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RESEARCH ARTICLE

Correlations of flavonoids content and antioxidant activity in bee honey from Bosnia and Herzegovina

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ABSTRACT

In this study, it was performed identification and quantification of flavonoids (apigenin, chrysin, hesperetin, kaempferol, luteolin, naringenin, and quercetin) and flavonoid glycosides (rutin and vitexin) in total 49 samples of five different honey types from Bosnia and Herzegovina: meadow honey (MH, 22 samples), forest (FH, 10), acacia (AH, 7), chestnut (CH, 5), and heather honey (HH, 5). Additionally, evaluation of correlations between FC and total hydrophilic antioxidant score (antioxidant activity against both: $ROO^{\bullet}$ + $OH^{\bullet}$) in supernatants (*s*) and in bulk (noncentrifuged) solution (*b*) of these honey types was performed. Moreover, correlations between flavonoids content (FC) and previously reported antioxidant activity against both peroxyl and hydroxyl free radicals ($AC_{(ROO^{\bullet})}$ and $AC_{(OH^{\bullet})}$) for the same honey samples was examined. High performance liquid chromatography with photodiode array detector (HPLC-DAD) and isocratic elution mode was used as method of analysis. Flavonoids were extracted by solid phase extraction (SPE). The average contents of three flavonoids (chrysin, naringenin, and luteolin) in MH were statistically higher than in AH ($p^{**}<0.01$). Also, the average content of naringenin in FH was statistically higher than in CH ($p^{*}<0.05$). We observed a high (positive) linear correlation between FC and $AC_{(ROO^{\bullet})}$ in *s* of four honey types (FH, AH, HH, CH) ($R^2=0.920$). If we correlate FC and $AC_{(ROO^{\bullet})s}$ of three honey types (FH, AH, HH), linearity is very high ($R^2=0.968$), and for FH, AH, CH linearity is complete. The correlation between FC and $AC_{(ROO^{\bullet})}$ in *b* of the same honey types is similar, but lower. The correlation does not exist between FC and $AC_{(OH^{\bullet})}$ neither in *s* nor in *b* of five or four honey types, but for FC to both ($AC_{(OH^{\bullet})s}$ and $AC_{(OH^{\bullet})b}$) of three honey types (FH, AH, CH), linearity is moderate ($R^2=0.732$ and $R^2=0.696$, respectively).

Keywords: antioxidant activity, correlations, flavonoids, honey, HPLC-DAD.

INTRODUCTION

Flavonoids are a class of natural products that more impresses by their great variety and the number of their members than by the complexity of the constituents in the structure (Maleki et al., 2019). The multiplicity of possible modifications of flavonoids result in more than 7.000 different compounds (Akimoto et al., 2017; Ginwala et al., 2019). These compounds exhibit a wide range of biological effects, including antioxidative (Zheng & Wang, 2001; Gheldof et al., 2002), anti-inflammatory (Wu et al., 2006), anti-carcinogenic (Brusselmans et al., 2005; Angst et al., 2013), anti-microbial (Cushnie and Lamb, 2005) and other usefulness effects (López-Lázaro, 2009; Johnston, 2015; Kedika et al., 2016; Panche et al., 2016).

Study has shown that there is a connection between the individual flavonoids structural components and properties of scavenging, creating chelate complexes and antioxidant activity (Adekunle et al., 2012). Ivey et al. (2017) showed that higher intakes of specific flavonoids, and flavonoid-rich foods, was associated with reduced risk of total and cause-specific mortality. However, it is important to note that many of phenolic compounds, at specific concentration and conditions, can have harmful effects (Boots et al., 2007; López-Lázaro, 2009). Analysis of phenolic compounds in the honey is also used to test the honey type as well as its geographic origin (Ferreres et al., 1994).

The main groups of flavonoids in honey are flavones, flavonols and flavanones that differ in structural formulas and in the position of substituents in rings A, B and C (Pyrzynska and Biesaga, 2009; Lachman et al., 2010).

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Nowadays, the various methods have been developed for the determination of flavonoids and other phenolic compounds in honey product, such as reversed phase high-performance liquid chromatography (RP-HPLC) (Yao et al., 2004; Kurtagić et al., 2013), gas chromatography (Sanz et al., 2005) and capillary electrophoresis with different detectors (Aljadi and Kamaruddin, 2004). Two UV absorption bands are characteristic for flavonoids, one band with maximum in the range of 240-285 nm, is believed to arise from (A) ring in the flavonoid structure, and the other band with maximum in the range of 300-380 nm, probably come from the (B) ring:

Of all listed methods, the most used is HPLC method. It gives the opportunity of simultaneous identification and determination of flavonoids in different honey samples (Stefova et al., 2003; Petrus et al., 2011; Moniruzzaman, et al., 2014).

In the study of Al-Farsi et al. (2018) it was performed analysis and correlation between flavonoids and phenolic content, their color as well as antioxidant activity (AA) in 26 samples of the honey. It was found that Omani honey has richness in its color and phenolics compared to the other tested honey samples and that represent a good source of antioxidants. Ibrahimi and Hajdari (2020) investigated 100 honey samples from the various Kosovo region and examined the content of flavonoids and phenolics, but as well as the AA of these samples. Correlation analysis revealed that there is a positive correlation of phenolic and flavonoid contents with AA (Ibrahimi and Hajdari, 2020). Ciucure and Geană (2019) in their study found that all investigated honey samples had very similar qualitative, but different quantitative profile of flavonoids and phenolic acids, and bioactive characteristics, which is related with the honey floral source. Furthermore, honeys with rich color, such as honeydew, showed high composition of phenolics and bioactive characteristics which further implicated a potential therapeutic property compared to the other investigated floral honeys.

Furthermore in the study of Tkáč et al. (2022) was found that the origin and botanical source of honeys had significant effect on the biological value and quality of honey.

Due to the very valuable properties of the honey, there are a lot of studies due to their biological properties (Maleki et al., 2019), as mentioned before in this section. But there is still a lack of this kind of researchers for honey from various regions. One of the region in which is important to determine correlation of antioxidant activity and flavonoids content is Bosnia and Herzegovina. Bosnia and Herzegovina is a country with a various local beekeepers which are producing a honey. But there is not enough studies focusing on honey research and determination of its quality.

With purposes to give more information on Bosnian-Herzegovinian honey quality, objectives of this were identification and quantification of targets flavonoids apigenin, chrysin, hesperetin, kaempferol, luteolin, naringenin, and quercetin in even 49 samples of honey from different regions in B&H by high performance liquid chromatography with diode array detector (HPLC-DAD). Furthermore, content of flavonoids was compared to the antioxidant activity determined in previous study of Tahirović et al., (2017), which have used the same honey samples as in this research. In that research was found that analyzed honey samples showed significant antioxidant activity against hydroxyl and peroxyl free radicals (Tahirović et al., 2017). Considering results from mentioned research and current research the correlations between flavonoids content and antioxidant activity for the same honey samples was examined and evaluated by statistical analysis.

MATERIALS AND METHODS

Chemicals: All pure, standard flavonoids were purchased from the same manufacturer (Sigma Aldrich), and they were of the following purity: apigenin, ≥95% (HPLC), chrysin, ≥98% (HPLC), hesperetin, ≥95% (HPLC), kaempferol, 97% (HPLC), luteolin, ≥98% (TLC solid), naringenin, 98% (HPLC), quercetin, ≥94%, rutin, ≥94% (HPLC), vitexin, ≥95% (HPLC). Acetonitrile, methanol HPLC grade (purity ≥99.9%) and acetic acid, 99.8% p.a. were also obtained from Sigma Aldrich. Deionized water was produced on the instrument Milli-Q Water Purification System (Millipore Corporation). SPE-C18 cartridges for extraction (6 mL/500 mg), were purchased from Agilent Technologies, and 0.45 μm pore filter of regenerated cellulose obtained from Macherey - Nagel (Lot 8301).

Instrumentation

Chromatographic system Agilent Technologies LC 1200 (Fig. 2).

Preparation of standard solutions

Stock solutions (200 μg/ml) of flavonoids apigenin, chrysin, kaempferol, luteolin, hesperetin, naringenin, quercetin, and flavonoid glycosides rutin and vitexin were prepared in methanol. The working solutions in concentrations 2.5, 5, 10, 25 and 50 μg/ml were prepared.

Sampling

In the period from 2013 to 2015, 49 samples of bee honey were collected from different locations in Bosnia and Herzegovina. Because the impact of botanical origin on

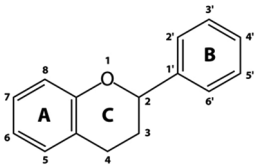

Fig 1. Basic structure of flavonoids.

Fig 2. HPLC chromatographic system. (Agilent Technologies LC 1200)

honey's quality was not the subject of this study, we did not specify the exact locations. A sample collection has been done randomly after a period of honey ripening. Various types of honey declared by the manufacturer namely meadow honey (22 samples), forest honey (10), acacia honey (7), chestnut honey (5), and heather honey (5 samples) were collected.

Sample preparation

The method of honey samples preparation for determination of content of flavonoids apigenin, chrysin, kaempferol, luteolin, hesperetin, naringenin, quercetin, and flavonoid glycosides rutin and vitexin was performed according to Kurtagić et al. (2013): 5 g of honey sample was dissolved in 10 ml of deionized water and then stirred vigorously. pH values of obtained honey solutions were measured at 25 °C. All pH values of samples were adjusted to 2.0 with HCl solution 1 mol/dm^3. The solutions are then passed through the pre-prepared solid phase extraction (SPE) - C18/500 mg column with a flow rate of 1 ml/min (Fig. 3). SPE columns are activated according to the confirmed procedure. Samples were extracted with 2.0 ml of methanol and 1.0 ml of acetonitrile with the same flow rate of solvent (1 ml/min). All prepared solutions for HPLC analysis were previously filtered through 0.45 μm pore filter of regenerated cellulose.

Conditions of chromatography

Isocratic chromatography was performed on the column Eclipse XDB - C18 with reversed phase (4.6 mm x 250 mm, 5 μm). Mobile phase consisted of the following components:

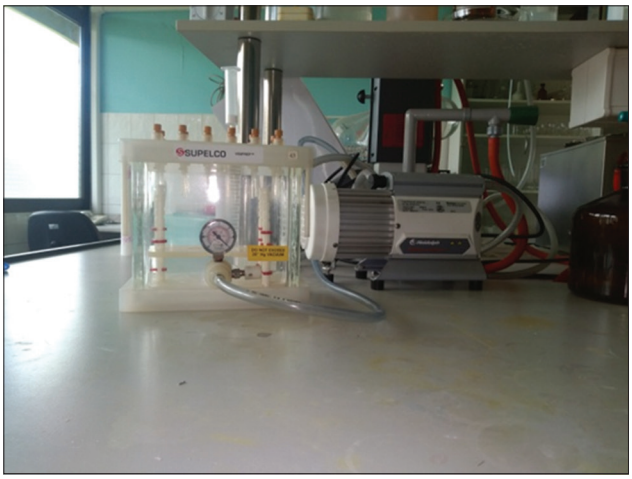

Fig 3. Apparatus for solid phase extraction (SPE).

5% aqueous solution of acetic acid and 99.9% methanol, in volume ratio 65: 35. Mobile phase was filtered through 0.45 μm pore filter. The following experimental parameters were used: flow rate of mobile phase was 0.5 ml/min, injection volume 20 μl and column temperature 35 °C. Quantitative and qualitative analysis of flavonoids after separation were done with DAD detector at 370 nm for kaempferol, quercetin, and its glycoside rutin, 340 nm for chrysin, luteolin and apigenin, and 290 nm for hesperetin, naringenin, and vitexin. After confirmation of the retention time (t_R) and UV spectra of standard substances, calibration curves with 5 points in the concentration range 2.5-50 mg/l were established. Linearity coefficients for the most of standard substances were $R^2 \geq 0.99$. All solutions were measured in triplicate. The stability of the two-month standard solutions, which were stored in the dark at +4 °C, were confirmed by HPLC analysis. Quantification of the target flavonoids was performed using method of external standard. The method used in this research was adapted in accordance with method used in the research of Chan et al. (2013).

Statistical analysis

Each sample of honey analysed in triplicate. The content of each flavonoid expressed in flavonoid mass per mass of honey (mg/kg$_{\text{of honey}}$) as average ± standard deviation, (S.D.). As statistical method, the Student's t-test was used in Microsoft Office Excel 2007. In case t-test value was <0.05, difference between flavonoid content for honey samples was statistically significant. For assessment of the correlations between total flavonoids content and antioxidant activity, coefficient of linearity (R^2) was used.

RESULTS AND DISCUSSION

a) Identification of flavonoids and glycosides

Flavonoids (kaempferol, naringenin, chrysin, apigenin, and luteolin) and glycosides (rutin and vitexin) were successfully

separated under the optimum chromatographic conditions. HPLC chromatogram of flavonoid standards is shown in the Fig. 2. The chromatograms of the samples showed a distinctly separation of flavonoids.

The retention times (t_R) of the flavonoids (kaempferol, naringenin, hesperetin, chrysin, apigenin, and luteolin) and flavonoid glycosides in standard solution and in honey samples are shown in the Table 1.

Naringenin, chrysin and luteolin were detected in all (five) types of analyzed honey (meadow, acacia, forest, heather, and chestnut). Naringenin was identified in 31 of 49 samples of honey, chrysin in 29, and luteolin in 15 samples of honey. Apigenin showed the lowest presence of the analyzed flavonoids. It was detected in 5 of 49 samples of honey, in 4 different types of honey (meadow, acacia, chestnut, and heather), but its glycoside vitexin was detected in most of the analyzed samples. Kaempferol was detected in 3 of 49 samples of honey, only in meadow honey. Hesperetin was detected in only one analyzed sample of honey (in acacia). Quercetin was not detected in any analyzed sample of honey by the used experimental conditions, but its glycoside rutin was detected in one third of the analyzed samples.

b) Quantification of flavonoids and glycosides

For the quantification of flavonoids, it was used area of the integrated peak in HPLC chromatogram of samples, and equation of calibrated curve for each flavonoid. For comparison of average content of flavonoid by the type of honey, we used only those with minimum three samples by the same type of honey. As statistical method, the Student's t-test was used.

By the total content (mg/kg$_{\text{of honey}}$) of five flavonoids (chrysin, naringenin, luteolin, kaempferol, and apigenin) and two glycosides found in analyzed honey samples the list was as in Table 2:

Chrysin

The highest average content of chrysin (mg/kg$_{\text{of honey}}$) was found in six samples of forest honey (2.57±1.76), followed by 13 samples of meadow honey (1.72±0.90), and six samples of acacia honey (1.10±0.74). The total content of chrysin, found in 25 of 49 samples of honey, was 5.39 mg/kg$_{\text{of honey}}$.

Naringenin

The highest average content of naringenin (mg/kg$_{\text{of honey}}$) was detected in 14 samples of meadow honey (1.58±1.25), followed by six samples of forest honey (1.06±0.76), five samples of chestnut honey (0.88±0.54), and four samples of acacia honey (0.75±0.77). The total content of naringenin, found in 29 of 49 samples of honey, was 5.27 mg/kg$_{\text{of honey}}$ (Table 2).

Table 1: The retention times of flavonoids and flavonoid glycosides in standard solution and in honey samples

Flavonoid/ glycoside	Standard solution (t_R) (min.)	Sample solution (t_R) (min.)
Vitexin	8.12	7.98
Rutin	10.18	10.61
Quercetin	28.18	-
Naringenin	30.88	31.15
Luteolin	35.33	33.99
Hesperetin	40.13	40.60
Kaempferol	53.05	52.85
Apigenin	59.70	60.31
Chrysin	86.70	86.75

Table 2: Total content of flavonoids and flavonoid glycosides in all analyzed honey samples

Flavonoid	Total content (mg/kg)
Chrysin	5.39
Vitexin	4.72
Naringenin	4.27
Luteolin	3.08
Rutin	1.37
Kaempferol	0.47
Apigenin	- *

*Apigenin was found in only five samples of analysed honey: In one sample of chestnut honey, in one sample of acacia honey, in one sample of meadow honey, and in two samples of heather honey. These values were not included in the statistical analysis.

Luteolin

The highest average content of luteolin (mg/kg$_{\text{of honey}}$) was found in four samples of meadow honey (1.64±0.84), followed by four samples of acacia honey (0.90±1.04), and four samples of forest honey (0.54±0.73). The total content of luteolin, found in 12 of 49 samples of honey, was 3.08 mg/kg$_{\text{of honey}}$ (Table 2).

Kaempferol

Kaempferol was found only in three samples of meadow honey with average content of 0.47±0.27 mg/kg$_{\text{of honey}}$, and this value included in the Table 2 as the total content for kaempferol (0.47 mg/kg$_{\text{of honey}}$).

Hesperetin

Hesperetin was detected in only one analyzed sample (acacia honey) with high content of 5.12 mg/kg$_{\text{of honey}}$, but it was not taken in the statistical analysis.

Apigenin

Apigenin was found in only five samples of analysed honey: in one sample of chestnut honey (0.71 mg/kg$_{\text{of honey}}$), in one sample of acacia honey (0.34 mg/kg$_{\text{of honey}}$), in one sample of meadow honey (0.31 mg/kg$_{\text{of honey}}$), and in

two samples of heather honey (0.28±0.15 mg/$kg_{of\ honey}$). These values were not included in the statistical analysis.

Because apigenin is flavon-aglycon of vitexin, which represented in most of the analyzed samples of honey (35 of 49), we supposed that in the period of harvesting of honey samples (May for acacia, and July-August for the other samples), biosynthesis of glycosides was very significant.

Vitexin

The highest average content (mg/$kg_{of\ honey}$) of vitexin was in 17 samples of meadow honey (1.67±2.05), followed by four samples of acacia honey (1.15±1.50), four samples of heather honey (0.84±0.97), four samples of forest honey (0.77±0.22), and the lowest average content of vitexin was found in three samples of chestnut honey (0.42±0.23). Total content of vitexin in all analysed honey sample was 4.72 mg/$kg_{of\ honey}$ (Table 2).

Quercetin

Quercetin was not detected in either sample of analyzed honeys at used experimental conditions. This compound is flavonol aglycon of rutin, which identified in 10 of 49 samples of honey (discussed in the next part of the manuscript).

Rutin

The highest average content of rutin (mg/$kg_{of\ honey}$) was found in four samples of acacia honey (0.57±0.64), followed by the average content of rutin in three samples of chestnut honey (0.52±0.30), and the average content of three sample meadow honey (0.28±0.27). The total content of rutin was 1.37 mg/$kg_{of\ honey}$ (Table 2).

Flavonoids in the honey samples were investigated by many authors. Lachman et al. (2010) were found that the main groups of flavonoids in honey samples were flavones, flavonols and flavanones. In our study, we analyzed the honey samples in Bosnia and Herzegovina to the presence of three flavones (apigenin, luteolin, and chrysin), two flavonols (quercetin and kaempferol), and two flavanones (hesperetin and naringenin). The most abundant were flavones: chrysin > luteolin > apigenin (Table 2). Flavonols and flavanones had a similar abundance (Table 2). The content of flavonoids, found in the investigated honey samples in our study, was very variable, according to the types of flavonoids and their quantities. Because quercetin was not detected in either sample of analyzed honey, we suggest other experimental conditions like gradient chromatographic elutions (Makawi et al., 2009; Kurtagić et al., 2013), or using the other detection system like HPLC coupled with coulometric electrode array detection and electrospray ionization mass spectrometry (HPLC/CEAD-ESI MS) (Petrus et al., 2011; Akimoto et al., 2017).

c) Comparison of flavonoid contents by the type of honey

Total contents of analysed flavonoids and flavonoid glycosides by the type of honey are shown in Table 3a (flavonoids: chrysin, naringenin, luteolin, kaempferol, and apigenin) and Table 3b (two flavonoid glycosides: vitexin and rutin). (it was taken in account only flavonoid/glycoside detected in minimum two samples of honey).

The average contents of three flavonoids (chrysin, naringenin, and luteolin) in meadow honey were statistically higher than in acacia honey ($p^{**}<0.01$). The average content of chrysin in forest honey was higher than chrysin content in acacia honey, but at the limit of statistical significance ($p=0.05$; Student's t-test). Naringenin content in forest honey was statistically significantly higher than naringenin content in chestnut honey ($p^{*}<0.036$; Student's t-test), and was higher than naringenin content in acacia honey, but at the limit of statistical significance ($p=0.05$; Student's t-test). The average content of luteolin in meadow honey was higher than luteolin content in forest honey, but at the limit of statistical significance ($p=0.05$).

The average content of rutin in four samples of acacia honey was similar to that of three samples of chestnut honey, but higher (non statistical) than the average content of rutin in three samples of meadow honey.

d) Correlations between flavonoid content and antioxidant activity of bee honey samples

Total flavonoid contents in five types of honey [meadow honey (MH), forest (FH), acacia (AH), heather (HH),

Table 3a: Total content of analysed flavonoids by the type of honey

Type of honey	Total flavonoids content (mg/kg)
MH 22[13(C), 3(K), 4(L), 14(N)]	5.41
FH 10[6(C), 4(L), 6(N)]	4.17
AH 7[6(C), 4(L), 4(N)]	2.75
HH 5[2(A), 2(C), 2(N)]	1.80
CH 5[2(C), 5(N)]	1.33

MH - meadow honey, FH – forest honey, AH – acacia honey, HH – heather honey, CH – chestnut honey. The number in front of the middle bracket indicates the total number of honey samples; The numbers in front of small brackets indicate the numbers of honey samples in which flavonoids apigenin (A), chrysin (C), kaempferol (K), luteolin (L), and naringenin (N) were detected and quantified.

Table 3b: Total content of two flavonoid glycosides by the type of honey

Type of honey	Total flavonoid glycosides (mg/kg)
MH 22[17(V), 2(R)]	2.04
AH 7[4(V), 4(R)]	1.72
FH 10[4(V), 2(R)]	0.99
CH 5[3(V), 3(R)]	0.94
HH 5[4(V)]	0.84

MH - meadow honey, AH – acacia honey, FH – forest honey, CH – chestnut honey, HH – heather honey. The number in front of the middle bracket indicates the total number of honey samples; The numbers in front of small brackets indicate the numbers of honey samples in which flavonoid glycosides vitexin (V), and rutin (R) were detected and quantified.

and chestnut (CH) honey] and belonging antioxidant capacity against peroxyl free radicals of both: hydrophilic low molecular fraction (supernatants, *s*) ($AC_{(ROO^{\bullet})s}$) and in bulk (noncentrifuged, *b*) ($AC_{(ROO^{\bullet})b}$) solution are shown in Table 4.

The correlations between total flavonoid contents (TFCs) in 3-5 types of honey and antioxidant capacity against peroxyl free radicals of both: hydrophilic low molecular fraction (supernatants) ($AC_{(ROO^{\bullet})s}$) and of bulk (noncentrifuged) solution ($AC_{(ROO^{\bullet})b}$) are shown in Table 5.

It is observed to exist low linear correlation between flavonoid content (FC) in five types of honey (MH, FH, AH, HH, CH) and antioxidant capacity against peroxyl free radicals in supernatants ($AC_{(ROO^{\bullet})s}$) of the same types of honey (R^2=0.308). If we omit MH, in that case linearity is high (R^2=0.920). If we correlate FC in only three types of honey (FH, AH, HH) and $AC_{(ROO^{\bullet})s}$ in supernatants of the same types of honey, linearity is very high (R^2=0.968), and for FH, AH, CH linearity is complete (R^2=1.000).

Table 4: Total flavonoid contents in five types of honey: meadow, forest, acacia, heather, and chestnut honey and belonging antioxidant capacity against peroxyl free radicals of both: hydrophilic low molecular fraction (supernatants, *s*) and in bulk (noncentrifuged, *b*) solution

Type of honey	Total flavonoids content (mg/kg)	$AC_{(ROO^{\bullet})s}$ (mM TE/g)*	$AC_{(ROO^{\bullet})b}$ (mM TE/g)*
MH 22[13(C), 3(K), 4(L), 14(N)]	5.41	5.62	7.61
FH 10[6(C), 4(L), 6(N)]	4.17	10.56	21.14
AH 7[6(C), 4(L), 4(N)]	2.75	7.16	7.87
HH 5[2(A), 2(C), 2(N)]	1.80	2.89	7.40
CH 5[2(C), 5(N)]	1.33	3.94	4.89

$AC_{(ROO^{\bullet})s}$-antioxidant capacity against peroxyl free radicals in supernatants $AC_{(ROO^{\bullet})b}$-antioxidant capacity against peroxyl free radicals in bulk (noncentrifuged) solution mM TE-millimole trolox equivalents *data previously reported (Tahirović et al., 2017) MH-meadow honey, FH-forest honey, AH-acacia honey, HH-heather honey, CH-chestnut honey. The number in front of the middle bracket indicates the total number of honey samples; The numbers in front of the small brackets indicate the numbers of detected and quantified flavonoids: C-chrysin, K-kaempferol, L-luteolin, N-naringenin, A-apigenin.

Table 5: The correlations between total flavonoids content (TFC) in 3-5 types of honey and antioxidant capacity against peroxyl free radicals of both: hydrophilic low molecular fraction (supernatants) ($AC_{(ROO^{\bullet})s}$) and in bulk (noncentrifuged) solution ($AC_{(ROO^{\bullet})b}$)

Number of the types of honey	Coefficient of linearity R^2 (TFC to $AC_{(ROO^{\bullet})s}$)	Coefficient of linearity R^2 (TFC to $AC_{(ROO^{\bullet})b}$)
$5_{(MH, FH, AH, HH, CH)}$	0.308	0.205
$4_{(FH, AH, HH, CH)}$	0.920	0.881
$3_{(FH, AH, HH)}$	0.968	0.863
$3_{(FH, AH, CH)}$	1.000	0.882

MH-meadow honey, FH-forest honey, AH-acacia honey, HH-heather honey, CH-chestnut honey.

If we correlate TFC and antioxidant capacity against peroxyl free radicals in bulk (noncentrifuged) solution ($AC_{(ROO^{\bullet})b}$) of the same types of honey, we have similar, but lower linearity (third column of the Table 5).

Total flavonoid contents in five types of honey: MH, FH, AH, HH, and CH and belonging antioxidant capacity against hydroxyl free radicals of both, hydrophilic low molecular fraction (supernatants, *s*) ($AC_{(OH^{\bullet})s}$) and in bulk (noncentrifuged, *b*) ($AC_{(OH^{\bullet})b}$) solution are shown in Table 6.

The correlations between TFCs in 3-5 types of honey and antioxidant capacity against hydroxyl free radicals of both: hydrophilic low molecular fraction (supernatants) ($AC_{(OH^{\bullet})s}$) and in bulk (noncentrifuged) solution ($AC_{(OH^{\bullet})b}$) are shown in Table 7.

The correlation does not exist between TFC and antioxidant capacity against hydroxyl free radicals either in supernatants ($AC_{(OH^{\bullet})s}$) nor in bulk (noncentrifuged) solution ($AC_{(OH^{\bullet})b}$) of five types of honey, but if we correlate TFC in only three types of honey (FH, AH, CH) and both antioxidant

Table 6: Total flavonoids content in five types of honey: MH, FH, AH, HH, and CH and belonging antioxidant capacity against hydroxyl free radicals of both: hydrophilic low molecular fraction (supernatants) ($AC_{(OH^{\bullet})s}$) and in bulk (noncentrifuged) solution ($AC_{(OH^{\bullet})b}$)

Type of honey	Total flavonoids content (mg/kg)	$AC_{(OH^{\bullet})s}$ (mM TE/g)*	$AC_{(OH^{\bullet})b}$ (mM TE/g)*
MH 17[13(C), 3(K), 4(L), 14(N)]	5.41	4.51	4.29
FH 10[6(C), 4(L), 6(N)]	4.17	7.59	5.35
AH 7[6(C), 4(L), 4(N)]	2.75	4.65	7.38
HH 5[2(A), 2(C), 2(N)]	1.80	8.46	11.94
CH 5[2(C), 5(N)]	1.33	4.72	1.66

MH-meadow honey, FH-forest honey, AH-acacia honey, HH-heather honey, CH-chestnut honey. mM TE-millimole trolox equivalents *data previously reported (Tahirović et al., 2017) The number before the middle bracket indicates the total number of honey samples; The numbers before small brackets indicate the numbers of detected and quantified flavonoids: A-apigenin, C-chrysin, K-kaempferol, L-luteolin, N-naringenin.

Table 7: The correlations between total flavonoids content (TFC) in 3-5 types of honey and antioxidant capacity against hydroxyl free radicals – hydrophilic low molecular fraction (supernatants) ($AC_{(OH^{\bullet})s}$) and bulk (noncentrifuged) solution ($AC_{(OH^{\bullet})b}$)

Number of the types of honey	Coefficient of linearity R^2 (TFC to $AC_{(OH^{\bullet})s}$)	Coefficient of linearity R^2 (TFC to $AC_{(OH^{\bullet})b}$)
$5_{(MH, FH, AH, HH, CH)}$	0.023 (negativelly)	0.039 (negativelly)
$4_{(FH, AH, HH, CH)}$	0.079	0.113
$3_{(FH, AH, CH)}$	0.732	0.696

MH-meadow honey, FH-forest honey, AH-acacia honey, HH-heather honey, CH-chestnut honey.

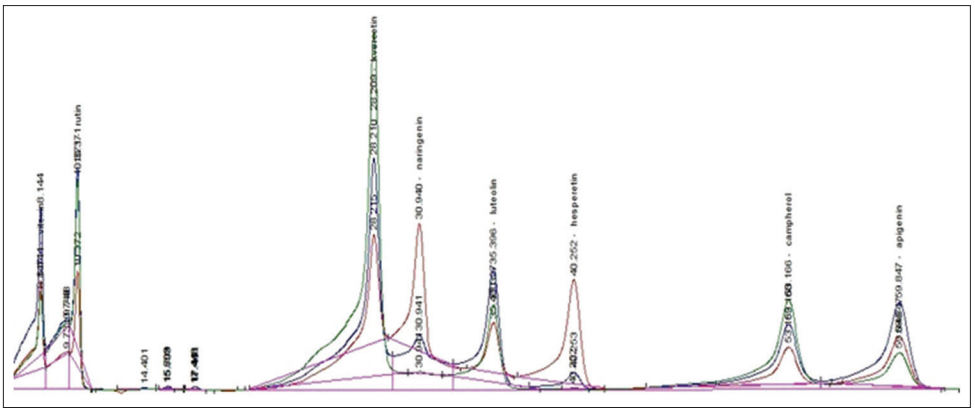

Fig 4. HPLC chromatogram of standard solution mixture containing rutin, kaempferol, quercetin, hesperetin, naringenin, vitexin, chrysin, apigenin and luteolin. The concentration of flavonoids and flavonoid glycosides were in the range 2.5-50 mg/l. (tR for chrysin was 86.70 min).

Table 8: Total flavonoids content in five types of honey: MH, FH, AH, HH, and CH and belonging total (against both, peroxyl and hydroxyl free radicals) hydrophilic antioxidant score of supernatants ($THAS_{(s)}$) and bulk (noncentrifuged) solution ($THAS_{(b)}$)

Type of honey	Total flavonoids content (mg/kg)	$THAS_{(s)}$ (mM TE/g)*	$THAS_{b}$ (mM TE/g)*
MH 17[13(C), 3(K), 4(L), 14(N)]	5.41	10.13	11.90
FH 10[6(C), 4(L), 6(N)]	4.17	18.15	26.49
AH 7[6(C), 4(L), 4(N)]	2.75	11.81	15.25
HH 5[2(A), 2(C), 2(N)]	1.80	11.35	19.34
CH 5[2(C), 5(N)]	1.33	8.66	6.55

MH-meadow honey, FH-forest honey, AH-acacia honey, HH-heather honey, CH-chestnut honey. mM TE-millimole trolox equivalents *data previously reported (Tahirović *et al*., 2017) The number before the middle bracket indicates the total number of honey samples; The numbers before small brackets indicate the numbers of detected and quantified flavonoids: (A)-apigenin, (C)-chrysin, (K)-kaempferol, (L)-luteolin, (N)-naringenin. *s* – supernatant fraction. *b* – bulk (noncentrifuged) solution

capacity ($AC_{(OH^{\bullet})s}$ and $AC_{(OH^{\bullet})b}$) of the same types of honey, linearity is moderate (R^2=0.732 and R^2=0.696, respectively).

Total flavonoid contents in five types of honey: MH, FH, AH, HH, and CH and belonging total (against both, peroxyl and hydroxyl free radicals) hydrophilic antioxidant score of supernatants $(THAS)_s$ and bulk (noncentrifuged) solution $(THAS)_b$ are shown in Table 8.

The correlations between TFCs in 3-5 types of honey and total hydrophilic antioxidant score of supernatants ($THAS_s$) and bulk (noncentrifuged) solution ($THASC_b$) are shown in Table 9.

If we correlate TFC and total hydrophilic antioxidant score (antioxidant activity against both: peroxyl and hydroxyl free radicals) in supernatants and in bulk (noncentrifuged) solution ($AC_{(THAS)s}$ and $AC_{(THAS)b}$) of the same types of honey, linearity is low for five types of honey (R^2=0.137

Table 9: The correlations between total flavonoids content (TFC) in 3-5 types of honey and total hydrophilic antioxidant score of supernatants ($THAS_{(s)}$) and bulk (noncentrifuged) solution ($THAS_{(b)}$)

Number of the types of honey	Coefficient of linearity R^2 (TFC to $THAS_{(s)}$)	Coefficient of linearity R^2 (TFC to $THAS_{(b)}$)
$5_{(MH, FH, AH, HH, CH)}$	0.137	0.080
$4_{(FH, AH, HH, CH)}$	0.929	0.200
$3_{(FH, AH, HH)}$	0.883	0.508
$3_{(FH, AH, CH)}$	0.964	0.995

MH-meadow honey, FH-forest honey, AH-acacia honey, HH-heather honey, CH-chestnut honey.

and R^2=0.080, respectively). If we omit MH, linearity is high for supernatants (R^2=0.929) and low for bulk solution (R^2=0.200). If we correlate TFC to $AC_{(THAS)s}$ and $AC_{(THAS)b}$ for only three types of honey (FH, AH, HH), linearity is also high for supernatants (R^2=0.883), moderate for bulk solution (R^2=0.508), and very high for FH, AH, CH (R^2=0.964 for supernatants, and R^2=0.995 for bulk solution).

CONCLUSION

In this study, it was performed identification and quantification of flavonoids (kaempferol, quercetin, hesperetin, naringenin, chrysin, apigenin, luteolin) in 49 samples of honey using HPLC-DAD as a method of analysis to separate, identify and to quantify flavonoids from honey samples. Also, we examined correlations between flavonoids content, determined in this experiment and antioxidant activity against both: hydroxyl and peroxyl free radicals, reported earlier for the same samples. Namely, it was established that B&H honey contains significant amounts of chrysin and luteolin (as flavones), and flavanone naringenin which is characteristic also for citrus honey. Flavonol quercetin was not detected in free form, but as a glycoside rutin in one third of the analyzed samples.

The most abundant flavonoid according to the type of analyzed honey samples was naringenin, then followed chrysin, luteolin, and the small abundant were apigenin, and kaempferol. Apigenin was significantly present in the form of glycoside vitexin (in most of the analyzed samples). The highest total content in all analyzed honey samples had chrysin, then followed naringenin, luteolin, and the smallest total content had kaempferol, and apigenin. The total average content of three flavonoids (chrysin, naringenin, and luteolin) in meadow honey was statistically higher than their content in acacia honey. The total average content of naringenin in forest honey was statistically higher than its content in chestnut honey.

Because we observed a high positive linear correlation between flavonoid content and both: antioxidant activity against peroxyl free radicals and total hydrophilic antioxidative score (antioxidant activity against both: peroxyl + hydroxyl free radicals) in supernatants of four honey types (forest honey, acacia, heather, and chestnut honey), it may be recommended to consume bee honey from B&H as a dietary supplement in order to preserve health.

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AUTHOR CONTRIBUTIONS

Research concept and design: Tahirović, I., Kurtagić, H. and Toromanović, J.; Collection of data: Tahirović, I., Kurtagić, H., Smječanin, N., Aldžić-Baltić, A. and Bajramović, Z.; Data analysis and interpretation: Tahirović, I., Kurtagić, H., Smječanin, N. and Čopra-Janićijević, A.; Writing the article: Tahirović, I., Kurtagić, H., Smječanin, N., Dizdar, M. and Buza, N.; Critical revision of the article: Tahirović, I., Kurtagić, H., Smječanin, N. and Toromanović, J.; Final approval of the article: Tahirović, I., Kurtagić, H., Smječanin, N., Aldžić-Baltić, A., Bajramović, Z., Toromanović, J., Čopra-Janićijević, A., Dizdar, M. and Buza, N.

REFERENCES

Adekunle, A. S., Aline, Afolabi and . Rocha. 2012. Determination of free phenolic acids, flavonoid contents and antioxidant capacity of ethanolic extracts obtained from leaves of mistletoe (*Tapinanthus globiferus*). Asian J. Pharm. Clin. Res. 5: 36-41.

Al-Farsi, M., A. Al-Amri, A. Al-Hadhrami and S. Al-Belushi. 2018. Color, flavonoids, phenolics and antioxidants of Omani honey. Heliyon, 4: e00874.

Akimoto, N., T. Ara, D. Nakajima, K. Suda, C. Ikeda, S. Takahashi, R. Muneto, M. Yamada, H. Suzuki, D. Shibata and N. Sakurai. 2017. Flavonoid search: A system for comprehensive flavonoid annotation by mass spectrometry. Sci. Rep. 7: 1243.

Aljadi, A. M. and M. Y. Kamaruddin. 2004. Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. Food Chem. 85: 513-518.

Angst, E., J. L. Park, A. Moro, Q. Y. Lu, X. Lu, G. Li, J. King, M. Chen, H. A. Reber, Go, G. Eibl and O. J. Hines. 2013. The flavonoid quercetin inhibits pancreatic cancer growth *in vitro* and *in vivo*. Pancreas. 42: 223-229.

Boots, A. W., H. Li, Schins, R. Duffin, Heemskerk, A. Bast and . Haenen. 2007. The quercetin paradox. Toxicol. Appl. Pharmacol. 222: 89-96.

Brusselmans, K., R. Vrolix, G. Verhoeven and J. V. Swinnen. 2005. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J. Biol. Chem. 280: 5636-5645.

Ciucure, C. T. and E. I. Geană. 2019. Phenolic compounds profile and biochemical properties of honeys in relationship to the honey floral sources. Phytochem. Anal. 30: 481-492.

Chan, C. W., B. J. Deadman, M. Manley-Harris, A. L. Wilkins, D. G. Alber and E. Harry. 2013. Analysis of the flavonoid component of bioactive New Zealand mānuka (*Leptospermum scoparium*) honey and the isolation, characterization and synthesis of an unusual pyrrole. Food Chem. 141: 1772-1781.

Ferreres, F., F. A. Tomás-Barberán, C. Soler, C. García-Viguera, A. Ortiz and F. Tomás-Lorente. 1994. A simple extractive technique for honey flavonoid HPLC analysis. Apidologie. 25: 21-30.

Gheldof, N., Wang and N. J. Engeseth. 2002. Identification and quantification of antioxidant components of honeys from various floral sources. J. Sci. Food Agric. 50: 5870-5877.

Ginwala, R., R. Bhavsar, D. G. I. Chigbu, P. Jain and Z. K. Khan. 2019. Potential role of flavonoids in treating chronic inflammatory diseases with a special focus on the anti-inflammatory activity of apigenin. Antioxidants. 8: 35.

Ibrahimi, H. and A. Hajdari. 2020. Phenolic and flavonoid content, and antioxidant activity of honey from Kosovo. J. Apic. Res. 59: 452-457.

Ivey, K. L., M. K. Jensen, J. M. Hodgson and A. H. Eliassen. 2017. Association of flavonoid-rich foods and flavonoids with risk of all-cause mortality. Br. J. Nutr. 117: 1470-1477.

Johnston, G. A. R. 2015. Flavonoid nutraceuticals and ionotropic receptors for the inhibitory neurotransmitter GABA. Neurochem. Int. 89: 120-125.

Kedika, B., K. Thotla, V. Noole and K. R. Chepyala. 2016. Research progress of chrysin derivatives with potential biological activities. J. Chem. Pharm. Res. 8: 1210-1222.

Kurtagić, H., S. Redžić and M. Memić. 2013. Identification and quantification of quercetin, naringenin and hesperetin by RP LC-DAD in honey samples from B&H. Glas. Chem. Tehnol. Bosne Herceg. 40: 25-30.

Kurtagić, H., S. Barudanović and V. Durmić. 2015. Determination of rutin, quercetin, naringenin and hesperetin in the honey from Bosnia and Herzegovina (B & H) in relation to the composition of pollen. J. Environ. Sci. Eng. A. 4: 615-622.

Lachman, J., M. Orsak, A. Hejtmankova and E. Kovarova. 2010. Evaluation of antioxidant activity and total phenolics of selected Czech honeys. LWT-Food Sci. Technol. 43: 52-58.

López-Lázaro, M. 2009. Distribution and biological activities of the flavonoid luteolin. Mini-Rev. Med. Chem. 9: 31-59.

Maleki, S. J., J. F. Crespo and B. Cabanillas. 2019. Anti-inflammatory

effects of flavonoids. Food Chem. 299: 125124.

Makawi, S. Z. A., E. A. Gadkariem and S. M. H. Ayoub. 2009. Determination of antioxidant flavonoids in Sudanese honey samples by solid phase extraction and high performance liquid chromatography. J. Chem. 6: S429-S437.

Moniruzzaman, M., C. Y. An, P. V. Rao, M. N. I. Hawlader, S. A. B. M. Azlan, S. A. Sulaiman and S. H. Gan. 2014. Identification of phenolic acids and flavonoids in mono-floral honey from Bangladesh by high performance liquid chromatography: Determination of antioxidant capacity. Biomed Res. Int. 2014: 737490.

Panche, A. N., A. D. Diwan and S. R. Chandra. 2016. Flavonoids: An overview. J. Nutri. Sci. 5: 47.

Petrus, K., H. Schwartz and G. Sontag. 2011. Analysis of flavonoids in honey by HPLC coupled with coulometric electrode array detection and electrospray ionization mass spectrometry. Anal. Bioanal. Chem. 400: 2555-2563.

Pyrzynska, K. and M. Biesaga. 2009. Analysis of phenolic acids and flavonoids in honey. Trends Anal. Chem. 28: 893-902.

Sanz, M. L., M. Gonzalez, C. De Lorenzo, J. Sanz and I. Martinez-Castro. 2005. A contribution to the differentiation between nectar honey and honeydew honey. Food Chem. 91: 313-317.

Stefova, M., T. Stafilov and S. Kulevanova. 2003. HPLC analysis of flavonoids. In: Encyclopedia of Chromatography. Marcel Dekker, Inc., New York, NY, USA. p1-7.

Tahirović, I., DŽ. Helbet, A. Gaštan, N. Buza, M. Dizdar, A. Topčagić, J. Toromanović, A. *Čopra-Janićijević* and H. Kurtagić. 2017. Hydrophilic antioxidant scores against hydroxyl and peroxyl radicals in honey samples from Bosnia and Herzegovina. In: A. Badnjevic (Eds.), IFMBE Proceedings. Vol. 62. Springer Nature, Singapore Pvt Ltd. p429-434.

Tkáč, M., L. Vorlová, I. Borkovcová and J. Golian. 2022. Physicochemical and bioactive characterization of beekeeper and market honeys. Emir. J. Food Agric. 34: 268-278.

Yao, L., Y. Jiang, B. D'Arcy, R. Singanusong, N. Datta, N. Caffin and K. Raymont. 2004. Quantitative high performance liquid chromatography analyses of flavonoids in Australian *Eucalyptus* honeys. J. Agric. Food Chem. 52: 210-214.

Zheng, W. and S. Y. Wang. 2001. Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food. Chem. 49: 5165-5170.