JOURNAL of APITHERAPY and NATURE

June/2024

Volume 7 Issue 1

dergipark.org.tr/tr/pub/jan
www.apitherapyandnature.com
e-ISSN:2667-4734

JUNE 2024

JOURNAL of APITHERAPY and NATURE

VOLUME 7 ISSUE 1

Publication Type: Peer-reviewed Scientific Journal

Publication Date: June 30, 2024

Publication Language: English

Published two times in a year (June, December)

www.dergipark.gov.tr/jan

www.apitherapyandnature.com

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Journal of Apitherapy and Nature/Apiterapi ve Doğa Dergisi, 7(1), 1-13, 2024
Research article/Araştırma makalesi

Apiterapi ve Doğa Dergisi
Journal of Apitherapy and Nature
www.dergipark.gov.tr/jan

Antimicrobial, Anti-quorum sensing, and Antibiofilm Potentials of Ethanolic Extracts from Lamium galeobdolon (L.) L. and Lamium purpureum L.

Lamium galeobdolon (L.) L. ve Lamium purpureum L. Etanolik Ekstraktlarının Antimikrobiyal, Anti-quorum sensing ve Antibiyofilm Potansiyelleri

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Received/Geliş Tarihi: 23/03/2024
Accepted/ Kabul Tarihi: 24/06/2024
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Doi: 10.35206/jan.1457624
e-ISSN: 2667-4734

Abstract

Bacteria act through a communication mechanism called quorum sensing (QS) to control pathogenicity. Biofilm formation is a process supported by the QS mechanism and is known to act a part in antibiotic resistance. In this study, antimicrobial, anti-QS and antibiofilm capacities of ethanol extracts obtained from *Lamium galeobdolon* (L.) L. and *Lamium purpureum* L. plants were determined. The antimicrobial activity of plant extracts was evaluated by well diffusion assay against various hospital isolates. The extracts have antimicrobial effects against all bacteria and yeasts. The 27.5 ± 0.71 mm inhibitory effect of *L. purpureum* against *Candida guilliermondii* yeast at a dose of 100 mg/mL is remarkable. The anti-QS potential of the extracts was evaluated by a well diffusion assay based upon violacein pigment inhibition. *L. purpureum* extract showed a higher level of anti-QS effect against *Chromobacterium violaceum* ATCC 12472 biosensor strain. Antibiofilm capacity against biofilm-forming *Escherichia coli* isolate was determined by the crystal violet staining method. *L. purpureum* extract showed an inhibitory effect of 89.66% against *E.coli* biofilm at the highest dose. Both plants have been shown to have anti-pathogenic properties. However, it can be said that the *L.purpureum* plant is a more highly anti-pathogenic plant compared to *L. galeobdolon*.

Keywords: Antibiofilm, Antimicrobial, *Chromobacterium violaceum* ATCC 12472, *Lamium galeobdolon*, *Lamium purpureum*.

Özet

Bakteriler, patojeniteyi kontrol etmek için quorum sensing (QS) adı verilen bir iletişim mekanizması yoluyla hareket eder. Biyofilm oluşumu QS mekanizması tarafından desteklenen bir süreçtir ve antibiyotik direncinde rol oynadığı bilinmektedir. Çalışmada *Lamium galeobdolon* ve *Lamium purpureum* bitkilerinden elde edilen etanol ekstraktlarının antimikrobiyal, anti-QS ve antibiyofilm kapasiteleri belirlendi. Bitki ekstraktlarının antimikrobiyal aktivitesi çeşitli hastane izolatlarına karşı kuyu difüzyon deneyi ile araştırılmıştır. Ekstraktlar, tüm bakteri ve mayalara karşı antimikrobiyal etki göstermiştir. *L. purpureum*'un 100 mg/mL dozunda *Candida guilliermondii* mayasına karşı 27.5 ± 0.71 mm'lik inhibitör etkisi dikkat çekicidir. Ekstraktların anti-QS potansiyeli, viyolasin pigment inhibisyonuna dayanan kuyu difüzyon deneyi ile değerlendirildi. *L. purpureum* ekstraktı, *Chromobacterium violaceum* ATCC 12472 biyosensör suşuna karşı daha yüksek seviyede anti-QS etkisi gösterdi. Biyofilm oluşturan *Escherichia coli* izolatına karşı antibiyofilm kapasitesi kristal viyole boyama yöntemiyle belirlendi. *L. purpureum* ekstraktı en yüksek dozda *E. coli* biyofilmine karşı %89.66 oranında inhibitör etki göstermiştir. Her iki bitkinin de anti-patojenik özelliklere sahip olduğu kanıtlanmıştır. Ancak *L. purpureum* bitkisinin, *L. galeobdolon* bitkisine göre anti-patojenik özelliği daha yüksek bir bitki olduğu söylenebilir.

Anahtar Kelimeler: Antibiyofilm, Antimikrobiyal, *Chromobacterium violaceum* ATCC 12472, *Lamium galeobdolon*, *Lamium purpureum*.

Abbreviations: ATCC, American Type Culture Collection; DMSO, Dimethyl sulfoxide; OD, Optical Density; MHB, Mueller-Hinton Broth; MIC, Minimum Inhibitory Concentration; PBS, Phosphate-buffered saline; QS, Quorum sensing; SD, standard deviation.

1. INTRODUCTION

Today, to combat infectious diseases, the search for new antimicrobial agents that will not cause resistance development continues. However, discovering new antimicrobial agents is not enough because the different strategies developed by bacteria bring microbial challenges. Quorum sensing (QS), a bacterial communication mechanism, causes planktonic cells to aggregate, form biofilms, and develop pathogenic properties. Gene expression regulated through QS causes changes in surface antigens, helping the bacteria to evade host immunity. Bacteria hiding in biofilms are resistant to harsh environmental conditions and nutrient limitations. By remaining dormant within the biofilm, they can hide from the immune system and cause acute infection. In short, bacterial adaptations such as QS and biofilms cause them to be more resistant to antimicrobial therapy by reducing the cellular function requirements that antimicrobials would interfere with or by modulating antimicrobial targets (Tamfu et al., 2020; Vestby et al., 2020; Mirghani et al., 2022; Sharma et al., 2023).

Considering their global use, therapeutic and industrial values, researching plant families with great therapeutic potential becomes important. One of these families that attracts attention with their medicinal plants is the Lamiaceae family. The *Lamium* genus, which is one of the less studied members of the Lamiaceae family compared to other genera, has annual or perennial forms. This genus, which is frequently visited by entomophile pollination representative bumble bee queens and honey bees, is a host for many insect species, making them ecologically valuable. Various species belonging to the *Lamium* genus, comprising nearly 30 species in Turkey, are utilized in public health for treating hypertension, astringency, trauma, paralysis, fractures, constipation, and gynecological diseases. Additionally, they are recognized for their antispasmodic, antiproliferative, antiviral, and anti-inflammatory properties. (Akkol et al., 2008; Bubueanu et al., 2019; Salehi et al., 2019). *Lamium galeobdolon* and *Lamium purpureum* are species belonging to the genus *Lamium*, and their chemical compounds differ qualitatively and quantitatively. These differences can be considered as the reason why the two species have different bioactive effects (Akkoyunlu & Dulger, 2019; Akkoyunlu & Dulger, 2022).

As far as is known, although there are antimicrobial studies on many *Lamium* species, there are almost no studies explaining anti-quorum sensing and antibiofilm effects. The objective of this study is to assess the antimicrobial effects of *Lamium galeobdolon* and *Lamium purpureum* plants against bacteria and yeasts, and their anti-quorum sensing and antibiofilm capacities as a precaution against resistance to antimicrobial treatment.

2. MATERIALS and METHODS

2.1. Plant Extraction Preparation

The above-ground parts of the plants were collected from Duzce province between March and May in 2023, according to the flowering periods of the plants. The collected plants were dried under suitable conditions and ground into powder in the shredder. 20 grams of the powdered plants were placed in the Soxhlet apparatus and extraction was performed with 200 mL of 96% ethanol for 8 hours. The extracts were passed through Whatman filter No.1 and ethanol was removed using a rotary evaporator at 55 °C. The resulting dried extracts were dissolved in dimethyl sulfoxide (DMSO) (Merck, Germany) to a final concentration of 100 mg/mL. The extracts were stored in a sterile dark glass bottle at + 4 °C (Dulger & Dulger, 2018).

2.2. Test Microorganisms Preparation

In vitro, antimicrobial research was conducted with five bacterial isolates (*Bacillus cereus*, *Escherichia coli*, *Listeria innocua*, *Staphylococcus aureus*, and *Streptococcus pyogenes*) and five yeast isolates (*Candida albicans*, *C. glabrata*, *C. guilliermondii*, *C. lypolitica*, *and C. tropicalis*) obtained from Duzce University Research and Application Hospital Medical Microbiology Laboratory. Bacteria were incubated in Nutrient Broth (Merck, Germany) at 35-37 ºC and yeasts in Malt Extract Broth (Merck, Germany) at 25 - 27 ºC for 24 hours. The turbidity of fresh cultures was regulated to McFarland 0.5 with sterile saline.

2.3. Antimicrobial Test

Well diffusion method was used to identify the antibacterial and antifungal activity of *L. galeobdolon* and *L. purpureum*. Fresh microorganism inoculum was coated on Mueller-Hinton agar (Merck, Germany) in three directions with sterile swabs. Wells (8 mm diameter) were made in the plates using a sterile tip. Based on previous studies, plant concentrations of 100 mg/mL and 50 mg/mL were chosen due to their effectiveness and being within safe limits and 50 µL were added to the wells. DMSO was used as a control. Bacteria were incubated at 35 - 37 ºC and yeasts were incubated at 25 - 27 ºC for 24 - 48 hours and the diameters of the transparent zones formed were measured using a caliper. Amikacin (Bioanalyse, Turkey) and Penicillin (Bioanalyse, Turkey) antibiotics were used for bacteria and Ketoconazole (Bioanalyse, Turkey) for yeasts to compare the antimicrobial activity levels of the plants. The experiments were conducted three times independently. The results were assessed using means and standard deviations (Dulger, 2022).

2.4. Anti-quorum Sensing Test

Well diffusion method was used to qualitatively detect the anti-QS potential of the plants. *Chromobacterium violaceum* ATCC 12472 biosensor strain was streaked continuously onto nutrient agar in three different directions. Wells were opened by puncturing with a sterile pipette tip. Crude extracts of plants were added to the wells in a volume of 50 µL, at a concentration of 100 mg/mL and 50 mg/mL, and incubated at 30 ºC for 24 hours. 1% DMSO was used as a control. Experiments were performed in two replicates. Measurements were made by calculating the turbidity zone around the wells where violacein production was prevented (Mulya & Waturangi, 2021).

2.5. Antibiofilm Test

100 µL of sterile Mueller-Hinton broth (MHB) (Merck, Germany) medium with 1% glucose was added to the wells to be studied on a flat-bottomed 96-well microplate. The sub-MICs of the extracts (100, 50, 25, 12.5, and 6.25 mg/mL) were added. A strong biofilm-forming *E. coli* isolate was used in this assay. 100 µL of fresh culture adjusted to 0.5 McFarland turbidity was pipetted into the wells, and the microplate was incubated at 37 °C for 48 hours. The positive control contained 100 µL of bacterial culture and 100 µL of medium. Only 200 µL of medium was used in the negative control. The experiment was performed in duplicate.

After incubation, the wells were washed three times with 200 µL of PBS and dried. Biofilms were fixed with 200 µL of 99% methanol for 5 min. The wells were inverted, their contents drained and dried. The wells were stained with 200 µL of 0.3% crystal violet dye for 15 minutes. Microplates were washed three times with distilled water. The dye bound to the biofilm mass was dissolved with 96% ethanol solution and the absorbance was established at 560 nm in a microplate reader (Robonik, India). Percent inhibition values of plant extracts were calculated with the following formula (Balli et al., 2019; Haney et al., 2021):

$$(\text{OD positive control} - \text{OD sample}) / \text{OD positive control} \times 100) \text{ (Equation 1)}$$

2.6. Statistical Analysis

The data were entered into Microsoft Excel version 365 and presented as mean ± standard deviation (SD) values. Analyses were conducted using IBM SPSS Statistics 27 software. Shapiro-Wilk normality test was applied to determine whether the data showed a normal distribution. The p-value obtained from the Shapiro-Wilk test was found to be greater than 0.05 (For *L. galeobdolon* p = 0.054; for *L. purpureum* p = 0.153). This result indicates that the data follow a normal distribution. To determine whether the inhibitory effect of the concentration groups was significant compared to the control group; a One-Sample t test was applied. The p-value obtained from the One-Sample t test was found to be less than 0.05 (for all p = 0.006). This result indicates that the inhibitory effect of the concentration groups is statistically significant compared to the control group.

3. RESULTS and DISCUSSION

3.1. Antimicrobial Activity

Table 1 shows the antimicrobial effects of *L. galeobdolon* and *L. purpureum* plants on 5 bacteria and 5 yeasts. The results show that plant extracts inhibit microbial growth in a dose-dependent manner (Figure 1). *L. galeobdolon* created the highest inhibition zone (> 15 mm) against *C. glabrata* at both doses. The plant gave better results than the penicillin antibiotic against *B. cereus*, *E. coli*, *S. aureus*, *S. pyogenes* bacteria. However, its effect on bacteria remained low compared to the amikacin antibiotic.

Another plant, *L. purpureum*, has more antimicrobial effects than *L. galeobdolon*. It is highly effective in inhibiting *C. guilliermondii* (≥ 25mm). It is noteworthy that it is as effective as the antibiotic amikacin in inhibiting the growth of *B. cereus*. Compared to the ketoconazole antibiotic used for *Candida* strains, the high dose of the plant showed a more inhibitory effect on the strains.

Table 1. Summary of Antimicrobial Activity of *Lamium galeobdolon* and *Lamium purpureum**

Microorganisms	***Lamium galeobdolon* L.**		***Lamium purpureum* L.**		**P**	**AK**	**KETO**
	50 mg/mL	*100 mg/mL*	*50 mg/mL*	*100 mg/mL*			
	Bacteria						
Bacillus cereus	12.0	13.5 ± 0.71	17.0 ± 1.41	20.0 ± 1.41	10.0	20.0	NT
Escherichia coli	13.5 ± 0.71	14.0 ± 2.82	14.5 ± 2.12	16.5 ± 2.12	7.0	25.0	NT
Listeria innocua	11.0 ± 1.41	12.0	15.5 ± 0.71	19.0 ± 1.41	24.0	30.0	NT
Staphylococcus aureus	12.5 ± 2.12	14.0 ± 5.65	14.5 ± 2.12	16.5 ± 2.12	12.0	26.0	NT
Streptococcus pyogenes	12.0	14.0 ± 1.41	17.0 ± 2.82	19.0 ± 2.82	9.0	29.0	NT
	Yeasts						
Candida albicans	11.5 ± 0.71	14.0 ± 2.82	16.0	18.0	NT	NT	10.0
Candida glabrata	15.5 ± 0.71	15.5 ± 2.12	15.0	16.5 ± 0.71	NT	NT	15.0
Candida guilliermondii	14.0	14.5 ± 4.95	25.0	27.5 ± 0.71	NT	NT	-
Candida lypolitica	13.5 ± 0.71	14.0 ± 1.41	15.5 ± 0.71	18.0	NT	NT	16.0
Candida tropicalis	**12.5 ± 0.71**	**12.0 ± 1.41**	**15.0**	**18.0**	**NT**	**NT**	**15.0**

**Contains zone diameter, which is the average value of three independent experiments (in mm). NT: Not tested; P: Penicillium (10 µg/mL); AK: Amikacin (30 µg/mL); KETO: Ketoconazole (20 µg/mL).*

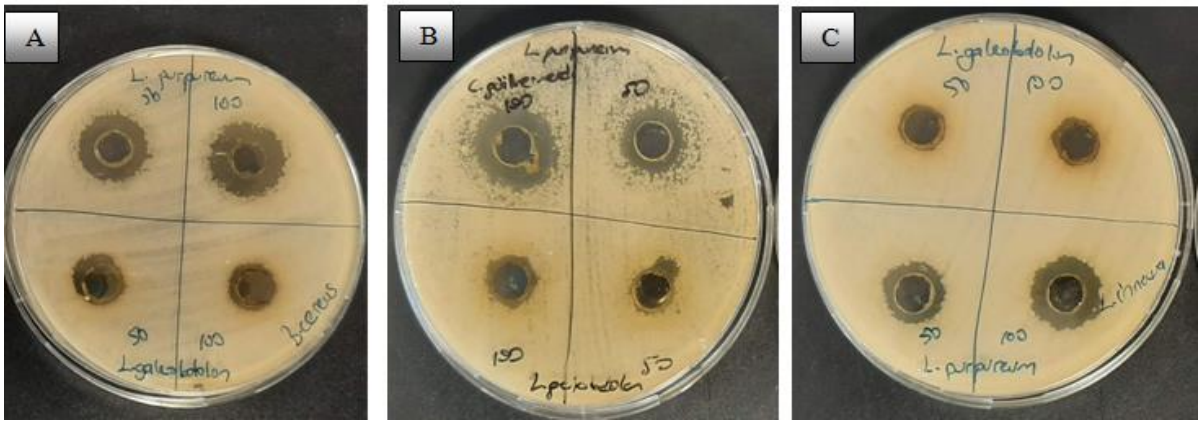

Figure 1. Antimicrobial activity of plant extracts against microorganisms samples (A: *B.cereus* B: *C.guilliermondii*; C: *L.innocua*) measured by well diffusion assay. It was studied in 3 repetitions and the inhibition zones were calculated by measuring with a caliper and presented as the inhibitory effect.

In a study conducted with 4 different *Lamium* species belonging to the genus, including *L. purpureum*, it was determined that all plants had moderate antimicrobial effects and were more effective against yeasts than bacteria. It has been stated that the solvent used also changes the antimicrobial capacity. It has been emphasized that the difference in antimicrobial effects between the species is due to the components they contain (Yalçın et al., 2007).

In a different study, it was stated that *L. galeobdolon* essential oil did not have an antibacterial effect against certain bacteria such as *B. cereus* and *S. aureus*, and had an antifungal effect against *C. glabrata* and *C. tropicalis* yeasts, but this was also weak (Akkoyunlu & Dulger, 2022). It has been stated in previous studies that solvent selection and extraction processes can influence the final results (Rios & Recio, 2005; Yalçın et al., 2007). Our study revealed that the antimicrobial effects of ethanol extracts from these plants are higher than those of the essential oils.

3.2. Anti-quorum Sensing Activity

C. violaceum ATCC 12472 bacteria, a biosensor strain, was used to identify the ability of plant extracts to inhibit the QS mechanism. This bacterial strain is the most popular microorganism used in elucidating QS mechanisms in recent years. Biosynthesis of violacein pigment governed by QS is considered an indicator in defining cell-to-cell signaling pathways (Dimitrova et al., 2023).

The opaque zones that appear around the plant extracts as a result of well diffusion experiments are an indication that only the QS mechanism is stopped without preventing

bacterial growth (Figure 2). The anti-QS effect was presented by calculating zone diameters in two replicate experiments. *L. purpureum* plant had a much greater inhibitory effect than *L. galeobdolon* (Table 2). It is noticed that the anti-QS effect increases depending on the concentration of plant extracts. While *L. galeobdolon* showed an inhibitory effect of 13.33 ± 1.53 at a high dose, *L. purpureum* showed a higher effect than this result even at a low dose (15.67 ± 1.53).

Table 2. Summary of Anti-quorum sensing Activity of *L. galeobdolon* and *L. purpureum*

Plants	**Effective concentrations (mg/mL)**	**QS inhibition zones (mm)**
Lamium galeobdolon	50	12.50 ± 0.58
	100	13.33 ± 1.53
Lamium purpureum	50	15.67 ± 1.53
	100	17.33 ± 0.58

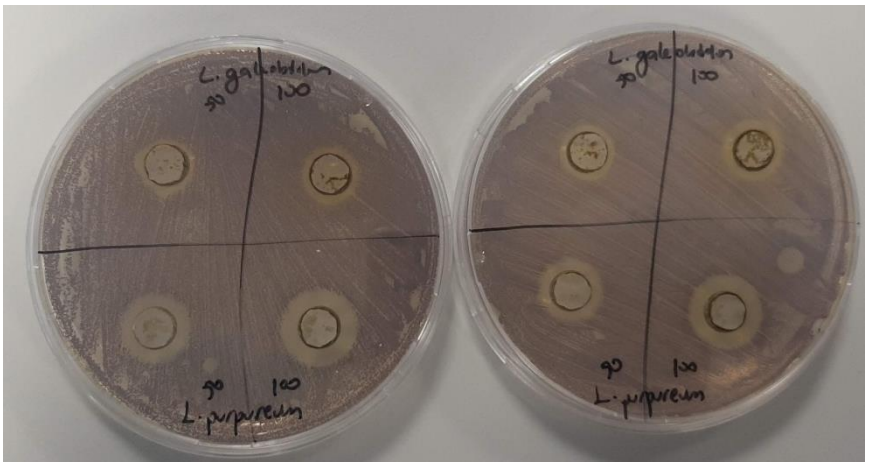

Figure 2. Demonstration of quorum sensing (QS) inhibitory activity of the plant extracts against *C.violaceum* ATCC 12472 biosensor strain

No anti-QS studies on *Lamium* species have been found in the literature. In a study conducted with 10 different plants, it was shown that plant essential oils belonging to the Lamiaceae family, such as *Salvia officinalis*, *Origanum vulgare*, *Clinopodium nepeta*, significantly inhibited the violacein pigment (D'Aquilla et al., 2023). In a different anti-QS study conducted on the pyocyanin pigment of *P. aeruginosa* bacteria, ethanol extracts of 23 plant species were studied. Among these plants, *S. officinalis*, two species belonging to the Lamiaceae family, showed high anti-QS effects, while *Solenostemon scutellarioides* species was ineffective (Elmanama & Al-Refii, 2017).

Attacking the QS mechanism, the key regulatory network of bacterial virulence has become a promising strategy against emerging antibiotic resistance. It was determined that the plant extracts we used in the current study inhibit the QS network of *C. violaceum* ATCC 12472 bacteria.

3.3. Antibiofilm Activity

The antibiofilm capacity *of L. galeobdolon* and *L. purpureum* extracts and the amount of biofilm biomass formed were determined by staining with crystal violet dye (Figure 3). Results calculated as the percentage of biofilm inhibition showed that the effect of plant extracts varied depending on species and dose (Figure 4).

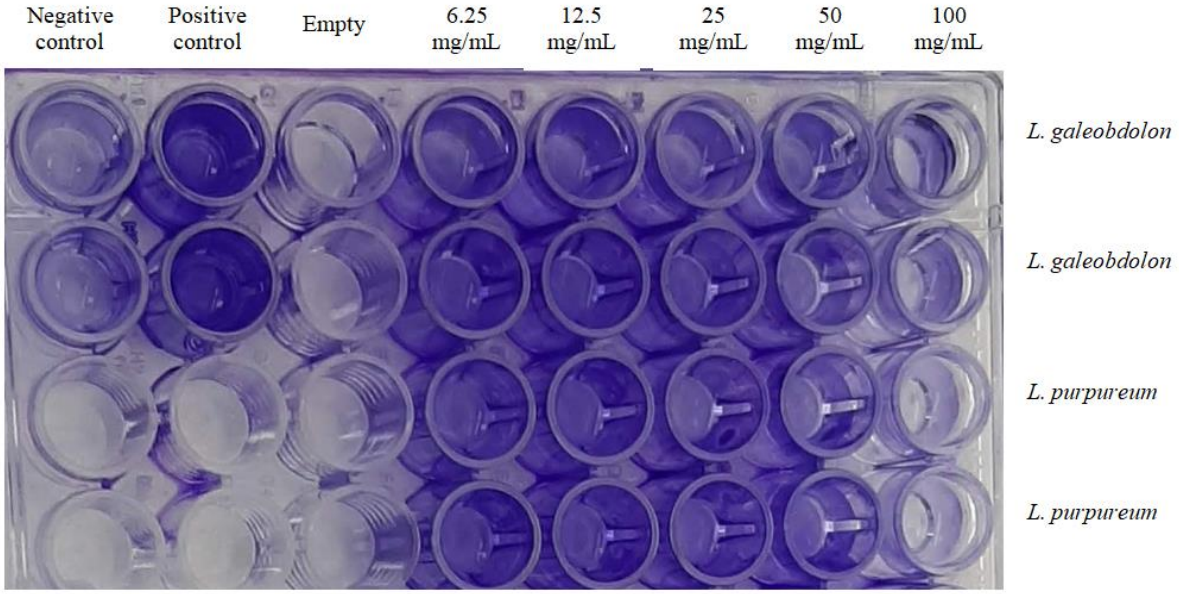

Figure 3. Images show crystal violet stained biofilm corresponding to controls and plant extracts.

L. galeobdolon plant exhibited lower antibiofilm effect than *L. purpureum* plant. *L. purpureum* inhibited biofilm formation by approximately 90% at high doses. Even at its lowest dose, biofilm formation remained below 50%. *L. galeobdolon* prevented biofilm formation by 64.64% at its highest dose, and biofilm formation was below 50% at all doses except the lowest dose. The antibiofilm effects of both plants are remarkable. Antibiofilm studies have not been described for these two species in the literature. However, in a study conducted with *Lamium album*, the most well-known member of this genus, ethanol, acetone, and ethyl acetate extracts were prepared and antibiofilm activity was evaluated against 2 reference strains and 9 clinical isolates obtained from wound swabs. The study found that the ethyl acetate extract was more effective against gram-positive biofilms, destroying *S. aureus* biofilm by up to 95% (Terzić et al., 2023).

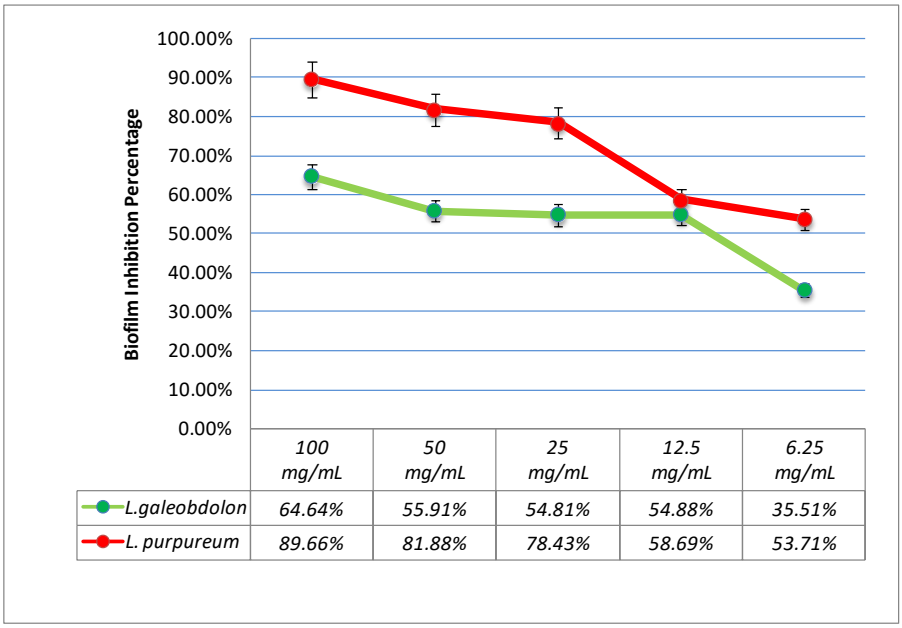

Figure 4. Antibiofilm activity of *L. galeobdolon* and *L. purpureum* extracts against *E. coli* biofilm. *Statistically different from the control ($p < 0.05$).

In treatment with *L. amplexicaule* extract, a different *Lamium* species, it significantly inhibited the Streptococcus mutans bacterial biofilm, which forms strong biofilms in the mouth, at a concentration lower than the MIC. The study indicates that this extract selectively prevents biofilm formation without inhibiting bacterial growth (Lee et al., 2019).

These results encountered in the literature are compatible with our study data. It is evident that *Lamium* species contain active biofilm inhibitory components. It can be said that there is a genus-wide effect against both Gram-positive and Gram-negative bacterial biofilms.

The bioactive properties of plants directly depend on the components they contain. These components depend on many factors such as the physiological development period of the plant, daily and seasonal changes during collection, the composition of the soil, and the type of extraction processes (Azwanida, 2015; Lin et al., 2019).

4. CONCLUSION

Biocontrol research on the use of plant extracts or pure compounds obtained from plants is gaining momentum today. The plants we used in our study are species with significant antibacterial, antifungal, anti-QS, and antibiofilm effects. It is extremely important to distinguish the biologically active natural components of these and many other plants, to determine the stability of crude extracts and to determine their cytotoxic effects before use. Our

study provides a basis for further studies and opens a motivating path for the discovery of potential antimicrobial agents, QS, and biofilm inhibitors.

DECLARATIONS

There is no conflict of interest between the authors.

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Journal of Apitherapy and Nature/Apiterapi ve Doğa Dergisi, 7(1), 14-27, 2024
Research article/Araştırma makalesi

Apiterapi ve Doğa Dergisi
Journal of Apitherapy and Nature
www.dergipark.gov.tr/jan

Usability of the phenolic profile analysis method developed in RP-HPLC-PDA in natural products

RP-HPLC-PDA'da geliştirilen fenolik profil analiz yönteminin doğal ürünlerde kullanılabilirliği

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Received/Geliş Tarihi: 02/02/2024
Accepted/ Kabul Tarihi: 29/05/2024
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Doi: 10.35206/jan.1430767
e-ISSN: 2667-4734

Abstract

The predominant majority of bioactive compounds in natural products are polyphenols. Reverse Phase High Performance Liquid Chromatography (HPLC) is the most employed analytical method for determining the polyphenol profiles of natural products. Analyses are conducted based on methods validated according to the number and type of phenolic standards used. In this study, it was aimed to determine 26 phenolic compound standards with HPLC-fotodiot array (PDA) detector, which is preferred for the separation of secondary metabolites commonly found in natural products. The analysis was carried out utilizing a C18 column (250 mm x 4.6 mm, 5 µm; GL Sciences) with a gradient program. The HPLC method was developed, determining the limit of detection within the range of 0.019-0.072 µg/mL, and the limit of quantification within the range of 0.063-0.239 µg/mL. All calibration curves exhibited linear corelations with R^2 values exceeding 0.994 across the specified range. The developed method has been optimized and validated by assessing detection and quantification limits, accuracy, repeatability, and recovery data suitable for phenolic analysis. It has been concluded that the optimized method allows for the rapid and reliable evaluation of the phenolic content of natural products and their quantitative determination.

Keywords: Phenolic content, Bee products, Plant, HPLC

Özet

Doğal ürünlerdeki biyoaktif bileşenlerin büyük çoğunluğunu polifenoller oluşturur. Polifenol kompozisyonlarının belirlemesinde en sık kullanılan analitik yöntemlerden biri Ters-Faz Yüksek Performanslı Sıvı Kromatografisidir. Analizler, kullanılan fenolik standartların sayısına ve türüne göre validasyonu yapılmış yöntemlere uygulanır. Bu çalışmada doğal ürünlerde yaygın olarak bulunan sekonder metabolitlerin ayrımında tercih edilen HPLC-fotodiyot dizisi (PDA) dedektörü ile 26 tane fenolik bileşen standardının belirlenmesi amaçlandı. Analiz C18 (250 mm x 4.6 mm, 5 µm; GL Sciences) kolonda gradient program kullanılarak yapıldı. HPLC yöntemi, tespit sınırını 0,019-0,072 µg/mL aralığında ve miktar belirleme sınırını 0,063-0,239 µg/mL aralığında belirleyecek şekilde geliştirildi. Tüm kalibrasyon eğrileri, belirtilen aralıkta R^2>0.994 değerleri ile doğrusal ilişkiler sergiledi. Oluşturulan metot optimize edilmiş, tespit ve nicelik sınırları, doğruluk, tekrarlanabilirlik ve fenolik analiz için uygun geri kazanım verileri değerlendirilerek doğrulanmıştır. Optimize edilmiş metot ile doğal ürünlerin fenolik içeriklerinin değerlendirilmesi ve kantitatif tayininin hızlı ve güvenilir bir şekilde yapılabileceği sonucuna varılmıştır.

Anahtar Kelimeler: Fenolik içerik, Arı ürünleri, Bitki, HPLC

Abbreviations: RP-HPLC, Reverse Phase High Performance Liquid Chromatography; PDA, Photodiode array; LOD, Limit of detection; LOQ, Limit of quantification

1. INTRODUCTION

Plants tend to protect themselves against harmful effects with secondary metabolites such as phenolic acids and polyphenols in their structures (Fang et al., 2007). These phenolic compounds, found in plants and transferable to animal products (bee products etc.), rank prominently among bioactive compounds (Rahsmi & Negi, 2020). Studies on phenolic compounds are increasing each day, primarily due to their bioactivities, including antioxidant properties as well as anti-inflammatory and antitumoral activities. Experimental and epidemiological studies also reveal the impact of phenolic compounds against degenerative diseases (Kumar et al., 2019).

Rhubarb (*Rheum ribes*), belonging to the Polygonaceae family, is a perennial herbaceous plant that grows at high altitudes and produces yellowish flowers in May and June. It has been reported that rhubarb, which contains various bioactive components, has metabolic benefits (Oktay et al., 2007). Knowing its phenolic content may contribute to elucidating these benefits. Among bee products, propolis and honey are two basic natural products, and various *in vitro* and in vivo studies are carried out on their phenolic contents (Ali & Kunugi, 2021; Ożarowski & Karpiński, 2023).

Various extraction methods are employed to obtain phenolic compounds from plant and bee products. Different solvents or solvent mixtures can be utilized depending on the selectivity of the bioactive compound to be extracted and the efficiency of the extraction method (Kumar & Goel, 2019). After extraction, meaningful content analyses can be achieved by identifying and quantifying the bioactive compounds obtained. However, despite numerous studies on the quantitative and qualitative analyses of bioactive compounds, it is an undeniable fact that the entire spectrum of these compounds, which has a wide range, has not been fully elucidated (Ignat et al., 2011; Kara et al., 2022). In this context, a broad network of research is aimed at developing quantitative and qualitative analysis methods for determining phenolic content (Liu et al., 2008).

While there are relatively simple spectroscopic methods available for the bulk quantification of phenolic compounds (such as the Folin-Ciocalteu reagent for total phenolic content determination), high-performance liquid chromatography (HPLC) enables both quantitative and qualitative analysis of individual phenolic compounds (Santos & MagalhÃes, 2020). In addition to addressing the diversity of phenolic compounds to be analyzed, HPLC methods are developed and utilized to gain advantages in terms of time and efficiency, enabling the determination of many analytes (Bae et al., 2015; Madrera & Valles, 2020; Michalaki et al., 2023). In methods developed for HPLC analysis, often two or more solvents are employed to determine the mobile phase for achieving the best separation. Generally, a gradient program is established to create optimal conditions for the mobile phase that will be used in the analysis. However, another crucial aspect of high analysis efficiency is the selection of a detector with high sensitivity, selectivity, and a broad analysis spectrum. In the literature, it is observed that detectors such as ultraviolet (UV), photodiode array (PDA), and tandem mass spectrometry (MS/MS) are preferred for phenolic content analyses (Michalaki et al., 2023; Kara et al., 2022; Zhang et al., 2023). Especially with the preference for PDA detectors, it is possible to operate within a wavelength range, and the spectra of analytes can be visualized. Thus, the PDA detector provides the opportunity to work with methods that offer high accuracy and sensitivity.

In the case of a research-based analytical method, it is essential to conduct reliability, repeatability, and applicability studies for its validation. In this context, the method's measurement range, quantitative and qualitative measurement limits, as well as accuracy and precision values, can be investigated (Can et al., 2015; Chaudhary et al., 2023; Sobral et al., 2017). Accuracy and precision assessment can involve the calculation of relative error and recovery percentage, along with the examination of various parameters. This study aims to

demonstrate the applicability of a chromatographic method for the analysis of phenolic content in plants and certain animal products (propolis, honey, etc.).

2. MATERIALS and METHODS

2.1 Extraction of Samples

In the context of our study, the rhubarb (*Rheum ribes*) was sourced from the Hakkari region, propolis from the Trabzon region, and honey from beekeepers in the Balıkesir region of Türkiye (Figure1). The samples were extracted with a solvent at a ratio of 1:10 (g/mL) for 24 hours, room tempruture at 200 rpm (Kara et al., 2022). The propolis sample was extracted with 70% EtOH, while the honey samples and rhubarb (*Rheum ribes*) were extracted with MeOH. Following extraction, a filtration process was conducted to remove solid particles, and the samples were divided into two parts for antioxidant analysis and phenolic content analysis. For phenolic analysis, the samples were subjected to the sample preparation procedure reported by Kara et al. (2022), and phenolic content analysis was conducted using the method developed and validated within the scope of this study. All antioxidant analyzes were performed in triplicate.

Figure 1. A: Propolis, B: Honey, C: Rhubarb (*Rheum ribes*)

2.2. Validation of the Phenolic Content Method

The validation of the developed method for phenolic content analysis included assessments of accuracy, precision, recovery, and analytical measurement limits. Accuracy, which expresses the closeness of the obtained value to the true value, can be examined through both absolute and relative error values. Relative error is calculated by dividing the absolute error by the true value. The closeness of the values obtained in the analysis is expressed as precision. The method's accuracy can be further examined through recovery and repeatability analyses.

Within the scope of the developed phenolic analysis method, each standard was studied in three repetitions at six different concentrations (38-1.188 ppm). Using the obtained

standard calibration curves, detection (LOD) and measurement (LOQ) limits were determined. The standard deviation at the lowest concentration and the slope of the curve were used to calculate these values. The ratio of standard deviation to slope was calculated by multiplying by 3.3 for the LOD value and by 10 for the LOQ value (Ribani et al., 2006).

2.3. Analysis Conditions of the Phenolic Content Method

The HPLC system used for phenolic content analysis consists of an LC-20AT liquid chromatograph (Shimadzu), SIL-20AC HT autosampler (Shimadzu), SPD-M20A diode array detector (Shimadzu), and an InertSustain C18 column (5 µm, 4.6 mm, 250 mm; GL Sciences). In the developed method, 70% acetonitrile (ACN)-ultrapure water (reservoir A) and 2% acetic acid (AcH)-ultrapure water (reservoir B) were used for the mobile phase. In the gradient program, a 50-minute analysis time was applied with the following composition: 0. min 82%(B), 5. min 81%(B), 10. min 73%(B), 14. min 62%(B), 25. min 35%(B), 40. min 10%(B), 40.01. min 82%(B), and 50. min 82%(B). For phenolic content analysis, the applied flow rate was 1 mL/min, column temperature was set at 30 °C, injection volume was 20 µL, and autosampler cell temperature was maintained at 20 °C. Within the scope of the analysis, four different wavelengths (250, 280, 320, 360) were utilized for the standards. The created phenolic content method involved the analysis of standards at specific wavelengths: protocatechuic acid, *p*-OH benzoic acid, vanillic acid, rutin, ellagic acid, daidzein at 250 nm; gallic acid, catechin hydrate, epicatechin, syringic acid, *t*-cinnamic acid, naringenin, hesperetin, chrysin, pinocembrin at 280 nm; chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, apigenin, caffeic acid phenethyl ester (CAPE) at 320 nm; and myricetin, luteolin, quercetin, rhamnetin, galangin at 360 nm. Standards were prepared by first dissolving them with a small amount of 100% MeOH and then making up their volume with 50% MeOH.

2.4. Antioxidant Capacity and Activity Analyzes

As part of the antioxidant capacity analyses, the total phenolic content (Slinkard & Singleton, 1977) and total flavonoid content (Fukumoto & Mazza, 2000) were determined. The Folin-Ciocalteu method was used for total phenolic content (TP). For this, 400 µL of a 1:10 diluted Folin-Ciocalteu reagent was mixed with 20 µL of the sample, 680 µL of distilled water, and 400 µL of 10% Na_2CO_3. After incubation for 120 minutes, the absorbance was measured at 760 nm wavelength. Gallic acid was used for the standard calibration curve, and the results were

expressed as mg of gallic acid equivalent per gram of the sample. Regarding total flavonoid content (TF), 250 µL of the sample was mixed with 2150 µL of MeOH, 50 µL of 10% $Al(NO_3)_3$, and 50 µL of 1 M NH_4CH_3COO solution. After a 40 minute incubation, the absorbance was measured at 415 nm wavelength. Quercetin served as the standard, and the results were reported as mg of quercetin equivalent per gram of the sample. In the assessment of antioxidant activity, we conducted analyses using the Fe(III) Reducing Antioxidant Power method (Benzie & Strain, 1999) and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activities (Molyneux, 2000). For Fe(III) Reducing Antioxidant Power analysis, 1500 µL of freshly prepared FRAP reagent (Kara et al., 2022) was mixed with 50 µL of the sample. After 4 minutes of incubation, the absorbance was measured at 593 nm wavelength. $FeSO_4.7H_2O$ was used as the standard, and the results were expressed as µmol of $FeSO_4.7H_2O$ equivalent per gram of the sample. A solution of 100 µM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) was prepared for DPPH· radical scavenging activity. The solution was mixed in a 1:1 (v/v) ratio with varying concentrations of the sample and incubated for 50 minutes. Subsequently, the absorbance was measured at 517 nm wavelength. The sample concentration corresponding to half of the concentration of the initial DPPH· radical in the medium (SC_{50}) was calculated, and the results were expressed in mg/mL. Trolox was used as standard.

3. RESULTS and DISCUSSION

For the validation of the method developed for the phenolic content analysis in plants and bee products, standards were tested in the range of 1,188-38,000 ppm, and the linearity of calibration curves, relative error, recovery of the method, LOD, and LOQ values were calculated (Table 1). When examining the recovery values at the standard concentration of 38 ppm, it is observed to be in the range of 97.803% to 103.948%. The low relative error of this method, both quantitatively and qualitatively applicable, suggests high precision and accuracy. When examining the LOD values of each standard, it was found that caffeic acid had the highest value at 0.072 µg/mL, while catechin hydrate had the lowest value at 0.019 µg/mL. The LOQ values are observed to be in the range of 0.071-0.239 µg/mL. Cayan et al. (2020) reported LOD and LOQ values of their method, established with 16 phenolic standards, in the ranges of 0.001–0.970 and 0.001–2.940 µg/L, respectively. Švecová et al. (2015) stated that the LOD and LOQ values of their method, developed with 12 phenolic standards, were in the ranges of 1.17-5.35 µg/L and 3.89-17.80 µg/L, respectively. In another study, the LOD and LOQ values for the HPLC analysis of 14 phenolic standards were found to be in the ranges of 0.009-0.192 µg/mL

and 0.027-0.582 µg/mL, respectively (Seal, 2016). Skendi et al. (2017) determined the LOD and LOQ values as 0.005-0.16 µg/mL and 0.01-0.48 µg/mL, respectively, in the method they developed for the analysis of 24 phenolic contents in plant samples. In a study by Aktaş Karaçelik and Şahin (2021), LOD and LOQ values for RP-HPLC-DAD analysis of 18 phenolic standards were found to be in the range of 0.019-0.2363 mg/L and 0.039-0.7162 mg/L, respectively. These valuable findings indicate that low amounts of phenolic content can be detected with RP-HPLC, but the limits may vary depending on the condition of the equipment.

Tablo 1. R^2, LOD, LOQ, relative error and recovery values of standards

Standards	**R^2**	**Limit of Detection (LOD) (µg/mL)**	**Limit of Quantification (LOQ) (µg/mL)**	**Relative Error**	**Recovery (%)**
Gallic acid	0.999	0.043	0.142	0.007	99.296
Protocatechuic acid	0.999	0.062	0.205	0.008	100.838
Chlorogenic acid	0.999	0.034	0.115	0.018	101.757
Catechin hydrate	0.999	0.019	0.063	0.011	98.917
P-OH benzoic acid	0.999	0.044	0.148	0.028	102.816
Epicatechin	0.997	0.033	0.109	0.028	102.843
Caffeic acid	0.999	0.072	0.239	0.027	102.716
Vanillic acid	0.998	0.043	0.143	0.022	97.803
Syringic acid	0.999	0.021	0.071	0.013	98.725
P-coumaric acid	0.999	0.064	0.214	0.028	102.830
Rutin	0.999	0.047	0.156	0.004	100.417
Ellagic acid	0.999	0.062	0.208	0.004	99.554
Ferulic acid	0.995	0.026	0.088	0.031	103.073
Myricetin	0.998	0.032	0.108	0.013	101.348
Daidzein	0.999	0.050	0.166	0.007	100.719
Luteolin	0.994	0.032	0.106	0.034	103.351
Quercetin	0.999	0.029	0.098	0.009	99.101
t-Cinnamic acid	0.999	0.021	0.071	0.008	99.242
Naringenin	0.996	0.026	0.087	0.008	99.181
Apigenin	0.999	0.037	0.123	0.040	103.948
Hesperetin	0.999	0.023	0.075	0.013	101.289
Rhamnetin	0.996	0.028	0.094	0.014	101.445
Chrysin	0.999	0.027	0.091	0.009	99.146
Pinocembrin	0.999	0.037	0.123	0.007	99.303
CAPE	0.999	0.025	0.083	0.035	103.456
Galangin	0.999	0.023	0.077	0.036	103.647

The chromatograms of the standards used in the developed phenolic analysis method in the scope of the study are presented in Figure 2.

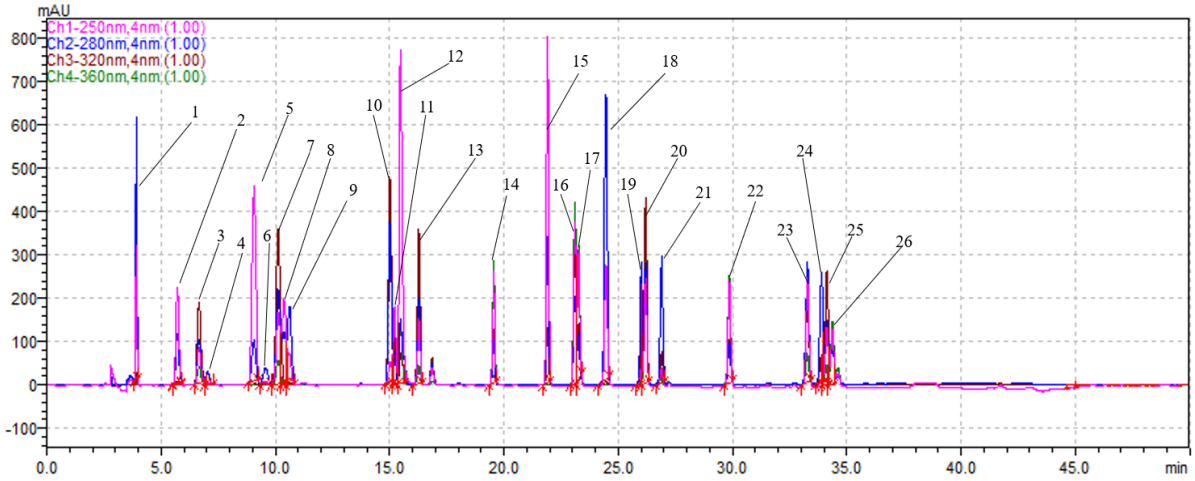

Figure 2. Phenolic Standard Chromatogram. 1. Gallic acid, 2. Protocatechuic acid, 3. Chlorogenic acid, 4. Catechin hydrate, 5. *p*-OH Benzoic acid, 6. Epicatechin, 7. Caffeic acid, 8. Vanillic acid, 9. Syringic acid, 10. *p*-Coumaric acid, 11. Rutin, 12. Ellagic acid, 13. Ferulic acid, 14. Myricetin, 15. Daidzein, 16. Luteolin, 17. Quercetin, 18. *t*-Cinnamic acid, 19. Naringenin, 20. Apigenin, 21. Hesperetin, 22. Rhamnetin, 23. Chrysin, 24. Pinocembrin, 25. CAPE, 26. Galangin

The developed phenolic content method has been applied to plant extracts and bee products, and the results are provided in Table 2. Quercetin has been reported as a significant component in the sample of rhubarb. (Mısır et al., 2023). Keser et al. (2020) reported that caffeic acid and ferulic acid were major components in the phenolic content analysis of the rhubarb sample (302.45 mg/g and 269.25 mg/g, respectively). Another study indicated the presence of 284.00 µg/mL gallic acid and 218.26 µg/mL rutin in the ethanolic rhubarb extract. (Abdulla et al., 2014). Meral (2017) reported that the phenolic content of rhubarb samples subjected to different drying conditions varied. The sample dried at 80 °C exhibited higher phenolic content (gallic acid: 353.5±2.4 mg/100g, rutin: 68.8±0.4 mg/100g, caffeic acid: 40.7±0.6 mg/100g). In the rhubarb sample used in our study, significant levels of ellagic acid (20.573 µg/g), ferulic acid (34.205 µg/g), and gallic acid (17.433 µg/g) were observed.

Kumazawa et al. (2004) conducted phenolic analysis on propolis samples collected from 16 different countries and reported that the samples contained high levels of chrysin and pinocembrin. In another study, propolis samples collected from 11 different regions of Türkiye were reported to be rich in chrysin, CAPE, pinocembrin, and rutin (Can et al., 2022). Halagarda et al. (2020), in their study on Polish honeys, reported that honey samples contained chrysin, caffeic acid, *p*-coumaric acid, pinocembrin, kaempferol, galangin, and apigenin. The phenolic contents of the propolis (chrysin, pinocembrin, CAPE, caffeic acid) and honey (ellagic acid, *p*-OH benzoic acid) samples used in our study have been elucidated with the new method.

Table 2. Phenolic content of rhubarb (*Rheum ribes*), propolis and honey samples

		Rhubarb (*Rheum ribes*)	**Propolis**	**Honey**
µg phenolic content /g sample	Gallic acid	17.433	-	-
	Protocatechuic acid	4.358	-	-
	Chlorogenic acid	-	-	-
	Catechin hydrate	-	-	-
	p-OH Benzoic acid	1.777	-	5.150
	Epicatechin	-	-	-
	Caffeic acid	-	1477.234	-
	Vanillic acid	-	-	-
	Syringic acid	1.762	-	-
	p-Coumaric acid	7.093	406.450	1.639
	Rutin	2.425	-	-
	Ellagic acid	20.573	-	17.903
	Ferulic acid	34.205	349.937	-
	Myricetin	-	-	-
	Daidzein	-	-	-
	Luteolin	-	-	-
	Quercetin	3.205	304.848	-
	t-Cinnamic acid	0.590	1128.909	0.563
	Naringenin	-	-	-
	Apigenin	-	361.103	-
	Hesperidin	-	-	-
	Rhamnetin	-	-	-
	Chrysin	1.300	6924.761	0.669
	Pinocembrin	-	9996.916	0.698
	CAPE	-	2652.344	-
	Galangin	-	-	-

* - : not detected

Antioxidant activity in various parts of the rhubarb collected from the Erzurum region of Türkiye was investigated using different solvents, and it was reported to exhibit antioxidant properties (Oktay et al., 2007). Ceylan et al. (2019) conducted a study where they examined the TP, TF, FRAP, and DPPH values per dry sample of rhubarb collected from the Erzurum region of Türkiye. They reported values of 112.82 ± 11.68 mg GAE/g, 2.50 ± 0.31 mg QUE/g, 42.50 ± 2.44 µmol Fe/g, and 0.11 mg/mL, respectively. Ozturk et al. (2007) extracted the stem part with aqueous methanol and expressed the TP and TF values as 35.71 ± 1.23 µg pyrocatechol equivalents/mg extract and 13.66 ± 0.75 µg QUE/mg extract, respectively. They stated that the extract at 100 µg/mL concentration had a radical scavenging activity of 87.07 ± 0.54%. The TP and TF values of the rhubarb sample collected from the Iğdır (Türkiye) region were found to be 18.644 mg GAE/g and 1.427 mg QUE/g, respectively (Mısır et al., 2023).

Studies on propolis have stated that the TP and TF values of propolis samples from different regions vary between 31.2±0.7- 299±0.5 mg/g and 2.5±0.8-176±1.7 mg/g,

respectively (Kumazawa et al., 2004). In honey, the TP, TF, FRAP, and DPPH analysis results have been shown to vary in the ranges of 16.02 ± 2.70 to 120.04 ± 18.56 mg GAE/100g, 0.65 ± 0.42 to 8.10 ± 2.56 mg QUE/100g, 0.59 ± 0.21 to 4.30 ± 0.13 µmol $FeSO_4.7H_2O$/g, and 12.56 ± 2.50 to 152.40 ± 6.20 mg/mL, respectively (Can et al., 2015). Antioxidant analyses were performed on the rhubarb (*Rheum ribes*), propolis, and honey samples, and the obtained results are presented in Table 3.

Table 3. Antioxidant results of Rhubarb (*Rheum ribes*), propolis and honey samples

	Rhubarb (*Rheum ribes*)	**Propolis**	**Honey**	**Trolox**
TP (mg GAE/ g)	1.742±0.078	124.847±0.152	0.392±0.005	
TF (mg QUE/ g)	0.343±0.012	31.566±0.052	0.029±0.007	
FRAP (µmol $Fe_2SO_4.7H_2O$/g)	17.494±0.107	5193.974±13.244	3.242±0.022	
DPPH SC_{50} (mg/mL)	6.968±0.098	0.033±0.004	83.256±0.126	0.006 ± 0.000

4. CONCLUSIONS

With this study, a method has been developed, validated, and applied for the determination of the phenolic content in plant and some animal samples (bee products, etc.). In our work, a goal was set to develop a method capable of analyzing 26 standards in a short period, as compared to methods created with different devices and different phenolic standards in the literature. When selecting standards, examples from the literature were reviewed, and commonly used phenolic standards were attempted to be identified. For the application of the developed method, rhubarb (*Rheum ribes*) was chosen as a plant sample, and two important bee products, propolis, and honey, were selected as animal products. With this study, a method was created for the phenolic content analysis of samples taken from different sources in a short time, less costly and with high sensitivity. Considering that natural products have rich phenolic content, studies can be carried out to develop easily applicable methods that can detect more phenolics.

DECLARATIONS

The authors declare that they have no conflicts of interest.

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Journal of Apitherapy and Nature/Apiterapi ve Doğa Dergisi, 7(1), 28-52, 2024
Research article/Araştırma makalesi

Apiterapi ve Doğa Dergisi
Journal of Apitherapy and Nature
www.dergipark.gov.tr/jan

Food Supplement Production from Propolis, Honey, and Mulberry Molasses and Its Optimization

Propolis, Bal ve Dut Pekmezli Takviye Edici Gıda Üretimi ve Optimizasyonu

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Received/Geliş Tarihi: 12/03/2024

Accepted/ Kabul Tarihi: 14/06/2024

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Doi: 10.35206/jan.1451422

e-ISSN: 2667-4734

Abstract

In this study, twelve different mixtures were formed by different ratios of honey (15%-78.5%) and propolis (1.5%-5%) to mulberry molasses to increase its antioxidant, phenolic compound, and mineral values, and the physical and chemical properties of these mixtures and pure products were investigated. In the analysis conducted in the study, it was determined that among the twelve different mixtures, the highest antioxidant activity was determined to be in sample A1, with a value of 400.4 ± 1.0 mg/100 g $FeSO_4$, whereas the lowest activity was found in sample B4, with a value of 203.1 ± 0.4 mg/100 g $FeSO_4$. Regarding the total phenolic content, the highest activity was identified in sample A1, with the highest value of 184.3 ± 0.9 mg GAE/100 g, while the lowest was noted in sample B4, with a value of 110.3 ± 0.05 mg GAE/100 g. Furthermore, in the determination of sugar components, the Fructose/Glucose ratio was observed to be highest in sample C4, at 1.16 ± 0.03, and lowest in sample B1, at 0.95 ± 0.003. In this study, it was determined that an increase in the percentage of propolis in the mixtures led to an increase

in the antioxidant and phenolic compound levels, thereby imparting antioxidant properties to the product. Additionally, it was observed that the nutritional content was enriched because of the increase in glucose and fructose amounts as the percentage of honey increased.

Keywords: Mulberry Molasses, Propolis, Honey, Health, Food Supplement.

Özet

Bu çalışmada, dut pekmezine; antioksidan, fenolik madde ve mineral değerlerinin artırılması amacıyla farklı oranlarda bal (%15-78,5) ve propolis (%1,5-5) eklenerek 12 ayrı karışım oluşturulmuş ve bazı fiziksel ve kimyasal özellikler araştırılmıştır. Çalışma kapsamında yapılan analizlerde, 12 farklı karışım arasında en yüksek antioksidan aktivitesinin A1 örneğinde 400,4 ± 1,0 mg/100 g $FeSO_4$ değeriyle, en düşük aktivitesinin ise B4 örneğinde 203,1 ± 0,4 mg/100 g $FeSO_4$ değeriyle gözlemlendiği tespit edilmiştir. Toplam fenolik madde içeriği açısından en yüksek aktivite A1 örneğinde en yüksek 184,3 ± 0,9 mg GAE /100 g değeriyle, en düşük ise B4 örneğinde 110,3 ± 0,05 mg GAE /100 g değeriyle belirlenmiştir. Ayrıca, şeker bileşenlerinin belirlenmesinde Fruktoz / Glukoz oranının en yüksek olarak C4 örneğinde 1,16 ± 0,03, en düşük olarak ise B1 örneğinde 0,95 ± 0,003 olduğu gözlenmiştir. Bu çalışmada, karışımlarda propolis yüzdesinin artmasıyla antioksidan ve fenolik madde miktarının arttığı tespit edilmiş ve böylece ürüne antioksidan özellikler kazandırılmıştır. Ayrıca, bal yüzdesinin artmasıyla glukoz ve fruktoz miktarlarındaki artış sonucunda besinsel içeriğin zenginleştiği görülmüştür.

Anahtar Kelimeler: Dut Pekmezi, Propolis, Bal, Sağlık, Takviye Edici Gıda.

Abbreviations: HPLC, High performance liquid chromatography; TPTZ, Tripyridyl Triazine; GAE, Gallic acid equivalent; ICP-OES, inductively coupled plasma optical emission spectrometer.

1. INTRODUCTION

Rapid shifts in living and working conditions have led to changes in dietary habits, causing malnutrition and unbalanced nutrition. The fast pace of life has increased reliance on dietary supplements to meet nutritional needs not adequately met by daily diets (Coşkun & Velioğlu, 2020). Diet plays a significant role in health (Işık, 2014), and food supplements help meet nutritional needs by adapting to changing eating habits (Karataş & Şengül, 2018). Adequate nutrition involves consuming a variety of nutrients from both animal and plant foods and their proper utilization by the body (Zohoori, 2020). Consuming antioxidant-rich foods is emphasized, especially in developed countries, to support health (Güngör, 2007).

Molasses, a sweet, dense liquid obtained from fresh or dried fruits using traditional or industrial methods (Figure 1), is characterized by high sugar content, predominantly glucose and fructose (Batu, 1993; Karaca, 2009). It provides quick energy and is used traditionally for various diseases due to its high iron content (Yılmaz, 2012; Bayrak & Aygün, 2018).

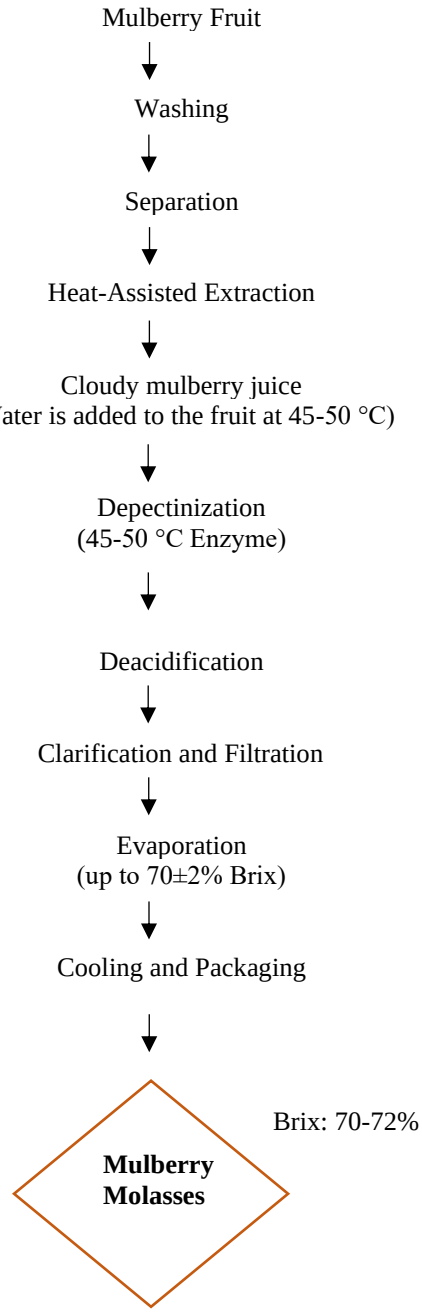

Figure 1. Mulberry Molasses Production Flow Diagram

Mulberry fruits, known for their low-calorie content and health benefits, are abundant in polyphenols, minerals, and vitamins, supporting overall health (Özbalcı et al., 2023). Mulberries are rich in essential minerals like potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg), sulfur (S), and iron (Fe) (Akbulut et al., 2007). They also contain essential fatty acids—linolenic, linoleic, and arachidonic acids—vital for cellular membrane integrity, optimal brain and nervous system function, and the synthesis of eicosanoids, which regulate blood pressure, viscosity, and immunity (Pawlovski et al., 1996; Simopoulos and Salem, 1996). Thus, incorporating mulberries into the diet ensures the provision of these critical fatty acids essential for bodily well-being.

Propolis is a natural product produced and utilized by honeybees (Apis mellifera) in the construction, adaptation, and protection of the hive (Cibanal & Sulaeman et al., 2019). Its composition varies depending on the source plant, containing approximately 45-55% resin, 23-35% waxes and fatty acids, 10% essential oils, 5% pollen, and 5% other organic substances and minerals (Burdock, 1998; Ertürk et al., 2013). Propolis contains over 300 compounds, including phenolics, terpenoids, lignans, stilbenoids, alcohols, benzaldehyde derivatives, and various minerals and amino acids, with fatty acids being the most abundant lipids (Huang et al., 2014; Crane & Walker, 1987). It has a wide range of biological activities, including antibacterial, antitumor, anti-inflammatory, antifungal, cytotoxic, immunomodulatory, and antioxidant properties (Krol et al., 2013; Kalsum et al., 2017).

Honey, another product of honeybees, is a functional food with protective and therapeutic properties against many diseases due to its various vitamins, minerals, organic acids, and enzymes (Alkın & Özmen, 2006; Dashora et al., 2011; Can, 2014; Molan, 2000). This rich content enables honey to have various positive effects on health (Sajtos et al., 2019 & Solayman et al., 2016). The mineral content of honey varies between 0.02% and 1%. The main minerals include potassium (K), calcium (Ca), phosphorus (P), and magnesium (Mg). Additionally, it contains trace elements such as iron (Fe), copper (Cu), zinc (Zn), selenium (Se), fluorine (F), and chlorine (Cl) (Can, 2014).Honey aids in the treatment of numerous diseases, including ulcers, stomach diseases, heart failures, palpitations, bone diseases, cough, allergies, bronchitis, anemia, throat pain, skin problems, and nervous system disorders. It also provides solutions to constipation, improves blood circulation, strengthens the heart, facilitates fat digestion, and heals wounds and burns (Molan, 2000). Thus, honey is a versatile natural product with a wide range of health benefits.

Natural products like propolis, honey, and molasses are rich in antioxidants and phenolic substances, protecting the body against oxidative stress and damage caused by free radicals (Karataş & Şengül, 2018).

Phenolic compounds, found in various plant sources, have numerous health benefits, providing protection against several diseases (Çağlar & Demirci, 2017; Khalil et al., 2020; Kolaç et al., 2017).

The principal objective of this scientific investigation was to comprehensively explore the physicochemical attributes of twelve distinct mixtures, each comprising varying proportions of honey (ranging from 15% to 78.5%), propolis (constituting 1.5% to 5%), and mulberry molasses to create a novel new product with desired properties. The study was meticulously designed to augment the inherent antioxidant capacity, phenolic compound content, and mineral profiles of these composite formulations. Through rigorous analysis of the samples, the researchers aimed to elucidate the intricate relationships between compositional variations and their multifaceted biological effects. Specifically, they probed the impact of altered ratios on antioxidant potential, and mineral composition. Furthermore, the investigation delved into the nutritional enrichment arising from fluctuations in glucose and fructose levels as the proportion of honey increased within the mixture.

2. MATERIALS and METHODS

2.1. Materials

In this research, dried mulberries procured directly from local producers in the Manisa region in July 2023 were employed for experimentation. These dried mulberries were utilized in the industrial production of mulberry molasses by SEM-AS Food Industry Trade Ltd. Co. The resulting mulberry molasses, identified as batch number 07S17, were transported to the laboratory in glass jars from the manufacturing facility. The acquired molasses samples were stored under controlled conditions in a dark environment at a constant temperature of 20 ± 2 ºC. All experiments were conducted in triplicate.

Propolis, in its raw form, was directly introduced into the mixtures, and a detailed analysis of the propolis samples was conducted using a pure water extraction method. The use of water in propolis extraction was preferred to comply with halal certification standards. 1 g of propolis sample was extracted with 100 mL of pure water to obtain a homogeneous mixture. The resulting mixture was dissolved in a stirrer overnight.

The flower honey utilized in this research, denoted as batch number 07S11, was produced by Semas Food Industry Trade Ltd. Co. and subsequently brought to the laboratory in glass jars. Furthermore, all analyses were conducted using deionized ultrapure water obtained from the Ultra-Pure Water System (Millipore, Synergy, Germany).

2.2. Method

The investigation involved the formulation of twelve different mixtures by introducing varying proportions of propolis (1.5-5%) and honey (15-78.5%) into mulberry molasses (as outlined in Table 1). These mixtures were prepared concurrently, and the outcomes were subsequently presented using the mean values derived from these formulations.

Table 1. Composition of mulberry molasses mixtures

MIXTURE	PROPOLİS (%)	HONEY (%)	MULBERRY MOLASSES (%)
Propolis	100 ± 0.01	0	0
Honey	0	100 ± 0.03	0
Mulberry Molasses	0	0	100 ± 0.01
A1 Mixture	5 ± 0.02	15 ± 0.03	80 ± 0.2
A2 Mixture	5 ± 0.01	35 ± 0.1	60 ± 0.01
A3 Mixture	5 ± 0.1	55 ± 0.3	40 ± 0.04
A4 Mixture	5 ± 0.003	75 ± 0.05	20 ± 0.02
B1 Mixture	2.5 ± 0.1	17.5 ± 0.03	80 ± 0.4
B2 Mixture	2.5 ± 0.02	37.5 ± 0.2	60 ± 0.01
B3 Mixture	2.5 ± 0.04	57.5 ± 0.07	40 ± 0.03
B4 Mixture	2.5 ± 0.03	77.5 ± 0.06	20 ± 0.004
C1 Mixture	1.5 ± 0.1	18.5 ± 0.07	80 ± 0.3
C2 Mixture	1.5 ± 0.03	38.5 ± 0.02	60 ± 0.01
C3 Mixture	1.5 ± 0.02	58.5 ± 0.1	40 ± 0.02
C4 Mixture	1.5 ± 0.01	78.5 ± 0.4	20 ± 0.006

2.2.1. pH Value

The pH assessment of the mixtures adhered to the TS 1728 ISO 1842 standard, employing the pH meter (Hanna, HI 2020, US) for the measurements (TS 1728 ISO 1842, 2001). The measurements were systematically executed with three replicates at a controlled temperature of 20 ºC.

2.2.2. Electrical Conductivity

The electrical conductivity of the mixtures was assessed using conductivity measurement instrument (Ohaus, St300c, US). Adhering to the TS 13366 Honey-Electrical Conductivity Determination standard, a 20% aqueous solution was prepared for each sample, and measurements were executed at 20 ºC. To

prevent the conductivity results from being influenced by the water used for dilution, deionized ultra-pure water (Millipore, Synergy, Germany) with a conductivity of 0.0006 mS/cm was utilized.

2.2.3. Determination of Soluble Solids Content (Brix°)

The analysis of soluble solids content, expressed in degrees Brix (º), employed a Refrakto Abbe (Ertick, Abbe-2, Germany) tabletop manual refractometer. Measurements were conducted at 20 ºC, and the results were expressed as a Brix° percentage.

2.2.4. Ash Determination

The determination of ash content was conducted using the gravimetric method, as detailed by Cemeroğlu (2010). Precision scale (RADWAG, AS220.R2, Poland) was utilized for sample analysis. Approximately 2 g of sample from each mixture, with an accuracy of 0.01 mg, was taken into porcelain crucibles brought to a constant weight, first burned with a bunsen burner flame, then heated in a muffle furnace at 550 ºC until it turned light gray-white, burned, and then weighed and the amount of ash was determined as a percentage with each mixture sample undergoing incineration, followed by weighing to ascertain the percentage of ash.

2.2.5. Hydroxymethylfurfural (HMF) Determination

The quantification of HMF involved weighing a 5 g mixture sample with a precision 0.01 mg and dissolving it in 100 mL ultrapure water. Following that, 2 mL of Carrez I —15 g potassium ferrocyanide (Merck, Darmstadt, Germany) was dissolved in pure water and water was added up to make 100 mL solution— and Carrez II — 30 g zinc acetate (Merck, Darmstadt, Germany) was dissolved in pure water and water was added to make 100 mL solution — reagents were added to the sample and the resulting solution was filtered through a 0.45 µm filter. After this, 2 mL of the sample solution were transferred to two separate test tubes and 5 mL of p-toluidine (Sigma-Aldrich, Munich, Germany) solution were added to each tube. Afterwards, 1 mL of barbituric acid (Sigma-Aldrich, Munich, Germany) was added to one tube (sample) and 1 mL of pure water was added to the other (blank), the tubes were thoroughly mixed. The absorbance values at 550 nm were measured using a spectrophotometer (SHIMADZU, UV-1900I, Japan). The obtained results were multiplied by the correction factor of 192 to calculate HMF (hydroxymethylfurfural) quantities in mg/kg (Güngör, 2007). The calculation formula for HMF content (Equation 1) is provided below.

$$HMF = A \times 192$$ (Equation 1)

2.2.6. Water Activity Determination

The water activity (a_w) of the mixture samples was measured using water activity determination device (Novasina Labmaster, 1119971, Switzerland) at room temperature.

2.2.7. Hunter Color Analysis (L, a, b)

The Hunter color values (L, a, b) of the homogenized mixtures were determined using color measurement device (Konica Minolta, CR-410, Japan). The values L (100: white, 0: black), a (+red, -green), and b (+yellow, -blue) were recorded.

2.2.8. Sugar Profile Analyses

The quantification of glucose, fructose, sucrose, maltose, and lactose in the mixture samples was performed using High-Performance Liquid Chromatography (HPLC) based on the DIN 10758 method. This method includes honey, jams, marmalades, molasses, confectionery, and fruit juices. 10000, 15000 and 20000 ppm standards of glucose, fructose and sucrose were prepared and injected into the HPLC device (SHIMADZU, Reservoir Tray, Japan), and the calibration curve was drawn. Then, 5 g of sample was weighed with a precision of 0.01 mg and dissolved in 40 mL of water. Following that, sample and 25 mL of methanol was taken into a volumetric flask and completed to 100 mL using water and the mixture were filtered through a 45 µm filter. Chromatographic conditions are given in Table 2.

Table 2. Chromatographic conditions for HPLC

Device	HPLC SHİMADZU, Reservoir Tray model
Mobile Phase	Water/Acetonitrile solution (20/80)
Detector	Agilent RID Detector, wavelength 284 nm
Column	Agilent Zorbax NH2 analytical column (4.6x250 mm, 5 µm)
Flow Rate	1.8 mL/min
Column Temperature	30 ºC
Injection Volume	20 µL

2.2.9. Antioxidant Assessment

The quantification of antioxidants in the samples was conducted employing the Ferric Reducing Antioxidant Power (FRAP) assay. The FRAP method relies on the reduction of the Fe(III)-TPTZ —2,4,6-tris(2-pyridly)-S-triazin— (Sigma-Aldrich, Munich, Germany) complex in the presence of antioxidants, forming the blue Fe(II)-TPTZ complex. The complex formed exhibits maximum absorbance in a spectrophotometer (SHIMADZU, UV-1900I, Japan) at 593 nm (Benzie IFF and Strain, 1996). A calibration curve was prepared using varying concentrations of $FeSO_4.7H2O$ (Sigma-Aldrich, Munich, Germany) (31.25-62.5-125-250-500-1000 µM) for calibration (Figure 3). A mixture of 3 mL of FRAP reagent—300 mM pH 3.6 acetate buffer (Merck, Darmstadt, Germany), a 10 mM TPTZ, and 20 mM $FeCl_3$ (Sigma-Aldrich, Munich, Germany) mixture in a ratio of 10:1:1— was combined with 100 µL of the sample. The results were compared against a standard $FeSO_4.7H_2O$, tested under the same conditions, and expressed as the µM $FeSO_4.7H_2O$ equivalent antioxidant power. Pipetting was performed as described in Table 3 (Can, 2014).

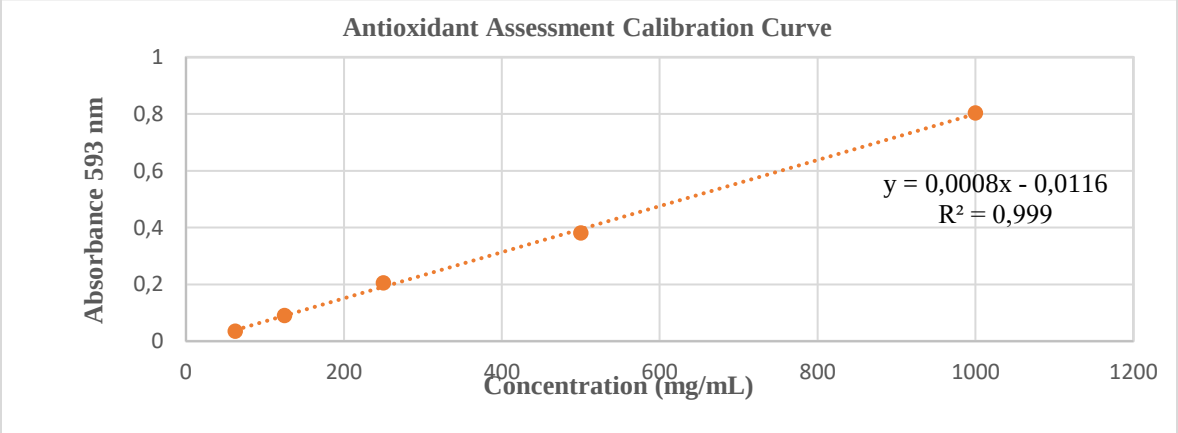

Figure 2. Antioxidant assessment calibration curve

Table 3. Pipetting procedure in FRAP determination.

	Blank MeOH	**Test (Sample)**	**Color Blank MeOH**	**$FeSO_4.7H_2O$**
FRAP Reagent	3 mL	3 mL	-	3 mL
Sample	-	100 µL	100 µL	-
$FeSO_4.7H_2O$(Variable Conc.)	-	-	-	100 µL
Methanol	100 µL	-	3 mL	-
In the 4th minute, absorbance is read at 593 nm.				

Color Blank MeOH: Color blank for the sample dissolved in methanol.

2.2.10. Total Polyphenol Analysis

The basis of the determination of total phenolic content relies on the redox reaction where phenolic compounds reduce the Folin-Ciocalteu (Sigma-Aldrich, Munich, Germany) reagent, an oxidative compound in a basic medium, converting them into their oxidized form. Following the reaction, the total amount of phenolic compounds in the sample is calculated by measuring the absorbance of the reduced reagent's resulting purple-blue color in a spectrophotometer (SHIMADZU, UV-1900I, Japan) at 760 nm. In the preparation of the standard curve, various concentrations of gallic acid (1; 0.5; 0.25; 0.125; 0.0625; 0.03125; and 0.015625 mg/mL) were utilized (Figure 3). The total polyphenol content was determined in terms of gallic acid (Sigma-Aldrich, Munich, Germany) equivalents (Slinkard, 1977; Singleton, 1999). The detailed procedures for the determination of total polyphenols are explained in Table 4.

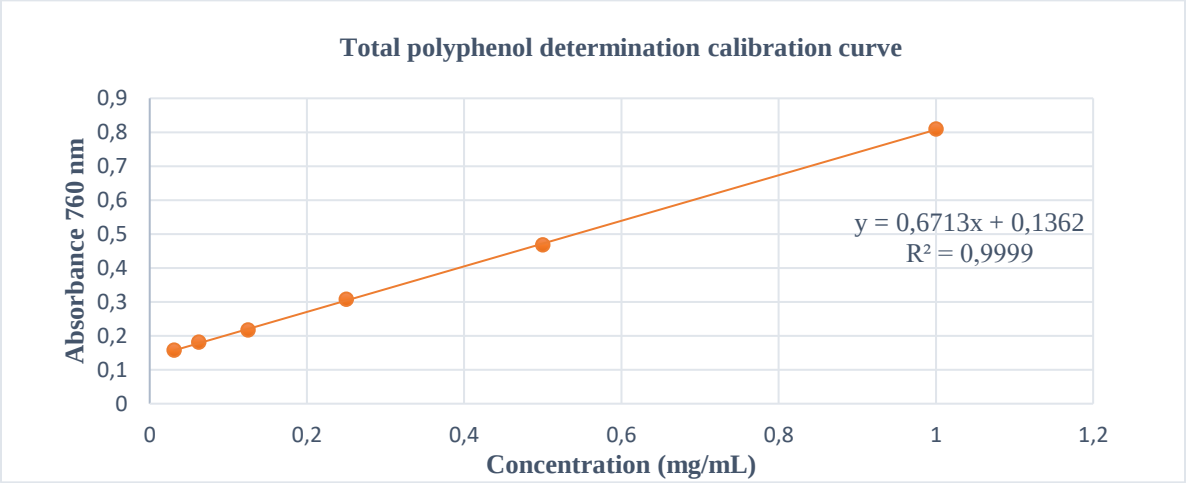

Figure 3. Total polyphenol determination calibration curve

Table 4. Pipetting procedure for total polyphenolic determination

	Blank	Standard	Sample
Distilled Water	700 µL	680 µL	680 µL
Standard (Various Conc.)	-	20 µL	-
Mixture Samples	-	-	20 µL
0,2 N Folin Reagent	400 µL	400 µL	400 µL
The tubes were mixed by vortex and after 3 minutes following chemical was added.			
%10 Na_2CO_3	400 µL	400 µL	400 µL
The absorbance was read against the blank at 760 nm.			

2.2.11. Aflatoxin Quantification (B1, B2, G1, G2)

To determine aflatoxin B1, B2, G1, and G2 in the mixture samples, samples were homogenized and prepared according to the AOAC 999.07 method (AOAC, 2007). For this purpose of 50 g of the sample, 5 g NaCl (Sigma-Aldrich, Munich, Germany), 100 mL deionized water, and 125 mL 70% methanol (Merck, Darmstadt, Germany), was stirred for 30 minutes at room temperature using a shaking extraction technique. Subsequently, the mixture was filtered first through filter paper and then through Whatman No. 4 paper before being analyzed using HPLC (SHIMADZU, Reservoir Tray, Japan). The chromatographic conditions
are shown in Table 5.

Table 5. Chromatographic HPLC conditions

Device	HPLC SHİMADZU brand, Reservoir Tray model
Mobile Phase	Water/Methanol/Acetonitrile (550/300/200)
Detector	Fluorescence Detector (360 nm- 440 nm)
Column Length	ODS-2 (C18 -250 mm-5µm- 4.6 mm)
Flow Rate	1 mL/min
Column Temperature	25 ºC
Injection Volume	100

2.2.12. Determination of Mineral Content

In the study, approximately 0.5 g of a homogeneous mixture was taken into a Teflon crucible, and 6 mL of pure HNO_3 (Sigma-Aldrich, Munich, Germany) and 3 mL of H_2O_2 (Sigma-Aldrich, Munich, Germany) were added. The samples were burned in a Milestone microwave oven, and the resulting ashes were diluted with distilled water to a volume of 25 mL. The mineral elements in the samples, including calcium (Ca), sodium (Na), phosphorus (P), potassium (K), magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn), and manganese (Mn), were determined using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) (Thermo ICAP, 7400, Japan) (Yıldız et al., 2009).

2.2.13. Determination of Titratable Acidity

The titratable acidity of samples were determined according to TS 1125 ISO 750.For this, 25 mL of the mixture samples were taken and diluted to 250 mL. A 50 mL aliquot of this solution was titrated with a

standardized 0.1 N NaOH (Merck, Darmstadt, Germany) solution using phenolphthalein as an indicator (Anonymous, 2002).

3. RESULTS AND DISCUSSION

Different ratios of honey (15%-78.5%) and propolis (1.5%-5%) were added to the mulberry molasses, creating 12 separate mixtures. Some physical and chemical properties related to propolis, honey, and mulberry molasses, along with the analysis results of these mixtures, are presented in Tables 6 and 7. The mineral analysis results for propolis, honey, mulberry molasses, and the 12 mixture samples are provided in Table 8.

Table 6. Analysis Results of Propolis, Honey and Mulberry Molasses

Composition Element	Honey	Propolis	Mulberry Molasses
pH Value	4.98 ± 0.2	6.61 ± 0.02	5.06 ± 0.03
Electrical Conductivity (mS /cm)	0.381 ± 0.006	1.079 ± 0.007	2.860 ± 0.01
Water Soluble Dy Matter (% Brix°)	78.7 ± 0.3	0.4 ± 0.003	71.8 ± 0.2
Ash (%)	0.3 ± 0.006	4.14 ± 0.01	1.99 ± 0.1
HMF Analysis (mg / kg)	18.4 ± 0.08	-	25.1 ± 0.5
Determination of Water Activity (a_w)	0.604 ± 0.006	-	0.703 ± 0.009
Hunter Color Analysis	L:18.8 ± 0.1 a:2.59 ± 0.03 b:3.6 ± 0.05	L:17.94 ± 0.05 a:1.91 ± 0.03 b:4.68 ± 0.06	L:16.4 ± 0.2 a:0.01 ± 0.001 b:0.75 ± 0.07
Glucose (%)	31.59 ± 0.07	-	30.85 ±0.05
Fructose (%)	36.12 ± 0.08	-	29.97 ± 0.04
Fructose / Glucose	1.14 ± 0.02	-	0.97 ± 0.03
Antioxidant (mg/ 100 g $FeSO_4$)	120.6 ± 1.2	180.5 ± 1.3	389.5 ± 1.7
Total Phenolic Substance (mg GAE /100g)	121.5 ± 1.4	97.83 ± 0.1	675.95 ± 1.9
Determination of Aflatoxin (B1.B2.G1.G2) (µg/kg)	0	0	0
Titratable Acidity (Citric acid equivalent) (g /100 mL)	0.5 ±0.03	-	0.308 ± 0.007

Table 7. Mineral Analysis Results of Propolis, Honey and Mulberry Molasses and 12 Mixture Samples

Mineral (mg/kg)	Honey	Propolis	Mulberry Molasses	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4
Ca	20 ± 1.2	38 ± 0.3	95.6 ± 0.03	93 ± 0.5	81.2 ± 0.5	64.1 ± 0.02	51 ± 0.4	82,2 ± 0.05	71.5 ± 0.1	68.1 ± 0.2	61.4 ± 0.03	74.2 ± 0.02	67.3 ± 0.03	62.1 ± 0.4	58.4 ± 0.07
Na	9.1 ± 0.7	6.2 ± 0.02	58.4 ± 0.02	56.8 ± 0.02	52.1 ± 0.06	48.4 ± 0.01	42.3 ± 0.7	48.7 ± 0.03	41 ± 0.4	38.2 ± 0.1	30.8 ± 0.02	45.7 ± 0.04	43.3 ± 0.05	38.4 ± 0.02	35.1 ± 0.3
K	4.1 ± 0.03	55.4 ± 0.04	432.9 ± 0.09	428.8 ± 0.7	422.2 ± 0.8	410.6 ± 0.07	402.8 ± 0.3	398.7 ± 0.5	381.3 ± 0.06	374.2 ± 0.6	355.4 ± 0.3	354.2 ± 0.06	336.1 ± 0.4	325.8 ± 0.5	312.5 ± 0.6
Mg	1.2 ± 0.02	1.5 ± 0.02	68.1 ± 0.1	67.3 ± 0.3	65.1 ± 0.3	61.8 ± 0.05	58.1 ± 0.07	65.7 ± 0.03	62.4 ± 0.2	59.4 ± 0.06	57.1 ± 0.4	58.1 ± 0.07	54.5 ± 0.2	48.3 ± 0.03	47.2 ± 0.05
Cu	0.25 ± 0.001	0.4 ± 0.03	4.32 ± 0.02	4.07 ± 0.04	3.45 ± 0.05	2.85 ± 0.3	2.02 ± 0.04	3.56 ± 0.04	3.21 ± 0.05	2.14 ± 0.01	1.87 ± 0.01	3.12 ± 0.003	3.01 ± 0.06	2.77 ± 0.01	2.64 ± 0.04
Fe	0.47 ± 0.004	0.65 ± 0.006	1.25 ± 0.09	1.02 ± 0.08	1 ± 0.08	1.01 ± 0.04	0.94 ± 0.2	1.01 ± 0.2	0.98 ± 0.08	0.95 ± 0.02	0.81 ± 0.03	0.94 ± 0.007	0.92 ± 0.02	0.81 ± 0.03	0.77 ± 0.08
Zn	0.14 ± 0.01	1.62 ± 0.007	1.2 ± 0.04	1.15 ± 0.04	1.07 ± 0.03	0.95 ± 0.08	0.56 ± 0.04	1.1 ± 0.3	0.87 ± 0.05	0.77 ± 0.01	0.69 ± 0.01	0.84 ± 0.02	0.71 ± 0.01	0.66 ± 0.05	0.54 ± 0.06
Mn	0.05 ± 0.008	0.57 ± 0.002	0.4 ± 0.01	0.4 ± 0.02	0.35 ± 0.04	0.21 ± 0.05	0	0.45 ± 0.07	0.43 ± 0.2	0.37 ± 0.01	0.34 ± 0.05	0.41 ± 0.04	0.38 ± 0.02	0.27 ± 0.07	0.22 ± 0.4
P	0.8 ± 0.01	7.1 ± 0.07	55 ± 0.6	42.2 ± 0.6	41.9 ± 0.06	41.3 ± 0.1	40.3 ± 0.3	41.7 ± 0.01	41.5 ± 0.3	40.5 ± 0.3	40.1 ± 0.02	41.4 ± 0.08	40.7 ± 0.1	40.2 ± 0.2	39.8 ± 0.1

3.1. pH Values of Mixture Samples

The pH values of the mixture samples examined in the study have a significant impact on the flavor and robustness properties of molasses. The samples of propolis, honey, and mulberry molasses, as well as the 12 different mixtures that were examined, had pH values ranging from 4.98 ± 0.2 to 6.61 ± 0.02. Based on the obtained data, the pH value of propolis was found to be 6.61 ± 0.02, while honey to be 4.98 ± 0.2, and mulberry molasses to be 5.06 ± 0.03 (Table 6). The product A1, whose pH value is 5.56 ± 0.04, was found to have the highest value among the prepared mixtures. In contrast, the mixture with the lowest value, C4, the 12th product, has a pH value of 5.33 ± 0.01 (Table 8).

Table 9. Average Analysis Results of Mixtures A (A1, A2, A3, A4), Mixtures B (B1, B2, B3, B4) and Mixtures C (C1, C2, C3, C4)

Composition Element	**A1**	**A2**	**A3**	**A4**	**B1**	**B2**	**B3**	**B4**	**C1**	**C2**	**C3**	**C4**
pH Value	5.56 ± 0.04	5.52 ± 0.02	5.46 ± 0.04	5.41 ± 0.1	5.53 ± 0.02	5.5 ± 0.03	5.44 ± 0.07	5.36 ± 0.06	5.54 ± 0.02	5.49 ± 0.03	5.4 ± 0.02	5.33 ± 0.01
Electrical Conductivity(mS /cm)	2.470 ± 0.02	9.26 ± 0.01	1.046 ± 0.01	1.163 ± 0.01	2.280 ± 0.003	0.596 ± 0.004	1.047 ± 0.3	2.276 ± 0.002	2.180 ± 0.003	1.162 ± 0.003	0.875 ± 0.004	0.469 ± 0.006
Water Soluble Dry Matter(% Brix^{o})	73 ± 0.3	74.5± 0.4	71.1 ± 0.6	77.5 ± 0.6	72.9 ± 0.3	74.5 ± 0.2	75.9 ± 0.1	77.4 ± 0.05	73 ± 0.4	74.6 ± 0.04	76 ± 0.6	77.8 ± 0.07
Ash (%)	1.68 ± 0.02	1.26 ± 0.01	0.84 ± 0.005	0.71 ± 0.001	1.57 ± 0.01	1.18 ± 0.003	0.76 ± 0.008	0.62 ± 0.03	1.69 ± 0.04	1.27 ± 0.06	0.85 ± 0.04	0.65 ± 0.03
HMF Analysis (mg / kg)	19.3 ± 0.2	20.7 ± 0.1	22.3 ± 0.1	24.2 ± 0.06	22.9 ± 0.03	23.8 ± 0.2	25.2 ± 0.3	27.9 ± 0.01	26.8 ± 0.05	27.1 ± 0.3	27.4 ± 0.05	28.5 ± 0.06
Determination of Water Activity (a_W)	0.693 ±0.005	0.675 ±0.006	0.657 ± 0.001	0.631 ± 0.004	0.694 ± 0.004	0.671 ± 0.006	0.654 ± 0.005	0.63 ± 0.09	0.691 ± 0.002	0.674 ± 0.005	0.651 ± 0.004	0.629 ± 0.007
Hunter Color Analysis L a b	L:16.53 ± 0.06 a:0.18 ± 0.04 b: 0.91 ± 0.07	L:16.8 ± 0.03 a:0.49 ± 0.07 b:1.21 ± 0.05	L:17.34 ± 0.09 a:0.8 ± 0.04 b:1.61 ± 0.03	L:18.1 ± 0.07 a:1.38 ± 0.02 b:2.13 ± 0.03	L:17.68 ± 0.04 a:0.23 ± 0.01 b:0.88 ± 0.03	L:17.63 ± 0.07 a:0.24 ± 0.02 b:0.79 ± 0.01	L:17.0 ± 0.04 a:0.29 ± 0.03 b:1.16 ± 0.06	L:17.7 ± 0.03 a:1.24 ± 0.2 b:2.02 ± 0.09	L:16.57 ± 0.01 a:0.09 ± 0.02 b:0.8 ± 0.07	L:16.59 ± 0.03 a:0.1 ± 0.02 b:0.86 ± 0.05	L:16.61 ± 0.02 a:0.01 ± 0.04 b:0.91 ± 0.02	L:17.33 ± 0.01 a:0.46 ± 0.03 b:1.62 ± 0.06
Glucose (%)	30.72 ± 0.07	30.86 ± 0.03	31.56 ± 0.06	31.83 ± 0.02	31.14 ± 0.08	31.46 ± 0.05	31.78 ± 0.02	31.82 ± 0.04	30.47 ± 0.006	30.57 ± 0.001	30.65 ± 0.05	30.79 ± 0.03
Fructose (%)	29.54 ± 0.04	30.26 ± 0.2	32.99 ± 0.2	34.46 ± 0.01	29.44 ± 0.05	31.24 ± 0.01	32.15 ± 0.04	33.53 ± 0.06	30.54 ± 0.005	32.87 ± 0.002	33.90 ± 0.002	35.57 ± 0.004
Fructose/Glucose	0.96 ± 0.04	0.98 ± 0.03	1.04 ± 0.02	1.08 ± 0.03	0.95 ± 0.003	0.99 ± 0.004	1.01 ± 0.05	1.05 ± 0.04	1.00 ± 0.2	1.08 ± 0.03	1.11 ± 0.02	1.16 ± 0.03
Antioxidant (mg/ 100 g FeSO4)	400.4 ± 1.0	392.6 ± 0.8	298.6 ± 0.02	264.2 ± 0.4	361.4 ± 0.1	303.5 ± 0.8	282.9 ± 0.7	203.1 ± 0.4	345.3 ± 0.04	275.1 ± 0.06	235.7 ± 0.2	222.0 ± 0.04
Total Phenolic Substance(mg GAE /100g)	184.3 ± 0.9	108.3 ± 0.07	128.7 ± 0.002	118.7 ± 0.08	156.9 ± 0.06	140.7 ± 0.06	127.1 ± 0.3	110.3	133.1 ± 0.02	131.1 ± 0.08	125.5 ± 0.03	111.1 ± 0.07
Aflatoxin (B1.B2.G1.G2) (µg/kg)	0	0	0	0	0	0	0	0	0	0	0	0
Titratable Acidity (Citricacid equivalent) (g /100 mL)	0.732 ± 0.005	0.738 ± 0.001	0.748 ± 0.007	0.752 ± 0.004	0.756 ± 0.004	0.762 ± 0.003	0.766 ± 0.002	0.775 ± 0.02	0.824 ± 0.003	0.835 ± 0.002	0.842 ±0.01	0.859 ± 0.005

The pH values ranged from 4.98 ± 0.2 to 6.61 ± 0.02 across the samples, indicating a slight acidic to neutral pH environment. Propolis exhibited the highest pH value, likely due to its weakly acidic nature, while honey and mulberry molasses displayed lower pH values, indicative of their acidic properties. These variations in pH values could influence the sensory attributes and stability of the final product.

According to TS 12001 Mulberry Molasses Standard, it is known that the pH value determined for mulberry molasses should be between 5.0 and 5.5 (Anonymous, 1996). In particular, the use of natural ingredients such as propolis and honey in the molasses production process may cause changes in the chemical composition of molasses. Therefore, it is thought that the pH fluctuations in the results are caused by natural ingredients such as propolis and honey in the product. These variations in pH values could influence the sensory attributes and stability of the final product.

3.2. Electrical Conductivity

Contrary to metals, electricity in food is carried by ions, not electrons, and a food's conductivity is directly correlated with its physicochemical characteristics, including pH, Brix value, protein, phenolic substance, organic acid, and mineral content (Lee et al., 2013).

The electrical conductivity measurement results for propolis, honey, and mulberry molasses samples, and 12 different mixtures based on analysis range from 0.38 ± 0.006 to 9.26 ± 0.01 mS / cm. These values are directly associated with food's physicochemical characteristics. The analysis results show that, out of 12 different mixtures, the A2 sample has the highest electrical conductivity (conductivity value: 9.26 ± 0.01 mS / cm), and the C4 product, has the lowest electrical conductivity (conductivity value: 0.496 ± 0.006 mS / cm) (Table 8).

The measurements ranged from 0.38 ± 0.006 to 9.26 0.01 mS/cm, indicating diverse conductivity levels across the samples. Product A2 exhibited the highest electrical conductivity, possibly due to its higher mineral content or ion concentration, while product C4 displayed the lowest conductivity. These differences in conductivity could be attributed to variations in the composition and concentration of the ingredients, affecting the overall quality and stability of the product.

3.3. Amount of Water-Soluble Dry Matter (Brix^{o} Values)

Within the scope of the research, samples of propolis, honey, and mulberry molasses as well as the 12 mixtures had water-soluble dry matter (WSS) ranging from 0.4 ± 0.003 to 78.7 ± 0.3 Brix^{o}. Based on the obtained data, the C4 product had the highest water-soluble dry matter quantity (77.8 ± 0.07

Brixº) out of 12 different mixtures, while the A3 product had the lowest water-soluble dry matter amount (71.1 ± 0.6 Brixº) (Table 8).

The range of Brixº values observed (0.4 ± 0.003 to 78.7 ± 0.3) suggests significant variability in the concentration of soluble solids across the samples. Product C4 exhibited the highest Brixº value, likely due to its higher honey concentration, which contributes to increased sweetness and viscosity. In contrast, product A3 displayed the lowest Brixº value, indicating lower soluble solid content. These differences in Brixº values could influence the taste, texture, and nutritional content of the final product.

3.4. Ash Content Determination

The total mineral components that constitute ash are present in varying and minute amounts in every fruit. Most of the mineral components present in the fruit have formed water-soluble salts with organic and inorganic acids. Consequently, many of them pass into fruit juice during processing (Cemeroğlu, 1982). Within the scope of research, it was found that samples of propolis, honey, and mulberry molasses and 12 different mixtures of these samples contained ash contents at rates varying between 0.3 ± 0.006 % and 4.14 ± 0.01 % (Table 6 and Table 8). Furthermore, Table 8 revealed that the product with the highest ash amount, C1, had 1.69 ± 0.04 %, while the product with the lowest ash amount, B4, had 0.62 ± 0.03%. The observed ash content ranged from 0.3±0.006% to 4.14 ± 0.01%, indicating variations in mineral content across the samples. Product C1 exhibited the highest ash content, possibly due to its higher concentration of mineral-rich ingredients, while product B4 displayed the lowest ash content. It is thought that this increase in the amount of ash is due to mulberry molasses.

3.5. HMF (5-Hydroxymethylfurfural) Analysis

It isstated that heat treatment or long-term storage under inappropriate conditions generally causes an increase in the amount of HMF (5-hydroxymethylfurfural). In addition, it is known that high amounts of HMF exposure is cytotoxic and has irritation effect, and there are many studies supporting that HMF is genotoxic and has mutagenic and carcinogenic effects (Capuano and Fogliano, 2011).

Within the scope of this study, HMF results of the mixtures show values ranging between 18.4 ± 0.08 and 28.5 ± 0.06 mg/kg. According to the results obtained, the C4 product had the greatest HMF value, measuring 28.5 ± 0.06, while the A1 product had the lowest value, measuring 19.3 ± 0.2 (Table 8).

The observed HMF values ranged from 18.4 ± 0.08 to 28.5 ± 0.06 mg/kg, indicating varying degrees of heat exposure and potential risks associated with HMF formation. Product C4 exhibited the highest HMF value, suggesting prolonged heat exposure or inadequate storage conditions, while product A1 displayed the lowest HMF value. However, in this study, no evaluation was made on how storage conditions and duration affect the HMF content.

The study by Bozkurt and his team (1998) examined how HMF amounts changed when molasses samples prepared at different concentrations and pH levels were exposed to heat. This study revealed that the occurrence time of the browning reaction varies depending on various factors, and as condensation increases and the pH level decreases, the adaptation period decreases. Therefore, it was concluded that the HMF formation rate increased. It was determined that the browning reactions in molasses production played a critical role in the formation of color and taste, but it was emphasized that the formation of intermediate products such as HMF should be kept under control. Therefore, it is important to consider the impact of storage conditions on HMF formation and manage it appropriately.

3.6. Determination of Water Activity (Aw)

The amount of free water available for chemical reactions and microbiological development inside the food matrix is measured and defined as water activity. Water is the primary necessity for microorganisms to sustain their activity. In general, bacteria function at greater a_w values than molds and yeasts. Water activity is therefore a crucial factor to consider when predicting the microbiological and chemical deterioration of food during preparation and storage (Jay, Loessner and Golden, 2008). Generally, it is known that yeasts and molds operate at lower water activity values (range 0.61-0.88) than bacteria (>0.90) (Özbey et al., 2013).

Water activity values ranged from 0.604 ± 0.006 to 0.703 ± 0.009 among 12 different mixtures and samples of propolis, honey, and mulberry molasses, according to the research (Tables 6 and 8). B1 had the highest water activity among the mixtures, with 0.694 ± 0.004, while C4 had the lowest water activity, with 0.629 ± 0.007.

The observed water activity values ranged from 0.604 ± 0.006 to 0.703 ± 0.009, indicating differences in moisture content and potential for microbial growth across the samples. Product B1 exhibited the highest water activity, suggesting a higher risk of microbial spoilage, while product C4 displayed the lowest water activity, indicating better stability and shelf-life.

In addition, in the study conducted by Salik and his team (2021), it was determined that water activity values in mulberry molasses samples varied between 0.59 and 0.75. The values are compatible in the research.

3.7. Color Analysis (Hunter) Test

Color is an important parameter in foods. In the Hunter Lab color model, L represents lightness with values ranging from 0 to 100, a denotes the green-red axis with negative values indicating green and positive values indicating red, and b represents the blue-yellow axis with negative values indicating blue and positive values indicating yellow, collectively providing a comprehensive description of color appearance for precise measurement and analysis (Dobrzansk and Rybcyzynsk, 2002).

In this research, the color analysis of 12 different mixtures and samples of propolis, honey, and mulberry molasses were performed to determine their color parameters (L, a, and b). L values range from 18.8 ± 0.1 to 16.4 ± 0.2, a value from 2.59 ± 0.03 to 0.01, and b value from 4.68 ± 0.06 to 0.75 ± 0.07 (Tables 6 and 8). It was concluded that there was no discernible color change when the honey content increased. This demonstrates that switching to a specific honey ratio has no significant impact on color change.

3.8. Determination of Sugar Content

When the sugar profiles of these products are examined, it is seen that the fructose/glucose ratio is close to 1.0. Within the scope of this study, it was found that the Fructose / Glucose ratio was highest in C4 (1.16 ± 0.03) and lowest in B1 (0.95 ± 0.003) among 12 different mixtures, propolis, honey, and mulberry molasses (Table 8).

Product C4 had the highest sugar content among the 12 different mixtures examined. This feature can be considered as a suitable food alternative for consumption in case of fast and high energy requirements (Kolayli et.,2013; Ischayek and Kern,2006).

3.9. Determination of Antioxidants

Fruits, particularly berries and vegetables are rich sources of antioxidants, which are phenolic compounds with antimutagenic and anticarcinogenic qualities (Güngör, 2007). Tables 6 and 8 show that the mixture samples tested have antioxidant activity ranging from 120.6 ± 1.2 to 400.4 ± 1.0 mg / 100 g $FeSO_4$. It was found that, with 400.4 ± 1.0 mg/100 g $FeSO_4$, A1 had the highest antioxidant content, while B4 had the lowest, with 203.1 ± 0.4 mg/100 g $FeSO_4$ (Table 8). An increase in the

amount of antioxidants has been observed with the increase in propolis in Mulberry Molasses (Zab, 2021; Szajdek,2008). It can be considered that these products can be used as food additives.

3.10. Total Phenolic Substance

It was discovered that the total amount of phenolic compounds varied between 108.3 ± 0.07 and 184.3 ± 0.9 mg GAE /100 g among 12 different mixtures and the samples of propolis, honey, and mulberry molasses. Out of 12 distinct mixtures, product A1 had the highest amount of phenolic compounds (184.3 ± 0.9 mg GAE /100 g), while A2 had the lowest amount (108.3 ± 0.07 mg GAE /100 g) (Table 6 and Table 8). The mixture samples contain significant amounts of phenolic substances.

It was observed that phenolic substance increased with the increase in the amount of propolis, but decreased with the increase in honey (Saroğlu, Bayram and Özçelik.,2023).Among 12 different mixtures, the presence of 184.3 ± 0.9 mg GAE /100 g phenolic substance in product A1 shows that this product can be used as a food supplement.

3.11. Mineral Analysis

Minerals are essential food components that the body needs to consume on a regular basis. They serve a variety of purposes in the body, including structural support, influencing, and balancing physiological processes, and supporting the neurological and muscular systems. Sodium, potassium, calcium, and magnesium are known as macro minerals, and copper, iron, zinc, and manganese are known as micro minerals (Güngör, 2007).

Table 7 lists the mineral contents of 12 distinct mixtures and the samples of propolis, honey, and mulberry molasses. The results of the analysis show that mulberry molasses have a higher mineral content than other samples, with product A1 having the highest value out of 12 distinct mixtures.
It contains elements such as potassium, calcium, sodium, iron, phosphate, copper, and zinc, which are the richest minerals in the A1 product. In case of deficiency of these minerals, people may be advised to choose this food.

3.12. Determination of Total Acidity Content

Fruit varieties and types can have a different flavor, which is influenced by the acidity/sugar content of their structural makeup (Güngör, 2007). In the study, propolis, honey, mulberry molasses samples, and 12 distinct mixtures had total acidity levels ranging from 0.308 ± 0.007 to 0.859 ± 0.005 g /100 mL (Table 6 and Table 8). Based on the obtained data, product C4 had the highest total acidity (0.859

± 0.005 g/100 mL), while product A1 had the lowest total acidity (0.732 ± 0.005 g/100 mL) (Table 8). It is thought that there is no relationship between acidity value and pH in propolis, honey and mulberry molasses mixtures and the acidity in the products arises from the natural acidity coming from the mulberry fruit (Koyuncu,2004; Bozhüyük,2015; Krishna et al.,2020).

3.13. Aflatoxin Analysis

Aflatoxins, harmful toxins produced by certain fungi, were analyzed in the mixture samples. In the study, 12 different mixtures and samples of propolis, honey, and mulberry molasses were examined; no aflatoxin residue was discovered (Tables 6 and 8). These results assure the safety and quality of the mixtures, indicating compliance with regulatory standards and consumer safety requirements (Official gazette,2011).

4. CONCLUSION

In this research, mulberry molasses -which has a high nutritional value and carbohydratecontent- was combined with natural items that have rich vitamin and mineral content, like propolisand honey, to create a new food additive. Through their synergistic effects, these additives increasethe nutritional value while also contributing to the provision of bioavailability and bioactive substances. When propolis was added to mulberry molasses, there was a noticeable increase in its antioxidant capacity. Furthermore, an essential substitute for the recommended daily intake of potassium is provided by the observed rise in potassium (K) content of mixtures. It is believed thatthese mixture samples might be advised to be consumed, particularly when there is an increased requirement for minerals. Mulberry molasses have been given a more palatable taste and flavor profile with the addition of varying amounts of honey. Moreover, the product gains antibacterial, antioxidant, anti-inflammatory, and immune system-supporting qualities from the addition of propolis. It is advised to conduct more research on the different additive alternatives in light of these findings. For sensory analysis testing, products also need to be assessed by sensory analysts.Finally, it is believed that the products of mixing propolis and honey with mulberry molasses can be utilized as useful food additives or natural antioxidant.

ACKNOWLEDGEMENT

I would like to thank SEM-AS Tourism Food Industry Trade Limited Company General Manager Reşat GÜRKAN for his valuable contributions, Deputy General Manager Semih GÜRKAN for his support.

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Journal of Apitherapy and Nature/Apiterapi ve Doğa Dergisi, 7(1), 53-64, 2024
Research article/Araştırma makalesi

Apiterapi ve Doğa Dergisi
Journal of Apitherapy and Nature
www.dergipark.gov.tr/jan

An Important Value of Turkey: Lavender (Lavandula stoaches L.) Honey

Türkiye'nin Önemli Bir Değeri: Karabaş Otu (Lavandula stoaches L.) Balı

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Received/Geliş Tarihi: 16/04/2024
Accepted/ Kabul Tarihi: 20/05/2024
***Corresponding author** /Yazışılan yazar
Doi: 10.35206/jan.1469441
e-ISSN: 2667-4734

Abstract

Lavandula stoechas L., belonging to the Lamiaceae family, is locally known as "karabaşotu, karan, gargan, keşişotu" and is used in central nervous system diseases (epilepsy and migraine), in the treatment of various wounds and in reducing blood sugar. This plant is also used as analgesic, antiseptic, sedative, expectorant, cardiotonic and to heal eczema. *L. stoechas* is also a well-known good nectar source for honeybees. In this study, melissopalynological, total phenolic, and chemical compound analysis of *L. stoechas* honey produced in Kemalpaşa-İzmir was examined. As far as we know, this is the first study in which the chemical compounds of Turkish *L. stoechas* honey were investigated via GC-MS. As a result of the study, 10 plant taxa were determined in the honey and *L. stoechas* pollen was found to be dominant with 41.37%. *Sanguisorba* spp., Cistaceae, Ericaceae, Rosaceae, *Trifolium* spp., Asteraceae, Brassicaceae, Chenopodiaceae and *Echium* spp. plant taxa, respectively, were detected in the honey in important minor and minor proportions. In addition to melissopalynological analysis, total phenolic and chemical compound analyses were performed in the honey. As a result of total phenolic analysis 385.72±2.25 mgGAE/L was found as a total phenolic amount. On the other hand, acetic acids and esters (5.87%), aldehydes (3.05%), alcohols (1.59%), carboxylic acids and esters (18.10%), fatty acids and esters (27.22%), hydrocarbons (7.96%), ketones (14.98%), terpenes (2.56%) and other chemical compounds (6.63%) were determined by the GC-MS analysis. The most abundant volatiles were found pyruvic acid (16.22%), myristic acid (5.74%), stearic acid (17.95%), which belong to carboxylic acids, fatty acids, and their esters. As a result, carboxylic acids (18.10%) and fatty acids (27.22 %) were detected most abundant compounds in *L. stoaches* honey. These detected components can be used as markers of *L. stoaches* honey.

Keywords: Honey, Chemical Compounds, *Lavandula stoaches* L., Pollen Analysis, Türkiye

Özet

Lamiaceae familyasına ait olan *Lavandula stoechas* L., yöresel olarak "karabaşotu, karan, gargan, kesişotu" adlarıyla bilinmekte olup merkezi sinir sistemi hastalıklarında (epilepsi ve migren), çeşitli yaraların tedavisinde ve kan şekerinin düşürülmesinde kullanılmaktadır. Bu bitki aynı zamanda ağrı kesici, antiseptik, sakinleştirici, balgam söktürücü, kardiyotonik ve egzamayı iyileştirmek için de kullanılmaktadır. *L. stoechas* aynı zamanda bal arıları için iyi bir nektar kaynağı olarak da bilinmektedir. Bu çalışmada Kemalpaşa-İzmir'de üretilen *L. stoechas* balının melissopalinolojik, toplam fenolik ve kimyasal bileşik analizi incelenmiştir. Bildiğimiz kadarıyla bu çalışma, Türk *L. stoechas* balının kimyasal bileşiklerinin GC-MS ile araştırıldığı ilk çalışmadır. Çalışma sonucunda balda, 10 bitki taksonu belirlenmiş ve *L. stoechas* poleninin %41,37 ile baskın olduğu tespit edilmiştir. *Sanguisorba* spp., Cistaceae, Ericaceae, Rosaceae, *Trifolium* spp., Asteraceae, Brassicaceae, Chenopodiaceae ve *Echium* spp., taksonları balda sırasıyla önemli minör ve minör oranlarda bitkiler tespit edilmiştir. Melissopalinolojik analizlerin yanı sıra toplam fenolik ve kimyasal bileşik analizleri de yapılmıştır. Toplam fenolik analiz sonucunda toplam fenolik içeriği 385,7223±2,253298101 mgGAE/L olarak bulunmuştur. Diğer taraftan asetik asitler ve esterler (%5,87), aldehitler (%3,05), alkoller (%1,59), karboksilik asitler ve esterler (%18,10), yağ asitleri ve esterler (%27,22), hidrokarbonlar (%7,96), ketonlar (%14,98), terpenler (%2,56) ve diğer kimyasal bileşikler (%6,63) GC-MS analizi ile belirlenmiştir. En çok bulunan uçucu maddeler ise karboksilik asitler, yağ asitleri ve bunların esterlerine ait pirüvik asit (%16,22), miristik asit (%5,74) ve stearik asit (%17,95) olarak bulunmuştur. Sonuç olarak *L. stoaches* balında en fazla bulunan bileşiklerin karboksilik asitler (%18.10) ve yağ asitleri (%27.22) olduğu saptanmıştır. Tespit edilen bu bileşenler *L. stoaches* balının belirteçleri olarak kullanılabilir.

Anahtar Kelimeler: Bal, Kimyasal Bileşenler, *Lavandula stoaches* L., Polen Analizi, Türkiye

1. INTRODUCTION

Lamiaceae family is represented by 236 genera and 7280 species worldwide and is a plant family that is especially widespread in the temperate zone. These plants, which are generally found in the form of herbs, rarely shrubs and trees, have a quadrangular stem and a circular (verticillate) leaf arrangement extending along the stem. Its flowers are dioecious and zygomorphic. Their pollen usually has three or six colpus. Family plants, due to their essential oils, it is included in the composition of cosmetic products and is used in the preparation of various foods and beverages. Also, plant products have been used as a natural medicine against many different diseases for years The best known and economically important species are *Lavandula angustifolia*, *Lavandula stoechas*, *Lavandula latifolia*, and *Lavandula* x *intermedia* hybrid (Angiosperm Phylogeny Group, 2016; Çelik & Arslantürk, 2007; Çinbilgel & Kurt, 2019; Ez Zoubi et al., 2020).

Turkey is an important gene center of the Lamiaceae family. Lamiaceae family is represented by 45 genera, 565 species, and 735 taxa in the flora of Turkey. Also Lamiaceae

family is one of the highest endemic families in Turkey. The endemism rate is 45% (Gedik et al., 2016).

Lavandula stoechas L., belonging to the Lamiaceae family, is locally known as "karabaşotu, karan, gargan, keşişotu" (Küçük et al., 2019) and is used in central nervous system diseases (epilepsy and migraine), in the treatment of various wounds and in reducing blood sugar (Çelik & Arslantürk, 2007). It is also used as analgesic, antiseptic, sedative, expectorant, cardiotonic and to heal eczema (Küçük et al., 2019).

L. stoechas is spread over three continents (Africa, Europe, and Asia). It is growing around the Mediterranean basin, including in Morocco, Algeria, Tunisia, Spain, Greece, France, Italy, and Turkey. It is also found in Saudi Arabia and Iran (Ez zoubi et al., 2020). *L. stoechas* is also a well known good nectar source for honeybees and *L. stoechas* honey is especially produced in Portugal and Spain (Guyot-Declerck et al., 2002). In Turkey, this honey is produced in Kemalpaşa, Izmir, on the Aegean coast.

In this study, melissopalynological, total phenolic, and chemical compound analysis of *L. stoechas* (lavender) honey produced in Kemalpaşa-İzmir was examined. As far as we know, this is the first study in which the chemical compounds of Turkish *L. stoechas* honey were investigated via GC-MS.

2. MATERIAL AND METHODS

2.1. Collection of Samples

Pool sample material was collected in a glass jar from hives, which is in Kemalpaşa district of Izmir. It was sent to Hacettepe University Bee and Bee Products Center (HARÜM) for melissopalynological, total phenolic, and chemical compounds analyses.

Kemalpaşa is a district of Izmir province, located in the Aegean region in western Turkey, with a population of approximately 112,049 (Figure 1). Kemalpaşa district is adjacent to İzmir's Bornova, Buca, Torbalı, and Bayındır districts and Manisa's Yunusemre, Şehzadeler and Turgutlu districts. The distance to the city center is approximately 24 km. The district's latitude and longitude coordinates are 38.427679 and 27.418462, respectively. Its surface area is 681 square kilometers and its altitude value, which represents its height above sea level, is ~ 197 meters (Anonymus, 2021).

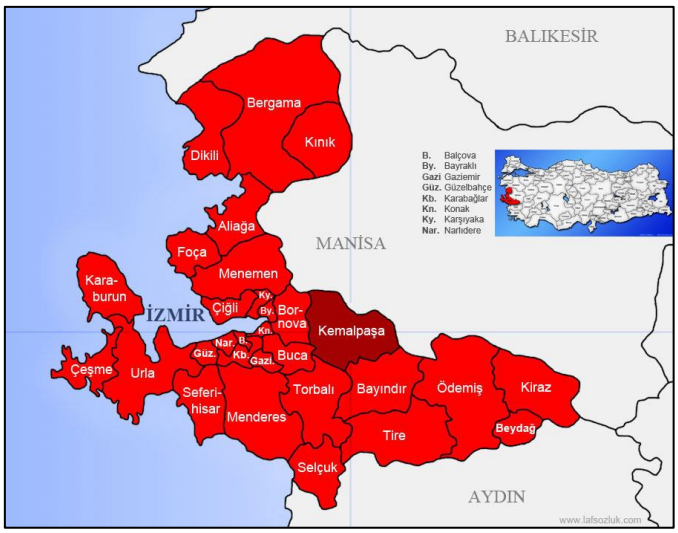

Figure 1. Kemalpaşa-İzmir region. Anonymus, (2021).

2.2. Melissopalynological Analysis

Melissopalynological analysis, which also means microscopic pollen analysis in honey, was carried out according to the method suggested by Sorkun, (2008) and Louveaux et al., (1978). Accordingly, 10 g of the honey sample, mixed well with the help of a glass drumstick, was weighed into a centrifuge tube, and 20 mL of distilled water was added and kept in a 45°C water bath for 10-15 minutes to ensure that the honey dissolved in water and became homogeneous. This solution, which then became homogeneous, was centrifuged at 3500 rpm for 45 minutes and then the supernatant was removed. Then, some sediment remaining at the bottom of the tube was treated with basic-fuchsine glycerin-gelatin and transferred to the slide. Finally, the slide was heated on the heating table at 30-40°C to melt the basic fuchsin-containing glycerin-gelatin. An 18x18 mm^2 coverslip was covered on this melted mixture. The inverted preparation was ready for examination after approximately 12 hours. Honey preparations were examined under a Nikon Eclipse E400 microscope. During the identification of pollen grains, pollen preparation libraries prepared from herbal references in the laboratory where the research was conducted, along with different reference sources were used (Sorkun 2008, Anonymus, 2000).

Starting from the upper left corner of the preparation, a total of 200 plant pollen grains were counted and identified. An immersion objective (x100) was used to identify the pollens, x20 and x40 size lenses were used to easily distinguish small-sized pollens during counting, and a microscopic picture of the *L. stoechas* pollen grains observed in honey was taken.

2.3. Total Phenolic Content

The total phenolic content of *L. stoechas* honey sample was determined by the Folin-Ciocalteu method (Özkök et al., 2010).

The stock was prepared by dissolving 25 mg gallic acid in 100 ml 70% methanol. Concentrations of 0, 25, 50, 75, and 100 mg/L were prepared from this stock. Standards were prepared by diluting 10 ml of the prepared concentrations with 70% methanol. 5 g of each honey sample was taken and dissolved in 50 ml of 70% methanol. 1 ml of each honey sample and the prepared standards were taken and placed in a test tube. 5 ml of 10% Folin Ciocalteu Reagent was placed on it. It was mixed with vortex. After 3 minutes, 4 ml of 75 g/l Na2CO3 solution was added within 8 minutes. The mixture was stirred for 1 minute. 15 min at 45°C was incubated in a water bath. Samples and standards were read on a UV Spectrophotometer at 765 nm.

2.4. Chemical Compounds Analysis

The chemical compounds of *L. stoechas* honey sample was determined by Gas Chromatography-Mass Spectrometry (GC–MS) analysis. For this analysis, Agilent 6890N GC system coupled with a mass selective detector MS5973 was used. Honey was dissolved in methanol and 1 µl of honey extract was injected into the GC-MS system to screen the chemical compounds. For the GC-MS experimental conditions Temiz et al., (2011) method followed. According to this, a DB 5MS capillary column (30 m x 0.25 mm x 0.25 µm) was used and the flow rate of the mobile phase (He) was set at 0.7 mL/min. In the gas chromatography part, the temperature was kept at 50° for 1 min. After this period, the temperature was increased to 150°C with a 10°C/min heating ramp and then kept at 150°C for 2 min. Finally, the temperature was increased to 280°C with a 20°C/min heating ramp and then kept at 280°C for 30 min.

Chemical compounds were identified by computer search using a reference to The Wiley Registry/NIST Mass Spectral Library, which is available in the data acquisition system of GC-MS.

3. RESULTS AND DISCUSSIONS

As a result of the study, 10 plant taxa were determined at the pool honey sample. In melissopalynological analysis, it was determined which plants the pollen found in the honey belonged to, and classification was made as dominant pollen (>%45), secondary pollen (%16-45), important minor pollen (%3-15) and minor pollen (<3%) according to the frequency of pollen grains. Generally, honey with more than 45% pollen from a plant is called unifloral or monofloral honey, and the honey is named after that plant. For example, astragalus, canola, clover, sainfoin honey. However, this is not the case for some plant species. Because while some plants can produce plenty of pollen, some plants can produce plenty of nectar. In this case,

when searching for monofloral pollen in honey, classification is made by taking into account the nectar and pollen production potential of the plant. For example, *Robinia* spp., *Citrus* spp., *Tilia* spp., *Lavandula* spp., and *Rosmarinus* spp. These species are plants that contain plenty of nectar but low pollen, and therefore, in order to be monofloral honey, it may be sufficient to have 10-20% pollen in the honey. On the other hand, *Castanea sativa*, and *Eucalyptus* spp. species produce abundant pollen, and in order to be monofloral honey, 70-90% of pollen must be present in the honey. If there is no dominant pollen in the honey and honey consists of secondary, minor, and trace pollens, such honey is called multifloral or mixed flower honey. (Louveaux et al., 1978; Terrab et al., 2003).

In our study, *L. stoechas* pollen was found to be dominant with 41.37% in the honey. Under normal conditions, 10 to 20% lavender pollen in lavender honey is sufficient for that honey to be dominant, but since the region is dense with *L. stoechas* plants, the pollen was detected at 41.37%. On the other hand, pollen grains belonging to *Sanguisorba* spp., Cistaceae, Ericaceae, Rosaceae, *Trifolium* spp., Asteraceae, Brassicaceae, Chenopodiaceae and *Echium* spp. plants, respectively, were detected in the honey at important minor and minor proportions (Table 1).

Table 1. Lavender honey melissopalynological and total phenolic acid content results

Honey type	Plant species in honey*	Total phenolic acid content (mgGAE/L)	Region
Lavender	*Lavandula stoechas*: 41.37 (D)** *Sanguisorba* spp.: 13.79 (IM) Cistaceae: 12.06 (IM) Ericaceae: 8.62 (IM) Rosaceae: 7.75 (IM) *Trifolium* spp.: 6.03 (IM) Asteraceae: 5.17 (IM) Brassicaceae: 2.58 (M) Chenopodiaceae: 1.72 (M) *Echium* spp.: 0.86 (M)	385.72±2.25	Kemalpaşa-İzmir

*>45%Dominant (D), 16-45% Seconder (S), 3-15% Important Minor (IM), <3% Minor (M)
**For lavender honey, 10-20% lavender pollen amount is sufficient for dominance.

At the same time, microscopic photographs of *L. stoechas* pollen, which is dominantly found in honey, were taken in equatorial and polar positions. Accordingly, *L. stoechas* pollen has medium-sized (26-50 µm), hexacolpate, stephanocolpate aperture and punctitegillate exine ornamentation (Anonymus, 2024) (Figure 2).

Figure 2. Plant photographs of *L. stoechas* L and Microscopic photographs of *L. stoechas* pollen (A.x20, B-C.x40, D-E.x100 Polar view, F-G.x100 Equatorial view) in lavender honey.

In addition to melissopalynological analysis, total phenolic and chemical compound analyses were performed. As a result of total phenolic analysis 385.72±2.25 mgGAE/L was found (Table 1). On the other hand, acetic acids and esters (5.87%), aldehydes (3.05%), alcohols (1.59%), carboxylic acids and esters (18.10%), fatty acids and esters (27.22%), hydrocarbons (7.96%),

ketones (14.98%), terpenes (2.56%) and other chemical compounds (6.63%) were determined by the GC-MS analysis in the *Lavandula stoaches* honey (Table 2).

Table 2. Chemical compounds of *Lavandula stoaches* honey

CHEMICAL COMPOUNDS	*Lavandula stoaches*
Acetic acids and esters (%)	
Lauryl acetate	0.42
Ethyl acetoacetate	3.87
n-Propyl acetate	0.16
Hexyl acetate	0.42
n-Butyl acetate	0.35
Nonyl acetate	0.65
Total	***5.87***
Aldehydes (%)	
Citral	0.04
Phenylacetaldehyde dimethyl acetal	0.90
2,4-Dimethylbenzaldehyde	0.19
2-Methyl-2-pentenal	1.15
2-Methylbutyraldehyde	0.77
Total	***3.05***
Alcohols (%)	
1-Decanol	0.33
Furfuryl alcohol	1.26
Total	***1.59***
Carboxylic acids and esters (%)	
Benzoic acid	0.53
Propionic acid	0.43
Pyruvic acid	16.22
2-Methyl-2-pentanoic acid	0.77
4-Methylpentanoic acid	0.07
3-Hexenoic acid	0.08
Total	***18.10***
Fatty acids and esters (%)	
Ethyl oleate	0.28
Myristic acid	5.74
Stearic acid	17.95
Lauric acid	0.61
Nonanoic acid	2.05
Isobutyl butyrate	0.59
Total	***27.22***
Hydrocarbons (%)	
n-Eicosane	0.43
n-Nonane	0.90
n-Undecane	6.63
Total	***7.96***
Ketones (%)	
3,4-Dimethyl-1,2-cyclopentadione	0.58
3,5-Dimethyl-1,2-cyclopentadione	0.52

CHEMICAL COMPOUNDS	*Lavandula stoaches*
Homofuronol	11.60
1-Methyl-2,3-cyclohexadione	0.36
6-Methyl-3,5-heptadien-2-one	0.28
5-Methyl-2,3-hexanedione	0.10
2-Octanone	1.1
Geranylaceton(isomer 2)	0.44
Total	***14.98***
Terpenes (%)	
Isoborneol (isomer 1)	2.56
Total	***2.56***
Others (%)	
Hexyl formate	0.31
Isoamyl propionate	0.81
2-Methoxy-3-methylpyrazine	1.68
5-Methylquinoxaline	1.83
Methyl-2-furoate	0.34
ɣ-Butyrolactone	0.50
5-hydroxy-2-decenoic acid delta-lactone	1.10
Propyl isovalerate	0.06
Total	***6.63***

Studies investigating the palynological and chemical properties of *L. stoaches* honeys are limited in the literature. Although Louveaux et al., (1978) say 10-20% lavender pollen is sufficient for monofloral lavender honey, the results of this study showed that more than 40% lavender pollen could be detected in lavender honey, depending on the density of lavender in the region. Castro-Vázquez et al., (2014) also found *L. angustifoliaxlatifolia* honey separetly from *L. latifolia* pollen grains in the honey samples but they didn't indicate the pollen percentage. On the other hand, Can et al., (2015) detected the highest percentage of *L. stoechas* pollen in honey as 53%.

Phenolic acids, also known as aromatic carbonic acids, are phytochemicals that have a basic antioxidant effect in honey (Özkök et al., 2010). Karabagias et al., (2019) found a total phenolic amount of 217 mgGAE/L, at the methanolic plant extract of *L. stoaches*. In our study, we determined 385.7 mgGAE/L in the *L. stoaches* honey. Because *L. stoaches* honey's total phenolic amount rate may be higher than just *L. stoaches* plant extract. As a matter of fact, similar to our study, Can et al., (2015) found the total phenolic amount 533.9 mgGAE/L in the *L. stoaches* honey.

Also, Karabagias et al., (2019) found 50 volatile compounds belonging to alcohols, aldehydes, ketones, norisoprenoids, and numerous terpenoids by the GC-MS at the methanolic

plant extract of *L. stoaches*. In our study, unlike Karabagias et al., (2019) work, we determined 45 volatile compounds by the GC-MS at the methanolic honey extract of *L. stoaches*. Acetic acids and esters, aldehydes, alcohols, carboxylic acids and esters, fatty acids and esters, hydrocarbons, ketones, terpenes, and other chemical compounds were found by the GC-MS analysis. The most abundant volatiles were pyruvic acid (16.22%), myristic acid (5.74%), stearic acid (17.95%), which belong to carboxylic acids, fatty acids, and their esters. As a result, carboxylic acids (18.10%) and fatty acids (27.22 %) were found most abundant compounds in *L. stoaches* honey. Özkök, (2019) also, found carboxylic acids (14.49%) and fatty acids (12.63%) in the mullein (*Verbascum* spp.) honey. Besides this, Karabagias et al., (2019) determined mostly essential oils in the *L. stoaches* plant extract. Ez zoubi et al., (2020) also emphasized the essential oil content of *L. stoechas* plants which is used for the preparation of traditional meals, herbal teas, and cosmetic purposes. On the other hand, Guyot-Declerck et al., (2002) compared the aromatic profiles of French (*L. angustifolia* and *L. angustifoliaxlatifolia*) and Portuguese (*L. stoechas*) lavender honey samples and they showed that there are major qualitative and quantitative differences between the honey types. They didn't find any marker specific to *L. stoechas* honey, but Portuguese lavender honey showed much lower concentrations of n-hexanal, n-heptanal, n-hexanol, and heptanoic acid than French lavender honey samples. In our study, we also determined 3-hexenoic acid, which is a carboxylic acid like heptanoic acid, in the *L. stoechas* honey. Castro-Vázquez et al., (2014) revealed the chemical composition of *L. angustifoliaxlatifolia* and *L. latifolia* by SPE/GC-MS, they found ɣ-nonalactone, farnesol, acetovanillone, 1- heptanol, decanal, 4-methoxyacetophenone and dehydrovomifoliol at the *L. angustifoliaxlatifolia* honey separetly from *L. latifolia* honey. In our study we couldn't determine these compounds at the *L. stoechas* honey.

4. CONCLUSION

In conclusion, lavender pollen frequency may vary depending on the lavender plant density of the region and the dominance can be determined to be over 20%. The total phenolic acid amount was found 385.7 mgGAE/L., and carboxylic acids, fatty acids, and their esters were determined mostly in the *L. stoaches* honey. The most abundant volatiles were found pyruvic acid (16.22%), myristic acid (5.74%), stearic acid (17.95%), which belong to carboxylic acids, fatty acids, and their esters. As a result, carboxylic acids (18.10%) and fatty acids (27.22 %) were detected most abundant compounds in *L. stoaches* honey. These detected components can be

used as markers of *L. stoaches* honey. Further extensive studies should be done about *L. stoaches* plant and it's honey to determine more specific characteristics.

ACKNOWLEDGEMENTS

I would like to thank Balyum Arı Ürünleri-Osman Yaşar for helping us obtain the honey samples for our study.

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