Antimicrobial and Antioxidant Activity of Natural Honeys of Different Origin

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Abstract
To examine the antimicrobial and antioxidant activity of 15 natural honeys, honey samples were collected from different locations of Slovakia, Poland and Serbia. For antimicrobial activity determination honey solutions were prepared at three concentrations: 50, 25 and 12.5 % (by mass per volume). The potential antimicrobial activity of selected samples against four species of bacteria (*Escherichia coli* CCM 3988, *Pseudomonas aeroginosa* CCM 1960, *Staphylococcus epidermis* CCM 4418, *Bacillus cereus* CCM 2010) and two species of yeasts (*Saccharomyces cerevisiae* CCM 8191, *Candida albicans* CCM 8216) was studied using the disc diffusion method. After incubation, the zones of inhibition of the growth of the microorganisms around the disks were measured. The strongest antimicrobial activity was shown at honey samples of 50 % concentration against *Escherichia coli*, *Pseudomonas aeroginosa* and *Staphylococcus epidermis*. Against *Saccharomyces cerevisae* and *Candida albicans* very low (at 50 %, 25 % concentration) or zero antifugal (at 12.5 % concentration) activity was determined. From the results obtained it was shown the variable ability of honey samples to scavenge stable free radical DPPH. $TEAC_{DPPH}$ values ranged between 0.1-1.0 $mmol.kg^{-1}$. As the antioxidative best source buckwheat honey was manifested and the lowest antioxidant activity was shown at acacia honey.

Keywords: antimicrobial activity, antioxidants, bacteria, DPPH, natural honey, yeasts

1. Introduction

Honey is a natural product with many attributes that are useful for humans. The consumption of honey is increasing because of its beneficial biological properties, including antioxidant and antibacterial activities. It is likely that these apian products have important biological properties inherited from specific floral sources, but it is necessary to conduct further chemical analyses to identify and characterize these biological attributes [1]. Honey has been widely accepted as food and medicine by all generations, traditions, and civilizations, both ancient and modern. For at least 2700 years, it has been used by humans to treat a variety of ailments through topical application, but only recently have the antiseptic and antimicrobial properties of honey been discovered. Honey has been reported to be effective in a number of human pathologies. A large number of *in vitro* and limited clinical studies have confirmed the broad-spectrum antimicrobial (antibacterial, antifungal, antiviral,

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and antimycobacterial) properties of honey, which may be attributed to the acidity (low pH), osmotic effect, high sugar concentration, presence of bacteriostatic and bactericidal factors (hydrogen peroxide, antioxidants, lysozyme, polyphenols, phenolic acids, flavonoids, methylglyoxal, and bee peptides), and increase in cytokine release, and to immune modulating and anti-inflammatory properties of honey; the antimicrobial action involves several mechanisms [2]. Results of Katiraee et al. (2013) showed a direct correlation between the type of plant nectar for honeys and antifungal properties. It seems that appropriate concentrations of honey can be used for controlling and treating of dermatophytosis and dermatomycosis [3]. On the other side antioxidant properties of various materials such as fruits and several plant products were subject of a lot of studies [4-5]. Therefore the purpose of this study was to examine the antimicrobial and antioxidant activity of 15 natural honeys originating from different sources and areas.

2. Materials and methods

Honey samples (Table 1) were collected from different locations of Slovakia (6 samples), Poland (5 samples) and Serbia (4 samples) and were obtained directly from local beekeepers.

Table 1. Investigated honey samples and their characteristics

No.	Sample	Origin
1	forest	Serbia (Milevici), altitude 800 m,
2	forest	Serbia (Babine), altitude 1250 m
3	forest	Serbia (Jabuka), altitude 1250 m
4	floral	Poland
5	heather	Poland
6	floral	Poland
7	floral (rape)	Slovakia (Nitra)
8	acacia	Slovakia (Nitra)
9	floral	Slovakia (Michalovce)
10	forest raspberry	Slovakia (Bystrá)
11	forest	Serbia, altitude 1000 m
12	buckwheat	Poland
13	floral	Poland
14	floral	Slovakia (Stupava)
15	honeydew	Slovakia (Relov)

Methods

Antimicrobial activity determination

Honey solutions were prepared in three concentrations: 50, 25 and 12.5 % (by mass per volume). The samples of each honey (10 g) and sterile water were stored at 37°C for 30 min before mixing, to facilitate homogenization. 50 % (mass per volume) solutions thus prepared were diluted to 25 % and 12.5 %. The samples were assayed immediately after dilution. The potential antibacterial activity of 15 selected natural honeys against four species of bacteria and two species of yeasts were studied using the agar well diffusion method.

Culture Media and Inoculum

The bacterial strains were purchased from the Czech Collection of Microorganisms (CCM). The strains of bacteria *Escherichia coli* CCM 3988, *Pseudomonas aeroginosa* CCM 1960, *Staphylococcus epidermis* CCM 4418, *Bacillus cereus* CCM 2010, *Saccharomyces cerevisiae* CCM 8191, *Candida albicans* CCM 8216 were maintained on Muller Hinton agar for bacteria and Sabuabord agar for yeast (MHA, SA, HiMedia). The concentration of microbial inoculum was determined by viable count serial dilutions within the range of 10^6 CFU/mL. The antimicrobial effect of the natural honey was tested in two experiments using the agar well diffusion method. The overnight microbial cultures were used for surface inoculation of Petri dishes containing 15 mL of Muller Hinton agar and Sabuabord agar. Each Petri dish was swabbed on with 0.5 mL of strain inoculum streaked thoroughly all over the surface of the agar. Subsequently, four equidistant wells, 6 mm in diameter each, were punched into the inoculated medium with sterile glass Pasteur pipettes and were filled up with 250 μL of honey using a precise eppendorf. All plates were

incubated at 37°C for bacteria and 25°C for yeasts and inhibition zones were measured after 24 hours. Six different strains of microorganism were tested in sets of plates, which were simultaneously processed for each strain. Both experiments were repeated three times, including two controls with plain methyl blue and sterilized distilled water every time. After incubation, the zones of inhibition of the growth of the microorganisms around the disks were measured. The mean values of both experiments and standard deviations were calculated.

Antioxidant activity determination

Samples (2 g) were mixed with 10 ml of distilled water. The prepared mixture was centrifuged for 5 minutes at 10 000 / min and a temperature of 20° C. Extracts prepared (20%) were stored during the experiments in a refrigerator at 4-6°C. Experiments were performed either directly, or samples were further diluted before measurement as needed.

DPPH test of honey

Solution of DPPH free radical was prepared daily prior to measurement by mixing 3.9 mg of DPPH (1,1-diphenyl-2-picrylhydrazyl) with 100 ml of 96% ethanol [6]. Exactly 1 ml of aqueous solution of honey was mixed directly in a cuvette with 1 ml of free radical DPPH. During the reaction time 10.5 minutes, reaction was monitored and the decrease in absorbance of the solution at 515 nm was monitored.

The results were converted into values of Trolox equivalent (TEAC) because of more objective comparison of radical-quenching activity with any possible dilution of the sample according to the following formula:

$$TEAC._{DPPH} = \frac{(A_{0(.\ DPPH)} - A_{t(.\ DPPH)}) \cdot V_{(.\ DPPH)}}{\varepsilon_{515} \cdot V_{(vzorky)}} \cdot \nu \cdot Z$$

Statistical evaluation

The mean values and standard deviations were calculated. Analysis of data was performed with analysis of variance (ANOVA), Fisher's least significant difference (LSD), and Pearson's correlation coefficient.

3. Results and discussion

The strongest antimicrobial activity was shown at honey samples of 50% concentration against *Escherichia coli* (forest samples, honeydew and heather sample) followed *Pseudomonas aeroginosa* (forest samples, honeydew and heather sample) and against *Staphylococcus epidermis* (forest samples and honeydew). Very low (at 50%, 25% concentration) or zero antifugal (at 12.5% concentration) activity was determined against *Saccharomyces cerevisae* and *Candida albicans*. At 50% of concentration of honey, antifugal activity was shown by forest samples and honeydew sample as well. Mean values of honey assessment are shown in the table 1.

The factors responsible for the antimicrobial activity of honey are high osmolarity, acidity, and particularly hydrogen peroxide [7]. The relative contribution of the peroxide and non-peroxide components in the total antibacterial activity of fresh honeys was investigated in the study of Elbanna et al. [8]. The antibacterial activity of honeys was mainly attributed to non-peroxide antibacterial agents, wherein their contribution was ca. 88%, while the contribution of H_2O_2 was only 12%. The contribution of the thermostable antibacterial components in honey was ca. 86.8%. The antibacterial activity of the fresh clover honey was compared with the effect of 16 antibiotics on indicator bacteria. Clover honey exhibited antibacterial activity comparable to that exhibited by the tested antibiotics [8]. Water dilution in our case had no positive effect on antimicrobial activity of honey. The highest antimicrobial activity at our samples was determined in 50 % concentration, less in case of 25% concentration (table 2) and the lowest at 12.5% (not shown). At 25% concentration of honeys no antifugal activity was detected (except honeydew sample) against *S. cerevisiae* and *C. albicans*.

Elbanna et al. [8] state that types of honey exhibited various degrees of antibacterial activity against different indicator bacteria. Different species of bacteria differed in their sensitivity to honey, wherein *Salmonella enteritidis* was the most sensitive followed by *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*, respectively. Compared to our results the most sensitive among selected microorganisms were *E. coli*, *P. aeroginosa* and *S. epidermis*. The inhibition zones of honey samples at *E. coli* varied

from 7.67 mm (floral) to 13 mm (forest sample), at P. *aeroginosa* from 6.00 mm (floral) to 12.67 mm (forest) and at *S. epidermis* from 6 (floral) to 11.67 mm (honeydew).

Table 2. The antimicrobial activity (mm) of honeys at 50% concentration against the tested strains

Sample[1]	*Escherichia coli*	*Pseudomonas aeroginosa*	*Staphylococcus epidermis*	*Bacillus cereus*	*Saccharomyces cerevisiae*	*Candida albicans*
1	11.33	11.00	10.00	8.00	1.67	2.00
2	13.00	12.67	10.67	10.00	2.67	1.00
3	11.00	11.00	11.00	10.33	2.00	1.33
4	10.33	9.67	9.00	7.00	ND	ND
5	11.67	11.00	9.00	8.33	ND	ND
6	7.67	6.67	8.00	5.00	ND	ND
7	9.00	8.00	8.00	8.00	ND	ND
8	8.00	6.67	7.33	7.33	ND	ND
9	8.00	6.00	7.67	8.00	ND	ND
10	9.00	10.00	8.00	8.33	ND	ND
11	12.00	11.67	10.67	11.00	2.00	2.33
12	8.00	8.00	8.00	9.33	ND	ND
13	9.00	8.00	6.00	7.67	ND	ND
14	7.67	8.00	7.67	7.00	ND	ND
15	12.67	11.67	11.67	9.00	1.66	2.00

[1]**1** forest (Serbia), **2** forest (Serbia), **3** forest (Serbia), **4** floral (Poland), **5** heather (Poland), **6** floral (Poland), **7** floral, rape (Slovakia), **8** acacia (Slovakia), **9** floral (Slovakia), **10** forest raspberry (Slovakia), **11** forest (Serbia), **12** buckwheat (Poland), **13** floral (Poland), **14** floral (Slovakia), **15** honeydew (Slovakia), ND- not detected

Among the tested honeys, forest samples (no. 1-3,11) and honeydew sample showed interesting antibacterial activity against all selected bacteria at 25% concentration of honeys as well.

In study of Zahoor et al. [9] honeys of *Acacia modesta*, *Prunus persica*, *Zizyphus sativa* and *Isodon rogosus* plants were tested against two Gram-positive bacterial strains (*Staphylococcus aureus* and *Bacillus cereus*), two Gram-negative bacterial strains (*Klebsilla pneumonia* and *Escherichia coli*) and two fungal strains (*Alternaria alternata* and *Trichoderma harzianum*) through agar well diffusion method. The tested honeys showed high antimicrobial activities to the tested bacterial and fungal strains. All the tested honeys were more active against Gram-negative bacterial strains than the Gram-positive bacterial strains. They showed lower activity against the tested fungal strains as compared to all the tested bacterial strains as it was confirmed in our case [9]. Antibacterial activity of all our samples of honeys was confirmed as the lowest at 12.5 % concentration of honeys (not shown).

Different monofloral honeys from Spain have been studied by León-Ruiz et al. [10] in order to determine their main functional and biological properties. Thyme honey and chestnut honey possess the highest antioxidant capacity, while chestnut honey showed high antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, others had no activity against *S. aureus* and showed very small activity against *E. coli*. Moreover it was found that the antimicrobial activity measured in chestnut honey was partly due to its lysozyme content. In addition the angiotensin I-converting enzyme (ACE) inhibitory activity was measured, and the ACE inhibition is one mechanism by which antihypertensive activity is exerted in vivo [10].

The antimicrobial activity of five Finnish honey products against important human pathogens *Streptococcus pneumoniae*, *S. pyogenes*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* were analyzed. Significant antimicrobial activity against all the tested pathogens was found from willow herb (*Epilobium angustifolium*), heather (*Calluna vulgaris*), and buckwheat (*Fagopyrum esculentum*) honeys [11]. Buckwheat

and heather honeys were also subjects of our research and we can confirm interesting antimicrobial activity (Table 2).

Table 3. The antimicrobial activity (mm) of honeys at 25% concentration against the tested strains

Sample[1]	*Escherichia coli*	*Pseudomonas aeroginosa*	*Staphylococcus epidermis*	*Bacillus cereus*	*Saccharomyces cerevisiae*	*Candida albicans*
1	8.00	5.00	6.30	5.00	ND	0.33
2	10.00	10.00	8.00	5.67	ND	ND
3	10.67	8.00	8.00	6.67	ND	ND
4	6.00	6.67	6.00	5.00	ND	ND
5	9.00	8.30	5.67	5.33	ND	ND
6	5.30	5.00	5.00	2.67	ND	ND
7	5.67	5.00	5.00	5.67	ND	ND
8	5.30	5.00	5.00	5.00	ND	ND
9	6.30	4.67	6.00	5.67	ND	ND
10	6.00	7.67	6.00	6.33	ND	ND
11	9.30	9.37	7.30	6.67	ND	ND
12	6.00	5.30	6.00	6.30	ND	ND
13	8.00	5.67	4.33	5.30	ND	ND
14	5.67	6.00	4.67	5.30	ND	ND
15	8.30	8.67	8.00	6.67	1.00	1.00

[1]**1** forest (Serbia), **2** forest (Serbia), **3** forest (Serbia), **4** floral (Poland), **5** heather (Poland), **6** floral (Poland), **7** floral, rape (Slovakia), **8** acacia (Slovakia), **9** floral (Slovakia), **10** forest raspberry (Slovakia), **11** forest (Serbia), **12** buckwheat (Poland), **13** floral (Poland), **14** floral (Slovakia), **15** honeydew (Slovakia), ND – not detected

The antioxidant activity of honey samples measured by the test against DPPH radical

Generally, antioxidant effect of our samples of honeys determined by DPPH method, increased in the order: acacia‹ floral‹ floral‹ floral‹ heather‹ floral‹ forest‹ floral‹ floral‹ honeydew‹ forest‹ raspberry‹ forest‹ forest‹ buckwheat.

Testing the effect of individual honey samples on the values of DPPH method by one-way analysis of variance showed that the sample has a highly statistically significant effect (Table 4).

Table 4. Analysis of variance (ANOVA) Table for DPPH (mmol.kg^{-1}) by Sample

Source of variability	Sum of Squares	Df	Mean Square	F-Ratio
Between groups	1.76	14	0.13	1139.16**
Within groups	0.00	15	0.00	
Total	1.76	29		

** statistically significant at $\alpha<0.01$; df - degree of freedom; n=30

Multi-species flowery honey samples from Slovak and Polish areas showed the lowest values of antioxidant activity. The results of DPPH method at flowery (multi-species) honeys originating from Poland ranged from 0.18 to 0.4 mmol.kg^{-1} $TEAC_{DPPH}$ while results of Slovak flowery honeys ranged from 0.19 to 0.37 mmol.kg-1, that are very similar values. Forest honeys originating from Serbia were in quenching DPPH radical more effective, values ranged from 0.34 to 0.85 mmol.kg^{-1}.

By further testing we observed the differences among samples of honeys. Several homogeneous groups were established and are shown in the Table 5.

Acacia honey in our study demonstrated antioxidant activity 0.13 mmol.kg^{-1} what was the lowest value among observed honeys. At assessing the antioxidant effect of herbal extracts in our previous study [12] acacia flower extract was also determined as the antioxidative weakest sample. In Slovenia with using the FRAP and the

DPPH method, results showed that the antioxidant activity varies depending on the different types of honey. Similarly in this study the honey from acacia belonged to the antioxidative weakest sample. Dark honeys were shown to be the antioxidative best samples [13].

Higher levels of antioxidant activity (0.66-0.85 mmol.kg^{-1}) have been detected at samples of forest honeys originating from Serbia from the same altitude 1250 m from Babine (n. 2) and Jabuka (n. 3) and are statistically different (Table 4). Dark honeys are often a rich source of vitamins and minerals, but their variation in the content of the various honeys is large [14].

On the other side, there were observed samples from the multi-species flowery group of honeys (no. 13,14) and floral rape (7), originating from different areas but were determined to be statistically in the same homogeneous group (Table 5).

Table 5. The mean values of DPPH method determined in honeys and homogeneous groups based on Fisher's test

Sample[2]	DPPH (mmol.kg^{-1}) Mean [1]		Conf. limits for Mean -95 %	Conf. limits for Mean +95 %
8	0.13	a	0.12	0.15
13	0.18	b	0.17	0.20
7	0.19	b	0.17	0.20
14	0.20	b	0.18	0.21
5	0.25	c	0.23	0.26
6	0.29	d	0.27	0.31
11	0.34	e	0.32	0.35
9	0.37	f	0.35	0.38
4	0.40	g	0.39	0.42
15	0.41	gh	0.39	0.42
1	0.43	h	0.41	0.45
10	0.50	i	0.48	0.51
2	0.66	j	0.64	0.68
3	0.85	k	0.84	0.87
12	0.99	l	0.97	1.01

[1]values in the same column with different letters are significantly different ($\alpha<0.05$)

[2]**1** forest (Serbia), **2** forest (Serbia), **3** forest (Serbia), **4** floral (Poland), **5** heather (Poland), **6** floral (Poland), **7** floral, rape (Slovakia), **8** acacia (Slovakia), **9** floral (Slovakia), **10** forest raspberry (Slovakia), **11** forest (Serbia), **12** buckwheat (Poland), **13** floral (Poland), **14** floral (Slovakia), **15** honeydew (Slovakia)

Honeydew honey from Slovakia in our study demonstrated antioxidant activity of 0.41 mmol.kg^{-1} that can be classified as effective antioxidant sample, in a position between the forest and floral honeys.

The highest antioxidant activity among our samples was detected buckwheat honey (0.99 mmol.kg^{-1}). Following the results of Ivanišová and Fikselová [15] the highest antiradical activity by DPPH method achieved also sample of buckwheat extract, confirming its higher values at other antioxidant method as well. High antioxidative value is mainly due to flavonoids containing, which represent a major group of natural antioxidants in buckwheat. Phenolic acids are also represented such as coumaric acid, vanillic, hydroxybenzoic, caffeic acid etc.

4. Conclusions

Honey has a potent antimicrobial activity against bacteria and fungi. The strongest antimicrobial activity was shown at honey samples of 50% concentration against strains of *Escherichia coli*, *Pseudomonas aeroginosa* and *Staphylococcus epidermis*. Very low (at 50%, 25% concentration) or zero antifugal (at 12.5% concentration) activity was determined against *Saccharomyces cerevisae* and *Candida albicans*. At 50% of concentration of honey, antifungal (anti-yeast) activity was shown by forest samples and honeydew sample as well.

Regarding the antioxidant potency, the samples of honeys which were found to be effective in antimicrobial activity (forest, honeydew) were found to be also effective in antiradical activity. Floral sources were observed to be low at

antioxidant effect as well as at antimicrobial one. Significant impact of locality has not been confirmed.

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