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Food Chemistry 100 (2007) 526–534
Food Chemistry
www.elsevier.com/locate/foodchem

Biological activities and chemical composition of three honeys of different types from Anatolia

Murat Küçük [a], Sevgi Kolaylı [a,*], Şengül Karaoğlu [b], Esra Ulusoy [a], Cemalettin Baltacı [a], Ferda Candan [c]

[a] Department of Chemistry, Faculty of Arts and Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey
[b] Department of Biology, Rize Faculty of Arts and Sciences, Karadeniz Technical University, Rize, Turkey
[c] Department of Chemistry, Faculty of Arts and Sciences, Cumhuriyet University, 58140 Sivas, Turkey

Received 19 April 2004; received in revised form 11 October 2005; accepted 11 October 2005

Abstract

In this study, we investigated and compared some chemical properties and in vitro biological activities of three different types of Turkish honey. The first two honey samples were monofloral from chestnut and rhododendron flowers, collected from the east Black Sea region, and the third sample was the heterofloral form of astragalus (*Astragalus microcephalus* Willd.), thyme (*Thymus vulgaris*) and other several mountain flowers, collected from Erzincan in Eastern Anatolia. The chemical properties of the honey samples, such as total moisture, ash, total protein, sucrose, invert sugar, diastase activity, hydroxymethylfurfural content and acidity, were determined. Total phenolics, superoxide radical- and peroxynitrite-scavenging activities, and ferric reducing/antioxidant power measurements were used as antioxidant capacity determinants with ±-catechin, butylated hydroxytoluene, ascorbic acid, and trolox® used as reference. The antimicrobial activity was studied by the agar diffusion method, using eight bacteria and two yeasts. The mineral contents were also determined by an AAS method. The chestnut flower honey had the highest phenolic content, superoxide radical-scavenging activity and reducing power, while the heterofloral honey sample exhibited the highest peroxynitrite-scavenging activity. The antioxidant activities were also found to be related to the sample concentrations. The mineral content of the chestnut honey was much higher than the others. The samples showed moderate antimicrobial activity against some microorganisms, especially *Helicobacter pylori* ATCC 49503, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Candida tropicalis* ATCC 13803 and *Candida albicans* ATCC 10231. The honey samples studied proved to be a good source of antioxidants and antimicrobial agents that might serve to protect health and fight against several diseases.

Keywords: Honey; Antioxidant; Antimicrobial; Superoxide; Peroxynitrite; Rhododendron; Chestnut; Astragalus; Thyme

1. Introduction

Honey is nectar collected from many plants and processed by honey bees (*Apis mellifera*). The composition of honey is variable, owing to the differences in plant types, climate, environmental conditions, and contribution of the beekeeper (Anklam, 1998; Azeredo, Azeredo, de Souza, & Dutra, 2003). Honey has been reported to contain about 200 substances and is considered as an important part of traditional medicine (White, 1979). It has been used in ethnomedicine since the early humans, and in more recent times its role in the treatment of burns, gastrointestinal disorders, asthma, infected wounds and skin ulcers has been "rediscovered" (Al-Mamary, Al-Meeri, & Al-Habori, 2002; Orhan et al., 2003).

Free radicals were a major interest for early physicists and radiologists and much later found to be a product of normal metabolism. Today, we know well that radicals cause molecular transformations and gene mutations in many types of organisms. Oxidative stress is well-known

* Corresponding author. Tel.: +90 462 377 24 87; fax: +90 462 325 31 96.
E-mail address: skolayli@ktu.edu.tr (S. Kolaylı).

0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.10.010

to cause many diseases (Storz & Imlay, 1999), and scientists in many different disciplines became more interested in natural sources which could provide active components to prevent or reduce its impacts on cells (Ulubelen et al., 1995; Yan, Murphy, Hammond, Vinson, & Neto, 2002). Nitric oxide ($NO^{\bullet}$), a radical, commonly produced by the aerobic oxidation of arginine, is an important intercellular messenger molecule involved in a wide range of physiological processes. The direct combination of $NO^{\bullet}$ with $O_2^{\bullet-}$, which is produced in the respiratory system as a result of incomplete reduction of O_2, has been recognized to produce peroxynitrite anion ($ONOO^-$), which, under physiological conditions, decomposes rapidly to yield oxidizing intermediates capable of damaging biological targets (Groves, 1999; Ischiropoulos & Al-Mehdi, 1995). The production of $ONOO^-$ in vivo can have pathological consequences by oxidizing or nitrating proteins, lipids, and nucleic acids (Groves, 1999; Haenen, Paquay, Korthouwer, & Bast, 1997; Tsuda, Kato, & Osawa, 2000).

Antioxidants, which can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important in the prevention of many diseases (Halliwell, Gutteridge, & Cross, 1992). The number of antioxidant compounds synthesized by plants as secondary products, mainly phenolics, serving in plant defence mechanisms to counteract reactive oxygen species (ROS) in order to survive, is currently estimated to be between 4000 and 6000 (Havsteen, 2002; Peterson & Dwyer, 1998; Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999; Wollgast & Anklam, 2000). The phenolic content and composition of plants and the products produced from them depend on genetic and environmental factors, as well as post-harvest processing conditions (Cowan, 1999; Vaya, Belinky, & Aviram, 1997). The antioxidant activities of phenolics are related to a number of different mechanisms, such as free radical-scavenging, hydrogen-donation, singlet oxygen quenching, metal ion chelation, and acting as a substrate for radicals such as superoxide and hydroxyl. A direct relationship has been found between the phenolic content and antioxidant capacity of plants (Al-Mamary et al., 2002; Robards et al., 1999). Honey contains a variety of phenolics and represents a good source of antioxidants, which makes it a good food antioxidant additive and increases its usability potential in ethnomedicine (Aljadi & Kamaruddin, 2004; Al-Mamary et al., 2002; Beretta, Granata, Ferrero, Orioli, & Facino, 2005).

Several methods have been developed, in recent years, to evaluate the antioxidant capacity of biological samples (Rice-Evans, Miller, & Paganga, 1997; Schlesier, Harwat, Böhm, & Bitsch, 2002). The total phenolic content of natural samples, such as plants and honey, reflects, to some extent, the total antioxidant capacity of the sample (Beretta et al., 2005). The most widely used antioxidant methods involve the generation of oxidant species, generally radicals, and their concentration is monitored as the present antioxidants scavenge them. Radical formation and the following scavenging are applied in 2,2-diphenyl-1-picrylhydrazyl (DPPH)- and superoxide radical-scavenging activity measurements (Gülçin, Büyükokuroğlu, Oktay, & Küfrevioğlu, 2003). In radical-scavenging activity, the higher extract concentration required to scavenge the radicals means the lower antioxidant capacity. Ferric-reducing/antioxidant power (FRAP) is another widely used antioxidant activity measurement method, which has been used for the assessment of antioxidant and reducing power of many different samples, including honey (Aljadi & Kamaruddin, 2004) and plant exudates (Gülçin et al., 2003).

The three Turkish honeys studied were rhododendron, chestnut and heterofloral origin. Rhododendron (*Rhododendron ponticum*) honey, also locally known as mad or wild honey, is collected from Black Sea Region. The symptoms of poisoning due to the consumption of large amounts of this honey include sudden severe vertigo, arterial hypotension, and bradycardia. The honey contains acetylandromedol, formerly called andromedotoxin, which originates from *R. ponticum* as the active agent (Sutlupinar, Mat, & Satganoglu, 1993). *R. ponticum* is a purple flowered ever-green shrub, which is known as one of the invasive plants in the Black Sea region, as well as in many Mediterranean countries and the British Isles, threatening the forest life (Erfmeier & Bruelheide, 2004; Eşen, Zedaker, Kirwan, & Mou, 2004). The second honey tested was mainly of chestnut, *Castanea sativa* Miller, flower origin. The chestnut honey is believed to be a good ethno-remedy for asthma and respiratory diseases (Orhan et al., 2003). The chestnut honey contains a volatile constituent, 3-aminoacetophenone, which serves as a marker to identify the type of the honey (Bonaga & Giumanini, 1986). The third honey sample was a heterofloral one, with the floral sources being thyme (*Thymus vulgaris*), astragalus (*Astragalus microcephalus* Willd), and, to a lesser extent, various mountain flowers. The genus *Thymus* (Lamiaceae) is represented in Turkey by several species, of which about half are endemic (Karaman, Digrak, Ravid, & Ilcim, 2001; Tumen, Baser, Demirci, & Ermin, 1998). Numerous antioxidant investigations have been carried out on *T. vulgaris* (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003). *T. vulgaris*, containing thymol as one of its major components, is used as sweetener and appetizer in foods (Yan et al., 2002). The plant has also been reported to possess antibacterial, antifungal, and anti-inflammatory activities (Karaman et al., 2001; Saez, 1998). Thyme honeys of various origins have been studied for their physicochemical properties (Terrab, Recamales, Hernanz, & Heredia, 2004) and chemical components (Tan, Wilkins, Holland, & McGhie, 1990). *Astragalus* species comprise the largest genus in Turkey, where it is represented by ca. 400 species in 62 sections (Aytaç, Ekici, & Açık, 2001). The roots of various *Astragalus* plants are used as antiperspirants, diuretics, and tonic agents. They have been used in the treatment of diabetes, nephritis, leukemia, and uterine cancer (Pistelli, Bertoli, Lepori, Morelli, & Panizzi, 2002).

Several types of honey are produced in Turkey, although detailed investigations on their chemical and bio-

logical properties are very limited (Sorkun, Doğan, & Başoğlu, 2001). Anklam (1998) reported that there is no direct parameter measured 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. An example is that diastase activity and hydroxymethylfurfural (HMF) content are widely used as an indication of honey freshness although they do not provide any information about its effectiveness from the point of view of health benefits.

The current study was designed to assess the chemical composition, including minerals, and in vitro biological activities, in terms of antioxidant, antibacterial, and antifungal activities of three Turkish honey samples, as well as to evaluate their nutritional and medicinal potentials.

2. Materials and methods

2.1. Chemicals and instruments

All of the reagents used were of analytical grade. L-Ascorbic acid, ±-catechin, 5-hydroxymethyl-2-furfuraldehyde (HMF), sucrose, maltose, fructose and glucose were purchased from Sigma Chemical Co. (Steinheim, Germany) and butylated hydroxytoluene (BHT) was supplied by Applichem (Darmstadt, Germany). Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and trichloroacetic acid (TCA) were obtained from Merck Co. (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent was purchased from Fluka Chemie GmbH (Switzerland).

An ATI-Unicam UV-2 UV–Vis spectrophotometer (Cambridge, UK) was used in all absorbance measurements. A Nüve EN 400 incubator (Ankara, Turkey) was used for incubations at 35–40 °C. A Sanyo (CFC-free) Medical Freezer (Sanyo Electric Co. Ltd., Japan) was used for storing the samples until tested. A Denley BS400 Centrifuge (England) was used for centrifugations.

2.2. Samples

The type and region of the honey samples, as well as the family, scientific, common, and local names of the plants that form the basic flora of the honey samples, are shown in Table 1. All three honey samples were collected in May–July, 2003, and the tests were performed within the two months following collection. Honey samples were obtained from a single experienced producer who provided the three authentic samples: chestnut and rhododendron honeys as unifloral, to the extent of natural limitations, and heterofloral honey, the major nectar source being thyme and astragalus. Chestnut and rhododendron honeys were collected in the east Black Sea region at 500 and 1800 m, respectively. They were collected in the fields where no other major contributing plant species exist in abundance. The heterofloral honey was collected from Otlukbeli plateau near Erzincan Province at 1500 m.

The artificial honey (80% sugar, w/v), serving as a blank, was prepared according to the method of White (1979) by dissolving 4 g of fructose, 3 g of glucose, 0.8 g of maltose, and 0.2 g of sucrose in distilled water to make a solution of 10 ml final volume, and the solution was mixed for 1 h at 80 °C. The antioxidant activity was calculated by subtracting the values obtained for the blank from that of each sample. All the tests were performed in triplicate unless indicated otherwise.

2.3. Chemical analyses

Moisture, ash, acidity, sucrose, and invert sugar were determined according to an AOAC method (1990). Total protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (1951). HMF was determined by a method in which the UV absorbance of a clarified aqueous honey solution is determined against a reference solution of the same honey in which the 284 nm chromophore of HMF is destroyed by bisulphate (White, 1979). Diastase activity was determined by the method of Horwitz (1980). The principle of the method is that a buffered mixture of soluble starch and honey solution is incubated and the time required to reach a specified end-point is determined spectrophotometrically. The diastase activity is expressed as ml of 1% starch hydrolyzed by the enzyme in 1 g of honey in 1 h, called the diastase number.

2.4. Determination of antioxidant activity

2.4.1. General

The antioxidant capacity of honey samples was examined by comparing to that of the known antioxidants, BHT, trolox® and ascorbic acid, by employing the following four complementary in vitro assays: total phenolic content (Slinkard & Singleton, 1977), superoxide radical-scavenging assay (Robak & Gryglewski, 1988), peroxynitrite-scavenging activity (Ischiropoulos & Al-Mehdi, 1995) and ferric reducing/antioxidant power (FRAP) assay (Oyaizu, 1986).

Table 1
Characterization of the honey samples

Type of honey	Family	Scientific name	Common name	Local name	Region
Monofloral	Ericaceae	*Rhododendron ponticum*	Wild rose	Komar	Trabzon, East Black Sea
Monofloral	Castanea	*Castanea sativa* Mill.	Chestnut	Kestane	Trabzon, East Black Sea
Heterofloral	Fabaceae	*Astragalus microcephalus* Willd	Astragalus	Geven	Erzincan, Northeastern Anatolia
		Thymus vulgaris L.	Thyme	Kekik	

2.4.2. Determination of total phenolic contents

Total soluble phenolic contents of the samples were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) by using ±-catechin as a standard. Briefly, 0.1 ml of catechin and sample solutions (various concentrations for the standard and 20% methanolic solution for the honey samples) was diluted with 5.0 ml distilled water. 0.5 ml of 0.2 N Folin-Ciocalteu reagent was added, and the contents were vortexed. Following a 3-min incubation, 1.5 ml of Na_2CO_3 (2%) was added, and, after vortexing, the mixture was incubated for 2 h at 20 °C with intermittent shaking. The absorbance was measured at 760 nm at the end of the incubation period. The concentration of total phenolic compounds was calculated as milligrammes of catechin equivalents per 100 g of honey sample, by using a standard graph.

2.4.3. Superoxide radical-scavenging activity

Superoxide radicals generated by the xanthine–xanthine oxidase system were determined by spectrophotometrically monitoring the product of the reaction with nitroblue tetrazolium salt (NBT) (Robak & Gryglewski, 1988). The aqueous extract (0.1 ml) of various concentrations was added to the reaction mixture containing 100 μM xanthine, 600 μM NBT, 0.05 U/ml of xanthine oxidase, and 0.1 M phosphate buffer (pH 7.4) making up to a final volume of 2.0 ml. Following the incubation at 25 °C for 10 min, the absorbance was read at 560 nm, and compared with that of the control in which the enzyme, xanthine oxidase, was absent. The results were expressed as the concentration of the test sample giving 50% reduction in the absorbance of control, 50% inhibitory concentration in mg/ml (IC_{50}).

2.4.4. Peroxynitrite-scavenging activity

Peroxynitrite ($ONOO^-$) was synthesized according to the method of Koppenol, Kissner, and Beckman (1996) and quantified spectrophotometrically prior to use. The method used in the literature to determine peroxynitrite-scavenging activity (Ischiropoulos & Al-Mehdi, 1995) was slightly modified for the honey samples.

A 0.8 ml aliquot of 0.1 M KH_2PO_4–K_2HPO_4 buffer (pH 7.4) was mixed with 0.1 ml of sample/standard solutions of various concentrations and 0.8 ml of 2.5 mM tyrosine solution prepared in 0.1 M phosphate buffer (pH 7.4). 0.1 ml of peroxynitrite stock solution (37 mM) and then 0.1 ml of 0.1278 M HCl solution, an equal amount of which was equivalent to the basicity of peroxynitrite solution, were added to the mixture while rapidly vortexing. The peroxynitrite and then the equivalent HCl solutions additions were made previous to tyrosine addition for blanks. The mixture was then incubated at 37 °C for 15 min. The pH of the solution incubated was brought to 9.5–10 with 2 ml of 0.2 M sodium borate. Distilled water was added to the mixture to give a final volume of 4 ml and the absorbance at 430 nm was measured. The absorbance was plotted against honey concentration, and the honey concentration (mg/ml) giving 50% inhibition (IC_{50}) was determined.

2.4.5. Ferric reducing/antioxidant power assay (FRAP)

The reducing power of the ethanolic extracts was determined according to a modified version of ferric reducing/antioxidant power (FRAP) assay of Oyaizu (1986). Different concentrations of honey samples (1.0%, 5.0%, and 10%) and ascorbic acid (1.0 mg/ml), for comparison, were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml 1% potassium ferric-cyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50 °C for 20 min. After the incubation period, 2.5 ml 10% trichloroacetic acid were added and the mixture was vortexed. Following centrifugation at 3000 rpm for 10 min, 2.5 ml of the supernatant were mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Higher absorbance value means higher reducing power of a sample.

2.5. Determination of trace metals

The samples were digested by the wet oxidation method, by treating the samples with HNO_3 and H_2O_2, and analyzed using an ATI-Unicam 929 model atomic absorption spectrometer (Cambridge, UK) and Jenway PFP-7 flame photometer (England). The minerals were quantified against standard solutions of known mineral concentrations that were analyzed concurrently. The amounts of Na, K, Ca, Fe, Cu, Zn, Mn and Cr metal ions were determined at the mg/kg (ppm) level.

2.6. Determination of antimicrobial activity

2.6.1. Test microorganisms

All test microorganisms, eight bacteria and two yeasts, were obtained from Refik Saydam Hıfzıssıhha Institute (Ankara, Turkey) and were as follows: *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Helicobacter pylori* ATCC 49503, *Moraxella catarrhalis* ATCC 25238, *Escherichia coli* ATCC 35218, *Enterobacter cloacae* ATCC 13047, *Pseudomonas aeruginosa* ATCC 10145, *Candida tropicalis* ATCC 13803 and *Candida albicans* ATCC 10231.

2.6.2. Agar-well diffusion method

A simple susceptibility screening test, using the agar-well diffusion method, was employed (Perez, Pauli, & Bazerque, 1990). Each microorganism was suspended in brain heart infusion (BHI) broth and diluted to ca. 106 colony forming unit (cfu) per ml. They were ''flood-inoculated'' onto the surface of BHI agar and Sabouraud Dextrose agar (SDA) (Difco, Detroit, MI) and then dried. For *C. albicans* and *C. tropicalis*, SDA was used. Five-millimetre diameter wells were cut from the agar, using a sterile cork-borer, and 50 μL of the solutions (50% in methanol) were delivered into the wells. The plates were incubated for 18 h at 35 °C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganism. Methanol served as negative control. The results were expressed in terms of the diameter of the inhibition zones:

<5.5 mm, inactive; 5.5–9 mm, very low activity; 9–12 mm, low activity; 12–15 mm, average activity; and >15 mm, high activity.

3. Results and discussion

The three authentic honey samples were studied in terms of chemical properties and biological activities and the tests were performed in triplicate. The chemical compositions of the three Turkish honey samples are given in Table 2. When each parameter of the chemical composition was compared to the values set by the Turkish Standards Institute (TSE) and Council of the European Union (CEU), all of the values were found to be within accepted ranges.

The moisture contents of all the samples were below 20%, the maximum value allowed by TSE and CEU (Anklam, 1998; White, 1979). Heterofloral honey had a lower moisture than other honeys. Abu-Tarboush, Al-Kahtani, and El-Sarrage (1993) have reported that moisture content is related to the source plant's floral type. The moisture content of honey is an important factor, contributing to its stability against fermentation and granulation during storage. The mean acidity values of all the honey samples were within the required limits (below 40 meq/kg), indicating the absence of undesirable fermentations. The acidity of honey is due to the presence of organic acids, particularly gluconic acid, pyruvic acid, malic acid and citric acid, in equilibrium with lactones or esters and inorganic ions, such as phosphate and chloride (Anklam, 1998). Like moisture, total acidity was lower in the heterofloral honey sample (29.4 meq/kg) than the others. The variation in acidity among honey samples may be attributed to the plants' floral types. Relatively little work has been published on the composition of organic acids in various types of honeys (Anklam, 1998; El-Sherbiny & Rizk, 1979). The ash content and water-insoluble fractions of all the samples were within the allowed ranges.

Diastase activity and hydroxymethylfurfural (HMF) content are widely used as an indicator of honey freshness. HMF can be formed by hexose dehydration in acid media or by Maillard reaction. Heating and storage temperature and duration cause an increase in HMF level (Tosi, Ciappini, Ré, & Lucero, 2002). A high quality honey is expected to have high diastase activity but low HMF content. All three samples showed an HMF level lower than the upper limit set (40 mg/kg). A positive correlation was observed between HMF levels and acidities of the three samples. Chestnut honey had the highest acidity, accompanied with the highest HMF level. Since the collection periods for all three samples were the same, the difference in HMF values was attributed to the difference in acidities. Diastase activity in honey is closely related to heat treatment, but has not been related to the origin of a sample (Anklam, 1998). However, we found considerably higher diastase activity in rhododendron honey (23.0 ± 2.1), indicating the floral type as an important factor for diastase activity, probably indirectly. Heterofloral and chestnut honey provided similar average diastase activities of 17.9 ± 1.3 and 17.7 ± 1.4, respectively.

Protein contents of the heterofloral and chestnut honey were also very close (0.16 and 0.17 g/100 g) and higher than the rhododendron honey. This variation may be attributed to the type of flora. Honey contains about 0.2% protein, as α-amylase, invertase, catalase, glucose oxidase, and phosphatase, which is related to plant origin and pollens and nectars (Anklam, 1998).

Sugars represent the main components of honey. Reducing sugars (or invert sugar), mainly fructose and glucose, have been found to be the major constituent of honey (Mendes, Brojo Proença, Ferreira, & Ferreira, 1998). A high sucrose concentration of honey, most of the time, means an early harvest of honey because sucrose has not been fully transformed to glucose and fructose by the action of invertase. The mean percentage of sucrose of all the honey types was below the maximum allowable limit of 5% proposed by TSE and CEU.

The averages of mineral contents (mg/kg of honey) are shown in Table 3. The mineral content of the chestnut honey, in general, was higher than the other two honeys. The chestnut honey was especially rich in potassium, calcium, and manganese. The mineral content of the chestnut honey was also found to be higher when compared to the results of an earlier investigation on flower honeys from southeastern Anatolia (Yılmaz & Yavuz, 1999). No chromium above the detection limit was observed in the samples.

Table 2
Chemical parameters of the honey samples

Parameter	Heterofloral	Chestnut	Rhododendron	Normal values TSE
Moisture (%, w/w)	17.0 ± 1.5	19.7 ± 1.7	19.0 ± 1.3	Max. 20
Ash (%, w/w)	0.20 ± 0.04	0.50 ± 0.03	0.24 ± 0.03	Max. 0.6
Total acidity (meq/kg)	29.4 ± 1.5	36.7 ± 1.9	33.6 ± 2.1	Max. 40
Total protein (%, w/w)	0.16 ± 0.02	0.170 ± 0.009	0.065 ± 0.006	Max. 0.2
Sucrose (%, w/w)	1.47 ± 0.05	2.87 ± 0.06	3.34 ± 0.05	Max. 5
Invert sugar (%, w/w)	65.8 ± 5.3	66.8 ± 6.2	65.9 ± 5.9	Min. 60
HMF (mg/kg)	19.2 ± 2.0	28.6 ± 1.9	24.1 ± 2.1	Max. 40
Diastase activity	17.9 ± 1.3	17.7 ± 1.4	23.0 ± 2.1	Min: 8.0
Water-insoluble fraction (%, w/w)	0.085 ± 0.004	0.05 ± 0.005	0.07 ± 0.003	Max. 0.1

Data are means ± SD of triplicate measurements.
TSE, Turkish Standards Institute; HMF, hydroxymethylfurfural.

Table 3
Metal ion concentrations (ppm or mg/kg) of the honey samples

Sample	Na	K	Ca	Fe	Cu	Zn	Mn	Cr
Chestnut	115	3818	900	2.64	0.42	0.68	9.69	ND
Heterofloral	163	500	160	2.41	0.09	0.54	0.59	ND
Rhododendron	73	2095	543	1.72	0.29	0.65	2.14	ND

ND, below detection limit (not detected).

Antimicrobial activities of the honeys were tested by using methanolic extracts of 50% concentration. The results of the tests of the three honey samples on ten microorganisms are given in Table 4. The honey extracts showed moderate inhibition against a few microorganisms. *H. pylori* and *S. aureus* were affected to a greater extent. The order of activity, in terms of overall activity against all the test microorganisms, though the differences were quite low, was: chestnut > heterofloral > rhododendron. This is in agreement with the findings of Bogdanov (1997). The antibacterial activity of honey is attributed to some physical (acidity, osmolarity) and chemical (H_2O_2, nectar, pollen, propolis) factors (Weston, 2000). For instance, Osato, Reddy, and Graham (1999) have demonstrated that in vitro osmosis determined the bactericidal effects of honey on *Helicobacter pylori*, which causes stomach ulcers. They also reported that a part of the antibacterial activity might be attributed to the components of plant origin. Our findings provide evidence for the contribution of floral origin to antibacterial activity since the osmotic contribution was similar from the three honeys and not so important because 50% methanolic dilutions were used in the tests.

The total phenolic substances were highest in the chestnut honey (Fig. 1). The polyphenol, contents were within the same range as the literature data on Yemen honeys (Al-Mamary et al., 2002). The chestnut honey phenolic content was the highest of all and at the higher limit of literature reports (Aljadi & Kamaruddin, 2004; Al-Mamary et al., 2002; Beretta et al., 2005). Most plants contain an extensive number of polyphenols and flavonoids and each plant tends to have a distinctive profile. The concentration and type of polyphenolic substances depend on the floral origin of honey and are major factors responsible for biological activities, including antioxidant, antimicrobial, antiviral, and anticancer activities. Catechin has been used as a standard for comparison or for quantitation in many investigations, including those about honeys of various origins (Al-Mamary et al., 2002; Wei & Zhirong, 2003).

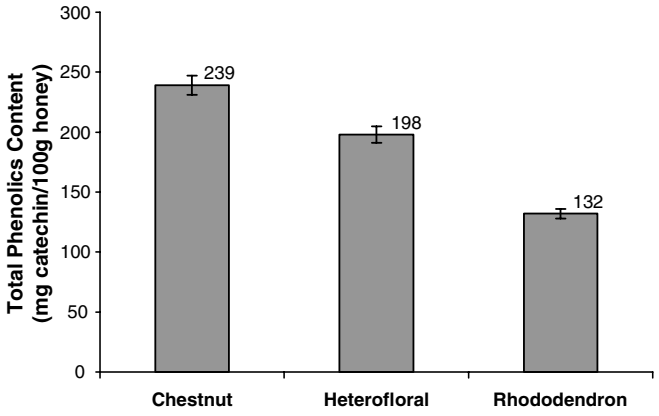

Fig. 1. Total polyphenolics contents of the honey samples expressed in terms of mg catechin/100 g honey.

There are many different antioxidant components of any natural sample, and it is relatively difficult to measure each separately. Several methods have been employed to determine antioxidant activity of biological samples, and the results are compared with those of reference antioxidant standards (Dorman et al., 2003; Rice-Evans et al., 1997).

The superoxide radical and peroxynitrite-scavenging activities of the honeys were investigated. The results were compared with those of reference antioxidants. A higher extract concentration required to scavenge the radicals means a lower antioxidant activity. Superoxide radicals were generated by the xanthine–xanthine oxidase and NBT systems in the tests (Robak & Gryglewski, 1988). The decrease of absorbance at 560 nm with the presence of antioxidants indicates the consumption of superoxide anions in the reaction mixture. Chestnut honey aqueous extract exhibited the highest superoxide scavenging activity when compared with the other two honeys (Fig. 2).

Peroxynitrite-scavenging activity of the samples was determined according to the peroxynitrite degradation by the system. Peroxynitrite-scavenging activity was higher in heterofloral honey (Fig. 3). Though the chestnut honey had the highest amount of phenolic substances, the

Table 4
Antimicrobial activity of the 50% methanolic extracts of chestnut, heterofloral, and rhododendron honeys from Anatolia

Microorganisms	Inhibition zone diameter (mm)[a]		
	Chestnut	Heterofloral	Rhododendron
Staphylococcus aureus	10	7	7
Enterococcus faecalis	7	–	–
Bacillus subtilis	7	7	7
Moraxella catarrhalis	–	7	7
Helicobacter pylori	10	8	9
Pseudomonas aeruginosa	–	–	–
Enterobacter cloacae	–	–	–
Escherichia coli	–	–	–
Candida tropicalis	7	9	–
Candida albicans	7	9	–

–, not active against tested microorganisms (<5.5 mm zone diameter); 5.5–9 mm, very low inhibition; 9–12 mm, low inhibition; 12–15 mm, average inhibition; and >15 mm, high inhibition.

[a] Data are the averages of duplicate tests.

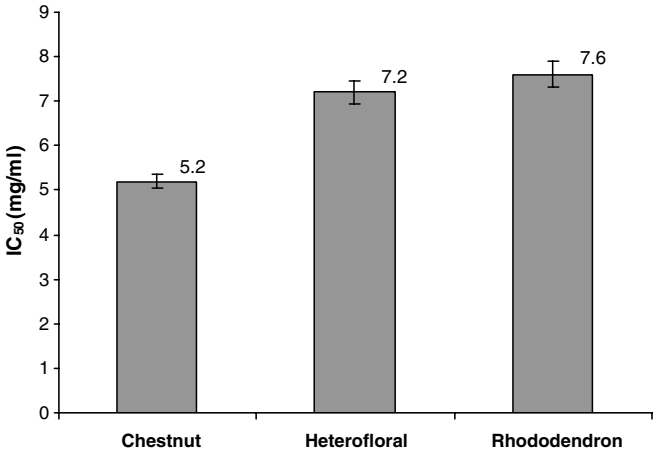

Fig. 2. Superoxide radical-scavenging activity of the honey samples. IC_{50} represents the mg/ml concentration providing 50% inhibition of radical formation or scavenging of available radicals. Ascorbic acid (IC_{50}: 1.2 mg/ml) and trolox® (IC_{50}: 0.02 mg/ml) were used as reference antioxidants for comparison.

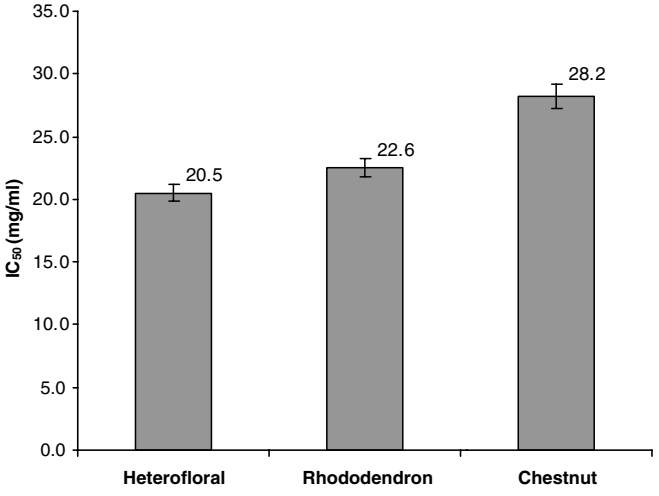

Fig. 3. Peroxynitrite scavenging activity of the honey samples. IC_{50} represents the mg/ml concentration providing 50% inhibition of peroxynitrite oxidation of tyrosine. trolox® (IC_{50}: 0.36 mg/ml) and butylated hydroxytoluene (BHT) (IC_{50}: 0.60 mg/ml) were used as reference antioxidants for comparison.

heterofloral honey showed the highest peroxynitrite scavenging activity. The explanation of this may be that the type of polyphenols in the samples may vary highly as do their radical-scavenging activities. It is well known that the type and position of the substituents of polyphenolics, such as flavonoids and anthocyanidins considerably affect their peroxynitrite-scavenging activities. Generally speaking, all three samples were potent scavengers of peroxynitrite and inhibitors of nitro-tyrosine formation (Fig. 3).

The reducing power test, in which the capacity of breaking radical chain reactions is reflected, is considered to be a good indicator of antioxidant capacity (Gülçin et al., 2003). Thus, this method was employed to determine the antioxidant capacity and, indirectly, total reducing potentials of

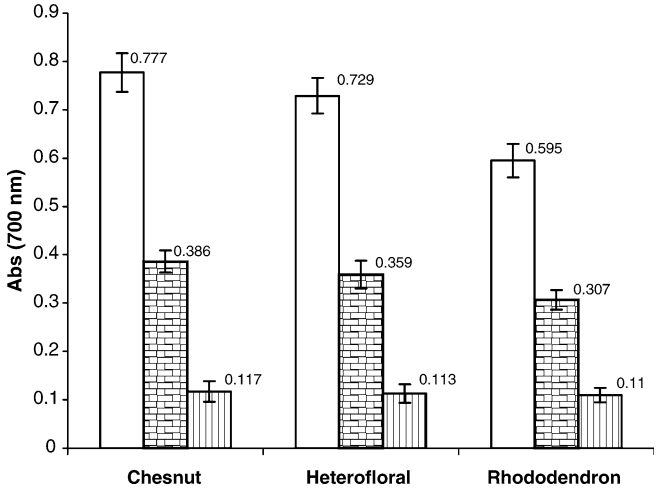

Fig. 4. Ferric reducing antioxidant power (FRAP) of the honey samples at three concentrations (▥: 1%; ▦: 5%; □: 10%). Ascorbic acid (Abs_{700nm}: 1.458 for 1.0 mg/ml conc) was used as reference antioxidant for comparison.

three different concentrations of the honey extracts and ascorbic acid, which was used as a reference standard. The reducing power measured for all honey types showed a concentration dependent pattern (Fig. 4). Hence, the method proved to be applicable. The increased absorbance is an indication of higher reducing power in this method. Among the samples, chestnut honey showed the highest activity. The order of reducing power for the honey extracts is as follows: chestnut honey > heterofloral honey > rhododendron honey. The total reducing power is the sum of the reducing powers of individual compounds present in a sample. The results of the total reducing power analyses correlated well with those of total phenolics and superoxide scavenging measurements.

There is not much information about the superoxide- and peroxynitrite-scavenging activities and reducing potential of honey samples in the literature. The comparison of the results of one investigation with another appears to be impossible. Though the honeys tested could be said to have quite a good level of antioxidant activity, it is not possible to compare our results with literature data, due to the lack of standardization in the methods. All three samples showed high antioxidant activity in the three antioxidant activity measurement tests when compared to reference antioxidants BHT, trolox® and ascorbic acid (Figs. 2–4), considering the concentrations (only 132–239 mg/100 g) of honey phenolics, the major total antioxidant activity contributors.

4. Conclusions

The three different Turkish honeys studied had high levels of polyphenolics and exhibited high antioxidant activity in three antioxidant assays, but did not possess any prooxidant character. The antioxidant activity showed a linear positive relationship with the extract concentration. The results of different antioxidant methods varied in relation

to the order of antioxidant capacity of the extracts. However, chestnut and heterofloral honey appeared to have higher antioxidant potential than had the rhododendron honey. The orders of the honey extracts, in terms of antioxidant capacity, with respect to different antioxidant activity measurement assays and of total polyphenolics, are as follows:

Total polyphenolics: chestnut honey > heterofloral honey > rhododendron honey.
Superoxide radical scavenging assay: chestnut honey > heterofloral honey > rhododendron honey.
Peroxynitrite scavenging assay: heterofloral honey > rhododendron honey > chestnut honey.
FRAP assay: chestnut honey > heterofloral honey > rhododendron honey.
When the sample concentration was increased, the activity in all three antioxidant methods also increased, as expected.
The order of the sample types, in terms of mineral concentration, was: chestnut > rhododendron > heterofloral.
Antimicrobial activity tests showed that the three honeys moderately inhibited especially *H. pylori*, *S. aureus*, *B. subtilis*, and *Candidas*, chestnut honey exhibiting the highest activity.

In conclusion, several antioxidant assays, mineral contents, and antimicrobial activity methods were utilized in order to evaluate the biological and chemical properties of Turkish honeys of three different types. The chestnut honey was found to have higher antioxidant and mineral contents. Low biological activities of rhododendron honey, as observed in this study, in comparison with chestnut and heterofloral honeys, reflect, once again, the need for a serious management system to stop the invasion by *R. ponticum* of the Black Sea and Mediterranean coasts (Erfmeier & Bruelheide, 2004; Eşen et al., 2004). In addition, the honey samples appeared to deserve further investigation of to their individual biologically active components, which may be an attractive source of nutraceuticals and medicinal ingredients.

Acknowledgments

We thank Karadeniz Technical University Research Fund for the financial support (Project No: 2003.111.002.6). We also thank Mr. Mehmet Kopal for providing the authentic honey samples.

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