

EVALUATION OF ANTIOXIDANT ACTIVITY OF *MELITTIS MELISSOPHYLLUM* L. EXTRACTS

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Abstract – The antioxidant activities of methanol and ethanol (10%, 30%, 50% and 96%) extracts of the aerial parts of *Melittis melissophyllum* L. were determined by DPPH, ABTS and FRAP assays. The content of flavonoids and phenols was also investigated. The total phenolic content in the extracts was determined using Folin-Ciocalteu assay; their amounts ranged between 63.5 and 111.7 mg GAeqv/g, while the concentrations of flavonoids were from 7.33 to 56.00 mg Queqv/g. IC₅₀ values of the DPPH scavenging effect were from 0.109 to 0.664 mg/mL. The DPPH scavenging effect of the extracts was determined and the obtained IC₅₀ values were from 0.109 to 0.664 mg/mL of solution. The values of ABTS radical activity were from 0.45 to 0.89 mg ascorbic acid/g. The FRAP value was within the range 0.160-0.382 mmol Fe/mg. The obtained values were analyzed by means of multivariate analysis, employing a hierarchical cluster analysis and between-groups linkage. The presented results confirmed that *M. melissophyllum* possesses good antioxidant properties and may serve as a promising source of natural antioxidants.

Key words: *Melittis melissophyllum*; Lamiaceae; extracts; phenols; flavonoids; DPPH; ABTS; FRAP.

INTRODUCTION

Reactive oxygen species (ROS) play important roles in the etiology of a number of pathological conditions (inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's disease, Alzheimer's disease etc. (Halliwell and Gutteridge, 1986; Halliwell, 1997; Bruneton, 1999). The screening of plant species to identify new antioxidants for health improvement have become very important in recent years. Antioxidants are defined as compounds present at low concentration compared to the oxidizable substrate that can significantly delay or prevent oxidation of that substrate (Halliwell, 1995). Lamiaceae species are a very important source of active natural compounds that differ widely in terms of structure, biological properties

and mechanisms of action. Phytochemical components, especially polyphenols (flavonoids, phenolic acids, phenylpropanoids and tannins) are known to be responsible for the free-radical scavenging and antioxidant activities of plants (Orhan et al., 2003; Ozgen et al., 2006; Krishnaiah et al., 2011; Prochazkova et al., 2011; Wojdylo et al., 2007; Capecka et al., 2005).

Melittis melissophyllum L. (Lamiaceae) is a perennial herb inhabiting shady places in western, southern, and central Europe (Ball, 1972), and is used in folk medicine, especially in Italy. The species, also known as "erba lupa", "erba limona" or "cedrina", was used as an antispasmodic, and against insomnia and eye inflammation in the traditional medicine of central Italy (Guarrera, 2005). It has been widely used

in the traditional medicine of central Serbia, due to its sedative properties, for the treatment of nervous anxiety and hysteria (Jaric et al., 2007). Leaves and inflorescences of this species are used as digestive agents and to treat cough and sore throat. Fresh and dry leaves of *M. melissophyllum* are used to prepare aromatic tea for drinking as a digestive and antispasmodic (Idolo et al., 2010). The dry calyx parts could be used as flavoring agents in food products as a mushroom aroma enhancer (Maggi et al., 2009). *M. melissophyllum* ssp. *melissophyllum* was characterized by high amount of the mushroom alcohol oct-1-en-3-ol and the phenolic coumarin with a characteristic sweet and creamy vanilla bean odor, playing a major role in the aroma of whole aerial parts (Maggi et al., 2011a).

Investigations of the potential protective action of different extracts of *M. melissophyllum* have shown that ether, chloroform, ethyl acetate and especially water extracts are good scavengers of free radicals *in vitro*. In addition, these extracts have hepatoprotective effects (Kaurinovic et al., 2011). Polyphenols exhibit many biological effects attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals. The chemical profile of the phenolic acid content of *M. melissophyllum* was identified (procatechic, chlorogenic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, *o*-coumaric and cinnamic acid) (Skrzypczak-Pietraszek and Pietraszek, 2012). The essential oil composition of *M. melissophyllum* was also investigated (Skaltsa-Diamantidis et al., 1991; Velasco-Negueruela et al., 2004; Baldini et al., 2009; Maggi et al., 2010; 2011a; 2011b).

According to available literature data, there is only one report dealing with the antioxidant activity of extracts of different polarity of *M. melissophyllum* from Serbia, applying a few radical scavenging and enzymatic assays (Kaurinovic et al. 2011). The aim of this study is to investigate the antioxidant capacity of various extracts of *M. melissophyllum* not previously analyzed using different methods (DPPH, ABTS and FRAP). Statistical analysis of the obtained data was

used to reveal the linkage/differences between analyzed samples and applied methods.

MATERIALS AND METHODS

Plant material

Aerial parts of the analyzed species were collected at the flowering stage in June 2011 (Jablanica, near Boljevac, Serbia). A voucher specimen has been deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac" (BEOU 16827), Faculty of Biology, University of Belgrade, Serbia.

Preparation of plant extracts

Methanol and ethanol (10%, 30%, 50%, 96%), ethyl acetate and chloroform (for FRAP assay) plant extracts were used in this experiments. Samples of air-dried and powdered aerial parts (10 g) of *M. melissophyllum* were extracted with a volume of 100 mL of different solvents. Mixtures were exposed to ultrasound for 60 min, left to stand in the dark for 24 h and filtered through Whatman No.1 filter paper. The solvents were removed by evaporation under reduced pressure at a maximum temperature of 40°C. The dried extracts were kept in the fridge at 4°C, protected against UV irradiation and dissolved in different solvents to a final concentration, just before conducting the assays.

Chemical reagents

Organic solvents (methanol, ethanol, HCl (concentrated hydrochloric acid), CH₃COOH (glacial acetic acid) were purchased from "Zorka Chemicals" (Šabac, Serbia). Gallic acid, 3-tert-butyl-4-hydroxyanisole (BHA) and 2,2-dyphenyl-1-picrylhydrazyl (DPPH), iron (III) chloride (FeCl₃×6H₂O), iron (II) sulfate heptahydrate (FeSO₄×7H₂O), sodium acetate (CH₃COONa×3H₂O), were obtained from Sigma Chemicals Co., St Louis, MO, USA. Folin-Ciocalteu phenol reagent was purchased from Merck, Darmstadt, Germany. Sodium carbonate anhydrous (Na₂CO₃), potassium acetate (C₂H₃KO₂), potassium peroxodisulphate (K₂O₈S₂) and L(+)-ascorbic acid

(Vitamin C) were purchased from AnalaR Normapur, VWR, Geldenaaksebaan, Leuven Belgium. Aluminum nitrate nonahydrate $[Al(NO_3)_3 \cdot 9H_2O]$, 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) was purchased from Fluka Chemie AG, Buchs, Switzerland. ABTS and quercetin hydrate were obtained from TCI Europe NV, Boerenveldsweg, Belgium. All chemicals were of analytical grade.

Determination of total phenolic content

The total phenolic content was determined according to the Singleton and Rossi method (1965) using gallic acid as standard. The dried plant extract was dissolved in the appropriate solvent to make a final concentration of 1 mg/mL. Extract solution was mixed with 1 mL 10% Folin-Ciocalteu's reagent; the mixture was kept for 6 min, followed by the addition of 0.8 mL of 7.5% Na_2CO_3 . After 120 min in the dark, the absorbance of the mixture was measured at 730 nm. The total phenolic content was expressed as mg gallic acid/g of dry extract (mg GA/g.d.e.)

Determination of flavonoid content

The determination of the total flavonoid content was carried out as described by Park et al. (1997). The reaction mixture contained the sample in a concentration of 1 mg/mL, $Al(NO_3)_3 \cdot 9H_2O$ and CH_3COOK was kept in the dark for 40 min. All the values are presented as means of triplicate analyses. The total flavonoid concentration in the different extracts was calculated from quercetin hydrate (Qu) calibration curve (10-100 mg/L) and expressed as quercetin equivalents (QuE)/g of dry extract.

Evaluation of DPPH scavenging activity

Antioxidant activity of methanol and ethanol extracts was evaluated by the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging method (Blois, 1958). Methanolic solutions (with starting concentrations of 50, 100, 150, 250, 500 μ g/mL of solution) of the investigated extracts (400 μ L) were added to 3.6 mL methanolic solution of DPPH radical (concentration of 0.04 mg/mL) and after shaking, the reaction mix-

ture was left to react in the dark for 30 min at room temperature.

The absorbance of the remaining DPPH radicals in the sample (A_1) was measured on 517 nm. Every sample and positive control (ascorbic acid-vitamin C and BHA) concentration were done in triplicate and the same was done with blank probes, which were prepared to contain methanol instead of the investigated sample (blank absorbance A_0). Percent of scavenging RSC (%) was calculated as follows:

$$RSC (\%) = (A_0 - A_1) \times 100 / A_0$$

From the obtained RSC (%) values, the IC_{50} value, which represents the concentration of extracts that caused 50% neutralization, was determined by linear regression analysis.

Evaluation of ABTS radical scavenging activity

The ABTS radical scavenging method by Rice-Evans and Miller (1994) and modified by Re et al. (1999) was used. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate and its reduction in the presence of hydrogen donating antioxidants is measured spectrophotometrically at 734 nm. The reaction mixture of 19.2 mg of ABTS was dissolved in potassium persulfate before the experiment. This solution was dissolved in distilled water in order to adjust the working solution absorbance to 0.7 at 734 nm. The sample concentration was 1 mg/mL. Reaction mixture was prepared by mixing 100 μ L of test sample and 4 mL ABTS. After 30 min incubation at 30°C in a water bath, the absorbance of the mixture was measured at 734 nm. The radical scavenging activity for each extracts was determined based on the linear calibration curve of ascorbic acid and was expressed as mg ascorbic acid/g of dry extract). All previously described assays were performed on a JENWAY 6305 UV VIS spectrophotometer.

Ferric reducing antioxidant power (FRAP) assay

The total antioxidant potential of methanol, 96% eth-

anol, ethyl acetate and chloroform extracts was determined using the ferric reducing ability of plasma FRAP assay by Benzie and Strain (1996) as a measure of antioxidant power. The assay is based on the reducing power of a compound (antioxidant). A potential antioxidant will reduce a ferric ion (Fe^{3+}) to a ferrous ion (Fe^{2+}); the latter forms a blue complex ($\text{Fe}^{2+}/\text{TPTZ}$), which increases absorption at 593 nm. Briefly, the FRAP reagent was prepared by mixing acetate buffer (200 μL , pH 3.6), a solution of 20 μL TPTZ and 20 μL FeCl_3 at 10:1:1 (v/v/v). The sample solution (200 μL) and the reagent (2.95 mL H_2O and 1 mL FRAP) were mixed thoroughly and incubated at 37°C. Absorbance was measured at 593 nm after 10 min using a Perkin Elmer type Lambda 15 spectrophotometer. A standard calibration curve was prepared using different concentrations of $\text{FeSO}_4 \times 7\text{H}_2\text{O}$. All solutions were freshly prepared. The results were expressed in $\mu\text{mol Fe}/\text{mg}$ dried extract.

Statistical analysis

Data obtained in the analysis of total phenols, total flavonoids and antioxidant characteristics were analyzed by means of multivariate analysis, employing a hierarchical cluster analysis and between-groups linkage. The distances between samples were calculated using Ward's method and square Euclidean distances. Standardization of the raw data was performed. The dendrogram similarity scales generated by the Statistica 7 program.

RESULTS AND DISCUSSION

Total phenolic content

The Folin-Ciocalteu procedure is a widely used method that provides a rapid evaluation of the phenolic content of plant extracts. Total phenolic content was determined for methanol and 10%, 30%, 50% and 96% ethanol extracts of the aerial parts of *M. melissophyllum*. The total phenolic contents of the examined plant extracts are presented in Table 1. Total phenols are expressed as gallic acid equivalents (GAE) per gram of dry extract. In this experiment, the highest phenolic content was found in the etha-

nol 96% extract (111.7 GAE/g) of *M. melissophyllum*, followed by 50% ethanol (99.1 GAE/g) and methanol extract (90.1 GAE/g). The lowest values of polyphenols were found in 10% and 30% ethanol extracts, respectively. All extracts contained a considerable amount of phenolic constituents. The antioxidant activity of the extracts of this species correlated well with the content of phenolic compounds. In a recent work (Skrzypczak-Pietraszek and Pietraszek, 2012), it was also reported that the antioxidant activity of extracts of *M. melissophyllum* using HPLC and HPLC/DAD was well correlated with the content of phenolic compounds.

Numerous reports about the antioxidant activity of Lamiaceae species and their phenolic content have been published. Polyphenolic compounds from different extracts from Lamiaceae species, such as *Salvia*, *Mentha*, *Sideritis* (Stagos et al., 2012), *Scutellaria baicalensis* (Li et al., 2008), *Melissa officinalis*, *Origanum vulgare*, *Mentha x piperita* (Capecka, 2005), *Thymus mastichina* (Barros et al., 2010), *Mentha* spp. (Nickavar et al., 2008), *Dracocephalum moldavica* (Dastmalchi et al., 2007), possess strong free radical scavenging activity. In addition, it was found that the antioxidant potential of *Thymus vulgaris* and *Origanum vulgare* extracts was a result of the presence of caffeic and *p*-coumaric acids (Wojdylo, 2007). Methanolic and aqueous extracts of *Salvia officinalis* were rich in phenolic acids and flavonoids, which are recognized as good antioxidants and hepatoprotective agents (Lima et al., 2007). The amount of polyphenols was also dependent on the extraction method. The samples for HPLC were subjected to enzymatic hydrolysis or extraction with methanol, which resulted in specific disruption of linkages or deglycosylation of phenolic compounds (Wojdylo et al., 2007).

Flavonoid concentration

The concentrations of flavonoids in different extracts of *M. melissophyllum* were determined spectrophotometrically with aluminum nitrate nonahydrate. The flavonoid content was expressed as quercetin hydrate equivalents. The total flavonoid contents from

Table 1. Total phenols and flavonoid content of *M. melissophyllum* extracts. Results are expressed as GAE/g and mg QE/g dry extract and averaged from three measurements (expressed as mean \pm SD).

Extracts	Total phenols (GAE/g)	Total flavonoids (QuE/g)
methanol	90.1 \pm 0.003	29.33 \pm 5.50
10% ethanol	63.5 \pm 0.004	7.33 \pm 0.57
30% ethanol	83.2 \pm 0.005	16.33 \pm 1.52
50% ethanol	99.1 \pm 0.001	25.00 \pm 1.00
96 % ethanol	111.7 \pm 0.004	56.00 \pm 0.00

(GAE/g) – Gallic acid equivalents/g

(QuE/g) – Quercetin equivalents/g

Table 2. Antioxidant activities of *M. melissophyllum* extracts determined by DPPH, ABTS and FRAP assays.

Extracts (1mg/mL)	IC ₅₀ DPPH (mg/mL)	ABTS (mg of ascorbic acid/g)	FRAP (μ molFe/mg)
methanol	0.116	0.66 \pm 0.00	0.3816
10% ethanol	0.664	0.45 \pm 0.00	-
30% ethanol	0.142	0.82 \pm 0.05	-
50% ethanol	0.109	0.89 \pm 0.17	-
96% ethanol	0.177	0.81 \pm 0.04	0.3829
ethyl acetate	-	-	0.1606
Chloroform (in MeOH)	-	-	0.3156

Table 3. Correlations between different assays (bolded correlations are significant at $p < 0.05000$), N=5.

	Total phenols (GAE/g)	Total flavonoids (QuE/g)	IC ₅₀ DPPH (mg/mL)	ABTS (mg ascorbic acid/g)	FRAP (μ mol Fe/mg)
Total phenols (GAE/g)	1.00	0.91	-0.77	0.79	0.58
Total flavonoids (QuE/g)		1.00	-0.52	0.49	0.79
IC ₅₀ DPPH (mg/mL)			1.00	-0.87	-0.37
ABTS (mg ascorbic acid/g)				1.00	0.05
FRAP (μ molFe/mg)					1.00

the examined plant extracts are presented in Table 1. The concentrations of flavonoids in plant extracts ranged from 7.33 to 56.00 mg QuE/g (mg of Qu per gram of dry extract). The highest flavonoid content was identified in the 96% ethanol extract and the lowest was in the ethanol 10% solution. Increasing the concentration of ethanol extracts (10, 30, 50%) leads to an enrichment in the content of flavonoids (29.00, 54.87, 112.33 GAE/g). The flavonoid content

depends on the used extraction solvents, as was recently reported by Kaurinovic et al. (2011). The water extract of *M. melissophyllum* leaf contained the largest amount of total flavonoids, while the lowest content was detected in the butanol extracts. A literature review showed the general dependence of flavonoid content and total antioxidant capacity in some Lamiaceae species (Nickavar et al., 2007; Shariffar et al., 2009). Flavonoids with the presence of a hydroxyl

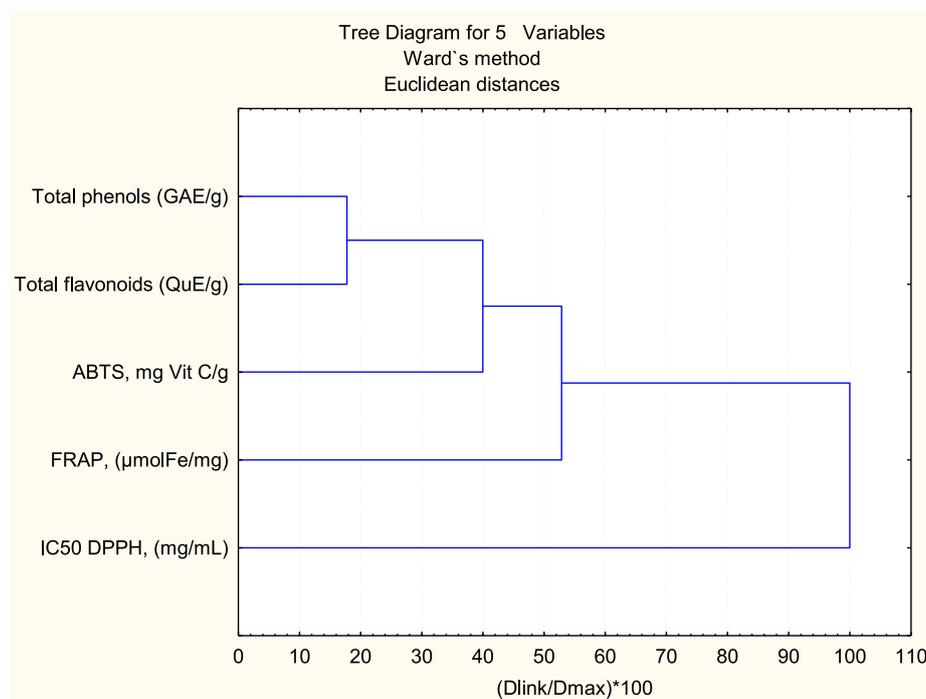


Fig. 1. Dendrogram of applied assays for antioxidant activity evaluation.

group in the molecule can act as proton donors and show good radical scavenging activity (Mensor et al., 2001; Li et al., 2008).

DPPH scavenging activity

The ability of the investigated extracts of *M. melissophyllum* to act as free-radical scavengers or hydrogen donors in the transformation of DPPH into its reduced form DPPH-H, was investigated. The free-radical scavenging capacity of the tested extracts was measured by DPPH assay and results are shown in Table 2.

All of the extracts were able to reduce the stable, purple-colored radical DPPH into the yellow-colored DPPH-H, reaching 50% of reduction with IC_{50} , as follows: 0.109 (mg/ml) for 50% ethanol, 0.116 (mg/ml) for methanol, 0.142 (mg/ml) for 30% ethanol, 0.177 (mg/ml) for 96% ethanol and 0.664 (mg/ml) for 10% ethanol solution. IC_{50} values of the synthetic antioxidants were 0.093 mg/ml and 0.054 mg/ml for

BHA and ascorbic acid, respectively. The 50% ethanol and the methanol extracts of *M. melissophyllum* exhibited the strongest inhibitory effects, as the IC_{50} was achieved with the lowest concentration. The lowest antioxidant potential was exhibited by the 10% ethanol extract. Comparison of the IC_{50} of the investigated extracts and BHA showed that all, except the 10% ethanol extract, showed similar antioxidant effects.

Numerous papers have documented the DPPH assay for antioxidant capacity determination when applied to Lamiaceae species, such as *Leonurus cardiaca*, *Lamium album*, *Marrubium vulgare*, *Stachys officinalis*, *Lamium purpureum*, *Galeopsis speciosa* (Mantle et al., 2000; Vander Jagt et al., 2002; Troullias et al., 2003; Matkowski and Piotrowska, 2006).

ABTS scavenging activity

The free-radical scavenging capacity of the tested extracts was measured by ABTS test and are shown

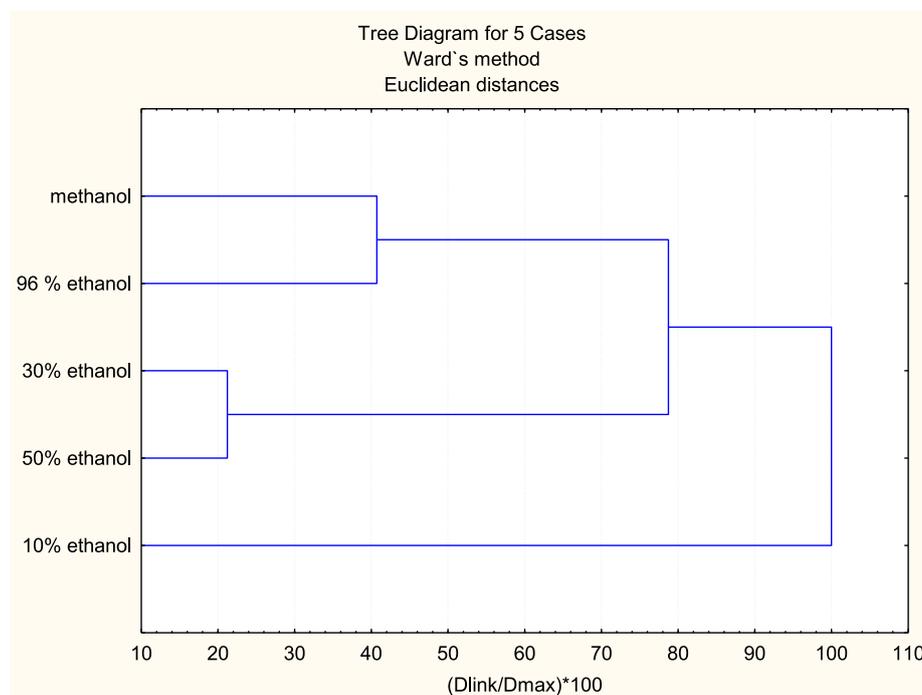


Fig. 2. Tree diagram of the extracts obtained by different solvents.

in Table 2. The values of ABTS antiradical activity ranged from 0.45 to 0.89 mg ascorbic acid/g. The highest activity was registered in 50% ethanol and the lowest in 10% ethanol extract. The 30% and 96% ethanol extracts also showed good activity, while the methanol extract gave an intermediate value. Stagos et al. (2012) determined the antioxidant capacity for methanol, ethanol and water extracts using the ABTS^{•+} scavenging assay and their results showed IC₅₀ values in the following order: *Salvia* > *Mentha* > *Sideritis*.

Ferric reducing antioxidant power (FRAP) assay

The results of total antioxidant capacity of the investigated extracts, measured by the FRAP method, are shown in Table 2, and were within the range 0.16-0.382 mmol Fe/mg. The reducing powers of 96% ethanol and methanol extract were almost equal (0.3816 and 0.3829 mmol Fe/mg). The lowest value was that of the ethyl-acetate extract (0.1606 mmol Fe/mg). The chloroform extract was dissolved in methanol

and the reducing capacity subsequently measured, and it was found to be 0.3156 mmol Fe/mg.

Recently, Hossain et al. (2010) published a paper dealing with the antioxidant capacity of six Lamiaceae herbs (rosemary, oregano, marjoram, sage, basil and thyme) using FRAP and ORAC methods. They found that drying, freeze-dried and vacuum oven-dried extracts as well as storage period, could affect the total phenol content and consequently antioxidant capacity.

Few statistical methods are used to classify the obtained data. Multivariate statistical methods are appropriate tools for the analysis of a complex data matrix, so the hierarchical cluster analysis (HCA) used in the present study represents a good choice for grouping the sets of available data by their similarities according to a set of selected variables. Statistica 7 offers all the tools for obtaining similarity dendrograms, including several distance options,

cluster methods and the means of standardization of the original data.

The data obtained applying different assays were statistically processed and correlations between them are presented in Table 3. The highest correlation was observed between total phenol and flavonoid contents ($R=0.91$), having in mind that flavonoids can be considered as members of the phenol group. Statistically significant correlations were registered for DPPH and ABTS assays ($R=0.87$), which was expected due to their similar mechanism of action as radical scavengers; for total phenols and ABTS ($R=0.79$) and total flavonoids and the FRAP assay ($R=0.79$). Hierarchical cluster analysis (HCA) was performed on the total phenols and total flavonoids content DPPH, ABTS, and FRAP assay. The dendrogram in Fig. 1 shows that total phenol and total flavonoid contents are quite homogeneous, while DPPH is in a separate cluster.

Clustering according to the type of extracts is shown in Fig 2. Based on the Euclidian distances, three cluster groups were noticed: cluster 1 (methanol and 96% ethanol extract); cluster 2 (30% and 50% ethanol extracts); cluster 3 (10% ethanol extract). These observations are in accordance with earlier ones.

CONCLUSION

The findings of the study revealed that extracts from wild-growing *M. melissophyllum* possess valuable antioxidant potential. Methanol and 50% ethanol extracts have proven to be good scavengers, estimated by both antiradical methods, DPPH and ABTS. The 96% ethanol and methanol extracts had a very high total reducing power, while the ethyl acetate extract had a lower reducing power potential. The application of statistical tools to the obtained data confirmed the high correlation between total phenolic and flavonoid contents with antioxidant activity, which is to be expected since flavonoids are significant and abundant constituents of a large and important group of secondary metabolites in plants - phenolic compounds.

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