ANTIOXIDANT ACTIVITY OF THREE DIFFERENT SERBIAN FLORAL HONEYS

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In this study, three Serbian honey samples (Acacia, Linden and "Homoljski med") were analyzed to determine their total phenolic, flavonoid and antioxidant content, as well as their in vitro antioxidant activity. The antioxidant activity of honeys was examined by different tests, including the reducing power and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. In addition, correlations between the antioxidant activity and total phenolic, as well as flavonoid content were also sought. The highest content of total phenolics (27.44 mg/100 g), flavonoids (9.78 mg/100 g), reducing power and DPPH free radical scavenging activity were obtained in the case of Linden honey. The EC50 values of the Linden honey, determined based on the reducing power and DPPH radical scavenging activity, were 24.17 mg/ml and 51.34 mg/ml, respectively. Also, the antioxidant content was highest for Linden honey, and it valued 5.45 mg QEAC/100 g (expressed as mg of quercetin equivalent antioxidant content - QEAC per 100 g of honey) and 7.82 mg AEAC/100 g (expressed as mg of ascorbic acid equivalent antioxidant content - AEAC per 100 g of honey). Also, a linear correlation was observed between the antioxidant activity and total phenolics, as well as total flavonoids.

KEY WORDS: Serbian floral honeys, phenolics, flavonoids, antioxidant activity

INTRODUCTION

Honey, a nectar collected from many plants and processed by honey bees (Apis mellifera), is one of the oldest and widely used food product. The composition of honey is variable, owing to the differences in plant types, climate, environmental conditions, and contribution of the beekeeper (1-3). Honey has been reported to contain about 200 substances (complex mixture of sugars, but also small amounts of other constituents such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemicals), and is considered to be an important part of traditional medicine (4, 5). Research indicates that honey has functional properties in human health promotion.

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which depend largely on the floral source of the honey. These properties could be associated to honey high osmolarity, antibacterial properties (6) and antioxidant capacity (7-9). Overall, honey serves as a source of natural antioxidants (5, 10, 11).

The antioxidants present in honey include both enzymatic: catalase, glucose oxidase, peroxidase and non-enzymatic substances: ascorbic acid, α-tocopherol, carotenoids, amino acids, proteins, organic acids, Maillard reaction products, and more than 150 polyphenolic compounds, including flavonoids, flavonols, phenolic acids, catechins, and cinnamic acid derivatives (5). The identification and quantification of antioxidant components, as well as their antioxidant activity of honey bee products have been reported in several studies (5, 12-14). Many studies indicated that the antioxidant activity of honey varies widely, depending on the floral source. The botanical origin of honey has the greatest influence on its antioxidant activity, while processing, handling and storage affect honey antioxidant activity only to a minor degree (8, 10, 14, 15). For these reasons the purpose of the present study was to determine the total phenolic and flavonoid contents, their antioxidant activity by different tests, including the reducing power and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay of three Serbian honey samples (Acacia honey, Linden honey and "Homoljski med"). Besides, a correlation between the antioxidant activity and total phenolic/flavonoid as well as antioxidant contents was also sought.

EXPERIMENTAL

Chemicals and instruments

The chemicals used for these investigations were Folin-Ciocalteu reagent (Fluka Chemical Co., Buchs, Switzerland), trichloroacetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, quercetin, rutin and gallic acid (Sigma Chemical Co., St. Louis, MO, USA). All other chemicals and reagents were of the highest analytical grade, obtained from Zorka (Šabac, Serbia).

The total phenolic, flavonoid, and antioxidant contents as well as antioxidant activity were determined using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

Honey samples

Three different types of Serbian honey were used for the experiment. The two honey samples were monofloral form of Acacia (Rudnik region) and Linden (Fruška Gora region) (obtained during 2009 from honey bee farm, Simonović, Beograd), and the third sample was the "Homoljski med" (protected geographical origin) multifloral honey form of Acacia (50%) and Meadow (50%) honey (obtained during 2009 from honey bee farm, Homoljmed, Žagubica).
Total phenolic content

The total phenolics were determined spectrophotometrically by the Folin-Ciocalteu method (16). The content of total phenolics was expressed as mg of gallic acid equivalents per 100 g of honey (mg GAE/100 g).

Total flavonoid content

Total flavonoids were measured by the aluminum chloride spectrophotometric assay (17). Total flavonoid content was expressed as mg of rutin equivalents per 100 g of honey (mg RE/100 g).

Reducing power

The reducing power (RP) of honey samples was determined by the method of Oyaizu (18). For this purpose, solution of honey (10-120 mg) in 1 ml of distilled water or 1 ml of distilled water (blank) was mixed with 1 ml of phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide K₃[Fe(CN)₆]. The mixture was incubated at 50 °C for 20 min and then rapidly cooled. Following this, 1 ml of trichloroacetic acid (10%) was added and the mixture was then centrifuged at 3000 rpm for 10 min. An aliquot (2 ml) of the upper layer, mixed with 2 ml of distilled water and 0.4 ml of 0.1% FeCl₃ was left to stand for 10 min. The absorbance of the mixture was measured at 700 nm against the blank.

The effective concentration (EC₅₀), assigned at 0.5 value of absorption, was used to define specific reduction capability. Quercetin (10-120 μg/ml) and ascorbic acid (10-120 μg/ml) were used as positive controls.

Radical scavenging activity and antioxidant content

The scavenging activity (SA) of honey samples for the DPPH radical was measured spectrophotometrically using the modified DPPH method (19). Honey samples were dissolved in methanol, and 1.5 ml of each sample or 1.5 ml of methanol (blank) was mixed with 3 ml of DPPH in methanol (0.02 mg/ml). The range of the investigated honey concentrations was 0.33-166.67 mg/ml. The mixtures were left for 15 min at room temperature and then the absorbances was measured at 517 nm against reference mixtures that had been prepared in a similar manner, by replacing the DPPH solution with methanol. The capability to scavenge the DPPH radicals, DPPH scavenging activity (SA) was calculated using the following equation:

\[ \text{SA (\%) = } 100 \times \frac{(A_0 - A_1)}{A_0} \]

where A₀ is the absorbance of the blank and A₁ is the absorbance of the sample.

The effective concentration (EC₅₀), defined as the concentration of honey required for 50% scavenging of DPPH radicals under experimental condition employed, was used to measure the free radical scavenging activity. Quercetin (0.33-16.67 μg/ml) and ascorbic acid (0.33-16.67 μg/ml) were used as positive controls.
The antioxidant content was evaluated as described by Meda et al. (19). Honey samples were dissolved in methanol (50 mg/ml) and 1.5 ml of each solution was mixed with 3 ml of a 0.02 mg/ml solution of DPPH in methanol. The blank for each sample consisted of 3 ml of a methanolic honey solution (50 mg/ml) with 6 ml of methanol. The mixtures were left for 15 min at room temperature and the absorbances were measured at 517 nm. The antioxidant content expressed as mg of quercetin equivalent antioxidant content (QEAC) per 100 g of sample and as mg of ascorbic acid equivalent antioxidant content (AEAC) per 100 g of honey was determined using standard calibration curves for ascorbic acid (0-1.67 μg/ml) and quercetin (0-1.67 μg/ml).

**Statistical analysis**

All measurements were carried out in triplicate, and presented as mean ± SD. Correlation and linear regression analyses were performed using Microsoft Office Excel 2003.

**RESULTS AND DISCUSSION**

The total phenolic contents in honeys were determined from the regression equation of gallic acid calibration curve, and expressed as mg of gallic acid equivalents per 100 g of honey. Similarly, total flavonoids in honeys were determined from the regression equation of rutin calibration curve, and expressed as mg of rutin equivalents per 100 g of honey. The contents of total phenolics and flavonoids are shown in Table 1. The highest content of total phenolics and flavonoids was in *Linden* honey.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Phenolics (mg GAE/100 g)</th>
<th>Flavonoids (mg RE/100 g)</th>
</tr>
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<tbody>
<tr>
<td><em>Acacia</em> honey</td>
<td>16.18 ± 0.65</td>
<td>2.65 ± 0.10</td>
</tr>
<tr>
<td><em>Linden</em> honey</td>
<td>27.44 ± 1.05</td>
<td>9.78 ± 0.38</td>
</tr>
<tr>
<td>&quot;Homoljski med&quot;</td>
<td>19.78 ± 0.78</td>
<td>2.00 ± 0.07</td>
</tr>
</tbody>
</table>

Our results of phenolic content in examined *Linden* honey was three times smaller than in *Linden* honey (~82 mg GAE/100 g) obtained from the Tongrentang (TRT) Pharmaceutical Cooperation (20). But, the total flavonoid content in *Linden* honey determined in our study was significantly higher than for the same type of monofloral honey reported by Chang et al. (~1.3 mg RE/100 g) (20).

The content of total phenolics in examined *Acacia* honey was considerably higher than in the *Acacia* honey (4.6 mg GAE/100 g) obtained from Langnese Honig KG, Bargteheide, Germany (21). In the study of Bertoncelj et al. (22) average result of total phenolics for ten samples of Slovenian *Acacia* honey was $4.48 \pm 1.48$ mg/100 g. The content of total flavonoids in the investigated *Acacia* honey was $2.65 \pm 0.10$ mg of RE/100 g. The *Acacia* honey from Burkina Faso had a total flavonoid content, expressed
as quercetin equivalents (QE), of 6.14 mg QE/100 g, which is considerably higher than that of French honeys (less than 1 mg QE/100 g) (19, 23).

The reducing power of the honey samples was measured by the method of Oyaizu (18). In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of the antioxidant substances in honeys. The reducing power may serve as a significant indicator of its potential antioxidant activity. At different concentrations (10-120 mg/ml), the honey samples showed reducing ability (Figure 1). The reducing power of honeys increased with increasing concentration.

![Figure 1. Reducing power of different concentrations of honeys](image)

The EC$_{50}$ values of reducing power of honey samples are shown in Table 2. Reducing power of honeys and controls (quercetin and ascorbic acid), based on the EC$_{50}$ values, exhibited the following order ascorbic acid > quercetin > *Linden* honey > *Acacia* honey > "Homoljski med". The results for reducing power demonstrate the electron donor properties of honey, thereby neutralizing free radicals by forming stable products. The outcome of the reducing reaction is the termination of the radical chain reactions that may otherwise be very damaging. Among the investigated honeys, the highest reducing power showed *Linden* honey.

The EC$_{50}$ values of reducing power in our study were higher than the EC$_{50}$ values of reducing power of three Portuguese honeys, which varied from 13.26 ± 0.20 to 48.95 ± 1.61 mg/ml (5). The presence of sugars in the honey samples which have a good reducing capacity (e.g. glucose and fructose), can also contribute to the reducing power of honey samples as well as phenolics (5).
Table 2. EC$_{50}$ (mg/ml) values of honeys and controls (quercetin and ascorbic acid) in the antioxidant activity evaluation assays: reducing power (RP) and scavenging activity (SA)

<table>
<thead>
<tr>
<th>Sample</th>
<th>EC$_{50}$ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RP</td>
</tr>
<tr>
<td>Acacia honey</td>
<td>100.80 ± 4.54</td>
</tr>
<tr>
<td>Linden honey</td>
<td>51.34 ± 2.31</td>
</tr>
<tr>
<td>&quot;Homoljski med&quot;</td>
<td>120 ± 5.40</td>
</tr>
<tr>
<td>Ascorbic acid*</td>
<td>0.0154 ± 0.0007</td>
</tr>
<tr>
<td>Quercetin*</td>
<td>0.0317 ± 0.0014</td>
</tr>
</tbody>
</table>

*Controls

The DPPH free radical scavenging activity of the honeys increased with increase in concentration. (Figure 2). The highest SA values were found for Linden honey, while the lowest was observed for Acacia honey.

![Figure 2. DPPH radical scavenging activity of honeys](image)

The EC$_{50}$ values, determined on the basis of DPPH radical scavenging activities of honeys, ranged from 24.17 ± 1.09 to 164.09 ± 7.38 mg/ml (Table 2). The highest DPPH free radical scavenging activity showed Linden honey. In the study of 27 samples of natural honeys, Meda et al. (19) found that the EC$_{50}$ values ranged from 1.63 to 29.13 mg/ml. The EC$_{50}$ for quercetin (0.86 ± 0.04 μg/ml) and ascorbic acid (1.71 ± 0.07 μg/ml) were similar to the values obtained in the study of Meda et al. (19) for quercetin and ascorbic acid, which were 0.87 ± 0.06 and 1.80 ± 0.43 μg/ml, respectively.
Using the standard curves of ascorbic acid \((r = 0.9996)\) and quercetin \((r = 0.9998)\), it was shown that the antioxidant contents in Acacia honey, Linden honey and "Homoljski med" were 1.00, 5.45 and 2.99 mg QEAC/100 g, and 1.43, 7.82 and 4.29 mg AEAC/100 g, respectively. The investigation of antioxidant contents of 18 multifloral honeys showed that the values varied from 4.27 to 17.30 mg QEAC/100 g, and from 10.20 to 37.87 mg AEAC/100 g (19), which are similar or smaller compared with our results.

![Graph A](image_url)

**Figure 3.** The correlation between reducing power \((1/EC_{50})\) and total phenolics/total flavonoids (A) and between scavenging activity \((1/EC_{50})\) and total phenolics/total flavonoids (B)
The correlation between the two sets of antioxidant contents determined in our study was 1. The correlation existed between the scavenging activity (1/EC_{50}) and QEAC (r = 0.9991) and between the scavenging activity (1/EC_{50}) and AEAC (r = 0.9992). Also, the correlation between the reducing power (1/EC_{50}) and QEAC (r = 0.8283), as well as between the reducing power (1/EC_{50}) and AEAC (r = 0.8281) was high. The correlation between the reducing power (1/EC_{50}) and total phenolics was 0.9003 and between the reducing power (1/EC_{50}) and total flavonoids was 0.9984 (Figure 3A). In the case of the scavenging activity (1/EC_{50}), the correlation coefficients were high for total phenolics and total flavonoids (r = 0.9946 and r = 0.8787, respectively) (Figure 3B). For the comparison, in the study of Meda et al. (19) the correlation between the two sets of antioxidant contents was 0.993. The same correlation was found between the scavenging activity (1/EC_{50}) and QEAC (r = 0.95) and between the scavenging activity (1/EC_{50}) and AEAC (r = 0.95). The correlation between the scavenging activity (1/EC_{50}) and total phenolics was 0.5, while between the total flavonoids and scavenging activity (1/EC_{50}) there was a negative correlation (19).

Many authors reported that total phenolic and flavonoid content are strongly linearly correlated with the antioxidant activity of honeys (5, 12, 24). This study indicated that the antioxidant activity was not fully due to phenolic compounds alone. Although individual phenolics may have substantial antioxidant potential, there may be synergistic or antagonistic interactions between phenolic and non-phenolic compounds. The other constituents (e.g., ascorbic acid, α-tocopherol, carotenoids), as well as the synergistic effect among them could possibly contribute to the total antioxidant activity. More studies are needed to evaluate as to which of the phenolic constituents are responsible for the antioxidant activity of honeys. Since the honeys are very complex mixtures of many different compounds with distinct activities, the role of non-phenolic compounds which are important for the antioxidant characteristics, also needs to be investigated. Amino acids are also one of the antioxidant components in honey. The antioxidant activity of some free amino acids (histidine, taurine, glycine, alanine) and their combinations have already been shown (25). It has been demonstrated that the correlation between radical scavenging activity and proline content was higher than that between radical scavenging activity and total phenolic content (19).

**CONCLUSION**

In this study, total phenolic, flavonoid and antioxidant content, as well as *in vitro* antioxidant activity of three Serbian honey samples (*Acacia, Linden* and "Homoljski med") were determined.

The content of total phenolics (27.44 ± 1.05 mg/100 g) and flavonoids (9.78 ± 0.38 mg/100 g) was highest in *Linden* honey.

The highest reducing power and DPPH free radical scavenging activity showed *Linden* honey. The EC_{50} values of the *Linden* honey, determined based on reducing power and DPPH radical scavenging activity, were 24.17 ± 1.09 mg/ml and 51.34 ± 2.31 mg/ml, respectively.
A linear correlation was observed between the reducing power and total phenolics \((r = 0.9903)\) as well as between the reducing power and total flavonoids \((r = 0.9984)\). In the case of scavenging activity, the correlation coefficients were high for total phenolics and total flavonoids \((r = 0.9946\) and \(r = 0.8787\), respectively).

More studies are needed to ascertain which of the phenolic constituents are responsible for antioxidant activity of honeys, and elucidate the role of non-phenolic compounds present in honeys with potent antioxidant characteristics.

**Acknowledgement**

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АНТИОКСИДАТИВНА АКТИВНОСТ ТРИ ВРСТЕ СРПСКОГ ЦВЕТНОГ МЕДА

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У овом раду су анализирани узорци три врсте српског цветног меда (барековог, липовог и "Хомолског") са циљем одређивања садржаја антиоксиданата, укупних фенолних јединења и флavoноида, као и њихове антиоксидативне активности. Антиоксидативна активност узорака меда испитана је спектрофотометријски 2,2-дифенил-1-пикрилхидразил (DPPH) тестом, а такође је испитана и њихова редукциона способност. Изведена је и корелациона анализа између антиоксидативне активности и садржаја фенолних јединења, односно флavoноида. Највећи садржај укупних фенола (27,44 мг/100 г), флavoноида (9,78 мг/100 г), редукционе способности, као и скевинџер активност на DPPH радикале добијена је за липов мед. EC50 вредности за липов мед, одређене на основу редукционе способности и скевинџер активности на DPPH радикале износе 24,17 мг/мл, односно 51,34 мг/мл. Такође, садржај антиоксиданата највећи је у липовом меду и износи 5,45 мг QEAC/100 г (изражен у мг еквивалената кверцетина - QEAC по 100 г меда) и 7,82 мг AEAC/100 г (изражен у мг еквивалената аскорбинске киселине - AEAC по 100 г меда). Значајна линеарна зависност утврђена је између антиоксидативне активности и садржаја укупних фенолних јединења, као и укупних флavoноида.

Кључне речи: српски цветни мед, феноли, флavoноиди, антиоксидативна активност

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