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IN VITRO ANTIOXIDANT ACTIVITY OF HONEYDEW AND MULTIFLORAL TYPES OF HONEY FROM SERBIA

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Antioxidant properties of fifteen multifloral and honeydew types of honey from Serbia were assessed by determination of ferric-reducing antioxidant power (FRAP assay) and 2,2-diphenyl-1-picrylhydrazyl free radical scavenging ability (DPPH assay), with respect to their total polyphenol content and colour intensity. The results of this study showed that total polyphenol content, antioxidant activity and colour intensity varied widely among different samples, even within the same type of honey. All investigated parameters were strongly dependent on geographic origin of samples, further emphasizing the importance of their detailed chemical characterisation. In general, polyphenolic content in investigated samples of honey, expressed as catechin equivalents, ranged from 480.2 to 1861.1 mg/kg. The ferric-reducing antioxidant power of honey tended to be lower in brighter and higher in darker samples, varying between 489.6 and 3089.8 μmol Fe(II) per 100 g of honey. The correlation between the colour and antioxidant activity never reached statistical significance in the case of honeydew type of honey, which was opposed to a trend observed in the case of multifloral honey.

KEY WORDS: honeydew honey, multifloral honey, antioxidant activity, polyphenol content, colour intensity

INTRODUCTION

Antioxidant activity is one of the most important physiological properties of foods. Regular use of nutrients with high antioxidant potential in everyday diet is supposed to have a major role in the prevention of a number of health disorders, whose etiology is closely related to the formation of reactive oxygen and nitrogen species during aerobic metabolism. Those nutrients are edible plants and plant-derived products, such as certain beverages and drinks, as well as dark chocolate, malt and honey (1-3).

Honey has been an element of traditional medicine for its dietary and healing properties since ancient times (4). However, more rigorous scientific investigations on chemical and biological properties of honey, including assessments of antibacterial, bacteriostatic, anti-inflammatory, wound and sunburn healing activities, were accomplished in the early 1970s. Recent views propose honey not only as a health-promoting nutrient,

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but also shed some light on its antioxidant properties. This makes honey more than just a nourishment of high energetic value, but a significant dietary source of antioxidants (5, 6).

It is important to note that the concentrations of antioxidants in honey tend to be lower than in many traditional sources of dietary antioxidants; therefore, honey itself may not be considered as their major source. Nonetheless, the pleasing taste of honey makes it readily consumed by individuals who may have very little antioxidant-containing fruits and vegetables, thus balancing their deficiency in regular diet (7-9). Since dietary antioxidants provide health benefits, floral source of honey is identified to be a very important factor in evaluating its potentials as an antioxidant-containing food supplement. This fact is largely recognised by nutritionists, as a growing number of studies that were focused on chemical profiles of antioxidants in honey from various floral sources can be found among the available literature data (5-14).

Serbia is a country with a long beekeeping tradition and favourable agro-ecological conditions (15). In vascular flora of Serbia, 3662 taxa, i.e. 3272 species and 390 subspecies, have been described, which makes Serbia one of the countries with the greatest floristic diversity and density in Europe, and, with this in regard, one of the countries with great potentials for further development of apicultural production (15,16). However, a dearth of information concerning the functional properties of different types of Serbian honey still exists. Therefore, the focus of this study was placed on a systematic survey on the relative levels of antioxidant activity in samples of multifloral and honeydew honeys produced in different sites in Serbia, with respect to their total polyphenol content and colour intensity.

EXPERIMENTAL

Chemicals and instruments

All reagents and solvents used in this investigation were of analytical grade. Folin-Ciocalteu reagent, 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ) and 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) were purchased from Sigma Aldrich GmbH (Steinheim, Germany); absolute ethanol, fructose and glucose from Merck (Darmstadt, Germany); maltose from Carlo Erba (Milan, Italy); (±)-catechin from Fluka AG (Buchs, Switzerland); ferric chloride hexahydrate, ammonium acetate and methanol from LaChema (Neratovice, Czech Republic); acetic acid, hydrochloric acid, sodium acetate trihydrate and ferrous sulfate heptahydrate from Zorka Pharma (Šabac, Serbia). All spectrophotometric measurements were performed on an Evolution 300 UV-VIS spectrophotometer (Thermo Scientific, Madison, WI, USA). Distilled, deionized water was obtained from a Simplicity 185 purification system (Millipore S.A., Molsheim, France).

Honey samples

For this study, fifteen honey samples of both types (Table 1) were collected directly from primary producers (beekeepers), after the 2012 harvest from different sites in Serbia, and stored refrigerated (at 4^{o}C) until processed. Prior to being analyzed, honey

samples were dissolved in warm distilled water (not exceeding 40°C) and sonicated for 5 min until a clear solution was obtained (test-solutions; 100 mg/ml). A sugar analogue (an artificial honey whose composition reflects the approximate sugar composition), consisting of 40% fructose, 30% glucose, 8% maltose, 2% sucrose and 20% water, was prepared to test and restrict the possible interference of the principal sugar components of investigated honey samples in all the proposed assays, and appropriately diluted, under the same conditions (6).

Total polyphenol content

Total polyphenol content (PC) was determined by the Folin-Ciocalteu method, as proposed by Beretta et al. (6). PC was calculated as catechin equivalents (mg/kg of honey), from the calibration curve of catechin standard solutions, covering the concentration range between 10 and 250 μg/ml.

Total antioxidant activity: FRAP assay

Total antioxidant activity was assessed by FRAP assay, as described by Beretta et al. (6). In brief, the test-solutions (100 μl) were transferred into the test tubes and 3.0 ml of freshly prepared FRAP-reagent (25 ml acetate buffer, 300 mmol/l, pH 3.6 + 2.5 ml 10 mmol/l TPTZ in 40 mmol/l HCl + 2.5 ml 20 mmol/l $FeCl_3 \cdot 6H_2O$) were added. The absorbances were recorded at 593 nm against blank, containing 100 μl of sugar analogue solution, after 30 min incubation at 37°C. FRAP values were determined against a calibration curve of $FeSO_4 \cdot 7H_2O$ solutions that covered a concentration range between 200 and 1000 μmol/l, and expressed as μmol Fe(II) per 100 g of honey.

Table 1. Investigated material: sites of production and type designation

Sample	Site of production	Type of honey
MF-A	Koceljeva, western Serbia	Multifloral
MF-B	Valjevo, western Serbia	Multifloral
MF-C	Knjaževac, eastern Serbia	Multifloral
MF-D	Kraljevo, southwestern - central Serbia	Multifloral
MF-E	Mt. Radan, southern Serbia	Multifloral
MF-F	Mt. Goč, southwestern - central Serbia	Multifloral
MF-G	Bukovik, Uvac gorge, western Serbia	Multifloral
MF-H	Donje Gorašće, southwestern Serbia	Multifloral
MF-I	Mt. Zlatar, western Serbia	Multifloral
MF-J	Mt. Zlatar, western Serbia	Multifloral
HD-A	Kraljevo, southwestern - central Serbia	Honeydew; oak
HD-B	Prokuplje, southern Serbia	Honeydew; oak + plum
HD-C	Bujanovac, southern Serbia	Honeydew; oak
HD-D	Kupinovo, Vojvodina	Honeydew; oak + willow + white poplar
HD-E	Vranje, southern Serbia	Honeydew; oak

Free radical scavenging activity: DPPH assay

The scavenging activity against DPPH radical was evaluated according to the method described by Beretta et al. (2005), with slight modifications. The assay mixture contained 1.9 ml of 130 μmol/l DPPH (final concentration: 83.3 μmol/l) dissolved in absolute ethanol, 1ml of acetate buffer solution (100 mmol/l, pH 5.5) and 100 μl of each test-solutions, including the one containing sugar analogue; the final volume was 3 ml (final concentration of honey: 33.3 mg/ml per sample). The mixture was shaken vigorously, and then incubated 90 min at 25^{o}C in a water bath in the dark. The absorbance was recorded at 517 nm against a blank (test-solution, containing all reagents except DPPH, in order to restrain the possible interference of honey colour). The percent inhibition was calculated against the control solution, containing absolute ethanol instead of test solutions or sugar analogue. Free radical scavenging activity (FRS, %) was calculated against the control solution, containing absolute ethanol instead of test solutions or sugar analogue, following the expression FRS (%) = $[(A_0 - A)/A_0] \times 100$, where A_0 and A were the absorbances of DPPH without or with the addition of test-solutions, respectively. The IC_{50} values, which denote the concentration of sample required to scavenge 50% DPPH free radicals, were calculated for active honey samples (at the given concentrations, those that expressed at least 50% inhibition) by probit analysis, from the calibration equation of transformed data (17).

Colour intensity

Honey samples were diluted to 50% (w/v) with warm water, sonicated for 5 min and filtered to eliminate large particles. The net absorbance (colour intensity - CI) was defined as the difference between the absorbances at 450 and 720 nm and expressed as mAU (6).

Statistical analysis

All analyses were carried out in triplicate and the data were expressed as means ± standard deviations (SD). Calibration curves were constructed by regression analysis of absorbances of catechin and $FeSO_4 \cdot 7H_2O$ standard solutions against their concentrations. The analysis of variance according to two-tailed Student's t-test was done to compare the PC, FRAP, DPPH and CI values of the different honey samples. Their mutual relations were tested by correlation analysis and expressed as Pearson's correlation coefficients. The differences between means at the 95% ($p < 0.05$) confidence level were considered statistically significant.

RESULTS AND DISCUSSION

The total polyphenol content (mg catechin/kg) of different honeys was investigated using the modified Folin-Ciocalteu assay, which is responsive both to phenol and polyphenol entities, as well as the other electron-donating antioxidants such as ascorbic acid, vitamin E etc. As shown in Table 2, the PC was particularly low in pale samples of both

types of honey, rising further in darker ones, some of which approached as much as 0.2% (1861.1 ± 17.3 mg catechin/kg). Although anticipated, the difference between average PCs in honeydew and multifloral honey was statistically insignificant at $p < 0.05$.

The assessment of the antioxidant activity in the investigated types of honey has been focused on direct measuring of antioxidants as reductants by the FRAP assay, which depends on the ability of analyte to reduce ferric 2,4,6-tris (2-pyridyl)-s-triazine [Fe(III)-TPTZ] to the ferrous 2,4,6-tris (2-pyridyl)-s-triazine [Fe(II)-TPTZ] complex at low pH. This complex has an intense blue colour that can be monitored at 593 nm.

Table 2. Total polyphenol content, antioxidant activity and colour intensity of investigated samples of honey

Sample	Total polyphenols[a]	FRAP-values[b]	FRS-activity[c]	IC_{50}[d]	Colour intensity[e]
Multifloral honey					
MF-A	1474.0±9.3	1386.9±2.7	57.7±1.5	3034.0	1627
MF-B	811.0±3.2	448.7±6.0	12.8±0.1	-	403
MF-C	858.6±11.4	243.3±1.5	10.2±0.1	-	555
MF-D	1090.0±6.0	326.9±2.0	9.7±0.3	-	250
MF-E	1714.8±14.2	1725.9±5.3	52.0±0.7	4475.0	1250
MF-F	621.0±1.7	1000.4±12.1	24.8±0.1	-	403
MF-G	1093.8±3.4	693.3±1.5	23.9±0.2	-	562
MF-H	1110.0±5.3	727.6±1.8	26.4±0.3	-	498
MF-I	1284.3±2.1	1139.8±2.2	25.2±0.3	-	555
MF-J	1074.3±6.9	1389.4±2.5	29.9±0.2	-	680
Honeydew honey					
HD-A	480.2±11.4	690.2±9.8	19.8±4.2	-	337
HD-B	1350.0±22.8	2360.2±66.5	61.2±3.5	3050.0	702
HD-C	1861.1±17.3	3089.8±67.8	90.1±2.0	1885.0	154
HD-D	520.9±16.4	489.6±28.7	10.8±2.0	-	67
HD-E	864.8±72.2	1304.0±79.1	21.9±1.6	-	25
Sugar analogue					
-	0.0	0.0	0.0	-	-

[a] Expressed as mg of catechin per 1 kg of honey. [b] Expressed as μmol Fe(II) per 100 g of honey. [c] Expressed as %. Final concentration of test-samples: 33.3 mg of honey per ml of sample. [d] Expressed as μg of honey per ml of sample. [e] Expressed as mAU.

As expected, the FRAP values of investigated types of honey have been found to vary in a wide range, between 243.3 ± 1.5 μmolFe(II)/100g (sample MF-C; multifloral honey from eastern Serbia) and 3089.8 ± 67.8 μmolFe(II)/100g (sample HD-M; honeydew honey from southern Serbia). Therefore, certain samples expressed a significant ability to reduce Fe(III) and, thus, ability to donate electrons (Table 2). This property suggested that their constituents may act as free radical scavengers, capable to transform reactive

free radical species into stable non-radical products. Compared to the samples of multifloral honey, the honeydew samples expressed slightly, but not significantly higher activity.

In the second assay system, the antioxidant activity has been evaluated by the ability of investigated types of honey to scavenge stable DPPH free radicals. Due to its unpaired electron, DPPH radical gives a strong absorption band at 517 nm. As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting discolouration is stoichiometric with respect to the number of electrons taken up (18). As shown by Table 2, several samples of both multifloral and honeydew honeys expressed a significant ability for scavenging free radicals and, thus, ability to prevent the initiation of free radical-mediated chain reactions by stabilizing reactive species before they can participate in deleterious reactions (e.g., the abstraction of hydrogen from susceptible polyunsaturated fatty acids).

It is believed that colour intensity is a reliable index of the presence of the pigments with antioxidant activity, such as carotenoids or Maillard reaction products, for instance (19). However, the colour could be due to specific pigments arising from handling, processing, and storage, and/or from biochemical reactions during honey maturation, which could lead to coloured components with no antioxidant activity. The absorbance of a 50% (w/v) honey solution varied from 250 mAU for the pale-yellow multifloral honey (sample MF-D; southwestern Serbia) to 1250 mAU for the dark-brown multifloral honey (sample MF-E; southern Serbia).

The correlation matrix (Table 3) shows, at $p = 0.05$, a high level of correlation between all the investigated parameters in the case of honeydew honey (correlation coefficients: $r = 0.911 - 0.996$; determination coefficients: $r^2 = 83 - 99\%$), except for the colour intensity parameter. The observed low correlation between the colour intensity, FRAP values, DPPH free radical scavenging activity and PCs (correlation coefficients: $r = 0.188 - 0.432$; determination coefficients: $r^2 = 4 - 19\%$) suggests that the honeydew honey colour is not a sound indicator of either polyphenol content (the colour is not a consequence of the presence of flavonoids and the other phenolic pigments alone), or the antioxidant activity. In other terms, the antioxidant activity of honeydew honey could not be estimated on the basis of the colour intensity alone, which is opposed to a trend observed in the case of multifloral honey (Table 3).

The high statistical significance of the correlation between the total antioxidant activity and PC ($r_{FRAP,PC} = 0.993$; $r^2_{FRAP,PC} = 98.6\%$) suggests that the reduction ability of honeydew honey samples depends predominantly on polyphenols. The correlations FRAP/DPPH ($r_{FRAP,DPPH} = 0.980$; $r^2_{FRAP,DPPH} = 96\%$) and DPPH/PC ($r_{DPPH,PC} = 0.977$; $r^2_{DPPH,PC} = 95.5\%$) further underline the contribution of polyphenol constituents to the total antioxidant activity of honeydew honey samples. Similar observation is valid for the samples of multifloral honey used in this investigation too, which fits well to the present knowledge about the features critical to the expression of antioxidant activity and DPPH free radical-scavenging ability in different types of honey (5-14,19,20).

Table 3. Correlation between total polyphenol content, antioxidant activity and colour intensity of investigated samples of honey (Pearson's correlation coefficients)

	Total polyphenols	FRAP-values	FRS-activity	Colour intensity
Multifloral honey				
PC	-	0.669	0.765	0.755
FRAP	0.669	-	0.879	0.719
FRS	0.765	0.879	-	0.931
CI	0.755	0.719	0.931	-
Honeydew honey				
PC	-	0.993	0.978	0.228
FRAP	0.993	-	0.980	0.319
FRS	0.978	0.980	-	0.333
CI	0.228	0.319	0.333	-

CONCLUSION

The aim of this study was to survey systematically the levels of antioxidant activity in samples of multifloral and honeydew honeys produced in different sites in Serbia, with respect to their total polyphenol content and colour intensity. The results suggest that there is a high level of correlation between polyphenol content, antioxidant activity and colour intensity for all the studied samples of multifloral and/or honeydew honey. However, the investigated parameters were predominantly dependent on the geographic origin of samples, implying a strong influence of floral composition and thus emphasizing the importance of their detailed chemical characterisation. The colour of multifloral honey is found to be a relatively reliable indicator of both the polyphenol content and the antioxidant activity, as opposed to the case of honeydew honey, where the correlation between the colour and the antioxidant activity never reached statistical significance.

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IN VITRO АНТИОКСИДАНТНА АКТИВНОСТ ШУМСКОГ И ЛИВАДСКОГ МЕДА ИЗ СРБИЈЕ

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У овом раду, испитивана су антиоксидантна својства петнаест узорака ливадског и шумског меда, применом Ferric-Reducing/Antioxidant Power и DPPH-free radical scavenging ability теста, у односу на садржај укупних полифенолних састојака и интензитет боје меда. Резултати овог испитивања су показали да су садржај укупних полифенола, антиоксидантна активност и интензитет боје изразито варирали од једног узорка до другог, чак и у оквиру сваке врсте меда понаособ. Сви испитивани параметри су били врло зависни од географског порекла узорака, наглашавајући важност детаљне хемијске карактеризације меда. Генерално, садржај укупних полифенола у испитиваним узорцима меда, изражен у еквивалентима катехина, кретао се у интервалу од 480,2 mg/kg до 1861,1 mg/kg. Способност редукције гвожђа била је нижа у светлијим врстама меда, а виша у тамнијим, варирајући између 489,6 и 3089,8 μmol Fe(II) на 100 g меда. Корелација између интензитета боје и антиоксидантне активности није имала статистичку значајност код узорака шумског меда, насупрот тренду који је уочен у случају ливадског меда.

Кључне речи: ливадски мед, шумски мед, антиоксидантна активност, садржај полифенола, интензитет боје

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