

## Investigation of Antioxidant and Antimicrobial Activity of Different Multifloral Honeys

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### A B S T R A C T

In this study, we purposed that antioxidant and antimicrobial characteristics of different nine different honey types belonging to Turkey flora. Samples were obtained from local markets. The antimicrobial activities of the samples against six bacteria and only yeast (*Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Candida albicans*) were assessed using disc diffusion method. As a result of these studies, it was determined that the studied samples did not have antimicrobial activities against these seven pathogens. Furthermore, antioxidant activities of the honey samples have been carried out by using six antioxidant test methods. These methods are DPPH free radical scavenging (DPPH) and hydrogen peroxide scavenging (HPSA) activities, ferric reducing antioxidant power (FRAP) and ferrous ion chelating activities (MCA). In addition, the total phenolic contents (TPC) and the total flavonoid contents (TFC) have been determined. The obtained activity values were compared to standard antioxidants (BHA, RUT and TRO). According to these results, sample 9 has more influential antioxidant activity than the other samples.

Keywords: Antimicrobial activity, Antioxidant activity, Multifloral Honey, Total flavonoid contents, Total phenolic contents

### Introduction

Honey is a flavorful natural product, which has high nutritive value and is consumed for its effects on human health, with antioxidant, bacteriostatic, anti-inflammatory and antimicrobial properties, as well as wound and sunburn healing effects [1]. Honey has been used as a food, medical product, treatment and prevention of wound infections since the earliest times. Recently, the bad effects on human health of drugs and antibiotics have directed the use of more traditional and natural nutrients. Honey, a rediscovered natural product, has also begun being used for numerous benefit effects. The floral diversity in Turkey allows a wide variety of honeys to be produced, with a lot of biochemical properties. Honey is known to

has valuable antioxidant activity [2]. Honey is rich in phenolic compounds, is food collected by the bees from the plants. The total phenolic content, total flavonoid component, DPPH, FRAP, MCA and HPSA in honey are strongly correlated with its antioxidant activity [3-5], so can be used as a reliable parameter to indicate antioxidant activities in honey.

The aim of this paper is to determine the antioxidant and antimicrobial activities of honey samples by using different antioxidant tests, and disc diffusion method, respectively. For determination of antioxidant activity, six different antioxidant activity assays were done in this study. DPPH radical and hydrogen peroxide scavenging activities are carried

out by widely used methods in the literature. Metal chelating activity assays were carried out by using ferrozine. Ferric reducing antioxidant power (FRAP) assays were applied owing to ferric/ferrous formation. Finally, total phenolic and

flavonoid contents were investigated via colorimetric methods. The obtained results showed that honey samples can be used for medicinal purposes as natural source according to antioxidant activity.

## Materials and Methods

### Samples

Honey samples (n=9) were collected from local markets in different province in Turkey. The samples are flower honeys that are classified of secretion, strain and comb honey.

### DPPH Free Radical Scavenging Activity Assay (DPPH)

DPPH free radical scavenging activities of honey samples were examined according to Blois method (1958) [6]. In this assay, sample solution (3.0 mL) and DPPH<sup>•</sup> (1.0 mL, 0.1 mM prepared in absolute ethanol) was mixed and vortexed. It was kept at the room temperature in a dark environment during 30 min. The absorbance values of samples/standards were recorded at 517 nm. The decrease in absorbance value of tube is a demonstration of high rate of free radical scavenging activity. The free radical scavenging activity of the sample is expressed as SC<sub>50</sub> (µg/mL).

### Hydrogen peroxide scavenging activity assay (HPSA)

Ruch method (1989) were used for determination of the hydrogen peroxide scavenging activity of samples [7]. In this assays, sample (3.4 mL) and H<sub>2</sub>O<sub>2</sub> (0.6 mL, 40 mM prepared with 0.04 M phosphate buffer (pH =7.4)) was mixed during 10 min. The absorbance value of the mixture was recorded at 230 nm against to blank sample. Phosphate buffer (0.04 M,

pH=7.4), not include hydrogen peroxide solution, was used as a blank sample. Decreasing in the absorbance value indicates that hydrogen peroxide scavenging activity of this sample is high. Hydrogen peroxide scavenging activity of sample is expressed as SC<sub>50</sub> values (µg/mL).

### Metal chelating activity assay (MCA)

The metal chelating activities of the samples/standard antioxidant materials were determined by following the Dinis Method (1994) [8]. The sample/standard solutions (0.4 mL) mixed with FeCl<sub>2</sub> (0.05 mL, 2 mM). The mixtures were kept during 10 min. Then they were filled to 4 mL by adding ferrozine (0.2 mL, 5 mM) and absolute ethanol (3.3 mL). The all tubes were forcefully shaken and the absorbance values of the samples were recorded at 562 nm. A decrease in absorbance value demonstrates a high level of metal chelating activity of samples/standards. The metal chelating activities of the samples/standards were calculated by using this formula:

$$\text{Ferrous Ions Chelating Activity (\%)} = [1 - (A_s/A_c)] \times 100$$

A<sub>c</sub>: Absorbance value of control; A<sub>s</sub> : Absorbance value of sample/standard.

*Ferric reducing antioxidant power (FRAP) assay*

Ferric reducing antioxidant activities of the samples/standards were carried out by using Oyaizu (1986) method [9]. Samples/standards (2.5 mL) were mixed with PBS (2.5 mL, 0.2 mol L<sup>-1</sup>, pH 6.6) and potassium ferricyanide (2.5 mL, 1.0%). The obtained mixtures were incubated at 50°C for 20 min and then TCA (2.5 mL, 10%) were mixed with the all tubes. 2.5 mL of this solution was mixed with distilled water (2.5 mL) and FeCl<sub>3</sub> (0.5 mL, 0.1%). The ferric/ferrous transformation was investigated via the absorbance values at 700 nm.

$$FRAP (\%) = (A_s/A_c) \times 100$$

A<sub>c</sub>: Absorbance of control, A<sub>s</sub>: Absorbance of samples.

*Determination of total phenolic contents (TPC)*

Total phenolic contents in the samples were examined according to the Slinkard and Singleton method (1977) using Folin C reactive [10]. A sample was taken in ethyl alcohol solution (1 mg/mL, 0.5 mL) and then deionized water (7.0 mL). 0.5 mL Folin C reagent was added and the content of the tube mixed thoroughly. After 3 minutes, Na<sub>2</sub>CO<sub>3</sub> (2.0%, 2.0 mL) was added and the sample was kept at room temperature and was shaken occasionally during 2 h. The absorbance values of samples/standards were recorded at 760 nm. Total phenolic contents of the samples were calculated with the gallic acid calibration curve (R<sup>2</sup>:0.9963).

*Determination of total flavonoid contents (TFC)*

Total flavonoid contents of samples were measured according to the aluminium chloride colorimetric method [11]. Ethyl

alcohol solution of the samples (1 mg/mL, 0.5 mL) were taken and deionised water (1.5 mL) was added. Then, AlCl<sub>3</sub>·6H<sub>2</sub>O (10.0%, 0.1 mL) and 1 M potassium acetate (0.1 mL) were added and was diluted using deionized water (2.8 mL). After incubation during 30 min, absorbance values of samples/standards were measured at 415 nm. The sample's total flavonoid contents were calculated with the the catechin calibration (R<sup>2</sup>:0.9950).

*Antimicrobial activity*

The agar disc diffusion method, which is one of the most preferred methods in determining the antimicrobial activity of honeys, was used. In this technique, a suitable trough with the test organism is used with a trough system in which the substance to be tested is located. At the end of the incubation period, if the substance is effective, inhibition zones are formed around the pits without significant growth. The diameters of the inhibition zones formed are measured and evaluated [12,13]. The microorganisms used in the antimicrobial activity test were obtained from the microbiology laboratory culture collection of the Department of Biology of Ordu University. Three Gram (+) bacterial strains (*Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*), three Gram (-) bacterial strains (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*) and a yeast strain (*Candida albicans*) were used for the determination of antimicrobial activity. All bacteria were grown at 37°C overnight in tryptic soy broth (TSB) medium. The species *Candida albicans* yeast was grown in Sabouraud Dextrose Broth at 27°C for 48 hours. Mueller Hinton Agar for bacteria and Sabouraud Dextrose Agar for yeast were used for the antimicrobial activity determination. The media used in the study

were first sterilized in autoclave (1.5 atmosphere pressure and 121°C, 15 min) and allowed to cool to 45-50°C. These media were then poured up to 20 ml with sterile pipettes into sterile petri dishes. The medium was expected to freeze by providing a homogenous distribution. The bacteria and yeast species, which were taken again by passaging from the stock culture, were left on the agar with 108 cells / ml microorganisms up to 200 µl and smeared with glass baguette. Then, blank

discs are placed on equal distances with each petri dish and 9 different kinds of honey 15 µl each, each petri dish on its own disc was dropped. All these seeding operations were carried out in the biosafety cabinet. Bacteria-cultivated petri dishes were incubated for 48 hours at 25°C, while the yeast-planted petri dishes for 24 hr at 37°C. At the end of the period, inhibition zones formed on the media were measured in mm with a ruler.

## Results and Discussion

Antioxidant molecules give hydrogen or electron to DPPH radical so it changes stable non-radical form as DPPH-H, while antioxidants and DPPH<sup>•</sup> radical interact [14]. The SC<sub>50</sub> values (µg/mL) were found as Sample 9 (76.50), Sample 5 (91.59), Sample 1 (115.21), Sample 6 (121.18), Sample 3 (171.64), Sample 2 (199.68), Sample 4 (225.02), Sample 7 (271.15) and

Sample 8 (275.48). BHA, RUT and TRO results have been determined as 18.56, 10.56 and 190.51, respectively. When compare the DPPH activities of honey samples with standards, samples show effective radical scavenging activities (Table 1).

**Table 1.** DPPH and hydrogen peroxide scavenging activities of samples and standards in terms of The SC<sub>50</sub> values (µg/mL).

	S1	S 2	S3	S4	S5	S6	S7	S8	S9	BHA	RUT	TRO
<b>DPPH</b>	115.21	199.68	171.64	225.02	91.59	121.18	271.15	275.48	76.50	18.56	10.56	190.51
<b>HPSA</b>	129.97	90.55	81.61	62.32	193.65	197.24	76.10	57.70	185.4	8.76	17.78	26.46

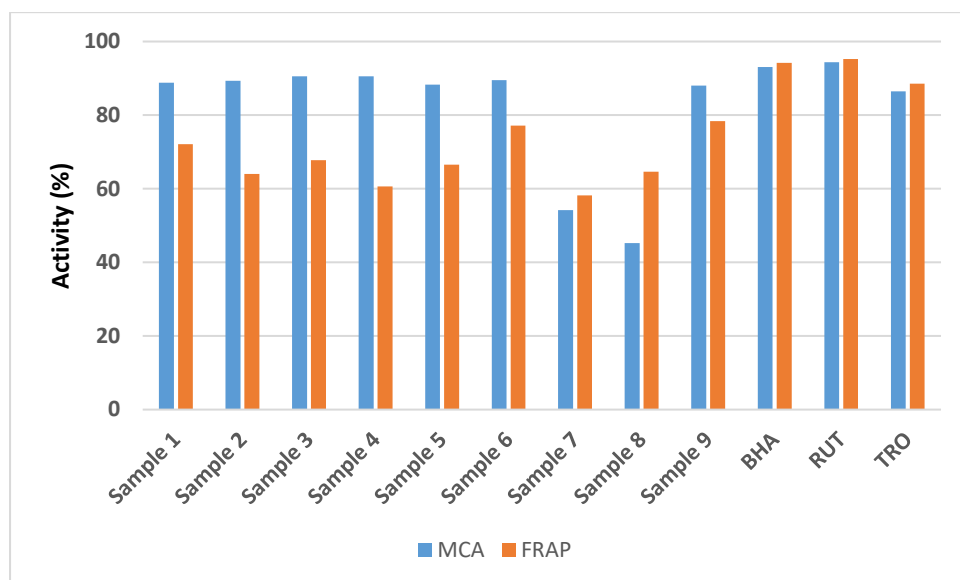
Hydrogen peroxide is produced in living aerobic organisms. Though it is not very reactive, it can be toxic for cells owing to the higher OH<sup>•</sup> concentration. Also, increasing hydrogen peroxide levels to cells in culture can bring about oxidative DNA damage. Therefore, scavenging hydrogen peroxide is very vital for protection of pharmaceuticals and food systems [15]. Hydrogen peroxide scavenging activity of

samples and standards in terms of SC<sub>50</sub> values (µg/mL) present in Table 1. The hydrogen peroxide scavenging activities of samples and standards decrease as follow BHA > RUT > TRO > Sample 8 > Sample 4 > Sample 7 > Sample 3 > Sample 2 > Sample 1 > Sample 9 > Sample 5 > Sample 6. According to these obtained results, all standards have higher activity than samples but samples (especially Sample 8 and

Sample 4) show effective activities when we compared to samples results.

In metal chelating activity assays, ferrozine and  $\text{Fe}^{2+}$  form strong red complex. In the presence of other chelating agents, the red

color intensity of complex disrupted and new complex forms between chelating agents (samples/standards) and  $\text{Fe}^{2+}$  [16]. Metal chelating activity results were compared in Figure 1.



**Figure 1.** Comparison of metal chelating and ferric reducing antioxidant power activities of samples and standards.

In ferric reducing antioxidant power assay, the presence of reductant components in the samples/standards cause the reduction of the  $[\text{Fe}(\text{CN})_6]^{3-}$  complex to the  $[\text{Fe}(\text{CN})_6]^{4-}$ . Therefore,  $\text{Fe}^{2+}$  can be determined by measuring the formation of Perl's Prussian Blue ( $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ ) at 700 nm [16]. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The highest reducing power was obtained for the Sample 1 and the lowest for the Sample 8. The other results were given in Figure 1.

The phenolic components in samples shows antioxidant activity owing to their free radical and active oxygen species scavenging activities. Flavonoids which are very important plant compounds have active hydroxyl groups that show antioxidant activity [11]. According to obtained results, the gallic acid and catechin equivalents of total phenolic and flavonoid contents of samples range from 184.85-26.57 mg GAE/g and 20.26-3.13 mg CAE/g, respectively. Total flavonoid contents were found as 58.77, 43.30 and 22.91  $\mu\text{g}$  of catechin equivalents/g, respectively (Table 2)

**Table 2.** Total phenolic and flavonoid contents of samples.

	S1	S2	S3	S4	S5	S6	S7	S8	S9
<b>TPC (mg GAE/g)</b>	106.20	79.33	83.83	41.28	149.00	98.50	57.45	26.58	184.90
<b>TFC (mg CAE/g)</b>	15.09	16.35	5.52	5.27	19.88	13.83	3.63	3.13	20.26

The three Gram (+) and three Gram (-) bacteria and one yeast were used for determination of antimicrobial activities of honey samples. In this assay, no samples show antimicrobial activity against the used microorganisms.

The study showed that *S. aureus* were the most sensitive microorganisms and *Bacillus subtilis*, *Staphylococcus lentus*, *Klebsiella pneumoniae* and *Escherichia coli* were each moderately sensitive to the antimicrobial activity of honey extracts. Nevertheless, no antimicrobial activity was observed in the test with *Pseudomonas aeruginosa* [17].

Using quantitative bactericidal assay with neutralization of individual honey bactericidal compounds, confirmed that MGO (methylglyoxal) is indeed the major component for the activity of manuka honey against *S. Aureus* but additional compounds contributed to the activity against *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* [18].

## Conclusion

Natural sources or their products are widely used as primary source for the alternative medicine in the world. They have numerous advantages because of the fewer side effects. Honey can be helpful for the treatment of various diseases due to the presence of antioxidant activities and useful components for the human body. Thereby, the results clearly show that honey may be used as natural sources in medical and other

Similarly, Escuredo et al. (2012) analyzed antibacterial activity by MIC in 23 honeys from Galicia, against gram negative and gram positive bacteria and reported a greater inhibitory activity against *B. cereus* [19].

All types of honey (n=24) showed antibacterial activity against the four bacteria tested (*Shigella dysenteriae*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Bacillus cereus*). The antibacterial activity was more effective with gram negative bacteria than with gram positive ones. Consequently, more efficient results occurred against *S. aureus* and *B. cereus* [20]. The obtained results of our samples are different with the literature. We can say that the differences can originate from the flora, storage temperature and conditions, honey's main components. In addition, the discrepancy may be resulted from to be commercial products of honey samples. These results can indicate that honey samples could be obtained with chemical processes.

sectors. In the future studies, active components can be determined via chromatographic methods. Although the antimicrobial activities of the honey samples could not be determined against seven pathogens, it was determined that they exhibited antioxidant activity.

## Farklı multifloral balların antioksidan ve antimikrobiyal aktivitelerinin araştırılması

**Öz:** Bu çalışmada, Türkiye florasına ait dokuz farklı bal tipinin antioksidan ve antimikrobiyal özelliklerinin belirlenmesini amaçladık. Örnekler yerel marketlerden elde edilmiştir. Örneklerin antimikrobiyal aktivitesi altı bakteri ve bir mantar (*Clostridium perfringens*, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* ve *Candida albicans*) kullanarak disk difüzyon methodu ile belirlendi. Bu çalışmaların sonucunda, çalışılan örneklerin yedi patojene karşı antimikrobiyal aktiviteye sahip olmadığı belirlendi. Ayrıca balların antioksidan

aktiviteleri altı antioksidan test metodu kullanarak gerçekleştirildi. Bu metodlar DPPH serbest radikal giderme (DPPH) ve hidrojen peroksit giderme aktiviteleri (HPSA), demir indirgeme gücü (FRAP) ve demir iyon şelatlama aktiviteleridir (MCA). İlâveten, toplam fenolik içerik (TPC) ve toplam flavonoid içerikler belirlendi. Elde edilen değerler standard antioksidanlarla (BHA, RUT and TRO) karşılaştırıldı. Bu sonuçlara göre, 9 numaralı örnek diğer örneklerden daha etkili antioksidan aktiviteye sahiptir.

**Anahtar Kelimeler:** Antimikrobiyal aktivite, antioksidan aktivite, multifloral bal, toplam flavonoid içeriği, toplam fenolik madde içeriği

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