Evaluation of the antioxidant potential of the mad honey collected from the black sea region in Turkey

Research Article

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ABSTRACT

Objective: Mad honey (*Rhododendron honey*) is produced by honeybees from Rhododendron flowers and contains grayanotoxins, antioxidants, flavonoid and phenolic properties. The mad honey samples are collected from Turkey's Black Sea region by local residents and are sold as mad honey in free-market conditions. This study was planned to evaluate the total phenolic content, total flavonoid content and total antioxidant capacity and protein profiles with SDS-Page electrophoresis determined the mad honey samples collected from seven different locations from Black Sea region by local residents. Material and Method: Total phenolic content was determined by the modified Folin-Ciocalteu method. Total flavonoid content was determined by colorimetric method using aluminium chloride ($AlCl_3$). Total antioxidant activity was calculated according to the free radical scavenging effect of 1,1-diphenyl-2-picryl hydrazyl (DPPH). Protein profiles evaluated with SDS-Page electrophoresis. Results and Conclusions: In Turkey, in the Black Sea Region, antioxidant potential of the honey samples known as mad honey, collected from seven different locations by local people, were evaluated. The mean total phenolic contents of the mad honey samples were found as 285,44±118,43 (125.85 to 471.18) mg GAE/kg honey, the mean total antioxidant activities were found as 29,68±7,2 (21.71 to 35.03) mg AAE/kg honey and the mean total flavonoid contents were found as 27,26±4,79 (19.93 to 39.18) mg QE/kg honey. The results revealed that the mad honey samples examined in this study were a good source of antioxidant, flavonoid and phenolic content at varying levels depending on the characteristics of the region where they were collected.

Keywords: Mad Honey, total flavonoid, total phenol, total antioxidant, protein profile

INTRODUCTION

Honey has been used in medicine since ancient times. The composition of honey is closely related to the differences in environmental factors, the variety of flowers from which honey is produced, and the environmental and climatic conditions of the region where honey is collected. Honey obtained from plants of the Rhododendron flowers is known as "mad honey". Flowers of the Rhododendron species are commonly found in Spain, Portugal, Japan, Brazil, the United States, Nepal, England, and Turkey. 'Mad honey' is common in the Black Sea region of Turkey. Especially Hopa, Kastamonu, Zonguldak, Rize, Ordu, Tokat and Sinop are the provinces where grayanotoxin-containing honey is detected. It is stated that consuming 50-100 g of mad honey at once may cause intoxication. Intoxication symptoms may occur within 20 minutes to 24 hours after consumption of mad honey. The main symptoms that can be seen in mad honey intoxication, depending on the amount of honey taken, are diarrhea, sweating, dizziness, temporary loss of consciousness, syncope, ECG disorders, blurred vision, hypotension and bradycardia.

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In patients with bradycardia and severe hypotension, atropine sulfate and intravenous saline infusion are administered as an early treatment (Salici and Atayoğlu 2015; Demir et al., 2011; Sayın et al., 2012). In the event that a complete atrioventricular blockade develops, a temporary pacemaker is applied in patients. Except for the case from Lanping County (Southwest China), the prognosis for mad honey poisoning is reported to be very good. Except for a few cases identified in the 1800s, it is stated that death from mad honey intoxication is rare in the modern medical literature today. Although the mortality rate is low, it is stated that mad honey consumption can cause arrhythmias that are life-threatening and difficult to differentiate (Shrestha et al., 2015; Dubey et al., 2009; Zhang et al., 2017; Ullah et al., 2018).

In addition, the bioactivity and antioxidant effects of each honey type are different from each other. As an alternative medicine Rhododendron honey consumption against various diseases has become very popular in recent years. It is stated that this honey has an antioxidant property that has been shown to be beneficial in the defense of cells with degeneration, despite the risk of toxic effects (Salici and Atayoğlu 2015; Demir et al., 2011). Mad honey is used as an alternative treatment method for various diseases among people, unlike the normal honey consumed for breakfast. For this purpose, it is used as an alternative medicine in cases of hypertension, diabetes, influenza infection, gastrointestinal disorders, abdominal pain, arthritis, sexual dysfunction, viral infections, skin diseases, pain and heat stress (Zhang et al., 2017; Lue and Wood 2000; Bobiş et al., 2018). In addition, it is well known that Rhododendron plant species have antiradical bioactivities such as antidiabetic, anti-inflammatory, antioxidant, analgesic, antimicrobial, cytotoxic and insecticidal (Erejuwa et al., 2014; Zhang et al., 2017).

The composition of honey varies according to the type of flowers from which honey is obtained and the environmental conditions of the region where they are grown. It is generally accepted that there are more than 180 contents in the composition of honey. The main ingredients that give honey its sweetness are fructose (38%) and glucose (31%). In addition to disaccharides and trisaccharides are also found in honey in varying proportions. Include sucrose, maltose, trehalose, erlose, maltotriose, masculine, hybridose, centose 3-a5, isomaltosylglucose, 1-kestose, isomaltotriose, panose, isopanose, and teanderose (Rahman 2013; Bonta et al., 2008). Apart from carbohydrates, honey also contains enzymes, amino acids, proteins (0.1%-3.3%), flavonoids and phenolic acids. There are 26 amino acids reported in honey; among them, proline is the main additive, accounting for 50-85% of the total amino acids (Hermosin et al., 2003). Some of the flavonoids and phenolic contents identified in honey are kaempferol, quercetin, chrysin, pinobanksin, luteolin, apigenin, pinocembrin, genistein, hesperetin, p-coumaric acid, naringenin, gallic acid, ferulic acid, ellagic acid, syringic acid and caffeic acid, vanyl (Erejuwa et al., 2014; Ahmed and Othman 2013; Bobiş et al., 2021). There may also be toxic contents in honey that can be harmful to human health when consumed in excess of certain amounts, originating from nectars containing grayanotoxins collected from the flowers of plants such as *Rhododendron ponticum* or *Azalea pontica*, and causing the collected honey to be called "Mad Honey" (Jansen et al., 2012). Other toxic contents most frequently encountered in honey are hyoscyamine from Datura sp. (Miraldi et al., 2001), hyoscine from Hyoscamus niger (Jaremicz et al. 2014), saponin from Serjania lethalis (Ekabo et al., 1996) and strychnine from Gelsemium sempervirens (Zhang et al., 2013).

A large number of phenolic contents in the content of mad honey perform their antioxidant effects by defending the cells against the attacks

of free radicals, and by preventing some metabolic effect processes that these may cause. In this way, the balance between oxidants and antioxidants in a healthy condition of body are well maintained. However, this balance can disrupt due to oxidative stress caused by oxidants, free radicals. Biopolymers, including nucleic acids, proteins, carbohydrates, and polyunsaturated fatty acids are the main target for oxidants. Also, antioxidants can effectively protect cells against such damage. Therefore, they are vital for the homeostasis of cells and tissues (Buratti et al., 2007; Alzahrani et al., 2012; Tezcan et al., 2011; Narimane et al., 2017).

Evaluation of the total phenolic content, total flavonoid levels, and 2,2 difanyl-1-picrylhydrazil (DPPH) radical scavenging test results are the most preferred methods in demonstrating the antioxidant property of a content.

The antioxidant properties of mad honey and *Rhododendron ponticum* extracts have been evaluated in many studies in vivo and in vitro. Ersan et al., (2018) determined the grayanotoxin I and III levels of the mad honey samples they collected, and experimentally administered these honey samples to rats at a single dose of 12.5 mg/kg (acute), at a dose of 7.5 mg/kg for 21 days (subacute) and at a dose of 5 mg. /kg dose for 60 days (chronic). As a result of their studies, they stated that acute, subacute and long-term use of mad honey may cause significant changes in oxidative stress markers and enzyme levels, and mad honey may have genotoxic results in rats. They stated that careless use of mad honey can cause significant side effects. Sahin et al., (2018) state that the effectiveness of mad honey is significantly higher than normal honey and propolis in the healing model of wounds and fractures they have experimentally created in rats. Bilir et al., (2018) concluded that *Rhododendron ponticum* extracts were effective on Du145 and PC3 prostate cancer cells in vivo.

In this study, mad honey samples collected from seven different locations in the Black Sea region of Turkey will be physically examined first, and then in order to evaluate the antioxidant properties of these honey samples, the antioxidant capacities of the honeys will be determined by determining the total phenolic and total flavonoid contents levels, and 2,2 diphanyl-1-picrylhydrazil. (DPPH) will be evaluated by applying radical scavenging tests. Despite the potential risk of toxic effects, mad honey is evaluated as a potential curative agent in clinical trials due to its high bioactivity. It is thought that the studies to be carried out on this subject will contribute to the diversification of the usage areas of mad honey collected from the Black Sea region, and will contribute to the up-to-date demonstration of the antioxidant properties of mad honey. The results to be obtained from this study will guide the analyzes to be made in this area.

MATERIAL and METHOD

Mad honey samples collected from seven different locations in the Black Sea region of Turkey were brought in dark plastic containers. Collected honey samples were stored at -40^{o}C until analysis. In order to evaluate the total antioxidant activity of honey samples, honey samples were taken out of the deep freezer before the measurements of total phenolic content and total flavonoid content levels of honey samples and they were kept in an environment at room temperature for the samples to melt. Honey samples that melted and reached room temperature were diluted 1/3 with deionized water. In order for the dilution to be homogeneous, a vortex device was used for mixing. Afterwards, honey samples diluted in a homogenized manner were centrifuged at 4000 rpm for 10 minutes and made ready for chemical analysis.

In order to determine the protein profiles of mad honey samples, honey samples were first diluted using equal volumes of distilled water.

Acetone precipitation method was used to determine the protein levels of honey samples. For this purpose, one volume of honey was taken in a centrifuge tube and mixed with 4 mL of ice-cold acetone. The mixture was vortexed thoroughly and incubated in -20 °C deep freezer for 2 h. Then the tubes were centrifuged at 8,000 rpm at 4 °C for 10 min. The pellet obtained was air dried for 30 min and re-solubilised in 0.2 M phosphate buffer and kept at 4 °C (Bocian et al. 2019).

Protein quantification

The protein concentration of all the samples after precipitation was determined according to the method of Lowry et. al., (1951). BSA was used as the standard and the absorbance was read at 620 nm in a UV/VIS spectrophotometer.

SDS-Page

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was preformed according to the method of Laemmli (1970). Honey proteins were resolved on 12% separation gel with 5% stacking gel. When the bromophenol blue in the loading sample reached the lower end of the gel, it was removed and transferred to staining tray with Coomassie brilliant blue. The gel was kept in the staining tray overnight. The stained gel was determined for about 1-2 h and the process was repeated until the bands in the gel were clear, photographed and analyzed in a transilluminator.

Total Antioxidant

In order to calculate the total antioxidant activity of mad honey samples, the indirect method was used in the prepared extracts and in this way, the free radical scavenging effects of 1,1-dipheny l-2-picrylhy-drazyl (DPPH) were determined in honey samples. For this, extracts were prepared in 0.1 mM DPPH methanol and 1 mL and 3 ml of methanol-mixed extract were added to the measuring tubes. Measurements were performed in a spectrophotometer modified at 520 nm, using ascorbic acid as a reference, according to the method of Meda et al., (2005) and Dimins et al., (2010).

The antioxidant activity of mad honey samples was expressed as the percent inhibition of DPPH and the calculation was carried out with the formula given below.

AA[%] = (Abs cont-Abs sample)/Abs cont X 100).

Total Phenolic Content Concentration

The levels of total phenolic content concentrations were measured using the Folin-Ciocalteu method modified by Beretta et al. (2005). For this, 900 µl of distilled water was added to 100 µl of extract and 5 ml of Folin-Ciocalteu reagent solution was added on it, 4 ml of Na_2CO_3 (75 g/L) was added to this mixture after 4 minutes, and this final mixture was left to incubate for 2 hours. At the end of the incubation period, the activity was determined by reading the mixture at 750 nm in the spectrophotometer. The total amount of phenol in mad honey samples was calculated as equivalent to mg gallic acid in 100 g extract (Bertoncelj et al. 2007 Beretta et al. 2005, Diminis et al. 2010).

Total Flavonoid Concentration

The levels of total flavanoid content concentrations of mad honey samples were determined by Dewanto et al. (2002), using aluminum chloride, was evaluated colorimetrically. For this, 1.25 ml distilled water, 75 µl 5% NaNO3 and 150 µl 10% $AlCl_3$ were added to 250 µl of mad honey sample. This mixture was allowed to incubate for 2 hours. At the end of the incubation period, the activity level was determined by reading the mixture at 765 nm in the spectrophotometer.

Statistical Analysis of Data

The assays were carried out in triplicate, and the results were expressed as mean values and the

standard deviation (SD) using Microsoft office Excel 2007. For statistical assessment of SDS Page electrophoresis bands prior to testing statistical significance all data were tested with Kurskal Wallis by using SPSS 12 statistical programme.

RESULTS

The mean total phenolic content (mg GAE/kg), total flavonoid content (mg QE/kg) levels and Total Antioxidant (mg AAE/1 g honey) values determined in mad honey samples are shown in Table 1.

In table1 results of the honey samples and that samples the mean total phenolic content level of mad honey samples was 285.44±118.43 (125.85-471.18) mg GAE/kg honey; the mean total antioxidant activity was 29.68±7.2 (21.71-35.03) mg AAE/kg honey; and the mean total flavonoid content level was 27.26±4.79 (19.93 - 39.18).mg QE/kg honey

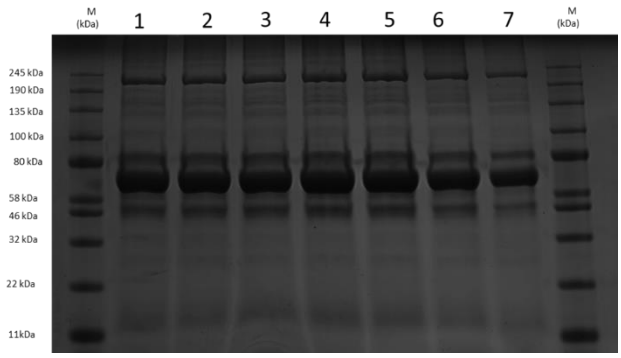

Figure 1. Mad Honey samples protein profile evaluation with SDS-PAGE electrophoresis

Table1. The mean total phenolic content (mg GAE/kg), total flavonoid content (mg QE/kg) levels and Total Antioxidant (mg AAE/1 g honey) values determined in mad honey samples (n=7).

Sample No	Total Phenolic Content (mg GAE/kg) X ±Sx	Total Flavonoid Content (mg QE/ kg) X ±Sx	Total Antioxidant level (mg AAE/1 g honey) X ±Sx
1	125,85±8,20	28,26±4,76	21,64±4,93
2	242,48±23,36	29,95±4,77	36,7±1,78
3	415,6±2,12	26,15±4,06	35,04±6,50
4	256,82±14,58	21,28±5,42	34,92±3,81
5	220,84±11,12	21,71±5,13	21,71±6,79
6	265,28±3,17	35,03±4,91	35,03±2,59
7	471,18±5,67	28,47±3,48	22,71±7,20

DISCUSSION

The Oxidants and antioxidants levels are well balanced in organism. However, this balance can change due to oxidative stress caused by free radicals, which are destructive for cellular components nucleic acids, proteins, carbohydrates and fatty acids. Antioxidants can effectively protect cells against such damage with radical scavenging activity. Therefore, they are important parts for the homeostasis of cells and tissues. Most of the phenolic compounds in the content of mad honey show antioxidant properties by defending the cells against the attacks of free radicals and preventing the progression of metabolic effect processes. (Buratti et al., 2007; Alzahrani et al., 2012; Tezcan et al., 2011; Narimane et al., 2017).

The antioxidant properties of mad honey and *Rhododendron ponticum* extracts have been evaluated in many in vivo and in vitro studies. Ersan et al., (2018) determined grayanotoxin I and III levels in mad honey and administered these honeys to rats for 12.5 mg/kg single dose (acute), 7.5 mg/kg 21 days (subacute) and 5 mg/kg 60 days (chronic) periods. As a result of their studies, they stated that acute, subacute and chronic use of mad honey has significant effects on oxidative stress markers and enzyme levels and may lead to genotoxic results. They stated that careless use for medical purposes may cause significant side effects. Sahin et al., (2018)

determined that the positive effect of mad honey on the healing model of wounds and fractures they created experimentally in rats was significantly higher than that of normal honey and propolis. Bilir et al., (2018) concluded that *Rhododendron ponticum* extracts were effective on Du145 and PC3 prostate cancer cells.

In various studies, the total phenolic contents in mad honey samples were 0.160-0.606 mg Gallic acid equivalents (GAE)/g (Sahin et al., 2015), 215.43-846.22 mg Gallic acid equivalents (GAE)/kg (Koca et al., 2015), 0.24-141.83 mg GAE /100 g (Silici et al., 2010). Lachman et al., (2010) reported that the total phenolic substance levels in 40 honey samples varied between 0.216-0.900 mg GAE/g. In our study, the average total phenolic content level in mad honey samples collected from 7 different locations of the Black Sea region was measured as 285.44±118.43 (125.85 to 471.18) mg GAE/kg honey (Table 1) and this result was found to be consistent with the above values. Ozkok et al., (2010) reported that they found the total phenolic content of pine honey as 35.36-365.94 mg GAE/kg in their study. The mean total antioxidant activity of 7 mad honey samples in our study was 29.68±7.2 (21.71 to 35.03) mg ascorbic acid equivalent (AAE)/kg honey (Table 1). This result was consistent with Silici et al., (2010) detected antioxidant activities in mad honey (12.76-80.80 mg ascorbic acid equvalent /g) levels. Ozkok et al., (2010) determined the total flavonoid level in pine honey as 4,80-22.80 mg QE/kg. The mean total flavonoid content in mad honey samples in our study was determined as 27.26±4.79 (19.93 to 39.18) mg Quercetin equivalents QE/kg honey (Table 1). Total phenolic content, total flavonoid content and total antioxidant activity levels determined in 7 mad honey samples used in our study were found to be compatible with the data reported by various researchers above.

Despite the reports in various studies that the properties of proteins in honey samples collected from various regions vary according to the type of plant flora and environmental factors, as a result of the evaluation of SDS-Page electrophoresis bands in mad honey samples in our study; It was revealed that there was no significant difference between the bands in mad honey samples taken from 7 different regions. In addition, it was concluded that the changes in the levels of total phenolic content, total antioxidant content and total flavonoid content in mad honey samples in this study did not cause any change in the protein levels of honey samples, which was similar to the results of other studies on this subject (Nath et al., 2018; Bocian et al. 2019).

CONCLUSION

In this study, mad honey samples collected from 7 different regions of the Black Sea region were primarily evaluated physically. Then, the total phenolic substance, total antioxidant activity, and total flavonoid content and protein profiles of these mad honey samples were revealed. In this way, it was aimed to enable the diversification of the usage areas of mad honey produced in the Black Sea region. Despite its toxic effect potential, noticeable antioxidants activity, rich in phenolics and flavonoid content in mad honey has been evaluated as a potential substance for clinical trials that may be useful in the field of alternative medicine due to its high bioactivity.

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