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Determination of phenolic content, antiradical, antioxidant and antimicrobial activities of Turkish pine honey

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RESEARCH ARTICLE

Abstract

Pine (*Pinus brutia* Ten.) honey obtained from Mugla (n=12) and Marmaris (n=8) in the southwest of Turkey were evaluated for total phenolic content by the Folin-Ciocalteu method, for potential antioxidant capacity using phosphomolybdenum assay and by the 1,1-diphenyl-2-picrylhydrazyl method for antiradical activity. The results of the study showed that total phenolic content of honey obtained from Mugla and Marmaris ranged from 62.01 and 68.78 mg/100 g honey as gallic acid equivalent, respectively. The antioxidant capacity of honey obtained from Mugla and Marmaris were 16.82 and 22.16 mg ascorbic acid equivalent/g honey, respectively. The antiradical activity of honey samples were assayed *in vitro* by measuring the inhibition of the scavenging activities of pine honey collected from Mugla and Marmaris which were found to be 44.05 and 57.49%, respectively. Pine honey did not show antimicrobial activity at 5, 10 and 25% concentrations. The samples showed their highest antimicrobial activity against some microorganisms, especially *Escherichia coli* and *Pseudomonas aeruginosa.* However, *Aeromonas hydrophila, Yersinia enterocolitica, E. coli* O157:H7*, Saccharomyces cerevisiae* and *Candida albicans* were the most resistant microorganisms. The results revealed that the pine honey studied proved to be a good source of antioxidant and antimicrobial agents which might serve to protect human health.

Keywords: antimicrobial activity, antioxidant capacity, antiradical activity, pine honey

1. Introduction

Honey is a natural sweet substance produced by *Apis mellifera* L. bees from the nectar of plants or excretions of plant-sucking insects on the living parts of plants, which bees collect, transform, deposit and leave in honeycombs to ripen and mature. Pine honey is mainly prepared from honeydew secreted by *Marchalina hellenica* (Gennadius). This honeydew is restricted to *Pinus brutia* Ten. and *Pinus halepensis* Miller (Nikolopoulos, 1965) and produced only in Turkey and Greece. Pine honey is regarded as a honey of good quality by consumers. It has good nutritional value because of its high content of minerals (Tananaki *et al.*, 2007).

In recent years there has been renewed interest in the study of the biological properties of honey. Therefore the use of honey in the treatment and prevention of numerous diseases is well documented (Molan, 1992). Oxidative stress is well known to cause many diseases. It has been reported that honey serves as a source of natural antioxidants, which are effective in preventing deteriorative oxidation reactions in foods (Chen *et al.*, 2000; Oszmianski and Lee, 1990). The components in honey responsible for its antioxidative effect are flavonoids (chrysin, pinocembrin, quercetin, galangin, kaempferol, hesperetin, myricetin), phenolic acids (caffeic, coumaric, ellagic, ferulic, chlorogenic), ascorbic acid, catalase, peroxidase, caretenoids and Maillard reaction products (Bertoncelj *et al.*, 2007). It is well known that the chemical properties of phenolic acids and flavonoids,

in terms of the availability the phenolic hydrogens such as hydrogen donating radical scavengers, comprise their antioxidant properties (Buratti *et al*., 2007; Havsteen, 1983). Antioxidant activities of honey are also influenced from their botanical origin as well as processing, handling and storage (Al-Mamary *et al*., 2002). Many researchers stated that antioxidant activity is strongly correlated with the content of total phenolics (Aljadi and Kamuriddin, 2004; Beretta *et al*., 2005). The antioxidant properties and antimicrobial activity of Turkish floral honey and rhododendron honey have been reported in several studies (Sagdic *et al*., 2013; Silici *et al*., 2010). Akbulut *et al*. (2009) determined biological activities of 15 different pine honey types. Ozkok *et al*. (2010) investigated phenolic contents of pine honey.

Honey has been used as an antibacterial agent since ancient times. The antibacterial activity of honey was reviewed extensively by Molan (1992) and this activity has been attributed to different factors such as osmolarity, acidity, hydrogen peroxide, catalase, nectar, propolis and some unidentified substances from certain floral sources. White *et al*. (1963) stated that the major antibacterial factor in honey is hydrogen peroxide, which is produced by glucose oxidase originating from the hypopharyngeal glands of honey bees (White *et al*., 1963). The non-peroxide antibacterial activity of honey is believed to be due to honey components derived from the floral source (Molan and Russel, 1988). Honey contains more than 150 polyphenolic compounds including flavonoids and phenolic antioxidants that are known to inhibit the growth of a wide range of Gram-negative and Gram-positive bacteria (Davidson, 1993).

Several types of honey are produced in Turkey, such as thyme, astragalus, citrus-tree, rhododendron, although detailed studies on their chemical and biological properties are very limited (Kucuk *et al*., 2007; Sorkun *et al*., 2001). It was reported that no direct parameter is used to determine the exact quality and biological activity of honey and that the routine chemical tests performed do not provide dependable information about the quality of honey. The aim of this study was to determine bioactive and antimicrobial properties of pine honey collected from Turkey.

2. Materials and methods

Honey samples

Twenty pine honey samples (*Pinus brutia* Ten.) were obtained from Mugla (n=12) and Marmaris (n=8) region in the south-west of Turkey in 2010. In order to ensure that the honey samples were as unifloral as possible, the melissoplaynological procedure was followed (Louveaux *et al*., 1978). The honey samples were characterised on the basis of the ratio between honeydew elements and pollen (HDE/P>3). All the samples were stored at 4 °C until analysis.

Chemicals and instruments

1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Aldrich (Steinhelm, Germany). All the other chemicals and reagents used were of analytical grade and were purchased from Merck (Darmstadt, Germany). An Agilent UV-Vis spectrophotometer (Agilent 8453; Agilent Technologies, Santa Clara, CA, USA) was used for absorbance measurements.

Determination of total phenolic content

The total phenolic contents of the samples were determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Results were calculated as mg gallic acid equivalents (GAE)/100 g of honey, by using a standard graph.

Determination of antiradical activity

The scavenging activity of honey samples against the radical DPPH was measured as described by Gyamfi *et al*. (1999) with some modifications. For this purpose, each honey sample (1 g) was dissolved in 4 ml methanol and the solution was then filtered through Whatman no. 1 paper (Whatman, Little Chalfont, UK). This solution (50 µl of honey samples) was mixed with 450 µl Tris-HCl and 1000 µl of 6×10^{-5} mM DPPH in methanol was then added. Methanol was used as control. After incubation at room temperature for 2 h, the absorbance was measured at 517 nm. The antiradical activities (%) of the samples were expressed according to the formula:

$$\text{Antiradical activity (\%)} = 100 \times ((\text{absorbance of control} - \text{absorbance of sample}) / \text{absorbance of control}) \quad (1)$$

Determination of antioxidant capacity

Antioxidant capacities of samples were evaluated by the phosphomolybdenum method according to Prieto *et al*. (1999) and expressed relative to that of ascorbic acid equivalents (AAE; mg/g honey). 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 blank. The samples were vortex-mixed and let to stand in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, their absorbance was measured at 695 nm.

Antimicrobial activity

The following fourteen microorganisms, containing twelve bacteria and two 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* NRRL E4463, *Yersinia enterocolitica* ATCC 1501, *Candida albicans* ATCC 1223, *Saccharomyces cerevisiae* BC 5461.

Y. enterocolitica and the test yeasts, namely *C. albicans* and *S. cerevisiae,* were grown in nutrient broth and malt extract 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 (Sagdic *et al*., 2006). 250 µl of each culture media was added to a flask containing 25 ml sterile nutrient agar or malt extract agar at 45 °C and poured into Petri dishes (9 cm diameter). Then the agars were 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* were incubated at 25 °C for 14-24 h in the inverted position. The other microorganisms were incubated at 35 °C for 18-24 h. The inhibition zones formed around the holes were measured in mm.

Statistical analysis

All assays were carried out in triplicate and the data were expressed as means ± standard deviations. One-way analysis of variance (ANOVA) followed by least significant difference test 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 in bivariate linear correlations.

3. Results and discussion

Total phenolic content of pine honey

Total phenolic content of the honey samples varied from 35.30 to 92.87 mg/100 g honey (Table 1). The samples collected from Marmaris (68.78 mg GAE/g honey) had higher phenolic contents than those obtained from Mugla (62.01 mg GAE/g honey). These findings were in agreement with the results found in honeydew honey from Lithuania (Baltrusaityte *et al*., 2007). The variation in phenolic content among pine honey samples may be due to environmental factors such as temperature and soil composition. Considerable differences in both composition and content of phenolic compounds have been previously found in unifloral honeys. Amiot *et al*. (1989) reported that dark coloured honeys contain more phenolic compounds than light coloured ones. Silici *et al*. (2010) found that total phenolic content ranged from 0.24 to 141.83 GAE/100 g in rhododendron honey. In another study, total phenolic content of Turkish honeys was detected as 1.50-108.21 mg GAE/100 g (Sagdic *et al*., 2013). Honeydew secretions from pine trees are produced by the same insect (*Marchalina helenica* L.) but phenolic content could be different because secondary plants may contribute to phenolic content. The results showed that our samples had higher phenolic contents than those of Ozkok *et al*. (2010) (3.54-15.56 mg GAE/100 g). In contrast to the last reference, Akbulut *et al*. (2009) found that total phenolic compounds in their study ranged from 234.9 to 394.0 mg/100 g for pine honey.

The antiradical activity and antioxidant capacities of pine honey

Mean antiradical activities of samples collected from Marmaris and Mugla were 57.49 and 44.05%, respectively. The largest group, containing about 80% of all tested samples, possessed the lowest radical scavenging activity, less than 50% DPPH radical. Only five honey samples were able to scavenge in the range of 53.28-87.24%. Baltrusaityte *et al*. (2007) stated that the antiradical activities of multifloral honey samples varied from 42 to 80.9% in DPPH radical scavenging assay (Baltrusaityte *et al*. 2007).

There were significant differences (P<0.05) among the pine honey samples in terms of antioxidant capacity. Antioxidant capacity of the honey samples varied from 10.07 to 43.84 mg AAE/g honey (Table 1). The pine honey samples obtained from Marmaris exhibited higher antioxidant activities compared with those obtained from the Mugla region in both antioxidant analyses. Sagdic *et al*. (2013) determined that 35 different Turkish honey samples had antioxidant capacity at levels of 51.92-114.39 mg AAE/g honey. It was reported that the highest antioxidant activities were observed in astragalus and chestnut honey compared to other honey types (honeydew, thyme and multifloral honey). Baltrusaityte *et al*. (2007) reported that honey samples had similar botanical composition, though their antioxidant activity was different. For instance willow honey exhibited quite strong antioxidant activity while rape honey did not show high antioxidant activity. It was suggested that botanical species as the main source of honey is not the only factor contributing to its antioxidant activity. Bee origin metabolism products which could have an effect on the antioxidant properties of honey should also be taken into account (Baltrusaityte *et al*., 2007). Gheldof *et al*. (2002)

Table 1. The total phenolic content, antioxidant capacity and antiradical activity of twenty pine honey samples.[1]

	Sample number	Phenolic content (mg GAE/100 g honey)[2]	Antioxidant capacity (mg AAE/ g honey)[3]	Antiradical activity (inhibition %)
Mugla (n=12)	1	56.70±2.16^{i}	15.58±0.26^{ij}	44.68±0.68^{hj}
	2	52.78±1.88^{j}	17.40±0.21^{g}	35.71±2.53^{k}
	3	69.00±0.84^{f}	18.90±0.04^{f}	43.48±1.64^{h}
	4	66.57±1.72^{fg}	20.27±0.08^{e}	47.27±0.29^{e}
	5	44.92±2.36^{k}	15.78±0.05^{i}	47.19±0.58^{e}
	6	46.35±2.81^{k}	13.44±0.08^{l}	33.36±0.86^{k}
	7	73.91±1.22^{e}	19.97±0.04^{e}	49.68±1.32^{d}
	8	69.00±1.31^{f}	20.85±0.05^{d}	35.48±0.52^{k}
	9	57.73±1.21^{i}	16.59±0.06^{h}	43.70± 1.00^{h}
	10	81.19±0.76^{c}	15.25±0.11^{jk}	53.28±0.43^{c}
	11	61.74±1.69^{h}	15.03±0.06^{k}	46.46±0.91^{ef}
	12	64.20±2.15^{gh}	12.72±0.05^{m}	48.28±0.44^{cd}
	Ranges	44.92-81.19	12.72-20.85	33.36-48.28
	Mean±SD	62.01±10.88	16.82±2.69	44.05±6.17
Marmaris (n=8)	13	52.86±0.87^{j}	10.07±0.24^{o}	34.35±1.42^{jk}
	14	36.78±0.38^{l}	10.94±0.06^{n}	40.47±0.92^{i}
	15	92.87±0.37^{a}	12.75±0.07^{m}	43.83±1.32^{h}
	16	86.21±0.75^{b}	11.09±0.08^{n}	49.88±1.63^{d}
	17	91.74±3.37^{a}	12.59±0.11^{m}	62.28±1.48^{b}
	18	35.30±1.77^{l}	40.63±0.18^{b}	55.08±0.23^{c}
	19	77.34±3.6^{d}	43.84±0.43^{a}	87.24±0.16^{a}
	20	53.10±0.44^{j}	35.35±1.00^{c}	86.78±0.28^{a}
	Ranges	35.30-91.74	10.07-43.84	34.35-87.24
	Mean±SD	68.78±24.07	22.16±14.93	57.49±20.15

[1] Each value is presented as mean±SD (n=3). Means within each column with different letters differ significantly (P<0.05).

[2] Total phenolic content is expressed as gallic acid equivalents (GAE).

[3] Antioxidant capacity is expressed as ascorbic acid equivalents (AAE).

stated that the antioxidant components in honey could facilitate synergistic interaction. Moreover it was reported that phenolic compounds are the main components responsible for the antioxidant effects of honey; however non-phenolic antioxidants are also involved (Aljadi *et al.*, 2004; Amiot *et al.*, 1989). It seems that antioxidant activity appears to be a result of the combined activity of honey phenolics, peptides, organic acids and enzymes (Gheldof *et al.*, 2002). There has been limited information related to biological activities of pine honey in the literature. Pine honey (*P. brutia*) are produced only in Turkey and Greece. Therefore a comparison of the results is very difficult. Though the honey samples tested could be said to have quite a good level of antioxidant activity, it is not possible to compare our results with the literature data except for phenolic contents.

Antimicrobial activity of pine honey

The results of the pine honey tests on 14 microorganisms are given in Table 2. In general, the Gram-positive bacteria were more sensitive to the honey phenolic compound extracts than the Gram-negative bacteria. No inhibition was observed at 5, 10 and 25% concentrations. *E. coli* and *P. aeruginosa* were the most sensitive species among the test microorganisms. The results were in agreement with the findings of Bogdanov (1989). However, the honey samples did not show antimicrobial activity on the test yeasts *C. albicans* and *S. cerevisiae*.

Osata *et al*. (1999) demonstrated that *in vitro* osmosis determined the bactericidal effects of honey on *Helicobacter pylori*. They also reported that a part of the antibacterial activity might be attributed to components of plant origin. Most plants contain a large number of polyphenols and flavonoids and each plant tends to have a distinctive profile.

Table 2. Antimicrobial activity ranges of pine honey samples at two concentrations (inhibition zones; mm).

	Mugla (n=12)		Marmaris (n=8)	
	50%	75%	50%	75%
Aeromonas hydrophila ATCC 7965	0	0	0	0-8
Bacillus cereus FMC 19	0-7	0-8	0	0
Bacillus subtilis ATCC 6630	0-7	0-11	0-8	0-11
Escherichia coli	0-10.5	8-16	0-12	0-13.5
E. coli O157:H7 RS 932	0	0-10	0	0
Listeria monocytogenes 1/2B	0-12	0-14	0	0
Mycobacterium smegmatis RUT	0	0-7	0	0-9
Proteus mirabilis BC 3624	0	0-10	0	0-6.5
Pseudomonas aeruginosa ATCC 27853	0-10.5	8-14	0-8	8-10.5
Salmonella typhimurium NRRL E4463	0-9	0-11	0	0-8
Staphylococcus aureus ATCC 28213	0	0-13	0-5	0-15.5
Yersinia enterocolitica ATCC 1501	0	0	0	0-6
Candida albicans ATCC 1223	0	0	0	0
Saccharomyces cerevisiae BC 5461	0	0	0	0

Several authors have concluded that honey from certain plants has better antibacterial activity than that of others. It has been shown that there can be a large variation in the activity of different samples from the same plant source (Molan and Russel, 1988; Weston, 2000). There have been several studies in which dark honey from the conifers of the mountainous regions of central Europe was found to have particularly high antimicrobial activity. This honey is not from a nectar source but from honeydew. Chestnut honey has also been reported to have high antimicrobial activity, but it is dark in colour and thus is considered to be partly derived from honeydew. Another honeydew honey from manuka (*Leptospermum scoporium*) in New Zealand has also been found to have a high level of antimicrobial activity (Molan and Russel, 1988; Weston, 2000).

4. Conclusions

In conclusion, antioxidant tests, phenolic contents and antimicrobial activity testing methods were utilised in order to evaluate the biological properties of Turkish pine honey. The pine honey obtained from Marmaris had a higher phenolic content and better antioxidant activities when compared with those obtained from Mugla, Turkey. In addition, the honey samples appeared to be worthy of further investigation of their individual biologically active components. Turkish pine honey contains important phenolic content, showing a substantial antioxidant capacity which may be used as a natural source of compounds with these properties. Taken as a whole, these factors give honey unique properties as a dressing for wound: it leads to the rapid clearance of infections, rapid debridement of wounds, rapid suppression of inflammation, minimisation of scarring, and stimulation of angiogenesis as well as tissue granulation and epithelium growth (Molan and Betts, 2004).

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