Phenolic Composition of Pine (Pinus spp.) Honey from Turkey

Türkiye’den Elde Edilen Çam (Pinus spp.) Balının Fenolik Bileşimi

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Abstract

In this study, the phenolic component of pine honey which is a secretion honey type were studied. Total polyphenol content and total flavanoid contents of the pine honeys were ranged from 30 to 52 mg GAE/100 g and 0.86 to 1.58 mg QE/100 g, respectively. The phenolic composition of the honeys were analyzed by HPLC-UV assay with C18 column. The honey was found to be rich in protocatechuic acid, chrysin, caffeic acid phenyl ester, p-OH benzoic acid, catechine, luteolin and gallic acid.

Keywords: Honey, Pine, Phenolic Component, Anatolia, Turkey

Abbreviations: TPC, Total phenolic content; TFC, total flavonoid content; GAE, gallic acid equivalent; QE, quercetin equivalents.

1. INTRODUCTION

According to the sources of produced honey, there are two different types of honey as blossom and secretion. Blossom (flower) honeys are collected by honey bees (Apis mellifera) from flower nectars, and are the most produced honey species in the world. Secretion honeys are secreted not from flowers of plants, but only from leaves and trees with sugar containing stem. Honey bees are generally produced honey in two different ways. One of them is the production from the insects that live on trees as parasites such as pine honey. The other way is the secreted from the trees by sweating depending on the weather such as oak and cedar honeys (Kara, Can & Kolaylı, 2019;
Kolayli, Can, Çakir, Okan & Yildiz, 2018; Özkök & Silici, 2017). While pine honey is the most produced in Turkey, other Mediterranean countries such as Greece, Italy, Spain, and Portugal are also less produced. It has a relatively dark colored, turbid appearance, and characteristic smell and aroma. Also, pine honey is not easily crystallized (Can et al. 2015).

Polyphenols are secondary metabolites of plants and have many biological values such as anti-oxidant, anti-microbial, anti-inflammatory, antiviral, anti-repellent and anti-tumoral etc. (Bahramsoltani, et al. 2019; Joseph, Edirisinghe & Burton-Freeman, 2016). Polyphenols are the most important secondary metabolites of honeys and they are also responsible many characteristic features of honeys such as color, aroma, taste and biological activity.

In the literature, the studies with the polyphenols of pine honey are very limited. The aim of this study was determined the phenolic composition and phenolic markers of the pine honeys from Mugla region of Turkey.

2. MATERIAL AND METHODS

Ten pine honey samples were collected in Mugla around of near the Aegean Sea of Turkey in October, 2018. Physicochemical properties (moisture content, optical rotation, pH) and phenolic compounds and contents of honey samples were determined.

2.1. Physicochemical Properties

Moisture contents of these samples were measured by refractometer method (Atago, Tokyo, Japan). Optical rotation of the samples were measured by polarimetry (BetaPPP7, England).

2.2. Preparation of samples extraction for antioxidant analysis and phenolic compounds

For determination of total phenolic content, ethanolic extracts of honey samples were used. 10 g of honey was extracted using 90 % ethanol solvent for 24 hours at room temperature using a shaker (Heidolph Promax 2020, Schwabach, Germany). After incubation, extracts were filtered with Whatmann No: 4 filter paper and stored at 4ºC for further analysis. Extracts were divided into two parts. The first part for antioxidant tests and the second part are for phenolic component analysis.

2.3. Total Phenolic Content Determination

Total phenolic content was measured by Folin Ciocalteu method (Singleton, Orthofer & Lamuela-Raventós, 1999). Firstly, 680 µL pure water, 20 µL of honey ethanolic extract and 400µL 0.5 N Folin–Ciocalteu reagent were mixed, and vortexed. Then, 400 µL of Na2CO3 (10 %) was added into the tubes. After vortexing, the mixture was incubated for 2 h at 20°C with shaker. For calibration curve of gallic acid standard, different concentrations of gallic acid solution were prepared with the same analysis procedure. The reaction using the intensity of the blue color at 760 nm in spectrophotometer was read and the results
were expressed as mg gallic acid equivalent (GAE) / 100 g. All the measurements were performed in triplicate.

2.4. Total Flavonoid Determination
For determination of total flavonoid substance, Fukumoto and Mazza (2000)’ method was used with using quercetin standard (Fukumoto and Mazza, 2000). Firstly, 0.5 mL of the samples, 0.10 mL of 10 % Al (NO₃)₃ and 0.10 mL of 1 M NH₄CH₃COO was added to reaction mixture. This mixture was incubated at room temperature for 40 min and the absorbance was measured against a blank at 415 nm. Quercetin (0.03125-1 mg/mL) was used as a standard to obtain the calibration curve. The total flavonoid content (TFC) was calculated as mg of quercetin equivalents (QE)/100 g honey.

2.5. Analysis of Phenolic Components by RP-HPLC-UV
 Phenolic composition of the honey was determined in RP-HPLC-UV. For this purpose, a calibration curve was prepared in the study using 19 phenolic standards and phenolic compositions of the samples were determined according to these curve values. Gallic acid, syringic acid, p-OH benzoic acid, ferulic acid, caffeic acid, t-cinnamic acid, p-coumaric acid, catechin, epicatechin, rutin, daidzein, myricetin, luteolin, hesperetin, chrysin, pinocembrin, protocatechuic acid, caffeic acid phenethyl ester, resveratrol were used as phenolic standards.

The ethanolic extracts of honey samples were evaporated until dryness in a rotary evaporator at 40°C. The residue was dissolved in 15 mL acidified distilled water (pH 2). Liquid–liquid extraction was carried out with 5×3 mL diethyl ether and 5×3 mL ethyl acetate, consecutively (Kim, Tsao, Yang & Cui, 2006). Both diethyl ether and ethyl acetate phases were pooled and evaporated by rotary evaporation (IKA-Werke, Staufen, Germany) at 40°C. The pellet was suspended in 2 mL ethanol, filtered with syringe filters (RC membrane, 0.45 μm), and injected to HPLC.

HPLC (EliteLaChrom Hitachi, Japan) with UV detector was used for analysis and conducted by reverse phase C18 column (150 mm x4.6 mm, 5 μm; Fortis). It was carried out by applying a gradient program with acetonitrile, water and acetic acid. (Malkoç, Çakir, Kara, Can & Kolaylı, 2019b).

For HPLC analyses, the mobile phase (A) and (B) consisted of 2% acetic acid in water and acetonitrile: water (70:30), respectively. The sample injection volume was 20 μL, the column temperature was 30°C and the flow rate was 0.75 mL / min. Using of the programmed solvent began with a linear gradient held at 95% A for 3 min, decreasing to 80% A at 10 min, 60% A at 20 min, 20% A at 30 min and finally 95% A at 50 min. Three injections were used for each sample. All calibration values for phenolic components are between 0.998 and 0.999 (Çakir, Şirin, Kolaylı & Can, 2018).
3. RESULTS AND DISCUSSION

Specific optical rotation, moisture content, and pH value of the samples was given in Table 1. Optical rotation value is an important distinguishing feature for flowers and honey (Dinkov, 2003; Serrano, Rodríguez, Moreno & Rincón, 2019). Honeydew (such as pine and oak honey etc.) optical rotation is dextrorotary, while blossom honey is laevorotary (Cavrar, Yıldız, Sahin, Karahalil & Kolayli, 2013). It was determined that the optical rotation values of the studied honeys were ranged from 0.90 to 2.50. As a matter of fact, the positive rotation values of the studied honeys confirm that all honeys are secretion honeys.

Table 1. Physicochemical analyses of Pine honey from Mugla region.

|               | Min  | Max  | Mean    |
|---------------|------|------|---------|
| Specific optic rotation \( \alpha^{20} \) | 0.560 | 2.80 | 1.48±0.76 |
| Moisture (%)  | 14.00 | 20.30 | 17.50±2.60 |
| Ph            | 3.98  | 5.40  | 4.58±0.70  |

It was determined that the moisture amount of the honeys varied between 15% and 20.30% and the average value was 17.40%. The moisture values are found suitable with the recommended amount of water in the honey codex (Bogdanov et al, 1999).

It was determined that the pH values of the studied honeys were ranged from 3.98 to 5.58 and all the honeys had acidic medium. Moreover, the current values found were similar to the honey codex (Bogdanov et al, 1999). Acidic properties of honey earns honey a stronger antibacterial effect and bacteria cannot survive at this pH. It has been reported using capillary electrophoretic technique that gluconic acid, formic, malic, citric and succinic acids are major acids of honey (Kaygusuz et al. 2016). However, there are phenolic acids in honey, which is higher than these organic acids, and gallic acid, benzoic acid, p-OH benzoic acid, coumaric acid, syringic acid, valinic acid, ferulic acid are important sources of honey (Can et al. 2015; Ertürk, Şahin, Kolaylı & Ayvaz, 2014; Kolaylı et al. 2018).

The biological activity of honey, it consists of phenolic acids and flavonoids. Phenolic content of honey varies according to flora and geographical origin. In this study, total polyphenol values of pine honey were measured spectrophotometrically, and the results were given in Table 2. In general, honeys consist of between 20 and 150 mg gallic acid/100 g depending on honey species. Total polyphenol contents of the samples were found between 24.60 and 68.20 mg GAE/100 g of the samples and the mean value was 46.30 mg GAE/100g. All phenolic compounds in honey are measured by the total polyphenol method and phenolic acids, flavonoids, stilbenes, tannins are included in this family (Can, Baltas, Keskin, Yıldız & Kolaylı, 2017). Total flavonoid contents of the honey samples were changed from 0.80 to 2.10 mg QE/100 g. The flavonoids contents of pine honeys were indicated nearly high and the other study findings confirmed this situation (Can et al, 2015; Kolaylı, Baltas, Sahin & Karaoglu, 2017).
Table 2. Total phenolic contents of Pine honey from Mugla region.

| Total phenolic content (mg GAE/100g) | Min  | Max  | Mean±SD  |
|-------------------------------------|------|------|----------|
| Total phenolic content              | 24.60| 68.20| 46.30±10.30 |

| Total flavonoid content (mg QE/100 g) | Min  | Max  | Mean±SD  |
|--------------------------------------|------|------|----------|
| Total flavonoid content              | 0.80 | 2.10 | 1.46±0.78 |

Phenolic profile of the pine honey was determined using nineteen polyphenolic standards with high pressure liquid chromatography (HPLC-UV) (Malkoç, Kara, Özkök, Ertürk & Kolaylı, 2019a). The results were summarized in Table 3. Protocatechuic acid was the major phenolic compound of the studied phenolic compounds, and chrysin, p-OH benzoic acid and catechin are followed them. Haroun et al. (2012) reported that Turkish honeydew honeys (pine and oak) have been shown to contain protocatechuic acid in the range of 1639 to 5986 µg/kg honey. In the current study, protocatechuic acid was identified as the major component. The presence of protocatechuic acid as a major ingredient in pine honey might be considered to use as a characteristic indicator of honey's origin. The concentrations of protocatechuic acid 480.20 µg/kg honey for pine honeys. Another study was found concentrations of protocatechuic acid ranged from 3058 to 5967 µg/kg honey for pine honeys (Spilioti et al., 2014). Chrysin was observed to be main flavonoid in pine honeys. Chrysin content was found 210.30 µg/100 g in pine honey. In this study was determined that phenolic components were detected in different proportions in pine honey.

Table 3. Phenolic profiles analyses in HPLC-UV of Mugla pine honey (µg/100g)

| Phenolic acids | Gallic acid | Protocatechuic acid | p-OH Benzoic acid | Caffeic acid | Syringic acid | p-Coumaric acid | Ferulic acid | t-Cinnamic acid |
|----------------|------------|---------------------|-------------------|-------------|-------------|----------------|--------------|----------------|
|                | 33.20±5.80 | 480.20±105.30       | 98.45±22.08       | 28.60±12.56 | 24.10±6.20  | 17.80±10.20    | 40.66±4.05   | -              |

Flavonoids

| Chrysin | 210.30±56.07 |
|----------|----------------|
| Rutin | - |
| Myricetin | - |
| Daidzein | - |
| Resveratrol | - |
| Luteolin | 38.50±12.40 |
| Hesperetin | 18.06±3.40 |
| Pinocembrin | 33.60±4.80 |
| Caffeic acid phenethyl ester | 24.50±8.40 |

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