



ANTIMICROBIAL AND ANTIRADICAL ACTIVITY OF SLOVAKIAN HONEYDEW HONEY SAMPLES

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ABSTRACT

The aim of this study was to determine the antioxidant and antibacterial activities of polyphenolic mixtures isolated from nine Slovakian honey samples. Spectrophotometrically at 517 nm was measured DPPH scavenging activity of tested samples, while antibacterial activity was evaluated by using minimum inhibitory concentration (MIC) method against two Gram-positive strains (*Listeria monocytogenes* CCM 4699; *Staphylococcus aureus* CCM

3953) and three Gram-negative strains (*Pseudomonas aeruginosa* CCM 1960; *Salmonella enterica* CCM 4420; *Escherichia coli* CCM 3988).

At the concentration of 500 $\mu\text{g.mL}^{-1}$ all tested samples of phenolic mixtures showed good DPPH antiradical activity. Among them, sample from location of Zilina showed the highest activity (90.75 % and 94.56 %, respectively), while the lowest activity was observed for sample of location Brezno (44.47 % and 51.33 %, respectively). Other mixtures (for assay after 30 min and 60 min) showed high to medium antiradical activities (from 45.89 % to 86.59 %). The MIC values of tested polyphenolic mixtures were in the range of 32 $\mu\text{g.mL}^{-1}$ to 512 $\mu\text{g.mL}^{-1}$. The highest activity of isolated extracts was observed in a case of *S. aureus* (32-64 $\mu\text{g.mL}^{-1}$) and *P. aeruginosa* (64-128 $\mu\text{g.mL}^{-1}$).

Keywords: honey, polyphenolic mixture, antiradical activity, antimicrobial activity, pathogenic bacteria, Slovakia

INTRODUCTION

The composition of honey is rather variable and depends primarily on its floral source; however, certain external factors, such as seasonal and environmental factors and processing also play a role. Honey is a supersaturated solution of sugars, of which fructose (38%) and glucose (31%) are the main contributors, with phenolic compounds, minerals, proteins, free amino acids, enzymes, and vitamins acting as minor components (**Alvarez-Suarez et al., 2010**).

The major antibacterial properties are related to the level of hydrogen peroxide determined by relative levels of glucose oxidase and catalase (**Weston, 2000**). Honey differences in antimicrobial activity can be in part a reflection of these levels as well. Although the honey therapeutic action has been taken some attention by researchers, studies only have been done on screening the raw honey samples on antimicrobial activity (**Taormina et al., 2001; Basualdo et al., 2007**) and on antioxidant capacity (**Rauha et al., 2000**). Others studies have shown that individual phenolic compounds have growth inhibition on a wide range of Gram-positive and Gram-negative bacteria (**Davidson et al., 2005**). Phenolic compounds are one of the most important groups of compounds occurring in plants, comprising at least 8000 different known structures. These compounds are reported to exhibit

anticarcinogenic, anti-inflammatory, antiatherogenic, antithrombotic, immune modulating and analgesic activities, among others and exert these functions as antioxidants (Estevinho et al., 2008).

Many authors demonstrated that honey serves as a source of natural antioxidants, which are effective in reducing the risk of heart disease, cancer, immune system decline, different inflammatory processes, etc. (The National Honey Board, 2003). The components in honey responsible for its antioxidative effect are mainly flavonoids, phenolic acids, catalase, peroxidase, carotenoids and non-peroxidal components. The quantity of these components varies widely according to the floral and geographical origin of honey, processing, handling, and storage of honey (Gheldof and Engeseth, 2002; Turkmen et al., 2006). However the botanical origin of honey has the greatest influence on its antioxidant activity (Al-Mamary et al., 2002). In literature several studies for the identification and quantification antioxidant components of honeybee products are reported (Gheldof et al., 2002; Gomez-Caravaca et al., 2006). The studies for the evaluation of the antioxidant capacity of honey involved the use of different methods such as the ORAC (oxygen radical absorbance capacity), FRAP (ferric reducing/antioxidant potential), and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays (Aljadi and Kamaruddin, 2004).

The objectives of our study were determinations of antioxidant properties of honey extracts by testing of their scavenging effect on DPPH radicals, as well as antibacterial activities against two Gram-positive strains (*Listeria monocytogenes* CCM 4699; *Staphylococcus aureus* CCM 3953) and three Gram-negative strains (*Pseudomonas aeruginosa* CCM 1960; *Salmonella enterica* CCM 4420; *Escherichia coli* CCM 3988).

MATERIAL AND METHODS

Honey samples

The original honeydew honey samples produced in various Slovak regions were obtained from the Slovakian beekeepers (table 1). The samples were stored in the dark at a room temperature of 22 °C.

Table 1 Characteristics of analysed Slovakian honey samples from the harvest in the year 2010

| Location | Number of samples | Kind of honey | Sensory characteristics |
|--------------------|-------------------|---------------|----------------------------------------------------|
| Jaraba | 1. | honeydew | Dark amber, very dark, bright, viscous |
| Jaraba | 2. | honeydew | Light amber, medium dark, fine granulated, viscous |
| Liptovský Mikuláš | 3. | honeydew | Dark amber, very dark, bright, viscous |
| Myto pod Dumbierom | 4. | honeydew | Dark amber, very dark, bright, viscous |
| Brezno | 5. | honeydew | Dark amber, very dark, bright, viscous |
| Poprad | 6. | honeydew | Dark amber, very dark, bright, viscous |
| Zilina | 7. | honeydew | Light amber, medium dark, fine granulated, viscous |
| Zilina | 8. | honeydew | Light amber, medium dark, fine granulated, viscous |
| Martin | 9. | honeydew | Light amber, medium dark, fine granulated, viscous |

Methodology for isolation of flavonoids and phenolic acids from honey, using Amberlite XAD-2 resin

Purification of the Amberlite XAD-2 Resin

Prior to use, commercial Amberlite XAD-2 (Supelco, Bellefonte, PA, USA, pore size 9 nm, particle size 0.3-1.2 mm) resin was cleaned with methanol to ensure it was free from contamination. All the clean Amberlite XAD-2 resin was kept in a screw-capped plastic bottle and stored in the fridge because the resin is susceptible to microbial growth.

Washing of Amberlite XAD-2 resin (previously cleaned as above) after use for every five honey extractions

Solid Amberlite XAD-2 resin (200 g) was placed in a 500 mL beaker, covered with methanol, stirred with a stirring bar on a magnetic stirrer (with no heating) for 25 min, and filtered through a Buchner funnel. This process was repeated for more times.

Swelling (activation) of the Amberlite XAD-2 Resin

A solution of methanol (200 mL) and deionised water (200 mL) (equivalent to 1:1 volume) was added to cover 150 g of Amberlite XAD-2 resin in a flask, which was then stoppered to avoid contamination or loss of solvents. The flask was left to stand overnight to ensure complete swelling of the resin. No mixing or stirring was required as this disturbs the resin structure. The solvent was filtered off on a Buchner funnel using filter paper. Prior to use for extraction, this solid Amberlite XAD-2 resin was then washed with 400 mL of deionised water.

Extraction of phenolic compounds

The phenolic compounds (flavonoids and phenolic acids) extraction from the nine honey samples was carried out by method of **Yao et al. (2003)**, with some modification. The honey samples, about 100 g, were mixed with 500 mL of acidified water (pH 2 with HCL) and, after dissolution, filtered with a cotton filter for solid particles removal. Amberlite XAD-2 was added to the filtrate and afterwards, the mixture was stirred with magnetic stirrer for about 1 h at room temperature. The Amberlite particles were packed in a column, washed with acidified water (pH 2 with HCL, 400 mL) and with deionized water (400 mL) for sugar and other honey compounds removal. The adsorbed phenolic compounds were extracted from the Amberlite by elution with 300 mL of methanol, which was evaporated under reduced pressure. The residue, dissolved in a 10 mL of deionized water, was extracted for the phenolic compounds three times with 10 mL of diethyl ether. The combined extracts suffered evaporation and, after measuring the weight, extract was re-dissolved with methanol for antimicrobial activity assays and antioxidant test.

DPPH free radical scavenging activity

Free radical scavenging activity of the phenolic extracts of the various samples of honey was evaluated with some modification in accordance with method of **Takao et al. (1994)**. DPPH (2,2-diphenyl-1-picrylhydrazyl) (8 mg) was dissolved in methanol (100 mL) to obtain a concentration of 80 mg.mL⁻¹. Diluted solutions of honey samples and BHT (butylated hydroxytoluene) as standard (500 mg.mL⁻¹, 2 mL each in methanol) were mixed with DPPH (2 mL) and allowed to stand for 30 and 60 min for any reaction to occur. Absorbance was recorded at 517 nm using T80 UV/Vis Double Beam Spectrophotometer.

Antioxidant activity was expressed as percentage (%) of scavenging activity on DPPH radical:

$$\% = [(A_{\text{DPPH}} - A_{\text{sample}}) / A_{\text{DPPH}}] \times 100$$

DPPH radical scavenging activity of standard antioxidant, BHT was also assayed for comparison.

Antimicrobial activity

The isolated phenolic extracts from honey samples were studied for their antimicrobial activity. *In vitro* antimicrobial studies were carried out by the dilution method, as previously described (**Melliou and Chinou, 2005**), measuring the MIC values in 96-hole plates against two Gram-positive strains (*Listeria monocytogenes* CCM 4699; *Staphylococcus aureus* CCM 3953) and three Gram-negative strains (*Pseudomonas aeruginosa* CCM 1960; *Salmonella enterica* CCM 4420; *Escherichia coli* CCM 3988). The bacterial strains were purchased from the Czech Collection of Microorganisms (CCM). Stock solutions of the tested extracts and pure compounds were prepared at 10 mg.mL⁻¹, respectively. Suspensions of the microorganisms were prepared to contain approximately 10⁸ cfu.mL⁻¹ and then 100 µL of these suspensions were inoculated in plates containing agar medium. Serial dilutions of the stock solutions in broth medium (100 µL of Müller-Hinton broth), were prepared in a microtitre plate (96 wells). Then 1 µL of the microbial suspension (the inoculum, in sterile distilled water) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked and the plates were incubated as referred to above. To measure the MIC values, various concentrations of the stock, 512, 256, 128, 64, 32, 16, 8 µg.mL⁻¹ were assayed against the tested bacteria. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth (**Shahidi Bonjar, 2004**).

Standard antibiotic chloramphenicol was used in to control the sensitivity of the tested bacteria. For each experiment, any pure solvent used was also applied as a blind control. The experiments were repeated three times and the results were expressed as average values.

RESULTS AND DISCUSSION

Antioxidant properties of different Slovakian honey samples were evaluated using the phenolic extract obtained by extraction with Amberlite XAD-2 resin. Numerous tests have been developed for measuring of the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively. Clearly, matching radical source and system characteristics to antioxidant reaction mechanisms is critical in assessing the antioxidant capacity assay methods (**Prior et al., 2005**). To screen the antioxidant properties of the samples, one chemical assay was performed: scavenging activity on DPPH radicals (measuring the decrease in DPPH radical absorption after exposure to radical scavengers).

The honey phenolic compounds under study are the phenolic acids and flavonoids, which are considered potential markers of the honey botanical origin. Phenolic acids are divided in two subclasses: substituted benzoic acids and cinnamic acids. The flavonoids present in honey are divided in three classes with similar structure: flavonols, flavones and flavanones. These are important due to their contribution to honey colour, taste and flavor and also due to their beneficial effects on health. Moreover, honey phenolic compounds composition and, consequently, antioxidant capacity depend on their floral sources used to collect honey which are predominantly dependent on seasonal and environmental factors (**Al-Mamary et al., 2002; Yao et al., 2003**). So, different honey properties were expected since the composition of active compounds in honey from different locations should be different.

Flavonoids and simple phenolic derivatives are the most common polyphenols (**Brovo, 1998**). Flavonoids are the secondary metabolites of plants, with more than 5000 compounds having been identified by 1990 (**Brovo, 1998**). Structurally, flavonoids are derivatives of 1,3-diphenylpropane (**Sivam, 2002**) and are low molecular weight polyphenols based on the flavan nucleus, which is characterised by a C₆-C₃-C₆ carbon skeleton (figure 1) (**Peterson and Dwyer, 1998**). The three phenolic rings are referred to as A, B and C (pyran) rings (**Cook and Samman, 1996**). Biogenetically, the A ring usually comes from the acetate pathway, whereas ring B is derived from the shikimate pathway (**Brovo, 1998**). Flavonoids

occur naturally as glycosides (with sugar moieties), but occasionally occur as aglycones (without sugar moieties) (Peterson and Dwyer, 1998).

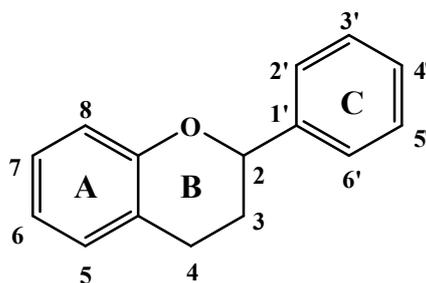


Figure 1 Generic structure of flavonoids

The structure of the B ring (Figure 1) is the primary determinant of the antioxidant capacity of flavonoids (Pannala *et al.*, 2001). Flavonoids such as quercetin, with 3',4'-OH substituents in the B ring and conjugation between the A and B rings, have antioxidant potentials four times that of Trolox, the vitamin E analogue. Removing the *ortho*-OH substitution or reducing the 2,3-double bond in the C ring decreases the antioxidant capacity by more than 50 % (Rice-Evans *et al.*, 1995, 1996, 1997). The carbonyl in the central ring (C ring) and the C2-C3 double bond could participate in radical stabilisation that increases antioxidant capacity, as occurs with quercetin.

There have been 33 flavonoids identified in honey, of which 11 are also found in the floral nectar, 9 in honeybee pollen, and 25 in propolis (Boudourova-Krasteva *et al.*, 1997). Moreover, there are over 70 other phenolics identified from honey and propolis (Bankova *et al.*, 1987; Joerg and Sontag, 1992). Compounds that have been identified include flavones such as chrysin; flavonols such as kaempferol; flavanones such as hesperetin; and phenolic acids. In the Northern Hemisphere where poplars (the source of propolis) are native, honeys show flavonoid profiles characterised by the presence of propolis flavonoids. Characteristic propolis-derived flavonoids pinocembrin, pinobanksin and chrysin are present in most European honeys in variable concentrations (Tomas-Barberan *et al.*, 2001).

At the concentration of 500 $\mu\text{g}\cdot\text{mL}^{-1}$ all tested samples of phenolic mixtures showed good DPPH antiradical activity. Among them, for measurement after 30 minutes sample 7 (location Zilina) showed the highest activity (90.75 %) in comparison to other samples, as well as standard BHT (89.46 %). Also, strong antiradical activity was observed in the case of phenolic mixture noted as 4 (location Myto pod Dumbierom) (after 30 min - 88.43 %; after 60 min - 92.88 %). The lowest activity was observed for sample 4 for location Brezno (44.47 %

and 51.33 %, respectively). Other phenolic mixtures (for assay after 30 min and 60 min) showed high to medium activities (from 45.89 % to 86.59 %).

Table 2 Inhibition of DPPH radicals (%)

| Sample (500 µg.mL ⁻¹) | after 30 min | after 60 min |
|-----------------------------------|--------------|--------------|
| 1. | 77.53 | 86.59 |
| 2. | 63.56 | 74.25 |
| 3. | 48.86 | 56.94 |
| 4. | 88.43 | 92.88 |
| 5. | 44.47 | 51.33 |
| 6. | 68.61 | 78.00 |
| 7. | 90.75 | 94.56 |
| 8. | 48.02 | 55.37 |
| 9. | 45.89 | 52.16 |
| BHT | 89.46 | 96.54 |

DPPH - 2,2-diphenyl-1-picrylhydrazyl, BHT - butylated hydroxytoluene

Table 3 Antimicrobial activities (µg.mL⁻¹) of the studied honey extracts

| Honey samples | | | | | |
|------------------------|-------------------------|------------------|----------------------|--------------------|----------------|
| number | <i>L. monocytogenes</i> | <i>S. aureus</i> | <i>P. aeruginosa</i> | <i>S. enterica</i> | <i>E. coli</i> |
| 1. | 256 | 32 | 64 | 256 | 256 |
| 2. | 512 | 64 | 128 | 512 | 256 |
| 3. | 256 | 32 | 64 | 256 | 256 |
| 4. | 512 | 32 | 64 | 256 | 512 |
| 5. | 512 | 64 | 128 | 512 | 512 |
| 6. | 256 | 32 | 64 | 512 | 256 |
| 7. | 512 | 16 | 64 | 512 | 256 |
| 8. | 256 | 32 | 64 | 256 | 256 |
| 9. | 512 | 64 | 128 | 512 | 512 |
| Chloramphenicol | 8 | 8 | 8 | 8 | 8 |

All studied honey extracts were tested for their antimicrobial activity against pathogenic Gram⁺ and Gram⁻ bacteria (Table 2). All samples showed very interesting antimicrobial profiles against five standard Gram⁺ and Gram⁻ bacteria strains, confirming the traditional reputation of honey as an antimicrobial agent. MICs values of antimicrobial activity of honey varied from 32 µg.mL⁻¹ to 512 µg.mL⁻¹.

Therefore, in this study nine produced honeys were screened for their antimicrobial activity against pathogenic strains. Our data showed that all honey samples tested, demonstrated antibacterial action. Furthermore, our results are in line with the studies of **Kumar et al. (2005)** and **Adetuyi et al. (2009)** that reported potent activity against Gram⁺ and Gram⁻ bacteria in India and Nigeria, respectively. Although not determined in this study, the antimicrobial activity in honey has been ascribed to factors such as high osmolarity, acidity, hydrogen peroxide and nonperoxide factors (**Taormina et al., 2001**). The antimicrobial capacity of phenolic compounds, in a general way, is well known (**Pereira et al., 2006; Rauha et al., 2000**). As previously described, individual phenolic compounds present in honey extracts were identified and quantified, but we chose to submit the entire extracts to antimicrobial activity studies. In fact, total food extracts may be more beneficial than isolated constituents, since a bioactive individual component can change its properties in the presence of other compounds present in the extract (**Borchers et al., 2004**), corresponding to a synergistic effect. The results of **Estevinho et al. (2008)** showed that honey phenolic compounds extracts obtained from the dark and clear honey have similar antimicrobial capacity inhibitory, with the exception of the *P. aeruginosa* microorganisms, but with different response degrees depending on the tested microorganism to honey extracts. The *Staphylococcus aureus* was the most sensitive microorganism, with lower MIC (0.4 mg.mL⁻¹). *Bacillus subtilis*, *S. lentus*, *K. pneumoniae* and *E. coli* were moderately sensitive to the antimicrobial activity of honey.

In general, the Gram⁺ bacteria were more sensitive to the honey phenolics compounds extracts than the Gram⁻ bacteria. Our results were in agreement with the data observed by **Mundo et al. (2004)** and **Agbaje et al. (2006)**. These authors also observed that *S. aureus* was the most sensitive bacteria to manuka and pasture honeys. Results of the inhibitory activity of raw honey against pathogens have been presented by **Taormina et al. (2001)** and **Basualdo et al. (2007)** which are similar to the results obtained in this work, that have been carried out in different experimental conditions. As expected, MICs chloramphenicol standards present lower MICs than honeys extracts. Usually, those pure active compounds reveal more activity than crude extracts (**Pereira et al., 2006**).

Moreover, the extracted phenolic compounds from other natural products, for example, table olives (Pereira et al., 2006), mushrooms (Barros et al., 2007), grape juice and wine (Mato et al., 2007), have lower antimicrobial effects comparatively to the ones obtained from phenolic compounds of honey extracts. These results show that honey is a natural product with therapeutic characteristics and that further studies should be carried out.

CONCLUSION

The present study demonstrates that honey phenolic compounds are partially responsible for honey antibacterial and antioxidant activity, corroborating the relevance of honey as a healthy alimentary product as a source of antioxidants. We have shown that extracts of phenolic compounds obtained from the dark honey samples show better antioxidant activity than the clear honey samples. These results also attributed to the differences in the phenolic compounds profile which are dependent of the honey geographical origin (*flora predominance*), and are in accordance with other works that state that dark honey samples have phenolic compounds with higher microbiological inhibitor properties, as well as stronger antiradical activities.

Acknowledgments: Work was funded by Grant Agency KEGA 053 SPU-4/2011.

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