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ANTIOXIDANT ACTIVITY OF SUNFLOWER AND MEADOW HONEY

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Abstract

Honey is a natural food produced by bees from the nectar of flowers and is a mixture of carbohydrates, amino acids, enzymes, vitamins and many bioactive compounds. Due to its special composition, honey is a functional food with antibacterial, anti-inflammatory and antioxidant properties. Studies have shown that honey's properties depend mainly on the vegetable source from which it is obtained, but also on the processing type and storage. This research aims to evaluate the antioxidant capacity of sunflower honey compared to meadow honey by assessing total phenolic content, total flavonoid content, total antioxidant activity, free radicals scavenging activity and reducing power. For the determination of total phenols and flavonoids, antioxidant capacity and reducing power, spectrophotometric methods were used. To assess free radicals scavenging activity, chemiluminometric methods were used. Meadow honey showed the highest concentration of polyphenols (143.29 ± 9.12 mg GAE / kg) and flavonoids (118.09±8.84 mg CE / kg). DPPH radical scavenging capacity was higher for sunflower honey (78.32 ± 5.11%) compared to meadow honey (45.12 ± 3.26%). The two honey types showed capacity to scavenge superoxide anion and singlet oxygen, with an inhibition rate of over 50%. In conclusion, sunflower honey and meadow honey presented important concentrations of polyphenols and flavonoids and the results suggest a relationship between honey type and total polyphenols and free radicals scavenging activity.

Key words: *antioxidant activity, chemiluminometric method, honey, phenols.*

INTRODUCTION

Honey played an important role for human civilization since its very beginnings. Honey is used in pure form or prescribed in different preparations. Honey is used in bakery (honey cookies), in the manufacturing of beverages by mixing with alcohol or it is incorporated into cosmetics products (Saxena *et al*., 2010).

Recent years have recorded a growing interest of consumers, researchers and food industry for honey because it may help maintain human health. It is widely accepted that honey plays an important role in preventing and treating different kinds of illnesses. The importance of honey as food and nutrient is primarily based on its high content of easily absorbed constituents.

A diet that provides carbohydrates, proteins, lipids, vitamins, and minerals in sufficient quantities to satisfy the body needs, is known by the concept of "functional food" which includes the potential of food to improve and promote health, and even to reduce the illnesses risk (Nagai and Inoue, 2004).

Honey is a natural complex liquid that contains more than 200 substances, of which many are known to have antioxidant properties. These substances include phenolic acids and flavonoids, enzymes (glucose oxidase, catalase), ascorbic acid, amino acids and proteins, organic acids, carotenoid-like substances, Maillard reaction products, vitamins and minerals (Beretta *et al*., 2005). The composition of honey is variable and it depends on the floral source and processing.

In this study sunflower honey (SFH) and meadow honey (MH) were subject to analysis. Honey samples used in this research were produced in a beekeeping farm from South-Eastern Romania.

MATERIALS AND METHODS

Total phenolic content analysis

Total phenolic content (TPC) in honey samples was determined according to Beretta *et al*. (2005) and Bertoncelj *et al*. (2007), with slight modification. A mass of 1 g of honey was diluted in 5 mL of distilled water. A volume of 500 µL of honey solution was pipetted into a 10 mL test tube which contained 4.5 mL

distilled water. Then, 0.2 mL Folin Ciocalteu reagent were added and the reaction mix was vortexed and left to stand for 2 min. In the end, 0.5 mL of 20 % (w/v) Na_2CO_3 solution were added. After 20 min, the absorbance was measured at 725 nm using V670 UV-VIS Jasco spectrophotometer. Blind control samples were prepared (aqueous solution of sugars: 40 % fructose, 30 % glucose, 8 % maltose and 2 % sucrose). The preparation and measuring procedure was the same as the one used for honey samples. The concentration of total phenolics was expressed as mg gallic acid equivalents per kg (mg GAE/kg) of honey.

Total flavonoid content analysis

Flavonoids are low molecular weight phenolic compounds that are vital components for the aroma and antioxidant properties of honey. Total flavonoid content (TFC) in honey samples was determined according to Blasa *et al*. (2006) and Kim *et al*. (2003). A blank was used to eliminate the interference of reducing sugars. Briefly, 0.1 g of insoluble polyvinylpyrrolidone (PVPP) was added to 5 mL of 75 mM phosphate buffer, pH 7.0, and moisturized at 4°C for 24 h. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. A volume of 5 mL of a honey solution (5 g of honey in 25 mL of phosphate buffer, pH 7.0) was added to the residual sediment, stirred for 30 min at 30°C and then filtered. This solution was used as blank. The determination of total flavonoids in honey samples started by mixing 1 mL of sample solution with 0.3 mL of 5 % $NaNO_2$ in a 10 mL test tube. After 5 min, 0.3 mL of 10 % $AlCl_3$ were added to the solution by mixing in a vortex. After 6 min of reaction, the solution was neutralized with 2 mL of 1 M NaOH. This solution was once more mixed in a vortex and transferred to a glass cuvette. The absorbance was measured using V670 UV-VIS Jasco spectrophotometer at 510 nm. The total flavonoid content was expressed as mg of (+)-catechin equivalents per kg (mg CE/kg) honey.

DPPH free radical-scavenging activity

DPPH scavenging activity was based on the method proposed by Ferreira *et al*. (2009). Briefly, 0.3 mL of honey extract was mixed with ethanolic solution containing DPPH radicals (0.004 g/100 mL, 2.7 mL). The mixture was vigorously shaken and left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorbance of the mixture at 517 nm. Ascorbic acid was used as reference. DPPH radical-scavenging activity (% Inhibition) was calculated as the percentage of DPPH discoloration using the following equation:

$$\% \, Inhibition = [(A_{Blank} - A_{Sample})/A_{Blank}] \times 100$$

Ferric reducing antioxidant power assay (FRAP Assay)

The FRAP assay was performed according to a modified method described by Benzie and Strain (1996). Briefly, 200 µL of diluted honey (1 g/5 mL) was mixed with FRAP reagent (1.5 mL). Then, the reaction mixture was incubated at 37°C for 4 min and its absorbance was read at 593 nm against a blank that was prepared with distilled water. Fresh FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6) with 1 volume of 10 mmol TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl containing 1 volume of 20 mM ferric chloride ($FeCl_3 \bullet 6H_2O$). The resulting mixture was then incubated at 37°C. A calibration curve was prepared using an aqueous solution of ferrous sulfate ($FeSO_4 \bullet 7H_2O$) at 100, 200, 400, 600 and 1,000 µM. FRAP values were expressed as micromoles of ferrous equivalent (µM Fe [II]).

Assay procedure for chemiluminescence emission kinetics and for quenching effects of honey samples on reactive oxygen species

For the evaluation of chemiluminescence (CL) quenching effects, all honey samples were diluted with dimethyl sulfoxide (DMSO) in ratio 1:100 (v/v). The CL measurements were performed at room temperature for 170 s (2min 50s), in test tubes (Φ12 x 75 mm), using a Berthold luminometer. The intensity of CL is given as the relative light units per second (RLU/s). Five measurements were made and an average value was calculated. The percentage of quenching effect against the reactive oxygen species was calculated using the following equation:

$$Quenching\ effect\ \%\ (S\ \%) = [(I_0 - I)/I_0] \times 100$$

where I_0 is the CL intensity generated by the reference system (control) (RS) and I represents CL intensity generated by the sample.

Hydrogen peroxide scavenging activity

H_2O_2 scavenging activity was assayed according to the method described by Papuc *et al*., (2012). Briefly, the reaction mixture consisted in 100 µL of luminol ($2.5x10^{-5}$M), 440 µL 30mM Tris pH 8.5 and 10 µL sample or 10 µL Tris (as RS). 50 µL of 30 mM H_2O_2 were added to start the CL reaction.

Hydroxyl radical scavenging activity

For hydroxyl radical scavenging effect assay, HO• was generated by a Fenton-type reaction system (Parejo *et al*. 2002). 50 µL $FeSO_4$ (0.4 mmol/L) and 50 µL H_2O_2 (1.5%) were incubated at 30°C for 2 min. Then, 50 µL sample or PBS (in control) and 600 µL luminol (LH_2) (0.1 mmol/L) were added into the mixture and the chemiluminescence intensity was measured.

Singlet oxygen scavenging activity

Singlet oxygen scavenging activity was performed as described by Voicescu *et al*. (2010). To evaluate the quenching effect of singlet oxygen, 6 µL of sample, 300 µL of 0.4% H_2O_2 in 100 mM acetate buffer (pH 4.5), 300 µL of 80 mM NaBr in acetate buffer and 0.8 mM luminol in acetate buffer were added. The mixture was incubated at 37°C for 10 min. The CL intensity was measured after adding 300 µL of a 10 µg/mL solution of horseradish peroxidase in acetate buffer.

Hypochlorite anion scavenging activity

Hypochlorite anion (ClO^-) scavenging activity was evaluated as described by Wada *et al*. (2007). Briefly, 900 µL of 0.53 mM luminol in 50 mM borate buffer (pH 9.5) were added to 6 µL of sample. After incubation at 37°C for 10 min, 300 µL of 40 µM NaClO in borate buffer were added to the mixture and then the CL intensity was measured.

Superoxide anion scavenging activity

O_2•- was generated in a pyrogallol autooxidation system (Zhao *et al*., 2003). The reaction mixture contained 50 µL of pyrogallol ($3.125x10^{-4}$ mol/L), 200 µL of carbonic acid/buffer saline solution (CBSS) pH 10.2 containing 0.1 mol/L EDTA, 10 µL of polyphenolic extract (DMSO was used in control) and 400 µL of luminol ($1x10^{-3}$ mol/L). The CL intensity was measured immediately for 170s.

RESULTS AND DISCUSSIONS

Because polyphenols are present in all plants, they are also found in honey. Honey contains complex mixtures of polyphenols depending on the climate, region, soil, pollution levels, storage and many other factors. These differences are possible because certain polyphenols are specific to particular plants and hence are found only in honey produced by bees from those plants. The TPC and TFC are presented in Table 1. It can be observed that the highest concentration of polyphenols was found in meadow honey (143.29 ± 9.12 mg GAE / kg), and over 80% of these compounds are represented by flavonoids. By comparison, sunflower honey flavonoids represent only 75% of the polyphenols concentration (84.51±6.11 mg GAE / kg).

Table 1. Total phenolic contents and total flavonoid content of sunflower (SFH) and meadow honey (MH)

Sample	TPC (mg GAE/100 g)	TFC (mg CE/100 g)
SFH	84.51±6.11	63.22±5.19
MH	143.29 ± 9.12	118.09±8.84

These results are similar to previous studies in which honey samples with high polyphenol concentrations also contained high flavonoid levels (Beretta *et al*., 2005; Vela *et al*., 2007; Khalil *et al*. 2011). Total phenolic content of SFH and MH are also within the reported range of Slovenian honey and Romanian honeys (Bertoncelj *et al*., 2007; Al *et al*., 2009). Al-Mamary *et al*. (2002) indicated that the determination of total phenolic content of honey is a good parameter for the assessment of its quality and possible therapeutic potential.

In evaluating the radical-scavenging potential of honey, the DPPH assay is frequently used because the antioxidant potential of honey has been shown to be directly associated with its phenolic and flavonoid contents (Beretta *et al.*, 2005).

Free radical scavenging activity by DPPH method was used to determine the antioxidant activity of honey. This method is specific because higher values mean higher antioxidant activity.

The highest percentage of DPPH inhibition was exhibited by SFH (78.32 ± 5.11 %); MH

showed a lower inhibition of DPPH free radical (45.12 ± 3.26 %) (Table 2).

FRAP is a widely used method for antioxidant determination and it has been used for the assessment of the antioxidant and reducing power of honey.

The FRAP assay gives an estimation of the antioxidants present in a sample based on its ability to reduce the Fe [III] to Fe [II]. The highest FRAP was recorded for MH (653.45 ± 49.46 µmols Fe (II)/kg), while SFH showed a lower FRAP (560.23 ± 44.71 µmols Fe II/kg) (Table 2).

Table 2. FRAP assay and DPPH scavenging activity of sunflower (SFH) and meadow honey (MH)

Sample	DPPH (% Inhibition)	FRAP (µmols Fe [II]/kg)
SFH	78.32 ± 5.11	560.23 ± 44.71
MH	45.12 ± 3.26	653.45 ± 49.46

Significant correlations were determined between TPC and TFC and antioxidant parameters. The strongest positive significant correlation was found between total phenolics and total flavonoids (R^2 =0.9287).

A strong positive correlation was also found between phenolics and DPPH (R^2 =0.7284), indicating that phenolics also contribute to the antioxidant capacity of honey. This statistically significant correlations are in agreement with previous findings of Saxena *et al*. (2010), Kishore *et al*. (2011), Khalil *et al*. (2011), Islam *et al*. (2012) and Maurya *et al*. (2014).

Luminol (LH_2) reacts with reactive oxygen species (ROS) to yield a compound in an excited electronic state which returns to ground state with production of light (chemiluminescence).

The decrease of CL intensity in time, under reference system (RS) signal, corresponds to the scavenging of ROS by an antioxidant, and the increase of CL intensity in time, upper RS signal corresponds to the formation of free radicals by a prooxidant (Papuc *et al*. 2012) .

Calculation of quenching effects 5 s after the start of luminol-superoxide anion reaction showed a remarkable antioxidant activity for SFH (85.68%) and MH (84.26%), for the dilution 1:100 (v/v) compared to ascorbic acid (AA) (23.86%) (Table 3).

Table 3. Percentage of quenching effect (Q %) against ROS, 5 s after the beginning of the reaction, of sunflower (SFH) and meadow honey (MH)

ROS	SFH	MH	AA
H_2O_2	42.55	70.93	39.75
·OH	78.79	17.34	65.81
$^1O_2$	54.93	65.20	67.67
ClO-	32.89	41.60	43.92
O_2·-	85.68	84.26	23.86

The results obtained after calculation of percentage of quenching effect after 5 s of reaction demonstrated that the two honey samples strongly scavenged hydrogen peroxide, the highest activity being recorded for MH extract (70.93 %).

Scavenging activity of SFH and MH and AA against reactive oxygen species, 5 s after the beginning of the reaction are shown in Figure 1 (hydrogen peroxide), Figure 2 (hydroxyl radical), Figure 3 (singlet oxygen), Figure 4 (hypochlorite anion) and Figure 5 (superoxide anion). Graphical representation of CL intensity depending on time demonstrates that, comparatively to reference system, all tested extracts have the capacity to scavenge reactive oxygen species.

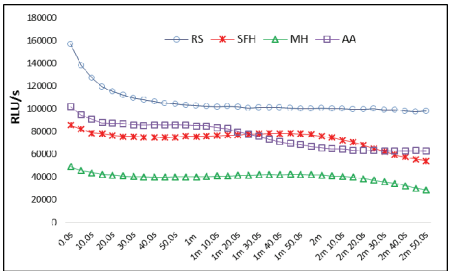

Figure 1. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by LH_2 - H_2O_2 system in Tris-HCl buffer pH 8.5

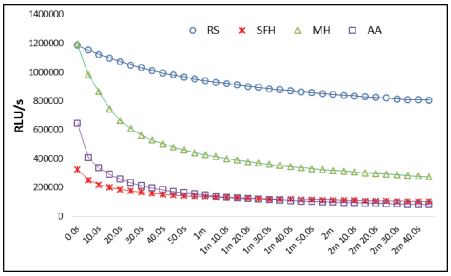

Figure 2. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by LH_2 - ·OH system in PBS buffer pH 7.4

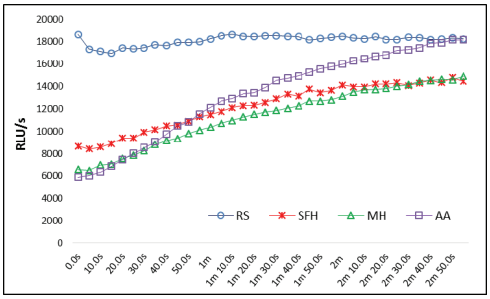

Figure 3. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by LH_2 - $^1O_2$ system in acetate buffer pH 4.5

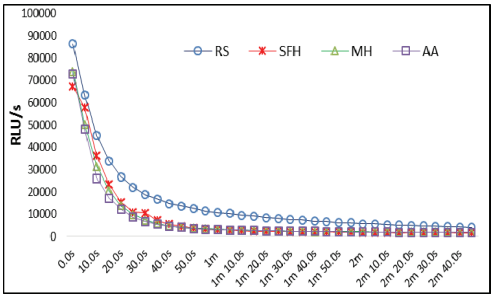

Figure 4. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by LH_2 – ClO- system in borate buffer pH 9.5

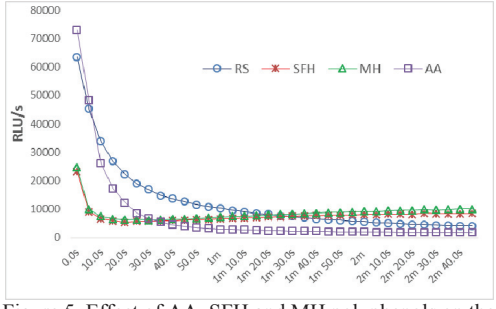

Figure 5. Effect of AA, SFH and MH polyphenols on the kinetics of the CL emission produced by LH_2–$O_2^{\bullet-}$ system in carbonic acid buffer/salt solution pH 10.2

The effect of meadow honey and sunflower honey on CL emission kinetics produced by luminol – singlet oxygen reaction was similar to the one of AA. Hydrogen peroxide (H_2O_2) scavenging activity was higher for MH than SFH and ascorbic acid used as reference. Superoxide anion and hypochlorite anion scavenging activities were very similar for the two honey samples.

CONCLUSIONS

In this study, the content in polyphenols and total flavonoids of two types of honey (sunflower and meadow honey) was investigated. Both honey assortments showed important concentration in polyphenols and flavonoids. Moreover, DPPH scavenging activity seemed to correlate with the concentration of honey phenolics and flavonoids.

The results suggested a relationship between honey total polyphenols and free radicals scavenging activity determined by chemiluminescent assay.

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