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EVALUATION OF THE PHENOLIC CONTENT, ANTIRADICAL, ANTIOXIDANT, AND ANTIMICROBIAL ACTIVITY OF DIFFERENT FLORAL SOURCES OF HONEY

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Thirty-five honeys were evaluated for total phenolic content by Folin-Ciocalteu method, for potential antioxidant activity using phosphomolibdenum assay and by the 1,1-diphenyl-2-picrylhydrazyl method for antiradical activity. The antimicrobial activity was studied by the agar diffusion method, using 12 bacteria and 2 yeasts. The means of the total phenolic contents of chestnut, honeydew, multifloral, thyme, and astragalus were 47 ± 18, 24.2 ± 0.6, 14 ± 11, 11 ± 6, and 9 ± 7 mg/100 g honey as gallic acid equivalent, respectively. The lowest antioxidant activity was observed in honeydew 70 ± 5 mg ascorbic acid equivalent/g honey while the highest content was observed in astragalus honey 86 ± 16 mg ascorbic acid equivalent/g honey. Correlation between the phenolic content and antioxidant activity were found to be statistically significant. Chestnut honeys (n = 5) exhibited maximum free radical scavenging activity with an average 68 ± 9%. The honey samples showed the highest antimicrobial activity against some microorganisms, especially* Escherichia coli *O157:H7,* Salmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes, *and* Proteus mirabilis*. On the other side,* Aeromonas hydrophila, Bacillus subtilis*, and* E. coli *were the most resistant microorganisms. The results revealed that the Turkish honeys studied proved to be a good source of antioxidant and antimicrobial agents that might serve to protect human health.

Keywords: ***Turkish honey, Antimicrobial activity, Antioxidant activity, Antiradical activity, Phenolic content.***

INTRODUCTION

Nowadays there is overwhelming evidence to indicate that free radicals cause oxidative damage to lipid, protein, and nucleic acids, and lead to many biological complications, including carcinogenesis, mutagenesis, aging, and atheroschlerosis.[1] Antioxidant compounds counteract these damaging effects. Honey contains mostly carbohydrates (70–80%), but also smaller amounts of a great variety of different other compounds. Especially, it contains different antioxidant substances, such as polyphenols, ascorbic acid and other organic acids, hydrogen peroxide, hydroxymethylfurfural, and amino acids.

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These compounds are found to a different extent in honey, depending mostly on the botanical origin of honey. Of all of these factors, only the polyphenol content correlates significantly to the antioxidant capacity of honey.[2] Beretta et al.[3] stated that different methods for measuring the antioxidant capacity of honey should be used for better characterization of honey antioxidant activity.

One of the beneficial features of honey is its antimicrobial activity. The antibacterial activity of honey was reviewed by Molan.[4] The main factor is the high osmotic activity of honey, which does not allow bacterial growth. There is a variety of different other antibacterial factors, such as hydrogen peroxide and organic acids, which originate both from the bee and from the plants.[4–6] The antioxidant properties and antimicrobial activity of Turkish honey have been reported in several studies.[7–11] The current study was designed to assess the total phenolic content and *in vitro* biological activities, in terms of antioxidant, antiradical, and antimicrobial activities of 35 Turkish honey samples from different regions of Turkey.

MATERIALS AND METHODS

Honey Samples

Honey samples were obtained directly from beekeepers throughout Turkey. The floral origin of the samples was specified by the beekeepers regarding hive location, season, and available floral sources. The honey samples were classified into five groups, namely, chestnut (*Castanea sativa*), thyme (*Thymus* spp.), astragalus (*Astragalus* spp.), honeydew, and multifloral honey.

Chemicals and Instruments

All of the chemicals and reagents used were of analytical grade and were purchased from Merck (Darmstadt, Germany). An Agilent 8453 spectrophotometer (Agilent Technologies, Waldbronn, Germany) was used for absorbance measurements. All tests were performed in triplicate.

Determination of Total Phenolic Content

The Folin-Ciocalteu method was used to determine total phenolic compounds.[12] Each honey sample (1 g) was dissolved in 4 ml of distilled water using a vortex-mixer and the solution was filtered though Whatman No. 1. This solution (40 μl) was mixed with 2400 μl of water and 200 μl of non-diluted Folin-Ciocalteu reagent and 600 μl of sodium carbonate (20% Na_2CO_3) was then added. After incubation at room temperature for 2 h, the absorbance of reaction mixture was measured at 765 nm against a methanol blank. Results were calculated as mg gallic acid equivalents (GAE)/100 g of honey using a standard graph.

Determination of Antiradical Scavenging Activity

The scavenging activity of honey samples for the radical 1,1-dihenyl-2-picrylhydrazyl (DPPH) was measured as described,[13] with some modifications. Briefly, each honey sample (1 g) was dissolved in 4 ml of methanol using a vortex-mixer and the solution was filtered through Whatman No. 1. An aliquot of 50 μL of honey samples were mixed with 450 μL of Tris-HCL and 1000 μL of 6×10^{-5} mM DPPH in methanol.

Methanol was used as a control instead of extract. The mixtures were left for 2 h at room temperature in the dark and the absorbances at 517 nm were measured using an Agilent 8453 spectrophotometer (Agilent Technologies, Waldbronn, Germany) using methanol as a blank. Antiradical activity (%) of the samples was calculated according to the formula:

Antiradical activity (%) =
100 × ((Absorbance of control − Absorbance of sample)/Absorbance of control).

Evaluation of Total Antioxidant Capacity by Phosphomolybdenum Method

The antioxidant activities of samples were evaluated by the phosphomolybdenum method according to Prieto et al.[14] and expressed relative to that of ascorbic acid. Briefly, an aliquot of 0.4 ml of the sample in methanol was mixed with 4 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Methanol was used as a blank instead of honey solution. The reaction mixture was vortex-mixed and let to stand in a water bath at 95°C for 90 min. Absorbance was measured at 695 nm. The antioxidant activity was calculated as ascorbic acid equivalents (AAE) (mg/1 g methanol extract).

Antimicrobial Activity

The 14 microorganisms containing 12 bacteria and 2 yeasts were used as test organisms: *Aeromonas hydrophila* ATCC 7965, *Bacillus cereus* FMC 19, *Bacillus subtilis* ATCC 6630, *Escherichia coli, E. coli* O157:H7 RS 932, *Mycobacterium smegmatis* RUT, *Proteus mirabilis* BC 3624, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 28213, *Listeria monocytogenes* 1/2B, *Salmonella typhimurium* NRRLE 4463, *Yersinia enterocolitica* ATCC 1501, *Candida albicans* ATCC 1223, and *Saccharomyces cerevisiae* BC 5461.

Test yeasts (*C. albicans* and *S. cerevisiae*) and *Y. enterocolitica* were grown in malt extract and nutrient broths at 25°C for 18 h, respectively. The other microorganisms were grown in nutrient broth at 35°C for 18 h. All test microorganisms in nutrient broth or malt extract broth were enumerated by using the serial dilution method. Their final cell concentrations were 10^6–10^7 cfu/ml. The agar well diffusion method was used to detect antimicrobial activity.[15] Then 250 μl of each microorganism was added into a flask containing 25 ml of sterile nutrient agar or malt extract agar at 45°C and poured into Petri dishes (9 cm diameter). The agars were then allowed to solidify at 4°C for 1 h. Five equidistant holes were made in the agar using sterile cork borers (Ø = 4 mm). The extracts (50 μl) were prepared at 5, 10, 25, 50, and 75% concentrations in physiological saline and were applied to the holes. *Y. enterocolitica*, *C. albicans*, and *S. cerevisiae* was incubated at 25°C for 14–24 h in the inverted position. The other microorganisms were incubated at 35°C for 18–24 h. At the end of the period, inhibition zones that formed on the medium were measured in millimeters (mm).

Statistical Analysis

All assays were carried out in triplicate and the data were expressed as means ± standard deviations. One-way analysis of variance followed by least significant difference was used to compare the data. Differences between means at the 95% ($P < 0.05$) confidence

level were considered statistically significant. Correlations were obtained by Pearson's correlation coefficient (r) in bivariate linear correlations.

RESULTS AND DISCUSSION

Total Phenolic Content

The results obtained showed that the total phenolic content (mg GAE/100 g honey) determined by the modified Folin-Ciocalteu method varied greatly among the honey types, as is apparent from Table 1. The total phenolic content of honey samples was between 1.50–108.21 mg GAE/100 g honey. The lowest values were determined in astragalus honeys, the average result of eight samples 9 ± 7 mg GAE/100 g honey. The total phenolic substances were highest in the chestnut honeys. The mean total phenolic content of honeydew, multifloral, and thyme honeys were 24.2 ± 0.6, 14 ± 11, 11 ± 6 mg GAE/100 g honey, respectively. The average total phenolic content was in close agreement with the results reported by Kucuk et al.[7] for chestnut honey. Similarly, Herken et al.[8] reported that total phenol content of certified and uncertified Turkish honey samples were between 9.14 and 19.85 mmol gallic acid equivalent/L. The total phenolic content was generally lower than the values 46 to 456 mg/kg measured by Gheldof et al.[2] with the same methods.

Antioxidant and Antiradical Activity of Turkish Honey Samples

For determination of the antioxidant capacity, phosphomolybdenum assay, a simple direct test that is used for antioxidant activity determination in honey,[16] and many different samples were used.[3,7,17] As can be seen in Table 1, there were significant differences among the honeys ($P < 0.01$). The antioxidant capacity for different honeys decreased in the order: astragalus > chestnut > multifloral > thyme > honeydew. The highest antioxidant capacity was reached by astragalus and chestnut honeys 86 ± 16 and 82 ± 2 mg AAE/g honey, respectively.

The DPPH method with the stable organic radical 1,1-dihenyl-2-picrylhydrazyl is used for determination of free radical scavenging activity. Due to remarkable differences in antioxidant properties, honeys were discriminated into several groups according to the ability of their extracts to scavenge free radicals used in the model reaction system. The largest group containing about 60% of all tested multifloral honey samples the lowest radical scavenging activity that was in the range 14.23–44.99%. Only 40% of all tested honey samples were able to scavenge more than 50% of DPPH. DPPH scavenging activity was lower in tyme and astragalus honeys. The chestnut honey had the highest amount of phenolic substances and showed the highest DPPH scavenging activity. Baltrusaityte et al.[18] reported that the radical scavenging activity of honey samples from Lithuania was from 31.1–86.9% in a DPPH reaction system. Bertoncelj et al.[19] used the DPPH method to determine free radical scavenging activity, and found that monofloral honeys, acacia, and lime honeys were the least active. The IC_{50} values were 53.8, 28.8, 10.0, 8.2, 7.4, 10.7, and 7.2 mg/ml for acacia, lime, chestnut, fir, spruce, multifloral and forest honeys in their study.

A significant correlation between the antiradical activity and total phenolic content was observed ($P < 0.05$). This statistically significant correlation was in agreement with the findings of other authors.[18,20,21] Also Gheldof et al.[2] found a significant correlation between antiradical capacity and total phenolic content of honey. The antioxidant and anti

Table 1 Total phenolic content, antioxidant, and antiradical activity of honey samples ($n = 35$) ($^*P < 0.01$).

	Total phenolic content (mg GAE/100 g honey)		Antioxidant activities of samples (mg AAE/g honey)		% Inhibition DPPH	
Parameters	Ranges	Mean ± SD	Ranges	Mean ± SD	Ranges	Mean ± SD
Chestnut ($n = 5$)	19.05−108.21	46.97 ± 17.52	75.64−87.18	82.36 ± 1.99	44.99−88.30	67.92 ± 8.58
Honeydew ($n = 5$)	22.42−25.68	24.21 ± 0.60	51.92−80.68	70.09 ± 5.12	50.96−76.47	62.58 ± 4.36
Thyme ($n = 8$)	3.55−21.86	11.22 ± 6.03	61.80−94.60	74.60 ± 3.49	20.57−64.12	35.37 ± 5.06
Astragalus ($n = 9$)	1.50−24.24	8.98 ± 6.89	71.39−114.69	86.19 ± 16.46	20.06−58.12	32.73 ± 4.42
Multifloral ($n = 8$)	5.09−32.76	14.09 ± 11.14	57.03−94.68	77.79 ± 3.90	14.23−89.65	41.35 ± 29.21

radical scavenging activity of honey samples tested varied in a wide range. First, the impact antioxidant activity of honey varies depending on the honey, because of the complicated chemical composition that varies between honeys from different floral sources. Also, chemical composition of honey depends on the composition of nectar where it originates from different plants as well as environmental factors, beekeeping practices, and storage conditions. Moreover, the differences in antioxidant activity between the tested honey samples most likely depend mainly on floral sources of honey. However, it suggested that botanical species as the main source of honey is not the only factor contributing to its antioxidant activity. The differences could be attributed to the presence of different compounds of antioxidants, such as flavonoids, phenolic acids, and other phenolic compounds that have different antioxidative effects.[22,23] Gazzani et al.[24] indicate that some phenolic compounds may react faster than others under the same conditions. In addition, the antioxidant components in honey probably had some synergistic interactions.[2] Therefore, it can be expected that the total antioxidant activity of the tested honey should be higher than the activity defined by the phenolic fraction. It was reported that phenolic compounds are the main components responsible for the antioxidant activity of honey, however, non-phenolic antioxidants are also involved.[19,25]

Antimicrobial Activity of Honeys

Antimicrobial activities of honeys were tested by using physiological saline extract of 5, 10, 25, 50, and 75% concentration. The concentration of honeys at 5, 10, and 25% had no inhibitory effect on the 14 microorganisms tested. The results of the test of honeys at 50 and 75% concentrations on 11 microorganisms are given in Table 2. The honey samples showed the highest antimicrobial activity against some microorganisms, especially *E. coli* O157:H7, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Proteus mirabilis*. On the other side, *Aeromonas hydrophila*, *B. subtilis*, and *E. coli* were the most resistant microorganisms. This is in agreement with the findings of Molan.[5] In addition, honey samples also had no inhibitory effects on *Yersinia enterocolitica* and two yeasts, *C. albicans* and *S. cerevisiae*.

In our study, each bacteria tested exhibited different sensitivities to each of the test honeys. Several authors have concluded that major antibacterial factor in honey is hydrogen peroxide, which results from the activity of catalase and glucose oxidase.[26,27] Non-peroxide factors may also contribute to antimicrobial properties of honey, such as lysozyme, phenolic acids, and flavonoids.[6] The peroxide and non peroxide originate both from the bee and from the plants.[4,6] Flavonoids and other phenolic components in nectar[28] have antioxidant capacity and inhibit growth of a wide range of Gram negative and Gram positive bacteria.[29]

CONCLUSIONS

Chestnut honey has the highest total phenolic content and showed the highest DPPH scavenging activity. Similarly, the thyme honeys have the lowest total phenolic content and displayed a lower DPPH scavenging activity. The highest antioxidant capacity was reached by astragalus and chestnut honeys 86 ± 16 and 82 ± 2 mg AAE/g honey, respectively. Phenolic content and antioxidant and antiradical activities of honey is strongly affected by floral sources. Additionally, different honey properties from the same floral

Table 2 Antimicrobial activities of honey samples (range of inhibition zones, mm).

		Microorganisms										
Sample number	% Concentration	*A. hydrophila*	*P. mirabilis*	*E. coli*	*E. coli* O157: H7	*S. typhimurium*	*S. aureus*	*L. monocytogenes*	*B. subtilis*	*P. aerugi-nosa*	*Mycobacterium smegmatis*	*B. Cereus*
Chestnut ($n = 5$)	50	0	0	0	0–6	0	0–6	0–6	0	0	0	0
	75	0	0–9	0	0–7	0–6	0–8	0–9	0–6	0	0	0
Honeydew ($n = 5$)	50	0	0	0	0–7	0	0	0	0	0	0	0–6
	75	0	0	0–6	0–15	0–8	0	0–6	0	0	0	0–7
Thyme ($n = 8$)	50	0	0–6	0	0	0–5.5	0	0	0	0	0	0
	75	0–6	0–14	0	0–6	0–8	0–6	0–8	0	0–6	0–6	0–6
Astragalus ($n = 9$)	50	0	0	0	0	0	0	0	0	0	0	0
	75	0	0–9	0–6	0–6	0–10	0–10	0–6	0	0–6	0–6	0–6
Multifloral ($n = 8$)	50	0	0–6	0	0	0–6	0–6	0	0	0	0	0
	75	0–5.5	0–9	0	0	0–8	0–7	0–6	0–8	0–12	0–12	0–6

0: Not detected.

sources were expected since the composition of active compounds in honey from different locations should be different. The honey samples at 75% concentration showed the highest antimicrobial activity against *E. coli* O157:H7, *S. typhimurium*, *S. aureus*, *L.monocytogenes*, and *P. mirabilis*. The antimicrobial effects of honeys were affected by peroxide factors, such as hydrogen peroxide, catalase, and glucose oxidase level,[26,27] and non-peroxide factors, such as lysozyme, phenolic acids and flavonoids.[27]

In conclusion, the phenolic compounds in honey may render it a good source of antioxidants beside their antiradical and antibacterial activity.[24] Therefore, these results indicate that honey has bioactive properties supporting human health. Additionally, antioxidant, antiradical, and also antibacterial activities of honeys may also be used as good parameters for the assessment of honeys quality.

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