Antioxidant activities of honeybee products and their mixtures

Duran Özkök* and Sibel Silici¹

Erciyes University, Safiye Cikrikcioglu Vocational School, Kayseri 38039, Turkey

¹Erciyes University, Agriculture Faculty, Department of Agricultural Biotechnology, Kayseri 38039, Turkey

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*Corresponding Author
Tel: +90-352-2076666
Fax: +90-352-4371383
E-mail: dozkok@erciyes.edu.tr

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Abstract In this study, the total phenolic content (TPC), antioxidant activity, and free radical scavenging activities (FRSA) of 70 samples comprising honeybee products (honey, pollen, royal jelly, and propolis) and their mixtures were determined. The TPC was determined in accordance with the Folin–Ciocalteu method, antioxidant activity with phosphomolybdenum, and FRSA with the 1,1-diphenyl-2-picryl hydrazyl (DPPH) assays. Honeybee propolis showed the greatest TPC, antioxidant activity, and FRSA followed by pollen, honey, and royal jelly, respectively. A positive correlation was observed between TPC and antioxidant activity of honey, pollen and mixed samples (respectively, $r=0.91$, $r=0.93$ and $r=0.92$) ($p<0.01$). Similarly, honey and mixed samples exhibited positive correlations with TPC and FRSA (respectively, $r=0.98$ and $r=0.92$) ($p<0.01$). It was concluded that honeybee products and their mixtures have antioxidant activity and FRSA and these effects may be attributed to their phenolic content.

Keywords: honeybee products, total phenolic content, antioxidant activity, free radical scavenging activity

Introduction

Air and environmental pollution, stress, and unhealthy nutrition have increased the formation of free radicals in the cells (1). Functional foods have come into prominence with their antioxidant characteristics being used in the prevention of some health problems, including cardiovascular diseases and cancer (1). Free radicals, which cause oxidation in the metabolism, are derived from external sources such as ozone, cigarette smoking, air pollutants, and industrial chemicals. It was indicated that oxidative stress created through the oxidation of free radicals may result in lipid peroxidation, enzyme deactivation, and aging (2). Thus, the search for effective natural compounds with antioxidant activity has been increased in recent years. Honeybee products like honey, bee pollen, royal jelly, and propolis are very rich in antioxidant compounds; hence, they have antioxidant and various other beneficial biological activities (3–5).

Honeybee products with already proven antioxidant characteristics, as reported by several studies, have a significant place among natural products (4,5). Honey is a natural, sweet food collected by honeybees from flower nectars or insect secretions. The antioxidant activity of honey has been mainly attributed to compounds such as phenolic compounds and enzymes (6). The presence of these compounds in honey varies according to the botanical and geographical origin, processing and others; the botanical origin of honey has great importance in terms of its antioxidant activity (7). Different monofloral

honeys have different phenolic contents (8). These phenolic substances are also used as a marker to identify the monofloral origin of honeys (9).

Bee pollen is a highly nutritious substance. It is commonly used to meet the growth and development needs of honeybees, to feed the broods, and to supply the protein required for royal jelly secretion. It is collected from flowers, mixed with salivary enzymes and honey and stored in brood cells. Royal jelly (RJ) is also a highly nutritious honeybee product. It is secreted from the hypopharyngeal glands of young (5–15 days old) bees and is effective in the differentiation of female eggs in queen bees (10). Propolis, another honeybee product, is a natural resinous hive product. Honeybees collect this resinous material from abundant plant sources around the hive, and mix it with saliva and beeswax (11). Propolis contains fatty acid derivatives, phenolics, including aromatic aldehydes and alcohols, flavonoids, naphthalene, and stilbene derivatives (12).

Honeybee products are composed of different nutrients and have different biological activities. In recent years, consumers have been using a combination of different foodstuffs to get the maximum benefit. Therefore, there is an increasing trend in the production of foodstuffs that include a combination of different products (2–5). The present study was aimed to investigate the antioxidant characteristics of honeybee products (honey, bee pollen, propolis, and royal jelly) and their mixtures with different compositions.

Materials and Methods

Chemicals The 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), 3,4,5-Trihydroxybenzoic acid (gallic acid; GA), Folin–Ciocalteu reagent, ascorbic acid (AA), and ethanol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sulfuric acid (H_2SO_4), sodium phosphate (Na_3PO_4), ammonium molybdate ($(\text{NH}_4)_2\text{MoO}_4$), sodium carbonate (Na_2CO_3), and methanol (MeOH) were obtained from Merck (Darmstadt, Germany).

Apparatus Spectrophotometric measurements were made using a Carry 100 UV visible spectrophotometer. Evaporation of organic solvents was performed with a rotavapor system, consisting of a rotary vacuum evaporator (Heidolph 4000 Laborota; Heidolph, Schwabach, Germany) with a water bath and a Heidolph Rotavac valve vacuum pump (Carl Zeiss AG, Oberkochen, Germany). The examination of the pollen slides was conducted with a Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan).

Samples Turkish unifloral honey samples ($n=20$) and bee pollen samples ($n=20$) from different botanical origins, royal jelly ($n=5$), crude propolis ($n=5$), and mixtures ($n=20$) were analyzed. The samples were collected by beekeepers between May and July of 2014 from apiaries located in different provinces of Turkey. The unifloral authenticity of honey and pollen samples was verified by pollen analysis. Citrus (Mersin), chestnut (Bursa), honeydew (Mugla), and multifloral (Sivas, Bingöl) honey samples and citrus (Mersin), chestnut (Bursa), clover (Panlyurfa), and multifloral (Sivas, Bingöl) pollen samples were obtained from beekeepers. The beehives were equipped with bottom-fitted pollen traps. Poplar type propolis was analyzed in our previous study (13). The pollen, royal jelly, and propolis samples were stored in a deep freezer (-20°C) and mixtures of honeybee products were stored in a refrigerator ($+4^\circ\text{C}$). Mixtures were supplied from Altiparmak Food Industry and Trade Co (Istanbul, Turkey). The ingredients of the mixtures were multifloral honey (MH), multifloral pollen (MP), royal jelly (RJ), and propolis (PR). The mixtures were combined as follows: MH+RJ (93% MH and 7% RJ), MH+RJ+PR (96.70% MH, 3% RJ, and 0.3% PR), MH+MP+RJ (85% MH, 10% MP, and 5% RJ) and MH+MP+RJ+PR (86.75% MH, 7.5% MP, 5% RJ, and 0.75% PR). Owing to the unlimited formulation of the mixtures, commercially available mixtures were preferred for the analysis. Honeybee products were diluted with methanol at ratios between 1/4 and 1/20.

Determination of TPC The Folin–Ciocalteu method was used to determine TPCs with some modifications (14). Each sample (1 g) was dissolved in 4 mL of methanol using a vortex-mixer and the solution was filtered through Whatman No. 1 filter paper. This solution (40 μL) was mixed with 2.4 mL of water and 200 μL of non-diluted Folin–Ciocalteu reagent, 600 μL Na_2CO_3 (20%), and 760 μL of water were then added. After incubation at room temperature in the dark

for 2 h, the absorbance of the reaction mixture was measured at 765 nm against a methanol blank and the results were calculated as mg GA equivalents (GAE/100 g of sample) (15).

Determination of antioxidant activity The samples were examined using the phosphomolybdenum method for antioxidant activities and expressed relative to that of ascorbic acid (16). Briefly, an aliquot of 0.4 mL of the sample in methanol was mixed with 4 mL of the reagent solution. Methanol was used as blank. The reaction mixture was vortexed and then left in a water bath at 95°C for 90 min. Absorbance was measured at 695 nm and the antioxidant activity was calculated as AA equivalents (mg AAE/1 g of sample) (15).

Determination of free radical scavenging activity (FRSA) The samples were evaluated using the DPPH assay with some modifications for FRSA (17). The sample (1 g) was dissolved in 4 mL methanol using a vortex-mixer and the solution was filtered (Whatman No. 1 filter paper). A 100 μL aliquot of samples was mixed with 450 μL Tris–HCl and 1,000 μL of 6×10^{-5} M DPPH in methanol. The mixtures were left in the dark for 2 h at room temperature and the absorbance at 517 nm measured in a spectrophotometer using methanol as blank. (15). The FRSA of the samples was calculated according to a previously reported formula (17).

FRSA (%):

$\text{Control Absorbance} - \text{Sample Absorbance} / \text{Control Absorbance} \times 100$

Results and Discussion

The TPC of honey samples varied between 57.59–261.71 mg GAE/100 g (Fig. 1). The least TPC was observed in citrus honey and the greatest value was observed in chestnut honey. Similar to chestnut honey, chestnut pollen also had the highest TPC (3570.78 $\pm$ 38.58 mg GAE/100 g) followed by citrus, mixed, and trifolium pollens, respectively (Fig. 2). Royal jelly (59.16 $\pm$ 5.92 mg GAE/100 g) had the second lowest TPC just before citrus honey. However, poplar-type propolis had the highest TPC (Table 1). The TPCs of the mixtures varied between 116.14–619.52 mg GAE/100 g (Table 2) with the lowest value in the royal jelly–honey combination and the highest value in the honey–pollen–royal jelly–propolis combination.

From the highest to the lowest, the antioxidant activities of citrus, chestnut, honeydew (pine honey), and flower honey were identified as 53.61 $\pm$ 5.28, 92.66 $\pm$ 4.81, 43.95 $\pm$ 2.44, and 45.36 $\pm$ 2.70 mg AAE/g, respectively (Fig. 3). With regard to antioxidant activity, chestnut pollen had the highest and the mixed pollen had the lowest value (Fig. 4). Among the mixtures of honeybee products, the mixture of royal jelly, honey, pollen, and propolis had the greatest antioxidant activity (72.98 $\pm$ 3.08 mg AAE/g) and the triplet mixture of royal jelly, honey, and propolis had the least antioxidant activity. The antioxidant activity of royal jelly and propolis was respectively

Fig. 1. TPC of honey samples. TPC, Total phenolic content; GAE, Gallic acid equivalent; Each value is expressed as a mean $\pm$ SD (values with different superscript letters (a-d) indicate that mean values are significantly different at $p<0.01$).

Fig. 2. TPC of bee pollen samples. TPC, Total phenolic content; GAE, Gallic acid equivalent; Each value is expressed as a mean $\pm$ SD (values with different superscript letters (a-d) indicate that mean values are significantly different at $p<0.01$).

observed as 59.02 ± 5.98 mg AAE/g and 267.37 ± 0.33 mg AAE/g.

As was the case in TPC and antioxidant activity, chestnut honey also had the highest FRSA (% inhibition) ($53.44\pm0.36\%$) followed by pine, citrus, and flower honey. Among the pollen samples, again chestnut pollen had the greatest FRSA ($91.00\pm0.19\%$). Interestingly, among all the investigated samples, royal jelly had the lowest ($3.03\pm0.16\%$) and propolis had the greatest ($96.13\pm0.08\%$) FRSA. In addition, of the mixtures, the combination including all products and the royal jelly–honey–pollen mixture had the greatest FRSA with values of $94.59\pm0.77\%$ and $91.88\pm0.62\%$, respectively.

Honey, bee pollen, and mixed samples exhibited positive correlations with TPC and antioxidant activity (respectively $r=0.91$, $r=0.93$, and $r=0.92$) ($p<0.01$). Similarly, honey and mixed samples exhibited positive correlations with TPC and FRSA (respectively $r=0.98$ and $r=0.92$) ($p<0.01$). However, a significant correlation was not observed between FRSA and antioxidant activity in pollen samples ($r=0.37$) ($p<0.01$).

The TPC of honey samples from different botanical and geographical origins varied between 57.59 – 261.71 mg GAE/100 g and the highest value was observed in chestnut honey. Previous researchers determined the TPCs, antioxidant, and FRSA of nectar and honeydew honeys collected from the geographical regions of different countries and

with different botanical origins. Al-Mamary *et al.* (8) investigated the antioxidant activity and TPC of four different Yemeni honeys (acacia and ziziphus) and three different exported honeys (USA, Sweden, and Iran) and reported the highest values for acacia (*Acacia ehrenbergina*) honey and indicated positive correlations between TPC and antioxidant activity. In the same study, the TPCs of diluted honey samples varied from 56.32 to 246.21 mg/100 g honey by the Folin–Ciocalteu method (8). In a study on honey obtained from Northeast Portugal, it was found that dark color honey had higher TPC than light colored honey (18). The researchers also reported FRSA (EC_{50} mg/mL) as 27.4 for dark-colored honey and 68.17 for light-colored honey. The TPC and flavonoid content of honey collected from different geographical regions of Italy were between 60.50 – 276.04 mg GAE/kg and 41.88 – 211.68 mg QE/kg, respectively (19). They indicated a significant correlation of TPC with antioxidant activity and FRSA. There are several studies in the literature investigating the bioactive characteristics of monofloral honey. For instance, the antioxidant activity of honey collected from Romania was reported in descending order as lime>poly flower>sea buckthorn >acacia (20). The TPC of seven honey samples produced in Slovenia was 44.8 mg/kg for acacia honey and 241.4 mg/kg for fir honey (21). The researchers also reported the lowest antioxidant activities for

Table 1. TPC, antioxidant activity, and FRSA of honeybee products

Samples	<i>n</i>	TPC ¹⁾ (mg GAE/100 g) ²⁾	Antioxidant activity (mg AAE/g) ³⁾	FRSA ⁴⁾ (% inhibition)
Multiple Honey	20	123.36 ± 21.21 ⁵⁾	58.89 ± 2.20	28.44 ± 4.02
Mixtures	20	361.47 ± 52.38	49.55 ± 3.74	70.36 ± 6.68
Bee pollen	20	2340.07 ± 199.32	42.37 ± 3.81	89.66 ± 0.39
Royal Jelly	5	59.16 ± 1.94	59.02 ± 1.96	5.72 ± 0.12
Propolis	5	32025.93 ± 673.55	267.37 ± 0.11	96.14 ± 0.03

¹⁾TPC, Total phenolic content.

²⁾GAE, Gallic acid equivalent.

³⁾AAE, Ascorbic acid equivalent.

⁴⁾FRSA, Free radical scavenging activity.

⁵⁾Each value is expressed as a mean $\pm$ SD.

Table 2. TPC, antioxidant activity and FRSAs of mixtures

Honeybee product mixtures	n	TPC ¹⁾ (mg GAE/100 g) ²⁾	Antioxidant activity (mg AAE/g) ³⁾	FRSA ⁴⁾ (% inhibition)
MH+RJ ⁵⁾	5	116.14±0.46 ^{a6)}	47.75±6.16 ^b	30.46±1.48 ^a
MH+RJ+PR	5	217.79±2.59 ^b	38.42±4.93 ^a	64.50±0.44 ^b
MH+MP+RJ	5	492.43±8.13 ^c	39.06±1.01 ^a	91.88±0.62 ^c
MH+MP+RJ+PR	5	619.52±1.99 ^d	72.98±3.08 ^c	94.59±0.77 ^d

¹⁾TPC, Total phenolic content.²⁾GAE, Gallic acid equivalent.³⁾AAE, Ascorbic acid equivalent.⁴⁾FRSA, Free radical scavenging activity.⁵⁾MH, multifloral honey; MP, multifloral pollen; RJ, royal jelly; and PR, propolis.⁶⁾Each value is expressed as a mean±SD (values with different superscript letters (a-d) within the same column are significantly different at $p<0.01$). **Fig. 3.** Antioxidant activity of honey samples. AAE, Ascorbic acid equivalent. Each value is expressed as a mean±SD (values with different superscript letters (a-c) indicate that mean values are significantly different at $p<0.01$). **Fig. 4.** Antioxidant activity of bee pollen samples. AAE, Ascorbic acid equivalent; Each value is expressed as a mean±SD (values with different superscript letters (a-c) indicate that mean values are significantly different at $p<0.01$).

acacia and lime honey and higher values for fir, spruce, and forest honey with dark colors and indicated significant correlations among these parameters (TPC and antioxidant activity). Complying with the current findings, the results of the above mentioned studies can be summarized as follows: a) TPC and antioxidant activity of honey can vary based on geographical and botanical origin. b) dark-colored honey has higher TPC, antioxidant activity, and FRSA. c) TPC of honey is positively correlated with antioxidant activity and FRSA.

Pollen has recently gained recognition as a functional food. Therefore, various studies have recently been performed to investigate the TPC, antioxidant activity, and FRSA of flower pollen, honeybee collected pollen, and stored pollen. Chestnut pollen had the highest TPC in this study. The bioactive characteristics of 12 honeybee pollens collected from Slovakia were investigated and the polyphenol content of rape (*Brassica napus*), poppy (*Papaver somniferum*), and sunflower (*Helianthus annuus* L.) pollens was reported as 1383.67, 817.33, and 691.67 mg/kg, respectively (22). In addition, the FRSA of these pollens was found as 86.25, 75.93 and 47.97 (% inhibition), respectively (22). Leja *et al.* (23) investigated the antioxidant activity and FRSA of honeybee pollens obtained from 12 different plants and reported the values as between 0.8–86.4 and 8.6–91.5%, respectively. The researchers indicated the highest and the lowest levels of total phenols for *Pyrus communis* and *Zea mays*

pollens and reported that TPC was not correlated with FRSA. The polyphenol content of different bee pollen samples was reported as between 7.4–9.7 mg/g and for pollen mixture the value was 8.2 mg/g. Kroyer and Hegedus (24) reported that the ethanol extract of bee pollen samples showed the highest FRSA. Morais *et al.* (25) carried out palynological analysis of bee pollens collected from a Portuguese Natural Park and identified eight different families and reported the TPC as between 10.5–16.8 mg GAE/g. It is reported that the greatest polyphenol concentration for methanol extract of bee pollen was obtained from *Salix spp.* as 16.4 mg GAE/g. This was followed by *Taraxacum officinale* (16.2 mg GAE/g), *Centaurea cyanus* L. (16.0 mg GAE/g), *Crataegus monogyna* (7.7 mg GAE/g), and *Capsella bursa-pastoris* L. (15.2 mg GAE/g). Mejias and Montenegro (26) reported the antioxidant activity (FRAP assay) and FRSA (DPPH assay) of lotus pollen and escallonia pollens produced in two different geographical locations of a volcano zone for lotus pollen as between 67.8–82.9 and 69.5–90.3 (mg AAE/g), respectively and between 112.1–139.3 and 119.9–140.3% for escallonia pollen, respectively. Furthermore, a study was conducted on the antioxidant activity of pollens collected by honeybees from the Sonoran Desert and the highest activity was reported for Mimosa pollen (27). As was the case for honey, positive correlations were identified between the TPC and antioxidant activity of pollen. Moreover, a study was conducted with 12

honeybee pollens with different botanical origins and Ferric ion reducing antioxidant power assay (FRAP) was used to examine for FRSA (28). The researchers indicated significant variations in these activities on the basis of floral origin and reported that a significant correlation was not observed between the TPC and antioxidant activity.

Some biomolecules and compounds in royal jelly were reported to have an antioxidant effect. For instance, albumin proteins in royal jelly also have antioxidative impacts (29). Protein and phenolic fractions of royal jelly have high antioxidant activity and FRSA against reactive oxygen species (30). In addition, phenolic compounds containing flavonoid and cinnamic acid derivatives were also identified in royal jelly. The antioxidant activity of royal jelly was tested in detail through in-vivo studies conducted on laboratory animals (5).

Among the honeybee products, propolis had the greatest antioxidant activity in our study. There are several studies about the antioxidant activity of propolis. The total polyphenol content of propolis samples collected from different countries was investigated and the values reported varied between 31.2 mg GAE/g (Thailand) and 299 mg GAE/g (China) (31). The researchers also indicated the existence of p-coumaric acid, ferulic acid, chrysine, and galangin-like phenolic compounds in propolis samples. Significant linear correlations were identified between TPC and FRSA and between TPC and reducing power. Moreover, the antioxidant characteristics of propolis collected from Erzurum (Turkey) were investigated and correlations were identified between the TPC and antioxidant activity of propolis (32). The researchers also identified caffeic acid, ferulic acid, ellagic acid, p-coumaric acid, gallic acid, ascorbic acid, p-hydroxybenzoic acid, quercetin, tocopherol, and vanillin in propolis samples. Furthermore, the TPC (colorimetric assay) of propolis samples collected from different regions of Portugal was found to be between 151–329 mg GAE/g and the FRSA to be between (EC_{50}) 6.22–52 μ g/mL (33).

Although there are several studies separately investigating the TPC, antioxidant activity, and FRSAs of honeybee products with different botanical and geographical origins, the number of studies assessing or comparing them together is very limited. The highest FRSA for propolis was reported and it was followed by royal jelly, buckwheat, and acacia honey (2). The high activity found in buckwheat honey was attributed to its dark color. The researchers attributed the higher antioxidant activity of propolis compared with that of other honeybee products to its flavone, isoflavone, quercetin, catechin, and isocatechin-like flavonoid contents. Honey, propolis and royal jelly samples were tested using the electrochemical method and a positive correlation between the TPC and antioxidant activity was identified (34). Free or glucosidal phenolic compounds in plants are composed of flavonoids, coumarins, and cinnamic acid derivatives (35). It was proven by scientific studies that all honeybee products contained phenolic substances since all these products were derived from the plants, even with some animal additives

through salivary enzymes. The phenolic compounds of honeybee products are usually in the form of flavonoids and they all have different phenolic contents. Such differences are mostly because of the honeybee species and sub-species as well as to climate, season, altitude-like environmental factors, and the type of plants from which nectar and pollen are supplied.

In conclusion, in recent years health protection through natural foodstuffs has been gaining popularity. People prefer to protect their health and try not to be sick instead of exposing themselves to the side effects of medicines and paying large sums for treatments. Currently, the concept of “adequate nutrition”, i.e., the sufficient supply of carbohydrates, proteins, fats, vitamins, and minerals, has come into prominence. Through optimum nutrition, it is possible to promote health, improve general well-being and reduce the risk of certain illnesses (36). A functional food with highly nutritious characteristics and positive impacts on the physiology and psychology of the organism can therefore be far superior to traditional foodstuffs. In this study, the mixture containing propolis had higher TPC and antioxidant activity. This was an expected outcome since propolis was reported to have the highest biologic activity in previous studies investigating honeybee products together. In recent years, environmental pollution, stress, and the consumption of take-away foods have increased free radical formation in the human body. It is well known that honeybee products like honey, pollen, propolis, and royal jelly are rich in antioxidants and they have various antioxidative impacts and beneficial functions in the human body. Therefore, honeybee products or their mixtures should be included in the daily diet for a healthy life. In this study, it was also concluded that honeybee products have high bioactive characteristics and the inclusion of propolis into mixtures may improve the bioactive characteristics of the mixtures.

Disclosure The authors declare no conflict of interest.

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