ABSTRACT

The composition and biological value of honey vary depending on its producing flora. The botanical, chemical and biological active properties of honey determine the geographical authenticity of the honey. In this study, geographic fingerprints of thirty *Astragalus* (*Astragalus microcephalus* Willd.) honeys from Erzincan region were investigated. The honey consists of *Astragalus* pollen more than 65% and others were *Salix* spp., *Cistaceae*, *Trifolium* spp., *Thymus* spp, *Rosaceae*, *Papaveraceae*, *Onobrychis* spp. etc. The characteristic color of the analyzed honeys was bright/light yellow. Hunter color values (L, a, b) were found to be as L: 72-78, a: 7-16 and b: 58-78, respectively. Total polyphenol content of the honeys was 38.20±4.60 mg gallic acid equivalent (GAE)/100 g, it was found to be rich in some phenolics such as chrysin, caffeic acid phenyl ester, myricetin, pinocembrin, luteolin, and gallic acid.

Key words: Honey, *Astragalus*, geographical fingerprint, Anatolia

ÖZ

Balın bileşimi ve biyolojik değeri üretilen floraya bağlı olarak değişir. Balın botanik, kimyasal ve biyolojik aktif özellikleri, balın coğrafi özgünliğünü (işaretini) belirlemektedir. Bu çalışmada Erzincan yöresine ait 30 adet geven (*Astragalus microcephalus* Willd.) balının coğrafi parmak izleri araştırıldı. Balın botanik içeriğinde%65’ten fazla *Astragalus* poleni ve azalan değerlerde *Salix* türleri, *Cistaceae*, *Trifolium* türeleri, *Thymus* türeleri, *Rosaceae*, *Papaveraceae*, *Onobrychis* türeleri vb. bulunmaktadır. Çalışılan balların karakteristik rengi parlak/açık san, Hunter renk değerleri (L, a, b) sırasıyla L: 72-78, a: 7-16 ve b: 58-78 olarak tespit edildi. Balların toplam polifenol içeriği 36,50±4,60 mg gallik asit eşiğini (GAE)/100 g olup temel olarak krisin, kafeik asit fenil ester, mirisetin, pinosembrin, luteolin ve gallik asit gibi bazı fenoliklerce zengin olduğu bulundu.

Anahtar Kelimeler: Bal, geven, coğrafi parmakizi, Anadolu
GENİŞLETİLMİŞ ÖZET

Amaç: Bal bileşimi ve biyolojik özellikleri ile toplandığı bölge florasına göre sınıflandırılan bir doğal şeker oranı yüksek üründür. Kuru ağırlığının %88 değerlerinden olusan bu doğal ürünü yapışında %2'nin altında bulunan çeşitli fenolik asitler, flavonaller, flavonaller, stilbenler, kalkonlar ve tanenler gibi polifenoller ile vitamin ve mineraller balın kimlikini oluştururan önemli ajanlardır. Balın tadi, renği, kokusu, aroması olusurun bu sekonder metabolit ajanlar aynı zamanda balın coğrafik işaretinden de sorumludurlar. Bileşimleri itibariyle kompoles bir yapıya sahip bulunan bal, çok kolay taşıgene edilebilir bir doğal ürün olarak kabul edilir. Balın coğrafik özelliklerini balın kompozisyonu belirler ve balın taşıgene önune geçilmesi amacıyla son zamanlarda balda coğrafik işaret kavramı gündeme gelmiştir. Yapılan çalışmalar belir bir bölgeye ait balların fiziksel, kimyasal ve biyolojik aktiv özelliklerini belirlienenek balın etiketlenmesi ile balda taşıgene azaltılmaktadır. Geven türleri (Astragalus spp.) Doğu ve Güneydoğu Anadolu'da yaygın olarak bulunur ve bal verim oldukça yüksekdir. Bu çalışma, unifloral (yuksek oranlı tek bitki kaynaklı) bal verimleri ve çalışılan balların ne kadar koyulu olduğunu gösterir. Ayni zamanda geleven bal örneği toplanmış ve laboratuvara gönderilmiştir. Numunelerin botanik kökenini belirlemek için melissopalinolojik analizler yapılmıştır. Numunelerin botanik kökenini belirlemek için melissopalinolojik analizler yapılmıştır. Numunelerin botanik kökenini belirlemek için melissopalinolojik analizler yapılmıştır. Numunelerin botanik kökenini belirlemek için melissopalinolojik analizler yapılmıştır. Numunelerin botanik kökenini belirlemek için melissopalinolojik analizler yapılmıştır.
collected area, these consist of 98% sugars of dry weight. Fructose and glucose are two main sugars of honey and it contains a small portion of other sugars like sucrose, maltose, arabinose, melezitose, melibiose, trehalose, e.g. (Kamal and Klein 2011). The agents that are responsible for the honey color, taste, smell and aroma are herbal oils and volatile compounds and different polyphenols and pigments. Polyphenols that are found in honey nearly 0.02–0.20% with depending on honey species also responsible for its biological activities (Can et al. 2015). In general, the dark-colored honey contains high polyphenols. Chestnut, heather, oak and pine honeys are dark colored and these have also high antioxidant capacities (Cavrar et al. 2013, Kaygusuz et al. 2016).

In recent years, the characteristic features of honey have been classified according to the flora of the region (Wang et al. 2009, Dinca et al. 2015). The flora of the area is determined geographic fingerprints of the honey. In the recent studies, geographic origins or authenticity of some honeys can be measured with different specific biomolecules, while some honeys require a wide of discriminant analysis (Wang et al. 2009, Cavazza et al. 2013, Dinca et al. 2015). For example, the geographic origin of Manuka honey is determined by methylglyoxal, while the chestnut honey is determined by the presence of p-aminoacetophenone (Bonaga et al. 1986, Adams et al. 2009, Cavazza et al. 2013). But anyway, if any honey types have received a geographical sign, it should define its all properties. Turkey has many different honey plants and honey species. Astragalus species (Astragalus spp.) are found widespread in eastern and southeastern Anatolia and has a high honey yield (Pinar et al. 2009). Erzincan meadow is in eastern Anatolia and has become prominent with apicultural activities. One of the dominant honey plants in the region is Astragalus. Geographical indication applications in Turkey’s honey production was evaluated and demonstrated the necessity of this kind of work (Alparslan and Demirbaş 2019). In this context, it is seen that geographic fingerprint studies belonging to different types of honey have been carried out (Gürbüz and Çelikel 2018). This study was carried out to illuminate the authentic structure of the Astragalus monofloral honey and to find the geographic fingerprint.

MATERIALS AND METHODS
Thirty Astragalus honey samples were harvested in the Erzincan area (39°34′46.8″N 39°45′33.6″E) by the Erzincan Beekeepers Association in August 2018 and sent to the laboratory for determination geographical fingerprints. Honeys were obtained from different beekeepers producing Astragalus honey in Erzincan. For determination of botanical origin of the samples melissopalynological analyses were carried out by microscopic assay (Louveaux et al. 1978). According to the counting results, the pollen rates were determined, and the rates were dominant pollen (45% and more), secondary pollen (16–44%), minor pollen (3–15%), trace pollen (3% and less).

For determination of antioxidant tests and phenolic analysis in honey, 10 g honey sample was weighed and shaken with 50 ml 70% ethanol into the falcon tube (Heidolph Promax 2020, Schwabach, Germany) for 24 hours at the room temperature. After shaking, the extracts were filtered with a filter paper (Heidolph Promax 2020, Schwabach, Germany) and finally, these were kept in the refrigerator at +4°C until future analysis. Besides phenolic content analysis, all prepared extracts were used to the other antioxidant tests and phenolic analysis.

Physicochemical Properties
Honeys supplied from different floral origins possess different color parameters. A Hunter spectrophotometer was used to measure the color values of the honeys (C400, Minolta, Osaka, Japan). Hunter L is darkness/lightness (0 black–100 white), a is greyness/redness, and b is b blueness/yellowness (Anupama et al. 2003).

The moisture refractometric values that come from the refractive index were identified by a refractometer (Atago, Tokyo, Japan). The conductivities and optic rotations of the honeys were measured by a conductivity meter (Hanna Instrument, HI 2030-02, Romania) and a polarimeter (BettaPP7, England), respectively. Proline content was determined with the reaction spectrophotometrically based on ninhydrin (Ough 1969) (Thermo Scientific EvolutionTM 201, UV-VIS spectrophotometer, USA).

Raw Honey Enzymes
Honey enzymes activities of invertase, glucose oxidase and diastase were measured with
spectrophotometric assays (Sahin et al. 2020). Invertase activity of the raw honey samples was determined by using p-nitrophenyl-α-D-glucopyranoside (p-NPG) as a substrate in Bogdanov's method (Bogdanov et al. 2002). Glucose oxidase activity was determined by the horseradish peroxidase/o-dianisidine method as previously described by Flanjak et al. (2016). Finally, diastase activity was measured according to Bogdanov et al., (1999)’s protocol.

Total Phenolic Content

Total amount of phenolic substance was measured by Folin Ciocâlteu method (Singleton and Rossi, 1965, Singleton et al. 1999). This method gives the response to all phenolics including phenolic acids, flavonoids, anthocyanins, and tannins etc. in the solution. The results were expressed as mg gallic acid equivalent (GAE) / 100g by using the intensity of the blue color at 760 nm readings.

Total Flavonoid Determination

Determination of total flavonoid substance was applied according to Fukumoto and Mazza (2000)’s procedure. The graph of different concentration of quercetin was performed to express the unit as mg quercetin equivalent (QE)/100 g honey.

FRAP Test

This method is based on the reduction of the Fe (III)-TPTZ complex to Fe (II)-TPTZ complex in the presence of the antioxidant substance (Benzie and Strain 1996). Variable concentrations of FeSO₄·7H₂O (from 31.25 to 1.000 μM) were used as standard. Blue form product was measured at 593 nm.

DPPH Radical Cleaning Activity Determination

This test is based on the investigation of the change in absorbance by reducing the purple DPPH radical by an oxidizing antioxidant. Antioxidant activity was given as 50% reduction of initial DPPH concentration expressing the amount of antioxidants spent for the SC₅₀ (mg/mL) (Molyneux, 2004).

Analysis of Phenolic Components by HPLC-UV

The phenolic profile of Astragalus honeys was determined by HPLC-UV. For this purpose, an individual calibration curve for each 19 phenolic standards was prepared. Also, the number of samples was determined according to these curve values. Catechin, epicatechin, rutin, daidzein, myricetin, luteolin, hesperetin, chrysin, pinocembrin, protocatechuic acid, syringic acid, gallic acid, p-OH benzoic acid, caffeic acid, ferulic acid, p-coumaric acid, t-cinnamic acid, caffeic acid phenethyl ester, and resveratrol were used as phenolic standards.

The ethanolic extract was evaporated until dryness with 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 et al. 2006). Both diethyl ether and ethyl acetate phases were pooled and dried 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 analyzes and conducted reverse phase C₁₈ column (150 mm x4.6 mm, 5 μm; Fortis) and acetonitrile, water and by applying a gradient program with acetic acid was carried out (Malkoç et al. 2019b).

For HPLC analyses, the mobile phase consisted of (A) 2% acetic acid in water and (B) acetonitrile: water (70:30). The sample injection volume was 20 μL, the column temperature 30°C and the flow rate 0.75 mL/min. The programmed solvent used 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 (Çakır et al., 2018).

RESULTS

Physicochemical parameters and melissopalynological analysis of the studied honeys are summarized in Table 1. According to the Hunter Lab values, the average of L value known as the darkness of the honey was determined as 73.40. While the range of “a” value changed from 6.95 to 15.55 (that’s meaning was almost greenness degree), “b” value changed from 58 to 78 (that’s meaning was almost yellowness degree). Optical rotation values of the honeys were negative; actually, these values could be as the evidence for the blossom honeys. The maximum of HMF level of honeys was observed to be 1.5 mg / kg. The proline values of the studied honeys were 585.5, 694.5 and 640 ± 54.50 mg / kg, respectively (min, max, mean).
Table 1. Physicochemical analyses of the *Astragalus* honey from Erzincan region.

| Parameter                          | min   | max   | mean       |
|------------------------------------|-------|-------|------------|
| pH                                 | 4.05  | 5.30  | 4.60±0.60  |
| Moisture                           | 14.20 | 18.30 | 16.40±1.36 |
| Conductivity (mS/cm)               | 0.28  | 0.46  | 0.34±0.15  |
| Optic rotation [α]°/²⁰              | -2.302| -2.88 | -2.40      |
| Color (Hunter Lab)                 | 71.98 | 78.80 | 73.40±3.20 |
| L                                  | 6.94  | 15.55 | 8.55±3.08  |
| a                                  | 58    | 78    | 64.40±8.60 |
| HMF (mg/kg)                        | Not detected | 1.50 | - | 40 mg/kg |
| Proline (mg/kg)                    | 585.5 | 694.5 | 640±54.50  |

Table 2 gives the activity of diastase, invertase and glucose oxidase enzymes. The average values of these enzymes were 18±3.80 DU, 152±12.30 U/kg, 3.36±1.22 µg H₂O₂/kg, respectively.

| Parameter               | min | max | mean       |
|-------------------------|-----|-----|------------|
| Diastase Unit (DU)      | 16  | 24  | 18±3.80    |
| Invertase (U/kg)        | 145 | 164 | 152±12.30  |
| Glucose oxidase (µg. H₂O₂/h.g) | 3.60 | 5.40 | 3.36±1.22 |

The results of total polyphenol content, total flavonoid content, DPPH and FRAP are given in Table 3. Total polyphenol value was found between 31 and 41 mg GAE/100 g and the mean value was 36.50 mg GAE/100g. Total amount of flavonoid substance of honey was found as 0.72 mg QE/100 g. DPPH and FRAP analysis results of honey were 146 ± 38 mg / ml (SC50), 204 ± 64 µmol FeSO₄.7H₂O / 100 g, respectively.

Table 3. Antioxidant capacity and activity of the *Astragalus* honey from Erzincan region.

| Parameter                      | min | max | mean       |
|--------------------------------|-----|-----|------------|
| Total Phenolic Content, TPC (mg GAE/100 g) | 31  | 41  | 36.50±4.60 |
| Total Flavonoid, TF (mg QE/100 g)       | 0.69 | 0.75 | 0.72±0.03  |
| Ferric reducing antioxidant power, FRAP (µmol FeSO₄.7H₂O/100 g) | 140 | 268 | 204±64 |
| DPPH radical scavenging activity (mg/ml) | 108 | 184 | 146±38 |
Phenolic profile of the honey samples was determined according to the 19 phenolic standards with HPLC-UV method (Malkoç et al., 2019b). The results are given in Table 4. As a result of the phenolic analysis, the major components were chrysin, myricetin, and caffeic acid phenethyl ester. Luteolin, pinocembrin, cinnamic acid, gallic acid, ferulic acid, and p-coumaric acid followed the major compounds. Besides these current ones, there were some compounds which were below the limits of detection such as catechin, caffeic acid, protocatechuic acid, and rutin.

Table 4. Phenolic profiles analyses with HPLC-UV of the Astragalus honey from Erzincan region (µg/100g)

| Standards                  | Detected       |
|---------------------------|----------------|
| Gallic acid               | 42.20±20.30    |
| p-Coumaric acid           | 15.20±3.40     |
| Ferulic acid              | 24.30±4.50     |
| Cinnamic acid             | 24.00±3.50     |
| Chrysin                   | 790±240        |
| Luteolin                  | 465±159        |
| Myricetin                 | 670±370        |
| Pinocembrin               | 320±48         |
| Caffeic acid phenethyl ester | 680±64     |
| Syringic acid             | -              |
| Catechin                  | -              |
| Epicatechin               | -              |
| Caffeic acid              | -              |
| Protocatechuic acid       | -              |
| Rutin                     | -              |
| Daidzein                  | -              |
| Resveratrol               | -              |
| Hesperetin                | -              |
| p-OH benzoic acid         | -              |

DISCUSSION

Moisture, pH and conductivity values were found similar to the type of blossom honeys and these were also explained as acceptable limits of the honey codex (Can et al. 2015, Malkoç et al. 2019a). There is a generalization about a light-colored honey class that L value is 50 or higher than 50. Hence Astragalus honey is light-colored honey in terms of the L results. When compared with the literature, it was seen that the L value was 88 in acacia honey, 78.5 in lime honey, and 78 in highland honey (Can et al. 2015). In the same study, the L value of Astragalus honey was reported as 74. The color of the honey is an important parameter in the geographical marking of honey, the color, odor, and aroma are the characteristic sensory identity for honey, as well. The Astragalus honeys are a relatively light color, actually, have greenness and yellowness.

Many studies have been shown that the optical rotation of honeydew honey such as pine honey, oak honey was positive (Can et al. 2015, Serrano et al. 2015).
2019, Degirmenci et al. 2020). There are many reasons such as the color of honey, the amount of HMF, and the amount of mineral content that are formed by various pigments, polyphenols, and non-enzymatic Maillard reactions (Marcucci, et al. 2019). There are studies showing the relationship between the polyphenol content and the darkness of the honey (Can et al. 2015). Chestnut honey, heather honey, and oak honey are dark honeys, their total polyphenol contents are approximately 100 mg GAE/100 g and their Hunter L values are below 50. The proline value of honey is a crucial quality parameter for honey, and it expresses its purity. The average proline value in Astragalus honeys was 680 mg/kg, and it was found to be higher than many honeys.

HMF is a Maillard reaction product, responsible for the freshness of honey and whether it is subjected to heat treatment. The low HMF value indicates that honey is a raw and/or a fresh honey (Turkut et al. 2018).

HMF is low in fresh honey. Prolonged storage or exposure to high temperatures increases the level of HMF. Studies have shown that the half-life of diastase activity decreases while the HMF level increases (Korkmaz and Küplülü 2017). The number of diastase enzyme that responsible for the hydrolysis of sugar in honey is at least 8 (Türk Gıda Kodeksi 2020). During the transfer of nectar from bee to bee, enzyme content increases. For this reason, diastasis level may change depending on the nectar source and colony (Can et al. 2015). Compared with different honeys, the diastase activity of Astragalus honey is significantly high and the HMF value is low, revealing the value of honey.

Some of the important properties to distinguish honey from other sweet products (jam syrup, etc.) are some enzymes in the honey. Diastase, invertase, and glucose oxidase are the main honey enzymes for honey. These values can be different in fresh and old honeys and these can affect with heating treatment. Especially, pasteurized honeys have almost low activity of diastase an invertase due to having heating treatment. Briefly, the high diastase and invertase activities imply that honey is raw honey that does not heat (Sahin et al. 2020). Unlike invertase and diastase activities, low glucose oxidase activity indicates high-quality raw honey (Bankar et al. 2009, Kamboj et al. 2019).

When compared with dark-colored flower honeys, Astragalus honey that does not have high polyphenol content and flavonoid content is a light-colored blossom species (Can et al. 2015, Heidari et al. 2013). Moreover, antioxidant capacity analyzes also show that Astragalus honeys have a moderate degree with comparison to other flower honeys. It cannot be inferred the means that Astragalus honey is worthless. (Marcucci et al. 2019, Degirmenci et al. 2020). Phenolics may vary depending on the geography of honey from which is obtained and type. Researchers say that honey is a valuable product for geographical markings (Sautier et al. 2018). Astragalus honey can have a strong potential to reveal its geographic fingerprint thanks to its characteristic phenolic components just like our investigation.

Gallic acid, p-coumaric acid, ferulic acid, cinnamic acid, chrysins, luteolin, myricetin, pinocembrin, caffecic acid phenethyl ester were observed in the examined Astragalus honeys. Chrysins, myricetin, caffecic acid phenethyl ester phenolics have been found to be major components. Syringic acid, catechin, epicatechin, caffeic acid, protocatechuic acid, rutin, daidzein, resveratrol, hesperetin and p-OH benzoic acid components were not found in Astragalus honey. The phenolic content of a honey is not only dependent on vegetation, but also importantly on the geographical location where honey is produced (Can et al. 2015, Mendes et al. 1998). The quality of honey cannot be fully understood by looking at the honey communiqué. In addition, the amount and composition of polyphenols are important in determining the quality.

CONCLUSION

Depending on the flora types, some differentiation in antioxidant capacity, physical properties, and enzyme inhibition properties of honey can be seen. Our study gives information about the geographic fingerprint of Astragalus honey obtained from Erzincaı region. By determining the geographic fingerprint of a product, different features of the region such as geological, phytogeographical and climate can be emphasized. Having some different phenolic compounds and strong enzymatic capacity, Astragalus honeys supplied from Erzincaı region have a claim to take a geographical fingerprint.
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