicals in scavenging free radicals and enhancing antioxidant capacity. Our results are in agreement with previous studies of lemon balm extracts (Firuzi *et al.*, 2005; Encalada *et al.*, 2011).

CONCLUSIONS

The findings of this study demonstrated notable variations in the phenolic and flavonoid content and antioxidant activity of lemon balm originating from different geographical locations. Such differences suggest that environmental factors, as well as the type of extraction solvent used, play an important role in determining the antioxidant potential of lemon balm. Future research should focus on the phenolic profiling of lemon balm samples cultivated in Bosnia and Herzegovina, the development of green extraction techniques for antioxidant phytochemicals, and the influence of cultivar and production methods on the chemical composition and bioactivity of lemon balm.

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SADRŽAJ BIOAKTIVNIH KOMPONENTI U UZORCIMA MATIČNJAKA (*Melissa officinalis* L.) UZGOJENIM U BOSNI I HERCEGOVINI

Sažetak

Listovi matičnjaka (*Melissa officinalis* L.) predstavljaju vrijedan izvor biološki aktivnih spojeva, posebno fenolnih jedinjenja. Ove supstance doprinose antioksidativnoj aktivnosti biljke, djelujući kao dio njenog prirodnog odgovora na stresne uslove okoline. Cilj ovog istraživanja bio je utvrditi sadržaj bioaktivnih spojeva i antioksidativnu aktivnost uzoraka matičnjaka uzgojenih u različitim područjima Bosne i Hercegovine. Sadržaj ukupnih fenola, flavonoida i antioksidativna aktivnost analizirani su u vodenim i etanolnim ekstraktima. Za određivanje ukupnih fenola i flavonoida korištene su Folin-Cioacalteau i AlCl_3 spektrofotometrijske metode, dok je za ispitivanje antioksidacijske aktivnosti primjenjena pFRAP metoda. Dobijeni rezultati ukazuju da lokalitet i sredstvo za ekstrakciju imaju značajan uticaj na sadržaj bioaktivnih spojeva, te njihov antioksidacijski potencijal.

Ključne riječi: *matičnjak, bioaktivni spojevi, antioksidacijska aktivnost, lokalitet, ekstrahirano sredstvo*