

Antioxidant, Antiinflammatory and Antimutagenic Activities of Various Kinds of Turkish Honey

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Çeşitli Türk Ballarının Antioksidan, Antiinflatuvar ve Antimutagenik Aktiviteleri

SUMMARY

Honey is used as an essential nutrient since ancient times and considered as a part of traditional folk medicine. In this study, a comparative study was carried out to assess antioxidant, anti-inflammatory and antimutagenic activities of six varieties of honey from different botanical and geographical region. According to the results, thyme and unbranded multifloral honey showed the highest antioxidant activity. All of the honey samples inhibited the LPS induced inflammation significantly at 200 mg/ml. Moreover, thyme and branded chestnut honey nearly neutralized the LPS effect. Ames test results showed no great differences between samples and the honey extracts with doses of 10 and 50 mg/plate were found to have a weak antimutagenic activity with or without S9 activation. The results of this study demonstrated that all honey samples showed antioxidant and anti-inflammatory activity although having different composition, botanical origin and processing. Accordingly, daily consumption of any kind of honey may be suggested to protect the body against the harmful effects of free radicals and oxidative stress related diseases.

Key Words: Honey, folk medicine, antioxidant, antiinflammatory, antimutagenic

ÖZET

Eski çağlardan beri esansiyel besin ögesi olarak tüketilen bal, geleneksel halk ilacının bir parçası olarak görülmektedir. Bu çalışmada, farklı botanik ve coğrafi bölgeden altı çeşit balın antioksidan, antiinflatuvar ve antimutagenik aktivitelerinin karşılaştırması amaçlanmıştır. Sonuçlara göre, en yüksek antioksidan aktiviteyi kekik ve markasız multifloral bal göstermiştir. Tüm bal örnekleri 200 mg/ml dozda, LPS ile indüklenen inflamasyonu anlamlı şekilde azaltmıştır. Kekik ve markalı kestane balı LPS'nin etkisini neredeyse nötralize etmiştir. Ames testinde numuneler arasında fark görülmemiş, 10 ve 50 mg dozlarda bal ekstraktlarının S9 ile veya S9 aktivasyonu olmaksızın zayıf antimutagenik aktiviteye sahip olduğu görülmüştür. Bu çalışmanın sonuçları, farklı kompozisyon, botanik köken ve işlemlere rağmen, tüm bal örneklerinin antioksidan ve antiinflatuvar aktiviteye sahip olduğunu göstermiştir. Buna göre, sağlığımızı serbest radikallerin zararlı etkilerine ve oksidatif strese ilişkilendirilen hastalıklara karşı korumak için bal tüketimi önerilebilir.

Anahtar Kelimeler: Bal, halk ilacı, antioksidan, antiinflatuvar, antimutagenik

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INTRODUCTION

Honey, the nectar of various types of plants processed by honeybees, is used as an essential nutrient since ancient times and is considered as a part of traditional folk medicine. Although honey types differ in their composition depending on their botanical origin and processing, honey is mainly composed of monosaccharide sugars which are glucose and fructose (55-85%), water (15-20%) and other ingredients (6%) such as flavonoids, phenolic constituents, vitamins and minerals (Yeşilada, 2015). Some of these vitamins found in honey include ascorbic acid, pantothenic acid, niacin and riboflavin; and minerals include calcium, copper, iron, magnesium and zinc (Ajibola et al., 2012). Also, proline, glutamic acid, alanine, phenylalanine, tyrosine and leucine are the most common protein and amino acid constituents of honey (Hermosin et al., 2003). Because of its high sugar content, important vitamin, mineral and some other bioactive constituents, honey is a valuable nutrient to investigate.

Besides its nutritional use, honey also exerts some important therapeutic effects. The medicinal importance of honey has also been known since

ancient times. The first written reference for honey was a Sumerian tablet that dates back to 2100-2000 BC, which mentioned honey's use as a drug and an ointment. Aristotle (384-322 BC) also referred to pale honey as being "good as a salve for sore eyes and wounds" (Mandal et al., 2011). One of the important therapeutic effects of honey is its antioxidant activity. Based on its antioxidative property, honey protects cell components from harmful effects of free radicals, and hinder age-related diseases associated with oxidative stress (Schramm et al., 2003). Antimutagenicity is another activity of honey that should be underlined (Wang et al., 2002). Honey has also been shown to have anti-inflammatory and antibacterial as well as wound-healing activities (Vallianou et al., 2014).

In this study, we aimed to investigate total antioxidant capacities of six kinds of Turkish honey and to compare their anti-inflammatory and antimutagenic activities.

Materials and Methods

Six kinds of honey samples were randomly purchased for the analysis. Sources and color of the honey samples were shown in Table 1.

Table1. Coding and sources of investigated Honey samples

	Sample name	Source	Color
A	Pine honey	Yerkesik and Marmaris region	Light amber
B	Unbranded chestnut honey	Eastern Anatolia	Dark amber
C	Citrus honey	Citrus trees' flowers of Mediterranean region	Extra white
D	Branded chestnut honey	Chestnut trees of Black Sea region	Amber
E	Thyme honey	Astragalus and Thymus species from Central and Eastern Anatolia	White
F	Unbranded multifloral honey	Black Sea region	Extra light amber

Preparation of Samples

For lyophilization process, 5 ml of honey were taken from each sample and then 75 ml of autoclaved distilled water was added to dilute honey samples in a volumetric flask. Lyophilizates were collected and stored in a dessicator at 4°C until analysis.

Total antioxidant capacity measurement

The total antioxidant activity of six types of honey was evaluated according to the procedure of Prieto et al. (1999). A 0.3 mL of sample extract was mixed with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the mixture were incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer (Thermo, Evolution 300). The analysis was performed in duplicate and the

antioxidant activity was expressed as mg equivalents of ascorbic acid (AAE) per g of dry extract. The calibration curve was prepared by using ascorbic acid (Sigma Co, USA) standards.

Determination of total phenolic content

The total phenolic content was determined based on Folin Ciocalteu assay (Samatha et al., 2012). The concentration at 10 mg/mL of honey lyophilizates was used. Folin-Ciocalteu reagent (Sigma, Switzerland) and sodium carbonate (Riedel-deHaen, Germany) (7.5%) were added to 0.5 mL of the honey samples and gallic acid standards (Sigma, Germany). After incubation for 90 min in the dark with intermittent shaking, the absorbance of the samples was measured at 765 nm (Thermo, Evolution 300). The analysis was performed in duplicate and the results were expressed as mg gallic acid equivalents (GAE) per g dry extract.

Determination of sugar content

Glucose, fructose and sucrose were prepared in methanol (1 mg/mL) as the standard solution. Honey samples (10 mg/mL) were prepared in methanol. For the chromatographic separation of glucose, fructose and sucrose, previously developed method by Puscas et al. was applied (2013). The chromatographic analysis was performed on 20 cm × 10 cm silica gel Thin layer chromatography (TLC) aluminum plates (Merck, Germany) developed twice with a mixture of ethyl acetate-pyridine-water-acetic acid, 6:3:1:0.5 (v/v/v/v) as mobile phase. The plate was developed to a distance of 90 mm at room temperature in a pre-saturated (30 min) twin trough chamber (Camag, Switzerland). After development, the dried plate was sprayed with vanillin/sulphuric acid reagent and heated at 110°C for 3 min. For the visualization, visible light was used and then the plate was documented by digital camera.

Mutagenicity assay

Standard mutagenicity assays in plate incorporation tests were carried out following the method of Maron and Ames (1983). Honey samples were tested at two different concentrations 10 and 50 mg/plate for mutagenicity assay in Salmonella strain TA98. To evaluate the impact of metabolites, similar experiments were also carried out by incubating bacteria and samples with liver S9 homogenate. An aliquot of 100 µL of overnight bacterial culture in nutrient broth and the samples dissolved in water with or without 500 µL of S9 was added to 2 mL of top agar with 0.5 mM histidine and biotine. The solution was mixed and poured onto minimal medium plates. Revertant colonies were counted after 48 h of incubation at 37 °C. Antimutagenicity effect of samples against known mutagens were carried out similarly using the same bacterial strain and the same sample concentrations with or without the liver S9 homogenate. 4-Nitro-o-phenylenediamine (NPD) and 2-Aminofloren (2-AF) were used as direct and indirect positive mutagens, respectively. For antimutagenicity assay, the percentage of inhibition was calculated according to the formula: $[(A-B)/(A-C)] \times 100$, where A= Number of revertants in the absence of sample, B= Number of revertants in the presence of sample and C= Spontaneous revertants. Inhibition rate of 40% or more was defined as strong antimutagenicity and 25-40% as moderate antimutagenicity. Inhibitory effects of less than 25% were considered weak and were not recognized as a positive result. The number of revertant colonies grown on plates containing the mutagen without sample was defined as 100% with 0% inhibition (Zengin et al., 2014).

Antiinflammatory effect

RAW 264.7 macrophages cells (ATCC, USA) were maintained in DMEM (Gibco, England),

supplemented with 10% FBS (Gibco, USA) and 1% streptomycin and penicillin (Gibco, USA) at 37°C in 5% CO₂. Anti-inflammatory activity of honey samples was evaluated by measuring the stable nitric oxide (NO) metabolite, nitrite with Griess assay (Kiemer and Vollmar, 1997). Briefly, RAW-264.7 cells were plated at the density of 2×10⁵ cell per well in a 96 well-plate and incubated for 2 hours at 37°C in 5% CO₂. Plated cells were pre-treated with three different concentrations of honey samples (50, 100 and 200 mg/mL) for 2 hours and then stimulated with 1 µg/mL of lipopolysaccharide (LPS) for additional 22 hours. The culture supernatant (50 µL) was mixed with Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma, USA) in 5 % phosphoric acid (Mettler, Switzerland)] and incubated at room temperature for 10 min. The absorbance of the mixture was determined at 570 nm using a microplate reader (Multiskan Ascent, Finland). The amount of nitrite in the test samples was calculated using sodium nitrite (Fluka Chemika, Germany) standard curve.

Statistical analysis

Results were expressed as the mean of triplicates ± standard deviation (SD). The differences among groups were evaluated with nonparametric Friedman-test and Wilcoxon signed-ranks test. For mutagenicity and antimutagenicity analysis data, Dunnett and Crisafio (1995) multiple comparisons were carried out. $p < 0.05$ was considered statistically significant.

Results and Discussion

One of the well known therapeutic effects of honey is its antioxidant activity (Schramm et al., 2003). Normally, body's own antioxidative enzyme system can neutralise the free radicals produced during normal cell activities and exposure to xenobiotics. As a result of increasing exposure due to air pollution, unhealthy nutrient habits etc., body's antioxidative enzyme system becomes inadequate and free radicals can cause inflammation, cardiovascular diseases and even different types of cancers (Singal et al., 1988). In our previous study, we have shown that honey addition to herbal teas substantially increased their antioxidant activity. As a supplemental treatment against oxidative stress related diseases, consuming herbal tea with honey at least once a day is recommended to protect the body against the harmful effects of free radicals (Özdatlı et al., 2014). However, the botanical origin, kinds, processing, handling and storage of the honey have influences on the antioxidant capacity of honey (Bertoncelj et al., 2007). In this study, a comparative research was carried out to assess the phenolic content and antioxidant activity of six varieties of honey from different botanical and geographical region. According

to our results, thyme honey and unbranded multiflora honey showed the highest antioxidant activity (Table 2). Although, thyme honey and unbranded multiflora honey have different botanical origin and collecting region, their antioxidant capacities were very similar. However, phenolic content of these two honey samples

were lower than expected. Similar studies comparing honey samples in context of their antioxidative properties showed a strong correlation between the antioxidant activities of honey samples and their total phenol contents (Akbulut et al., 2009; Alzahrani et al., 2012).

Table 2. The phenol content and antioxidant capacities of honey samples

	Pine honey	Unbranded chestnut honey	Citrus honey	Branded chestnut honey	Thyme honey	Unbranded multiflora honey
Total phenol content (mg GAE/g extract) ^A	5.5±1.2	8.09±2.0	0.88±0.4	10.93±2.2	4.95±1.1	3.26±0.8
Antioxidant capacities (mg AAE/g extract) ^B	29.33±3.4	27.01±2.4	35.58±3.2	33.09±2.2	36.22±4.2	36.12±3.1

^ATotal phenolic content was expressed as mg gallic acid equivalents (GAE) in 1 g of dried extract ± S.D.

^BTotal antioxidant capacity was expressed as mg ascorbic acid equivalents in 1 g of dried extract ± S.D.

In a previous study comparing the antioxidant activity of chestnut, rhododendron and heteroflora (thyme and astragalus) honey, chestnut honey with the highest amount of phenolic content was found to be more effective in superoxide scavenging. While the heteroflora honey showed the highest peroxynitrite scavenging activity. These results were interpreted as antioxidant activity may not be attributed only to the amount of polyphenols; other features such as type and position of the substituents of polyphenolics considerably affect their peroxynitrite-scavenging activities (Küçük et al., 2007).

Beretta et al. (2005) reported that honey samples with dark color have a higher amount of total phenolic compounds and a correlatively higher antioxidant activity. Similarly, branded and unbranded chestnut honey which had the darkest color, also had the highest phenolic content (Table 1). However, the antioxidant capacity of unbranded chestnut honey was the lowest among groups contrary to what was expected (Table 2).

The lower phenolic content than expected led us to make sugar content determination. Honey gets its

sweet taste mostly from monosaccharides which are glucose and fructose. Sucrose in other words table sugar is one of the well known disaccharide sugar in the worldwide. The disaccharide sugar is established by the combination of glucose and fructose with glycosidic linkage (Yeşilada, 2015). The amount of sucrose in honey products should be within certain limits. However, increasing demand, cost and limited availability of honey products has increased sucrose adulteration in honey. The most common methods for sucrose adulteration in honey are direct sucrose addition into honey or overfeeding bees with sucrose syrup (Puscas et al., 2013). Thin layer chromatography is one of the most commonly applied analytical techniques to screen sugar ingredients in honey products. In our study, the presence of glucose, fructose and also sucrose as an adulterant was investigated. According to the TLC chromatogram (Figure 1), all honey samples seems to contain glucose and fructose. Moreover, slight sucrose zones appeared with the same hRF value with sucrose standard. Therefore, the presence of sucrose could be considered as normal for this study. However, a quantitative analytical method should be applied to determine sucrose amount in these samples.

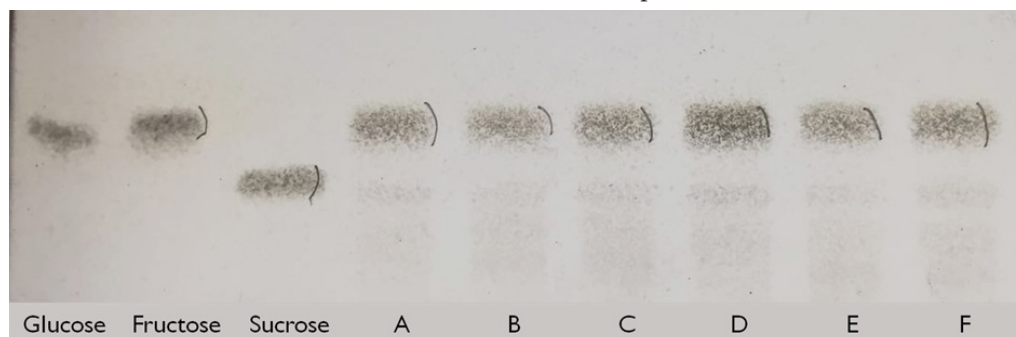


Figure 1. TLC determination of glucose, fructose and sucrose in honey products

A: Pine honey, B: Unbranded chestnut honey, C: Citrus honey, D: Branded chestnut honey, E: Thyme honey, F: Unbranded multiflora honey.

Another mentioned activity of honey is anti-inflammatory effect. In a study, following the consumption of water containing natural unprocessed honey for 15 days, the blood sampling results showed reductions in prostaglandin level. Therefore, it was thought that honey can lower the concentrations of prostaglandins in blood, hence exerts an anti-inflammatory activity (Al-Waili, 2003). In another study, the rats were treated with ethanol to cause an increase in plasma tumor necrosis factor alpha (TNF- α) which is an important modulator of apoptotic cell death in gastric mucosa, manuka honey showed similar effects was with the reference drug omeprazole and its gastroprotective effect estimated to be inhibition of the inflammatory cytokine (Almasaudi et al., 2016).

In our study, anti-inflammatory activity of honey samples were evaluated by measuring nitric oxide

levels in LPS-stimulated macrophage cells. Nitric oxide synthase is an enzyme that produces large amounts of NO from L-arginine. This normally plays an important role in the regulation of various physiological and pathological processes in our bodies such as neuronal communication, vasodilatation, and neurotoxicity. LPS is a good stimulator of macrophages and leads them to produce more pro-inflammatory cytokines by the expression of nitric oxide synthase enzyme. Therefore, inhibition of NO production in LPS-stimulated RAW 264.7 cells is one of the possible ways to screen various anti-inflammatory drugs (Joo et al., 2014; Kiemer et al., 2002). As shown in Figure 3, branded chestnut, thyme and unbranded multiflora honey samples inhibited the production of LPS induced NO in a dose-dependent manner. The strongest inhibition with branded chestnut and thyme honey samples at 200 mg/mL dropped the nitrite levels almost to the same levels with the control medium.

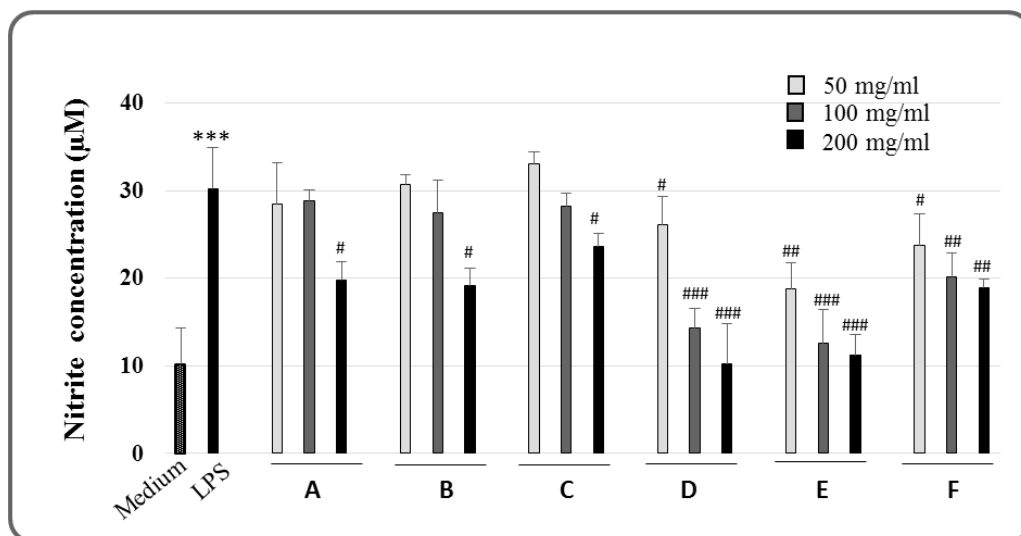


Figure 3. Antiinflammatory activity of different kinds of honey samples.

A: Pine honey, B: Unbranded chestnut honey, C: Citrus honey, D: Branded chestnut honey, E: Thyme honey, F: Unbranded multiflora honey. ***p<0.001 vs. medium control. *p<0.05; **p<0.01; ***p<0.001 vs. LPS control. LPS: lipopolysaccharide

On the other hand, in a study using the carrageenan-induced hind paw edema model in mice, a dose-dependent anti-inflammatory activity was found for honey-bee pollen mix formulation at the 500 mg/kg dose, but honey was found to be inactive against this model of inflammation (Küpeli Akkol et al., 2010). This may be due to the phenolic content of the multiflora honey which was found as 50.3 ± 0.9 mg GAE/100 g. In our results, the lowest phenolic content was approximately 2 times higher compared to the phenolic content of mentioned multiflora honey.

The mutagenic and antimutagenic activities of honey extracts with doses of 10 and 50 mg/plate

were investigated in our study. The revertant colony numbers observed in the mutagenicity assay were within normal values in TA98 strain. The average revertant colony numbers in negative control were 29±7 for TA98 with S9 and 31±6 without S9. None of the tested concentrations induced a significant increase in the revertant number of TA98 strain with or without S9 activation, indicating no mutagenicity to the tested strain. The results also showed that different honey samples at tested concentrations did not influence indicator bacteria viability, suggesting no toxicity in *S. typhimurium* strain TA98 with or without S9 metabolic system (data not shown).

Honey has many medicinal properties that should be underlined like antimutagenicity which is associated with its sugar content and phenolic components. As a common phenolic compound in honey, quercetin, p-coumaric acid and caffeic acid are examples of antioxidants that have previously displayed antimutagenic activity (Wang et al., 2002). All samples in that study exhibited significant antimutagenic

activity on TA98 strain in concentration from 10 µg/mL to 20 mg/mL. However, in our study, the percentage of inhibition were found to be below the 25% which was categorized as a weak antimutagenic activity with or without S9 activation (Table 3). This finding suggesting that the type, growth, preparation and environmental conditions may also have an impact on antimutagenic activity of final product.

Table 3. Antimutagenic activity of different honey samples in Salmonella strain TA98 with and without metabolic activation (S9) against known positive mutagens

		10 mg/plate		50 mg/plate	
Without S9		Number of revertant/plate	inhibition (%)	Number of revertant/plate	inhibition (%)
Negative control		31 ± 6			
Positive control		441.5 ± 20.5			
Honey Sample Dose (mg/plate) + Positive mutagen (control)	A	429.0 ± 15.6	3	399.5 ± 20.5	10.2
	B	422.5 ± 13.4	4.6	429.0 ± 15.6	3
	C	421.5 ± 16.3	4.8	414.0 ± 11.3	6.7
	D	433.0 ± 9.9	2.1	399.5 ± 20.5	10.2
	E	421.0 ± 25.5	5.0	401.0 ± 24.0	9.8
	F	418.0 ± 14.1	5.7	385.0 ± 38.2	13.7
With S9					
Negative control		29 ± 7			
Positive control		927.5 ± 17.7			
Honey Sample Dose (mg/plate) + Positive mutagen (control)	A	912.5 ± 19.1	3.6	896.5 ± 19.1	7.5
	B	870.5 ± 27.6	13.8	839.5 ± 20.5	21.3
	C	910.5 ± 13.4	4.1	907.0 ± 24	5.0
	D	917.0 ± 9.9	2.5	838.0 ± 21.2	21.6
	E	909.0 ± 11.6	4.5	844.5 ± 21.9	20.1
	F	910.5 ± 3.5	4.1	843.5 ± 23.3	20.3

*p<0.05; Positive mutagen vs positive mutagen plus different honey samples. Dunnett multiple comparisons test. Sterile distilled water (100 ml/plate) was used as negative control. 4-Nitro-o-phenylenediamine (NPD) (20 µg/plate) was used as positive control (mutagen) for S. typhimurium TA98 strain without S9 activation. 2- Aminofloren (2-AF) (5.0 µg/plate) was used as positive control (mutagen) for TA98 strain with S9 activation.

A: Pine honey, B: Unbranded chestnut honey, C: Citrus honey, D: Branded chestnut honey, E: Thyme honey, F: Unbranded multifloral honey.

Conclusion

In many studies, it was thought that phenolic content of honey has the major role in antioxidant activity and a strong correlation existed between phenolic content and antioxidant activity. According to our results, there was no correlation between phenolic content and antioxidant activity of honey. However, all of the samples lowered nitrite levels significantly at 200 mg/mL. Moreover, three of the honey samples nearly neutralised the LPS effect.

As a conclusion, although having different

composition, botanical origin and processing, all honey samples showed antioxidant and antiinflammatory activity in varying proportions. Therefore, daily consumption of any kind of honey may be useful in protecting the body against the oxidative stress related diseases.

Conflict of interest: None declared

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