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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF WATER SOLUBLE FRACTION OF PROPOLIS AND ITS ANTIBACTERIAL POTENTIAL ON BACTERIA CAUSING CONJUNCTIVITIS

Propolisten Suda Çözünür Bir Fraksiyonun İzolasyonu, Karakterizasyonu ve Bakteriyel Konjonktivite Karşı Potansiyel Etkinliğinin Belirlenmesi

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ABSTRACT

Propolis is a bee product with a variety of biological activities. Although chemical composition of propolis differs by the location but all propolis types possesses antimicrobial activity. The usage of propolis for apitherapeutic purposes has increased recently. But its ethanol solubility limits its usage in certain areas like ophthalmology. Main objective of this study is to isolate water soluble components of propolis and determination of its antimicrobial activity against two bacteria causing conjunctivitis namely *Neisseria gonorrhoeae* and *Haemophilus influenzae*. Isolation of water soluble fraction of propolis was carried out in two steps by using pectin-propolis micro beads. Isolated water soluble fraction and crude extract was examined by thin layer chromatography and HPLC analyses. Three main spots were screened on TLC plate after isolation. These spots could be explained by the presence of different class of compounds in the isolate. HPLC analyses showed that water soluble fraction contained phenolic acids, their esters and flavonoids like p-OH benzoic acid, *t*-cinnamic acid, pinocembrin and caffeic acid phenethyl ester. Low antimicrobial activity was achieved against tested microorganisms for the fraction. It can be concluded that isolation of water soluble fraction of the propolis extract could be a solution for its usage in restricted areas.

Keywords: Propolis, Water soluble fraction, Conjunctivitis, Antimicrobial activity

ÖZ

Propolis farklı biyolojik aktivitelere sahip doğal bir arı ürünüdür. Propolisin kimyasal bileşimi lokasyona göre farklılık gösterse de tüm propolis türleri antimikrobiyal aktiviteye sahiptir. Propolisin apiterapötik amaçlarla kullanımı son zamanlarda artmıştır. Ancak propolisin etanolde çözünürlüğü, oftalmoloji gibi bazı alanlarda kullanımını sınırlamaktadır. Bu çalışmanın temel amacı propolisin suda çözünen bileşenlerini izole etmek ve izolatların konjonktivite neden olan iki bakteriye karşı

antimikrobiyal aktivitesini tespit etmektir. Propolisin suda çözünür fraksiyonunun izolasyonu pektin-propolis mikro boncukları kullanılarak iki aşamada gerçekleştirildi. İzole edilmiş suda çözünür fraksiyon, ince tabaka kromatografisi ile incelendi ve ham ekstraktla karşılaştırıldı. İzolasyondan sonra TLC plakasında üç ana nokta tarandı. Bu lekeler izolatta fenolik asitler, flavonoidler ve kafeik asit esterleri gibi farklı sınıftaki bileşiklerin varlığıyla açıklanabilir. Suda çözünebilen fraksiyonun fenolik asitler, bunların esterleri ve p-OH benzoik asit, t-sinnamik asit, pinosembrin ve kafeik asit fenetil ester gibi flavonoidleri içerdiği belirlendi. Fraksiyon için test edilen mikroorganizmalara karşı düşük antimikrobiyal aktivite elde edildi. Propolis ekstraktının suda çözünen kısmının izolasyonunun kısıtlı alanlarda kullanımı için bir çözüm olabileceği sonucuna varılabilir.

Anahtar Kelimeler: Propolis, Suda çözünen fraksiyon, Konjonktivit, Antimikrobiyal aktivite

GENİŞLETİLMİŞ ÖZET

Amaç: Propolis arılar tarafından bitkilerin tomurcuk, yaprak ve benzeri farklı kısımlarından toplanan reçinemsi, rengi sarıdan koyu kahverengiye kadar değişen doğal bir arı ürünüdür. Propolis antimikrobiyal, anti-inflamatuvar, antikanser, antioksidan gibi farklı biyolojik aktivitelere sahip doğal bir arı ürünü olmakla birlikte propolisin kimyasal bileşimi ve biyolojik aktivitesi toplandığı bölgeye göre değişiklik göstermektedir. Propolisin kimyasal bileşimi toplandığı bölgeye göre farklılık gösterse de tüm propolis türleri antioksidan ve antimikrobiyal aktiviteye sahiptir. Apiterapi; arı zehri, bal, polen, arı sütü ve propolis gibi arı ürünlerinin hastalıkların tedavi edilmesi veya önlenmesi amacıyla kullanılması olarak tanımlanmaktadır. Propolisin apiterapötik amaçlarla kullanımı son zamanlarda artmış olmasına karşın yapışkan yapısından dolayı doğrudan kullanılması veya tüketilmesi mümkün değildir. Etanol, methanol, zeytinyağı gibi farklı çözücüler kullanılarak ekstrakte edilen propolis için en iyi çözücü %70'lik etanol çözeltisidir. Bu durum propolisin oftalmoloji gibi bazı alanlarda kullanımını sınırlamaktadır. Bakteriyel konjonktivit, *Staphylococcus aureus*, *Streptococcus pneumonia*, *Neisseria gonorrhoeae* ve *Haemophilus influenzae* gibi bakterilerin neden olduğu en sık karşılaşılan göz hastalıklarından biridir. Bakteriyel konjonktivit, gözün mukoza zarının enfeksiyonudur ve antibiyotik içeren göz damlaları kullanılarak tedavi edilir. Bu çalışmanın temel amacı, propolisin suda çözünebilen bileşenlerini ve konjonktivite neden olan gram negatif bakterilerden olan *Neisseria gonorrhoeae* and *Haemophilus influenzae*'ye karşı antimikrobiyal aktivitesini tespit etmek ve göz hastalıklarının tedavisinde ya da önlenmesinde kullanılabilecek yeni bir ürün geliştirilme potansiyelinin tespit edilmesidir.

Gereç ve yöntem: Propolisin suda çözünür fraksiyonunun izolasyonu, pektin-propolis mikro boncukları kullanılarak iki aşamada gerçekleştirildi. İlk olarak pektin-propolis boncuklar 1:100 oranında su içerisinde homojenize edilerek gece boyunca karıştırıldı. Elde edilen homojenat 4000 rpm'de 5 dakika santrifüj edildi ve süpernatan toplandı. İkinci olarak aynı hacimde etanol eklenerek süpernatanttan çözünmüş pektin çökeltildi ve karışım süzüldü. Daha sonra propolisin suda çözünebilen bileşenlerini içeren süzüntü evaporatörde kurutuldu. İzole edilmiş suda çözünür fraksiyon, ince tabaka kromatografisi ile incelendi ve ham ekstraktla karşılaştırıldı. Hem suda çözünür fraksiyonun hem de ham ekstraktın kimyasal bileşimi RP-HPLC-UV analizi ile belirlendi.

Bulgular: İzolasyondan sonra TLC plakasında üç ana nokta tarandı. Bu bantlar izolatta fenolik asitler, flavonoidler ve kafeik asit esterleri gibi farklı sınıftaki bileşiklerin varlığıyla açıklanabilir. Suda çözünen fraksiyonun fenolik asitler, bunların esterleri ve p-OH benzoik asit, siringik asit, t-sinnamik asit, hesperidin, pinocembrin ve kafeik asit fenetil ester (CAPE) gibi flavonoidleri içerdiği belirlendi. Genel olarak propolisler gram negatif bakterilerdense gram pozitif bakterilere karşı daha güçlü antimikrobiyal aktivite göstermektedirler. Bu çalışmada da gram negatif bakteriler olan *Neisseria gonorrhoeae* ve *Haemophilus influenzae* karşı düşük antimikrobiyal aktivite göstermiştir. Ancak bu her iki bakteri de nazlı üreme özelliği olan bakteriler olup etkinliğin yüksek olması beklenmekteydi. Bu sonuçun propolis bileşenlerinin düşük konsantrasyonundan dolayı fraksiyon için test edilen mikroorganizmalara karşı düşük antimikrobiyal aktivite elde edildiği düşünülmektedir. Fraksiyonda daha yüksek bileşenler elde edildiği takdirde etkinliğin yüksek olacağı düşünülmektedir. Bu konuda daha fazla çalışmaların gerektiği düşünülmektedir.

Sonuç: Propolis ekstraktının suda çözünen kısmının izolasyonunun kısıtlı alanlarda kullanımı için bir çözüm olabileceği sonucuna varılabilir. Bu ön çalışma niteliğinde olan bu çalışma geliştirilerek propolisli göz damlası veya göz temizleme solüsyonlarının hazırlanmasına temel oluşturabilir.

INTRODUCTION

Propolis is an important bee product with a variety of biological activities (Kolaylı and Keskin 2020). It is collected from different parts of the plants by honey bees. Biological activity with relation to chemical composition of a propolis depends on the flora of collection site (Keskin and Kolaylı 2018). Even though chemical composition of propolis samples differs by the location but all propolis samples around the world possess antimicrobial activity (Bouchelaghem 2022, Nichitoi et al. 2021, Stepanović et al. 2003,). Apitherapy is defined as the usage of bee products in the treatment either for curative or preventive purposes (Kolaylı and Keskin 2020). The usage of propolis for apitherapeutic purposes has increased recently. Many biological activities of propolis has been reported up to now (Bouchelaghem 2022, Keskin and Kolaylı 2018, Kolaylı and Keskin 2020, Nichitoi et al. 2021, Stepanović et al. 2003, Weis et al. 2022). But the usage of propolis in the treatment of eye diseases is not possible because of its resinous nature and its alcohol solubility.

Propolis contains many active substances and some of them are soluble in water but it is not possible to extract these substances from the raw propolis directly (Chua et. al. 2023). It is clear that new methods are required for this purpose. Although it is clearly stated in the literature that the best solvent for propolis extraction is ethanol but it is also a limiting factor for the usage of propolis in certain areas. When achieved to separate them, water soluble components of propolis have good potential to be used in restricted areas like bacterial conjunctivitis.

Bacterial conjunctivitis is an infectious inflammation of the conjunctiva which is a flexible transparent mucous membrane covering the *Bulbus oculi* (Azari and Arabi 2020). Due to direct exposure of the conjunctiva to the environmental factors, inflammation of the conjunctiva, either infectious or non-infectious, is seen commonly and one of the common reasons for visiting eye care clinics (Pisano et al. 2023). Viral and allergic reasons are the other common causes of conjunctivitis and usually cause mild inflammation and ocular discomfort that healed spontaneously (Chan et al. 2022). However, bacterial conjunctivitis has a relatively more severe course, and topical antibiotics, either drop or ointment, are generally required in the treatment (Mohammed et al. 2020, Banks et al. 2020, Stiles 2021). The high prevalence and frequent occurrence of this disease and the need for isolation of cases from school and work cause a huge economic impact on governments. In the United States, the estimated cost of bacterial conjunctivitis per year was $377 million to $857 million (Azari and Arabi 2020).

Topical antibiotic drugs are commonly used in ophthalmology clinics for various indications in addition to conjunctivitis (Aramă 2020). They are suggested for almost after any surgical intervention through the ocular surface. Increasing administrations of intravitreal anti-VEGF drugs for Age Related Macular Degeneration (AMD), diabetic macular edema and other retinopathies caused a serious increase in antibacterial drug use in the last decades (Xu et al. 2023). In addition to the cost of these drugs, the common use of topical antibacterial drugs may cause toxic and allergic reactions to the ocular surface and lead to an increase in antibiotic resistance (Aramă 2020). Therefore, various natural and herbal products are investigated for a possible antimicrobial activity to use in infectious inflammation instead of antibiotics.

Main objective of this study is to isolate water soluble components of propolis and identification of isolated compounds by HPLC analyses. Antimicrobial activity of the isolated compounds against two common pathogens of bacterial conjunctivitis (Devipriya 2020) namely *Neisseria gonorrhoeae* and *Haemophilus influenza* was also carried out.

MATERIAL AND METHODS

Bacterial strains were supplied from Refik Saydam Institutes (Ankara). Propolis was obtained by a local beekeeper in Bilecik city. Propolis was collected by using traps in the summer season of 2020. Pectin, ethanol, Na_2CO_3, and HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, catechin, syringic acid, *p*-coumaric acid, epicatechin, rutin, *t*-cinnamic acid, luteolin and ferulic acid were obtained from Sigma Aldrich

Chemie GmbH (Munich, Germany) and used as HPLC standards. Other chemicals used in the study were off analytical grade.

Preparation and Encapsulation of Propolis Extract

The propolis sample was extracted with 70% ethanol/water (v/v) in the ratio of 1:10 (g/v) (Keskin and Kolaylı 2018). Extraction was carried out for 48 h on a magnetic stirrer under constant stirring at 150 rpm. Finally, the obtained mixture was filtered and labeled as ethanol propolis extract (EPE).

Microencapsulation of propolis extract with pectin was carried out by using both ionic gelation and solvent exchanging methods (Keskin et al. 2019). 5% pectin solution was prepared by dissolving 5 g pectin in 100 mL water. In another beaker 1.72 g $CaCl_2$ was dissolved in 100 mL ethanol propolis extract. Pectin solution was added into ethanol propolis extract drop by drop under constant stirring. Finally obtained mixture was dried in a vacuum oven at 50 °C and obtained beads were grounded in a fine powder (Keskin et al. 2019).

Isolation of Water Soluble Fraction of Propolis

Isolation of water soluble fraction of propolis was carried out in two steps. Firstly, pectin micro-beads were homogenized in water at 1:100 ratios (1 g pectin beads/100 mL water). Obtained mixture was centrifuged at 4000 rpm for 5 min and supernatant was collected, precipitate was named as residue. Secondly dissolved pectin was precipitated from the supernatant by adding the same volume of ethanol. Then the mixture was filtered and the filtrate was collected and dried in a rotary evaporator (IKA-Werke, Staufen, Germany).

Examination of Water Soluble Fraction/ Thin Layer Chromatography

Isolated water soluble fraction was examined by thin layer chromatography. Commercially obtained silica gel was used. Mobile phase was composed of ethanol/chloroform in a 9.5/0.5 (v/v) ratio.

Determination of Chemical Composition by HPLC Analyses

Propolis samples, both water soluble fraction and ethanol propolis extract, were examined by using HPLC analyses. For this, the solvent of the samples was removed. Obtained residues were dissolved in acidified (pH 2) distilled water separately. 5 mL diethyl ether after then 5 mL ethyl acetate was used for re-extraction for 3 times for each. Obtained phases were separately combined and the solvents were evaporated in a rotary evaporator at 45 °C. The residues were re-dissolved in 2 mL of methanol and filtered with 0.45 µm filter. The filtrates were injected to HPLC. Nineteen phenolic standard compounds were analyzed using HPLC (Elite LaChrome; Hitachi, Tokyo, Japan) equipped with a reversed phase Fortis C18 column (Chromex Scientific, 150 mm* 4.6 mm, 5 µm). Mobile phase was composed of acetonitrile/water (7/3 ratio) and acetic acid 2%. A programmed gradient was applied. Reservoir A contained 2% acetic acid and reservoir B contained 7/3 ratio of acetonitrile/water. Elution of samples began with the eluent composed of 95% of reservoir A and 5% of reservoir B for 3 min. Then, the ratio of reservoir A was decreased gradually to 20% at the end of 30 min. Later, gradient program was shifted to starting point of 95% reservoir A for 20 min more. 20 µL of samples was injected individually at room temperature and flow rate was set as 0.75 mL/min (Can et al. 2015).

Determination of Antimicrobial Activity

Antimicrobial activity of the sample was determined by using agar well diffusion method. Chocolate and Blood agar was used for *Neisseria gonorrhoeae* and *Haemophilus influenza* respectively. Hemin and Vitamin K were added to both media at a rate of (%1) and used. Dried propolis samples were dissolved in ethanol at 100 mg/mL concentration as stock solution. Working solution of samples was prepared by tenfold dilution of the stock solution. A serial dilution of the propolis samples was achieved by diluting the working solution in the range of ½ to 1/16. After microorganisms were homogeneously seeded into the media with the help of swab sticks, wells (5 mm) were opened. Each dilution was tested three repetitions and in 50 microliter quantities. Petri dishes were placed in an incubator at 37 °C for 48-72 hours with microaerophilic conditions. Finally, zone diameter was measured as mm (Kolaylı et al. 2020). The analyses were performed three times, the results were presented as mean values and standard deviations.

RESULTS

Main objective of this study is to isolate water soluble compounds of propolis and to determine its potential as an antimicrobial agent. As mentioned above it is not possible to extract them from the raw propolis

directly. It is suggested that micro encapsulation of ethanol extract of propolis and then homogenization of the obtained beads in water may be applicable for this purposes.

Isolation of water soluble fraction was carried out in two steps by starting the homogenization of 10 g of pectin-propolis beads in 1L of water. Then the homogenate was centrifuged at 4000 rpm for 5 min and the supernatant (800 mL) was collected in a clean beaker. In the second step, the same volume of ethanol was added into the supernatant and dissolved pectin was precipitated. Obtained mixture was filtered to separate the pectin from the propolis active compounds. Finally, solvent of the filtrate was evaporated to dryness in a rotary evaporator and 2.16 g dry residue (water soluble fraction) was achieved. Obtained residue was examined by thin layer chromatography. The spots obtained were colorless in day light and they could be visualized under UV light (Figure 1). Identification of chemical compounds in ethanol propolis extract, water soluble fraction and residue was carried out by HPLC analyses and obtained results were summarized in Table 1. It was determined that water soluble fraction contained phenolic acids, their esters and flavonoids like p-OH benzoic acid, syringic Acid, t-cinnamic acid, hesperidin, pinocembrin and caffeic acid phenethyl ester (CAPE). Ethanol propolis extract, remaining residue and water soluble fraction were tested as an antimicrobial agent against two bacteria causing bacterial conjunctivitis. Antimicrobial activity was determined as the zone diameter (Figure 2) and results were summarized in Table 2. Antimicrobial activity of samples was defined as high (zone diameter > 15mm), moderate (zone diameter between 10-15 mm) and low (zone diameter < 10 mm).

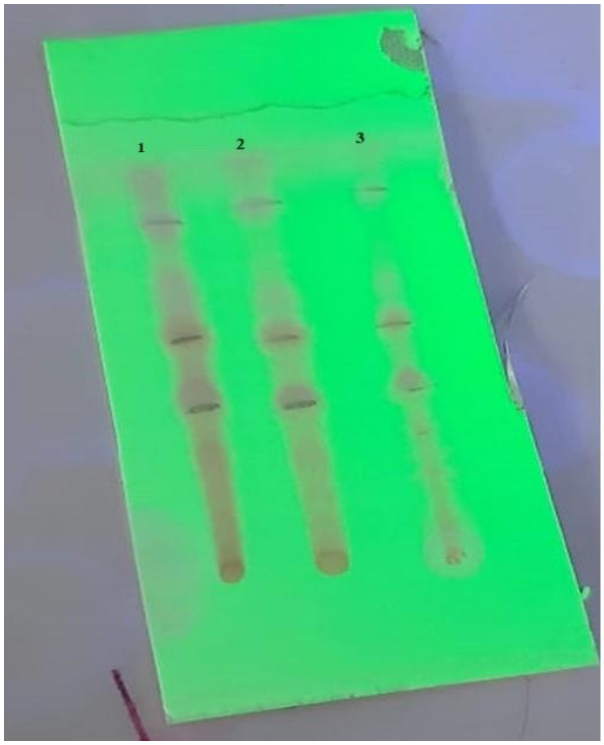

Figure 1. TLC image of samples.
Lines as 1,2,3, Represents ethanol propolis extract, Residue and Water Soluble Fraction respectively.

Figure 2. Antimicrobial activity of samples

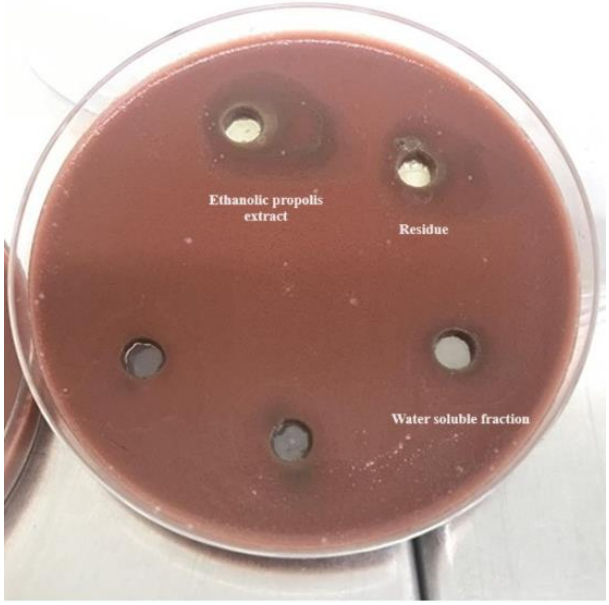

Figure 2. Antimicrobial activity of samples

Table 1. Phenolic composition of samples

	Ethanol Extract	Residue	Water Soluble Fraction
Gallic acid	-	-	-
Protocatequic acid	-	-	-
p-OH benzoic acid	91.45[a]	33.05[b]	7.52[c]
Catechin	-	-	-
Caffeic Acid	-	-	-
Syringic Acid	240.61[a]	35.16[b]	17.42[c]
Epicatechin	-	-	-
p-coumaric acid	-	-	-
Ferulic acid	-	-	-
Rutin	778.27[a]	287.88[b]	142.85[c]
Myricetin	489.39[a]	75.55[b]	49.79[c]
Resveratrol	-	-	-
Daidzein	-	-	-
Luteolin	-	-	-
t-cinnamic acid	157.09[a]	46.67[b]	30.08[c]
Hesperetin	64.30[a]	16.29[b]	24.29[c]
Chyrisin	-	-	-
Pinocembrin	1581.93[a]	601.35[b]	663.94[c]
CAPE	112.53[a]	21.38[b]	34.02[c]

Results were expressed as µg/ g sample. – means not detected.

Different letters represent signifcant diferences at $p < 0.05$ probability level

Table 2. Antimicrobial Activity of Samples

	Dilution	Concentration (mg/mL)	Zone Diameter (mm) *Neisseria gonorrhoeae*	*Haemophilus influenzae*
Water Soluble Fraction	1/10	20 mg/mL	8±0.2	8±0.2
	1/2	10 mg/mL	6±0.1	6±0.1
	1/4	5 mg/mL	nd	nd
Residue	1/10	20 mg/mL	12±0.3	8±0.2
	1/2	10 mg/mL	10±0.3	6±0.1
	1/4	5 mg/mL	8±0.2	nd
Ethanol Extract	1/10	20 mg/mL	12±0.3	20±0.5
	1/2	10 mg/mL	10±0.3	18±0.5
	1/4	5 mg/mL	8±0.2	10±0.3

* nd: Not detected

DISCUSSION

This is the first study reporting the fractionation of propolis extract after encapsulation. Propolis is highly resinous substances and not readily soluble in water. Many biological activities of ethanol-propolis extract have been reported up to now but there is a limit for the usage of this extract as a treatment agent especially in ophthalmology (Kubiliene et al. 2015). Main reason is its water insolubility. The application

of water based products for treatment is easier than other solvent based products. That is why isolation of water soluble components of propolis is still an attractive research area. Many attempts have been made for improving the water solubility of propolis up to now. In an early attempt, Ivanovska et. al. (1995) described the production of water soluble derivative of ethanol propolis extract by using L-lysine. Some phenolic compounds were purified from Mexican propolis by using chromatographic methods (Guzmán-Gutiérrez et al. 2018). It is a well-known fact that chromatographic methods require many experimental steps and usage of huge amount of organic solvents for purification (Hostettmann et al. 1986). The method we described in this paper is new and relatively easier for application and does not require the usage of such amount of organic solvents. This method is also easy to scale up for commercial purposes.

Water soluble fraction and propolis ethanol extract were analyzed by thin layer chromatography. Three main spots were screened on TLC plate after isolation. These spots could be explained by the presence of different class of compounds in the isolate like phenolic acids, flavonoids and caffeic acid esters. A chemo metric fingerprinting of Chinese propolis was developed by using TLC and it was reported that five main spots were obtained for Chinese propolis. R_f value for four of these spots were reported as the same with quercetin, kaempferol, chrysin and galangin (Tang et al. 2014). In a study it was reported that standardized TLC method could be developed for investigation of phenolic acids and flavonoids in propolis. The researchers reported that many spots were visualized for propolis samples where some of them representing the phenolic acids and flavonoids (Medić-Šarić et al. 2004).

Chemical structure of water soluble fraction, propolis ethanol extract and the residue was also identified by using HPLC analyses. Findings of this analyses support the findings of thin layer chromatography analyses. It is clearly seen from the Table 1 that water soluble fraction is rich in some phenolic acids, esters and flavonoids. Propolis sample used in this study was obtained from Bilecik province. When compared with the literature, it is clear that similar findings were also reported earlier (Keskin et al. 2019). It is also mentioned in literature that Anatolian propolis is rich in phenolic compounds (Özkök et al. 2021).

The findings of the present study showed that it is an easy and cheap way of fractionation of propolis extract. Propolis is a resin like substance and not readily soluble in water. It is stated in literature that water extract of propolis contains lesser compounds of propolis (Wieczorek et al. 2022). The resinous nature of propolis makes it unusable in the treatment of certain diseases like ophthalmology. Isolation of water soluble fraction of the propolis extract could be a solution.

It is clear from the results that the water soluble fraction showed lower antimicrobial activity compared to propolis ethanol extract. In general, propolis shows stronger antimicrobial activity against gram-positive bacteria than gram-negative bacteria (Kolayli et. al. 2020). In this study, low antimicrobial activity against the gram-negative bacteria *Neisseria gonorrhoeae* and *Haemophilus influenza* was achieved for the water soluble fraction. However, both of these bacteria have fastidious growth characteristics and the activity was expected to be high. This result is thought to be due to the low concentration of propolis components in the fraction resulting in low antimicrobial activity against the microorganisms tested. It is thought that the effectiveness will be higher if higher components are obtained in the fraction.

Our findings also showed that antimicrobial activity of residue was moderate, higher than water soluble fraction and lower than propolis ethanol extract. This result could be explained by the synergy between phenolic and non-phenolic compounds of propolis. It was stated in literature that propolis is composed of plant resins containing phenolic and non-phenolic compounds like plant waxes, hydrocarbons, alcohols and esters (Hossain et al. 2022). Although biological activities like antioxidant and antimicrobial activity of propolis extract have been associated with its phenolic content (Silva et al. 2012), our finding somehow is opposing this consideration. When the phenolic compounds were separated from the ethanol extract, decreased antimicrobial activity was achieved.

This is the first study reporting the separation of active compounds from the propolis ethanol extract by combining the microencapsulation and homogenization techniques. For improving the yield, the effect of pH and ionic strength of water should be studied. Also the type of encapsulant used for microencapsulation might help the yield of

separation. It is clear that more study is required for improving the yield.

Conclusion: Propolis is a highly resinous material composed of plant resins and bees wax. Its highly apolar nature makes it not readily soluble in pure water. Although propolis contains some water soluble components nevertheless extraction of these components from the raw propolis by using pure water is not possible because of the chemical structure of raw propolis. In this study water soluble components of propolis were separated in two steps. Obtained fraction is completely soluble in pure water. Antimicrobial activity of ethanol extract, water soluble fraction and the residue left was tested against two bacterial strains responsible for conjunctivitis. It could be concluded that despite of the low antimicrobial activity of water soluble fraction, it might be a step in the production of eye drop for the treatment of bacterial conjunctivitis.

Author contributions: ÖEM and ŞK:Conceptualization, Project administration, Formal analysis, Data curation, Writing - original draft; YK: Formal analysis; ŞAK: and MK: Formal analysis, Data curation and Writing - review & editing.

Conflict of interest: There is no conflict of interest between the authors.

Data availability: The data used in the present study could be obtained from the corresponding author upon request.

Ethics: No animal or human experiments were performed during the conduct of the study.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

PREFERENCE BEHAVIOR TOWARDS MINERAL ELEMENTS BY HONEYBEE

Bal Arısının Mineral Elementlere Yönelik Tercih Davranışı

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ABSTRACT

The study was conducted to investigate honeybee preferences for various micronutrients and their concentrations. throughout the summer of 2021 at the Plant Protection Research Institute, ARC, Giza, Egypt, specifically at the apiary of the Bee Research Department. Forager bees showed strong avoidance responses only to high mineral concentrations (2, 1, 0.5%, and 0.25%) for sodium, potassium, calcium, and magnesium chloride. On the other hand, Foragers bees recorded a high visitation number in low concentrations (0.1, 0.05, 0.025 %, and 0.0125%) for 4 minerals and tap water. The honeybee prefers dilute sodium chloride and its low concentrations (0.0125%), which recorded a higher visitation number among all mineral concentrations under the study. In contrast, the bees exhibited no discernible preferences for the calcium chloride solutions with a low visitation number of 0.1 and 0.05% compared with tap water. The visitation numbers are similar in magnesium and potassium at 0.05, 0.025, and 0.0125% but higher than tap water. The solution was consumed at a concentration of 0.0125 after 139 minutes, a concentration of 0.025 after 142.5 min., and a concentration of 0.05 after a time had passed 157.5 min. The preference factor for NaCl solution was recorded at a concentration of (0.0125) Thus, the bees' preference for this concentration is higher than their preference for tap water. The lowest preference factor (0.4) was recorded with a $CaCl_2$ solution with a concentration of (0.1). low consumption ratios were recorded for 0.0125% potassium chloride (indicating a preference for the test solution), and higher consumption ratios were reported for 0.1% calcium chloride (indicating avoidance of the test solution).

Key Words: Honeybee, Preference, Elements, Solutions, Concentrations

ÖZ

Çalışma 2021 yazında çeşitli mikro besinler ve bunların konsantrasyonlarında bal arısı tercihlerini araştırmak için Mısır'ın Giza kentindeki ARC Bitki Koruma Araştırma Enstitüsü'nde Arı Araştırma Bölümünün arı kovanlarında gerçekleştirildi. Yayılmacı arılar yalnızca sodyum, potasyum, kalsiyum ve magnezyum klorür için yüksek mineral konsantrasyonlarına (%2, 1, 0,5 ve 0,25) güçlü kaçınma tepkileri gösterdi. Öte yandan, toplayıcı arılar için 4 mineral ve musluk suyu için düşük konsantrasyonlarda (%0,1, 0,05, 0,025 ve 0,0125) yüksek bir ziyaret sayısı kaydedildi. Bal arısı için seyreltik sodyum klorür ve onun çalışma kapsamındaki tüm mineral konsantrasyonları arasında daha yüksek bir ziyaret sayısı kaydeden düşük konsantrasyonlar (%0,0125) kaydedilmiştir. Buna karşılık arılar, musluk suyuyla karşılaştırıldığında %0,1 ve %0,05 gibi düşük bir ziyaret sayısına sahip kalsiyum klorür çözeltileri için fark edilebilir bir tercih sergilemedi. Ziyaret sayıları magnezyum ve potasyumda %0,05, 0,025 ve 0,0125'te benzerdir, ancak musluk suyu ziyaret sayılarından daha yüksektir. Çözelti, 139 dakika sonra

0,0125 konsantrasyonda, 142,5 dakika sonra 0,025 konsantrasyonda ve 157,5 dakika geçtikten sonra 0,05 konsantrasyonda tüketildi. NaCl çözeltisi tercih faktörü (0,0125) konsantrasyonda kaydedilmiştir. Dolayısıyla arıların bu konsantrasyonu tercihi, musluk suyu tercihinden daha yüksektir. En düşük tercih faktörü (0,4), konsantrasyonu (0,1) olan bir $CaCl_2$ çözeltisi ile kaydedildi. Bunun yanında %0,0125 potasyum klorür için düşük tüketim oranları kaydedilmiştir (test solüsyonunun tercih edildiğini gösterir) ve %0,1 kalsiyum klorür için daha yüksek tüketim oranları rapor edilmiştir (test solüsyonundan kaçınıldığını gösterir).

Anahtar Kelimeler: Bal Arısı, Tercih, Elementler, Çözümler, Konsantrasyonlar

GENİŞLETİLMİŞ ÖZET

Amaç: Bu deney, bal arısının 8 konsantrasyondaki sodyum, potasyum, kalsiyum ve magnezyum klorür çözeltileri ve musluk suyu tercihini, birincil tercih deneyleri olarak 90 dakika boyunca gözlemlenen ortalama bal arısı ziyareti sayısına göre ayrı ayrı değerlendirmeyi amaçlamaktadır.

Gereç ve Yöntem: Tablo 1'den, yiyecek arayan bal arılarının yalnızca sodyum, potasyum, kalsiyum ve magnezyum klorür için yüksek mineral konsantrasyonlarına (2,0, 1,0, 0,5 ve 0,25) güçlü bir tepki göstermediği açıktır. Öte yandan yiyecek arayan arılar için dört metalin ve musluk suyunun düşük konsantrasyonlarında (%0,1, %0,05, %0,025 ve 0,0125) çok sayıda ziyareti kaydedildi. Bu nedenle, daha yüksek konsantrasyonlar ana tercih çalışması denemesinin dışında tutuldu. Arı ziyaretlerinin ortalama sayısına göre mineral konsantrasyonu tercihleri, NaCl, KCl, $MgCl_2$ ve **$CaCl_2$** için Tablo (2)'de gösterilmektedir.

Bulgular ve Sonuçlar: Bulgular, çalışma sırasında bal arılarının tüm tuz çözeltilerinin tüketiminin önemli ölçüde değiştiğini göstermektedir. Genel olarak tuz çözeltisi tercihi, tuzun türüne ve konsantrasyonuna göre belirleniyordu. Bal arısı seyreltik sodyum klorürü ve düşük konsantrasyonlarını (%0,0125) tercih etmekte, bu da çalışma kapsamındaki tüm mineral konsantrasyonları arasında daha yüksek bir ziyaret sayısı kaydedilmesine neden olmuştur (52,1 ± 13,3). Ziyaret sayısı %0,1 ve %0,05 gibi düşük olan kalsiyum klorür çözeltileri ise musluk suyuna kıyasla arılar tarafından özellikle tercih edilmemiştir. Ayrıca, ziyaret sayıları ortalama olarak magnezyum ve potasyum bakımından %0,05, 0,025 ve 0,0125 ile benzerdir ancak musluk suyu ziyaret sayılarından daha yüksektir. Zaman tercihle ters orantılıdır. Arılar çözeltiyi ne kadar çok tüketirse tercihi de o kadar az olur. Arılar çözeltiyi ne kadar az tüketirse tercih de o kadar fazla olur. Bu süre dakika olarak tahmin edildi. Bal arıları musluk suyuna kıyasla NaCl'yi ve konsantrasyonlarını tercih eder. Bal arıları seyreltilmiş sodyum klorürü ve onun düşük konsantrasyonlarını tercih eder. Çözelti, 139 dakika sonra %0,0125 konsantrasyonda, 142,5 dakika sonra %0,025 konsantrasyonda ve 157,5 dakika geçtikten sonra %0,05 konsantrasyonda tüketildi. Arıların solüsyonu tüketmesi için en uzun süre, sodyum klorür solüsyonunun %0,1 oranında konsantre edildiği zamandı ve 163,5 dakikaydı. Potasyum klorür çözeltisinin konsantrasyonda (%0,1) tüketim süresi 171,0 dakikaya, konsantrasyonda minimum çözelti tüketim süresi (%0,0125) ulaştı. 123,0 dakikaydı. Aynı durum, en düşük tüketim süresinin düşük konsantrasyonlar için, en yüksek tüketim süresinin ise yüksek konsantrasyonlar için olduğu kalsiyum ve magnezyum çözeltileri için de geçerlidir. NaCl çözeltisi tercih faktörü (0,0125) konsantrasyonunda kaydedilmiştir. Dolayısıyla arıların bu konsantrasyonu tercihi, musluk suyu tercihinden daha yüksektir. En düşük tercih faktörü (0,4), konsantrasyonu (0,1) olan $CaCl_2$ çözeltisi ile kaydedildi. Ek olarak Şekil (2)'de %0,0125 potasyum klorür için düşük tüketim oranları kaydedilmiştir (test solüsyonunun tercih edildiğini gösterir), %0,1 kalsiyum klorür için daha yüksek tüketim oranları rapor edilmiştir (test solüsyonundan kaçınıldığını gösterir). Son olarak, sonuçlar, çalışma sırasında bal arılarının musluk suyuna kıyasla tuz çözeltisini daha güçlü bir şekilde tercih ettiğini ve bu tercihin türe ve konsantrasyona bağlı olduğunu, arıların minerallerle ilgilendiğini ortaya koymaktadır.

INTRODUCTION

Whether for humans or animals, water is the most essential component of existence and cannot be ignored. Bees, like other insects, consume water to quench their thirst and provide their bodies with the water they need for bodily reactions to keep them alive. As the temperature rises in the summer, as in months 7, 8, and 9 their need for water increases. (Khan et al. 2021)

Nectar, which has about 60% water by volume (Nicolson 2008)**,** provides the water bees need. It has been observed that bees gather huge amounts of water throughout the summer to reduce heat stress and to maintain colonies at an ideal humidity level, which should not be less than 75% (Ellis et al. 2008), which is necessary for bee eggs to hatch without deformations and for the duration of the broods normal development.

An apiary with 100 colonies uses 350 liters of water per week, and a single hive typically uses half a liter of water per day while raising brood. The higher the temperature, the more water bees consume up to 3 liters per day in some cases. The bee needs five minutes to bring the water to the hive, dump it inside, and then store it in its body. The closer the distance and the hotter the environment, the more frequently it does this process 1 to 7 times each hour. (Abrol et al. 2012, Al-Kahtani et al. 2020, Chakrabarti et al. 2020)

No matter if it is surface, ground, or even rainfall collected in domestic wells, drinking water contains a variety of elements in the form of dissolved salts or suspended matter. It may be argued that calcium, magnesium, sodium, and potassium are the elements that are most concentrated in the majority of drinking water (Ricigliano 2020, Wright et al. 2018, Zhang and Xu 2015). They can be considered the four primary mineral components of drinking water. They are present in the form of salts that combine with sulfates, carbonates, chlorides, and other groups. Other elements, such as iron or manganese, or trace amounts of uncommon elements found in nature, such as cadmium, lead, and others, are present in water in smaller quantities (Hafeez et al. 2019).

According to research studies (Baumgartner and Roubik 1989, Bänziger et al. 2009, Ferry and Corbet 1996), sodium, magnesium, and potassium are important for the growth of larvae in honey bees. It is believed that salts from water may be a crucial component of the brood food provided by nurse bees (Herbert and Shimanukia 1978).

Perhaps because it is frequent and contains salts, honeybees prefer water runoff from cities or farms (Hooper, 1932; Butler, 1940). These preferences are not well understood, though. Honey bees have strong group-foraging preferences for water with particular salt concentrations, as demonstrated by (Butler (1940)**.** Foraging bees are drawn to the presence of other bees due to their social facilitation (Avarguès-Weber et al. 2015), making it challenging to distinguish between group and individual preferences for foraging. Little progress has been made since Butler (1940) in our understanding of the salt preferences of water foragers. When salt concentrations rise to a point where drinking the water becomes unpleasant, or when other polluting chemicals, like heavy metals or nitrates, have concentrations that are too high, the problem of salts in drinking water arises (Adgaba et al. 2020).

Higher levels of potassium and phosphate in nectar can repel nectar foragers (Afik et al. 2006, Hagler et al. 2011), additionally, a high enough concentration of salt can act as a punishing stimulus (Abou-Shaara 2012, Letzkus et al. 2006). Individual water foragers' salt preferences, however, remain unknown. It is crucial to comprehend these salt preferences to comprehend honey bee biology and, possibly, to develop salt additions that would prevent bees from collecting agricultural water contaminated with dangerous xenobiotics (Adgaba et al. 2020).

Honeybees frequently gather water from various unfavorable places, including puddles on top of cow dung and sewage effluent and rainwater gutters loaded with decomposing organic debris. They avoid using the pure water sources that are available in the apiary for their usage (de-Sousa et al. 2022).

The bee automatically extends its proboscis to drink if the concentration is suitable. Phosphate may discourage nectar foragers since Nacl, $Mgcl_2$, and Kcl are crucial bee nutrients (Afik et al. 2006).

The study aims to determine which ingredients in water honeybee foragers prefer. It also seeks to identify the concentrations that appeal to and repel them. To discover salt concentrations in bee-collected water, the research also aims to study the behavioral mineral selection of honey bees.

MATERIALS AND METHODS

The current study was conducted throughout the summer of 2021. Forty colonies with open-mated hybrid queens of the same age in preparation for the experiment. The general micronutrient requirements of sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), and magnesium chloride ($MgCl_2$) were tested to see which the honey bees preferred in eight concentrations for each mineral (2.0,1.0, 0.5, 0.25, 0.1, 0.05, 0.025, 0.0125% w/v) and tap water as a compared solution (Lau and Nieh 2016). There were three to four preference assays

conducted each week. For this, a 2-meter-long wooden table with salt solutions was set up. Tap water was also placed on the table for comparison, the distance between the water source and the colonies was 2 meters. Bees stand on the walls of the cup to drink water. A hundred (100) mL of the assigned solution was added to a plastic cup (125 mL) at the beginning of each trial, and the cups were then arranged randomly on the table each day. During the first trial, every mineral was placed individually in 8 concentrations. In the main preference experiment trial, the four minerals with different concentrations were placed randomly together in the table. The measurements were taken by continuously monitoring the bees throughout the day with a digital video camera Sony DSC-W810 (20.1 MPixels) placed 1 meter away from element solutions, The digital camera records the salt water and tap water data of the bees throughout the day via a memory card, throughout the summer, from mid-June to mid-September. All other water sources in the apiary area were closed the bees were guided to use these cups for drinking rather than looking for another source.

Test solutions contained different concentrations of NaCl, KCl, $MgCl_2$, $CaCl_2$ (ACS reagent grade compounds, 99.8% purity, Fisher Chemical in distilled water.

Primary experiments

This experiment aims to exclude solutions of elements that the bees do not prefer, through the total number of visits made to the solution within 90 minutes. We preferred an hour and a half because for the first 20 minutes, the bees are circling over the solutions and no decision on preference is made. The other hour, the bees decide on their preference, and during an hour and a half, the solutions are mostly available to bees. After that, a statistical analysis was performed for each solution element alone because the measurement was done at the element level in the primary experiment.

Main preference experiment:

In this experiment, solutions of elements were placed together 4 elements *4 concentration with tap water as compared to solution. It was repeated 15 times for 15 days.

Four concentrations of chloride salt solutions were stabilized in comparison to tap water using the following 17 treatments: hundred (100) ml of each concentration of 0.1%, 0.05%, 0.025%, and 0.0125 % w/v for NaCl, KCl, $MgCl_2$, $CaCl_2$, and tap water.

A digital camera recorded the number of worker bees visiting the solutions. The amounts of salt solutions and tap water consumed throughout the day from morning to afternoon are counted and used to track the bees' preferences for each treatment and the number of bees attracted to it.

To ascertain the honeybees' preference behavior, the locations of the solutions and their various concentrations varied daily.

Studying parameters:

1- Number of honeybee visitation observations per 90 minutes (Cairns et al. 2021).

2- different mineral solution concentrations in primary preference experiments.

3-The mean time (minute) for a honeybee to consume 100 mL of mineral solution.

4- Calculate the Preference index for bee visits **=**

The number of visits for each solution

The number of visits to tap water (as a control).

-When the preference factor equals one, the bees' preference for the element solution is equal to the bees' preference for tap water.

-When the preference factor is greater than one, the bees' preference for the element solution is greater than the bees' preference for tap water.

- But the preference factor is less than one, and the bees' preference for the element solution is weak compared to the control (tap water).

5- Calculate the preference index for solution consumption time =

The time the bees consume 100 ml of the element solution

The time the bees consume 100 ml of tap water (control)

That means the shorter the consumption time, the greater the preference.

6- Statistical analysis

Means were statistically evaluated using a completely random block design (RCBD) and a two-way ANOVA using the MSTAT program (Snedecor and Cochran, 1980) and Prisma software. To compare the data, Duncan's test was employed (Duncan, 1955).

Picture 1: Mineral preferences experiment table study

RESULTS

A. Primary experiments

Preference of foragers honeybees for different mineral concentrations individually

This experiment aims to evaluate the honey bee preference for sodium, potassium, calcium, and magnesium chloride solutions in 8 concentrations and tap water individually by the mean number of honeybee visits observed for 90 minutes as primary preference experiments. From Table 1, it is clear that forager bees showed strong avoidance responses only to high mineral concentrations (2.0, 1.0, 0.5, and 0.25%) for sodium, potassium, calcium, and magnesium chloride, with a significantly different response. On the other hand, Foragers bees recorded a high visitation number in low concentrations (0.1, 0.05, 0.025, and 0.0125%) for 4 minerals and tap water. Therefore, high concentrations were excluded from the main preference study experiment.

Table 1. The mean number of honeybee visitations observations per 90 minutes for eight mineral solution concentrations (%w/v).

Mineral concentrations	NaCl	KCl	$MgCl_2$	$CaCl_2$
2.0	2.0 ± 1.0	0.0 ± 0.0	0.7 ± 0.5	0.7 ± 0.0
1.0	7.0 ± 1.0	6.7 ± 0.6	4.0 ± 1.0	0.0 ± 0.0
0.5	6.7 ± 1.5	5.0 ± 3.0	11.7 ± 6.5	0.7 ± 0.6
0.25	36.7 ± 13.5	18.7 ± 7.5	34.0 ± 12.0	2.0 ± 2.0
0.1	61.7 ± 13.5	24.0 ± 2.0	48.0 ± 5.0	11.3 ± 1.5
0.05	43.7 ± 2.5	31.7 ± 0.6	73.7 ± 4.6	49.7 ± 6.5
0.025	48.7 ± 0.6[a]	40.0 ± 3.0	65.7 ± 7.5	31.0 ± 19.0
0.0125	23.0 ± 1.0	39.7 ± 0.6	59.0 ± 14.0	62.0 ± 1.0
Tap water	30.7 ± 1.5	29.0 ± 4.0	40.0 ± 13.0	92.5 ± 5.5
LSD $_{0.05}$	**8.9**	**4.5**	**11.7**	**9.6**

B. Main preference experiment:

1. Number of honeybee visitation observations

Mineral concentration preferences by the mean number of forger honey bee visits observed per 90 minutes are indicated in Table (2) for NaCl, KCl, $MgCl_2$, and $CaCl_2$ in four concentrations compared with tap water. The findings showed that during the study, honey bees' consumption of all salt solutions varied significantly. In general, one's preference for a salt solution was determined by the type and concentration of the salt. The honeybee prefers dilute sodium chloride and its low concentrations (0.0125%), which recorded a higher visitation number among all mineral concentrations under the study **(**52.1 ± 13.3). The calcium chloride solutions with a low visitation number of 0.1 and 0.05%, on the other hand, did not appear to be particularly preferred by the bees compared with tap water. In addition, visitation numbers are on average similar in magnesium and potassium at 0.05, 0.025, and 0.0125% but higher than tap water visitation numbers.

Table 2. The mean number of honeybee visitations observations per 90 minutes for four mineral solution concentrations (%w/v).

Salts	Mineral concentrations (w\v)				Tap water	LSD $_{0.05}$
	0.1	0.05	0.025	0.0125		
NaCl	35.6 ± 10.4 ^{c}	27.7 ± 7.1 ^{e}	45.2 ± 17.2 ^{b}	52.1 ± 13.3 ^{a}	24.5 ± 9.4	**10.9**
KCl	21.5 ± 8.0 ^{f}	32.2 ± 8.4 ^{d}	37.8 ± 14.6 ^{b}	37.8 ± 10.2 ^{b}		**9.4**
$MgCl_2$	33.2 ± 11.2 ^{d}	33.8 ± 8.1 ^{d}	33.2 ± 10.7 ^{d}	35.2 ± 10.8 ^{c}		**9.13**
$CaCl_2$	10.1 ± 4.8 ^{g}	16.3 ± 7.7 ^{g}	21.7 ± 6.2 ^{f}	28.8 ± 11.2 ^{e}		**7.4**
LSD $_{0.05}$	**8.2**	**7.4**	**11.1**	**10.0**		

This means that the rows and columns that have the same letter, are not significantly different at 0.05 level of probability.

2. The mean time (min.) for a honeybee is to consume 100 ml of mineral solution

Table (3) shows the time required to complete 100 ml of salt solutions of the elements. The time is inversely proportional to preference. The more time the bees consume the solution, the less the bees' preference. The less time the bees consume the solution, the greater the preference. This time was estimated in minutes. Honey bees prefer NaCl and its concentrations compared to tap water. Honeybees prefer diluted sodium chloride and its low concentrations. The solution was consumed at a concentration of 0.0125% after 139 minutes, a concentration of 0.025% after a time of 142.5 min., and a concentration of 0.05% after a time had passed 157.5 min. The longest time for bees to consume the solution was when the sodium chloride solution was concentrated at 0.1% was 163.5 min. The potassium chloride solution's consumption time at the concentration (0.1%) reached 171.0 min., and the minimum solution consumption time at the concentration (0.0125%) was 123.0 min. The same applies to calcium and magnesium solutions, where the lowest consumption time was for low concentrations and the highest consumption time was for high concentrations.

Table 3. The mean time (min) for honeybee is to consume 100 ml of mineral solution (%w/v)

Mineral solution	Mineral concentrations (w\v)				Tap water	LSD $_{0.05}$
	0.1	0.05	0.025	0.0125		
NaCl	163.5 ± 25.9 ^{b}	157.5 ± 19.0 ^{b}	142.5 ± 36.9 ^{d}	139.5 ± 22.4 ^{d}	199.8+48.1	**29.1**
KCl	171.0 ± 24.7 ^{b}	147.3 ± 23.1 ^{c}	138.0 ± 28.1 ^{d}	123.0 ± 24.3 ^{e}		**28.1**
$MgCl_2$	150.0 ± 23.5 ^{c}	156.0 ± 23.7 ^{b}	150.0 ± 18.7 ^{c}	142.5 ± 17.7 ^{d}		**25.8**
$CaCl_2$	190.5 ± 30.0 ^{a}	187.5 ± 32.6 ^{a}	175.5 ± 27.4 ^{b}	163.5 ± 29.5 ^{b}		**31.1**
LSD $_{0.05}$	**28.7**	**28.1**	**30.1**	**27.4**		

This means that the rows and columns that have the same letter, are not significantly different at 0.05 level of probability.

3. Visitation frequency ratios

Figure (1) shows the frequency of visits for different mineral solution concentrations by calculating the preference factor. The preference factor for NaCl solution was recorded at a concentration of (0.0125) Thus, the bees' preference for this concentration is higher than their preference for tap water. The lowest preference factor (0.4) was recorded with a $CaCl_2$ solution with a concentration of (0.1). In addition in Figure (2), low consumption ratios were recorded for 0.0125% potassium chloride (indicating a preference for the test solution), and higher consumption ratios were reported for 0.1% calcium chloride (indicating avoidance of the test solution).

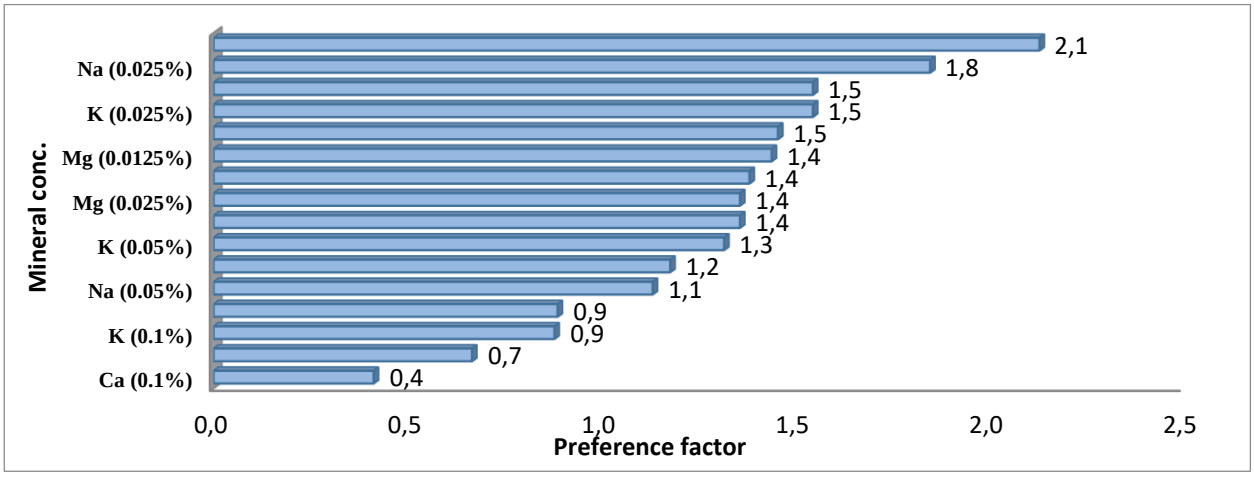

Figure 1. Visitation frequency ratios of different mineral solution concentrations.

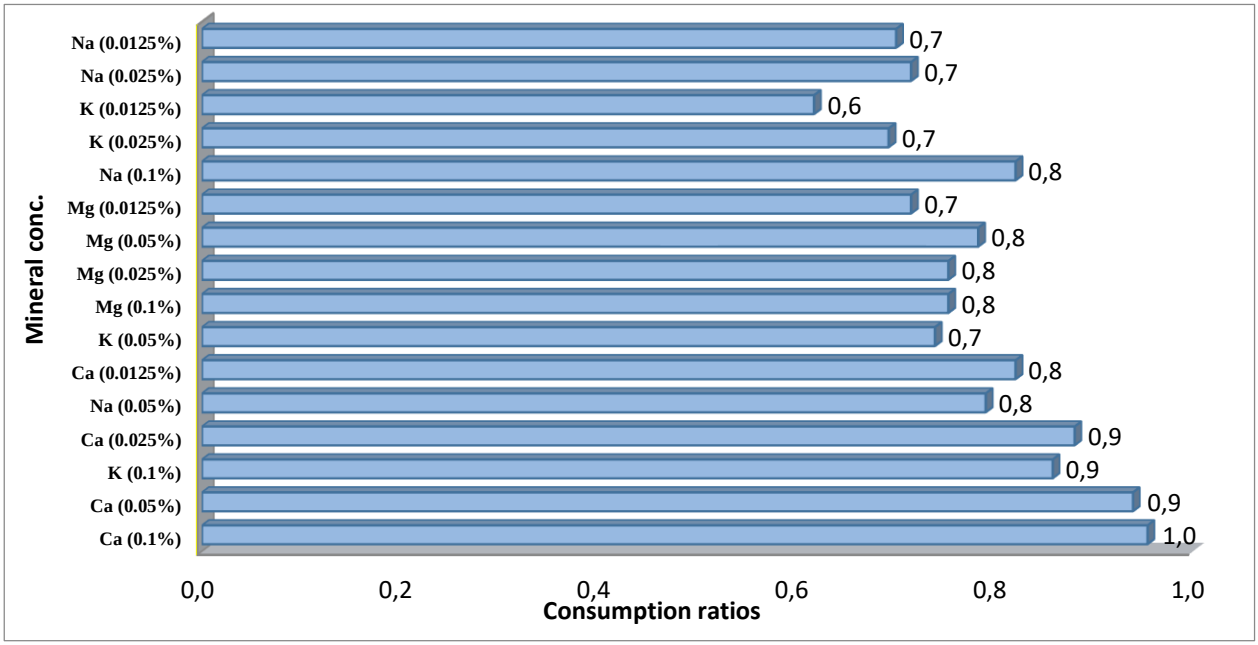

Figure 2. Consumption ratios for different mineral solution

DISCUSSION

The present study revealed that honey bees showed different micronutrient preferences. This confirms our theory that honey bees hunt for minerals lacking in their floral diet by foraging in contaminated water. Since these minerals are the most concentrated in honey bee products like honey, pollen, and royal jelly, four minerals in water solutions sodium (Na), potassium (K), calcium (Ca), and magnesium (Mg) were investigated. and because bees have a high need for these minerals additionally, sodium and potassium play important roles in the neurotransmission process in honeybees this finding is in line with the theories put forth by (Harrison 1987, Herrod-Hempsall 1931, Khan et al. 2021) mentioned that the instead of using the clean water source that is available in the apiary for their consumption, honeybees frequently collect water from a variety of undesirable sources, including runoff sewage, cow manure puddles, and gutters clogged with rotting organic debris. Therefore, it was crucial to identify

what draws bees. Bees prefer salty water to pure water, according to experiments already done. Honey bees like to feed on minerals for their physiological activities and functions, such as muscular movement (Chakrabarti et al. 2020, Day et al. 1990, Wang et al. 2013), honey bees preferentially consume various minerals and salts. In the experiment with the single element at gradual concentrations, it was observed that it prefers low concentrations before gradually moving to higher concentrations. Different salts seemed to appeal to different types of bees. Thus, the type of salt had a significant impact. It was noted that calcium is weakly preferred in comparison to sodium, potassium, and magnesium. These findings support the findings of (Butler (1940) and, Cairns et al. (2021), and others who concluded that the bees preferred low concentrations of the element solution and did not favor it at higher concentrations when it was introduced at various concentrations. The findings support the assertions made by (Avarguès-Weber et al. 2015, Letzkus et al. 2006).Our results confirm earlier studies that found honey bees to have a preference for Na in "dirty water" (Bonoan et al., 2016), with the highest proboscis extension reflex (PER) to 1.5% NaCl solutions (Lau and Nieh 2016) and a preference for 0.29% NaCl over distilled water (Butler 1940). Similar findings were published by Lau and Nieh (2016), who found that forager bees strongly preferred a particular concentration of potassium, sodium, magnesium, and phosphate over deionized water.

Honey bee PER responses significantly decreased above 0.75% of this compound, according to Butler (1940), Lau and Nieh (2016), who reported very few honey bee visitations at 1.42% Na_2HPO_4. According to research by Bonoan et al. (2016 and 2018), foraging preferences for water solutions with 1% NaCl and $MgCl_2$ followed changes in pollen. Using tamed honeybees, Lau and Nieh (2016) found that the concentrations of NaCl, KCl, and $MgCl_2$ in water solutions ranged from 0.1% to 1.5%. Except for high Na, bees rejected high mineral concentrations in sucrose solutions; only high Fe and Cu concentrations caused an increase in total water intake when compared to the control. The fact that bees did not favor 1000 ppm of K diets over sucrose alone was also unexpected (De Sousa et al. 2022). Although honey bees have been observed to favor solutions containing 1500 ppm of K over sucrose alone, these authors eventually discovered that the acceptance-rejection concentrations of nectar minerals are species- and concentration-dependent for K (Afik et al. 2014). This corroborates earlier studies (Butler 1940, Lau and Nieh 2016) in which honey bees demonstrated aversions to K concentrations exceeding 1.5%. Similarly, Bonoan et al. (2018) discovered that honey bees avoided calcium during the summer and drank less of it than they did of Na.

Such as bees have found that the most effective way to obtain a balanced diet is to forage on multiple resources simultaneously. Over several hours of our observation, bees were noticed when experimental solutions were placed, hovering around all the solutions and taking approximately 20 minutes to determine the preferred solution. When this solution ended, we thought it would take some time to reassess the other solutions, but when this solution ended, the next preferred solution was already approaching it. This indicates that the initial time (20 minutes) was spent evaluating all the solutions, organizing them to be preferred, and memorizing them for bees. The significance of micronutrients like calcium, magnesium, and sodium in honeybee diets has not received much attention from studies (Black 2006, Brodschneider & Crailsheim 2010), the micronutrient needs of honeybees vary depending on the season. Most observers typically attribute honeybees' preference for drinking water to the amount of salt present. However, there are at least four key components that are likely to be significant: sight, (Chakrabarti et al. 2020, Jaleel et al. 2020) description of the sense of water perception, the perception of numerous odor chemicals present in the water, and the sense of taste after being submerged. The honeybee prefers to consume the water in the time afternoon because of the high temperature in the summer season, the time it takes for one flight to collect water is significant, as the bee spends a minute or more taking the load of water and spends one minute flying a distance. Honeybees do not typically collect a lot of water in the morning, and the majority of the water sources are consumed at noon (Bänziger et al. 2009, Nawaz et al. 2020).

The water-collecting bee travels 400 meters, spends 2 to 3 minutes inside the hive, and makes 100 visits. It is crucial to understand the nutrient and mineral requirements of bees because this knowledge will help in the creation of a synthetic diet for honeybees. According to the needs of the honeybee colonies, enhances the colony's health and could improve beekeeping and assist in developing a full diet for honeybees (Khan et al. 2021).

Conclusion: The results showed that honey bees have a strong preference for salt solution compared to tap water depending on the type and concentration of the element. This study has implications in applied and basic sciences for understanding the mineral-selective behavior of honey bees and determining the appropriate and preferred concentrations of the mineral solution Na, K, Mg, and Ca, and they can self-select minerals based on concentration; they can control the intake of low concentrations and avoid high concentrations. Overall, collecting information about the minerals preferred by honey bees can help us better understand the nutritional ecology and overall health of honey bees.

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Declaration of interest: The authors declare that there is no conflict of interest.

Ethics: The Research was conducted in vitro and not with animals or humans.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

METHOD VALIDATION FOR DETERMINATION OF KYNURENIC ACID CONTENT IN NATURAL PRODUCTS BY RP-HPLC-UV

Doğal Ürünlerdeki Kynurenik Asit İçeriğinin RP-HPLC-UV ile Belirlenmesine Yönelik Yöntem Doğrulama

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ABSTRACT

Kynurenic acid (KYNA) is a metabolite with pharmacoactive properties found primarily in chestnut honey, linden, and other honeys. Considering the anti-inflammatory and immunosuppressive functions of KYNA, it can be seen that it has bidirectional effects on biological pathways. For this reason, determining and knowing the amount of honey, an important natural product can have a high impact on health. Studies on the detection of this metabolite in both natural products and animal tissues are ongoing, and it is important to develop fast and easily applicable methods. Within the scope of this study, a new method was created using an ultraviolet detector (RP-HPLC-UV) in reverse-phase high-performance liquid chromatography to determine the KYNA content of natural products (honey, chestnut pollen, and chestnut flowers) quantitatively in a short time. According to the data obtained, LOD and LOQ values were found to be 0.030 µg/mL and 0.092 µg/mL, respectively. At the same time, solutions of KYNA prepared in ultrapure water (UPW), 70% EtOH, EtOH and MeOH solvents were analyzed in this method, and it was found that UPW was the best solvent. The findings of this research can contribute significantly, particularly in the application of measuring KYNA, to distinguishing the botanical origin of chestnut honey.

Keywords: Kynurenic acid, Chestnut honey, HPLC-UV

ÖZ

Kynurenik asit (KYNA) başta kestane balı olmak üzere ıhlamur ve diğer bazı ballarda bulunan farmakoaktif özelliğe sahip bir metabolittir. KYNA'nın antiinflamatuar ve immünosüpresif fonksiyonu düşünüldüğünde biyolojik yolaklar için çift taraflı bir etkiye sahip olduğu görülebilir. Bu sebeple de önemli bir doğal ürün olan ballardaki miktarının belirlenmesi ve bilinmesi sağlık üzerinde yüksek bir etkiye sahip olabilir. Bu metabolitin hem doğal ürünlerde hem de hayvan dokularında tespitine yönelik çalışmalar devam etmekte olup, hızlı ve kolay uygulanabilir metotların geliştirilmesi önem arz etmektedir. Bu çalışma kapsamında doğal ürünlerin (bal, kestane poleni ve kestane çiçeği) KYNA içeriğinin kantitatif olarak kısa sürede belirlenmesi amacıyla ters faz yüksek performanslı sıvı kromatografisinde ultraviyole dedektörü (RP-HPLC-UV) kullanılarak yeni bir metot geliştirilmiştir. Elde edilen verilere göre LOD ve LOQ değerleri sırasıyla 0,030 µg/mL ve 0,092 µg/mL olarak bulunmuştur. Aynı zamanda KYNA'nın ultra saf su (UPW), %70 EtOH, EtOH ve MeOH çözücülerinde hazırlanmış çözeltileri bu metotta analiz edilmiş ve UPW 'un en uygun çözücüsü olduğu görülmüştür. Bu araştırmanın bulguları, özellikle kestane balının botanik kökeninin ayırt edilmesinde uygulanması açısından KYNA'nın ölçülmesine önemli ölçüde katkıda bulunabilir.

Anahtar Kelimeler: Kynurenik asit, Kestane balı, HPLC-UV

GENİŞLETİLMİŞ ÖZET

Amaç: Kinurenik asit (KYNA), C-4'te bir hidroksi grubu ile ikame edilmiş kinolin-2-karboksilik asit olan bir kinolinmonokarboksilik asittir. Yapılan bazı çalışmalarda, KYNA, özellikle kolit, kolon tıkanıklığı veya ülserasyonla ilgili olarak gastrointestinal sistemin çeşitli patolojilerinde olumlu özelliklere sahip olabileceği ifade edilmiştir. Temel olarak gastrointestinal sistemdeki olumlu özellikleri ve hipermotiliteyi azaltma yeteneği KYNA'nın gıdalardan alımı konusunda daha geniş bir araştırma yapılması ihtiyacını ortaya çıkarmaktadır. Örneğin ısırgan otu veya sarı kantaron, her ikisi de KYNA açısından zengin maddelerdir, sıklıkla sindirim sistemi hastalıklarının semptomlarını azaltmak için kullanılır. Bunların yanı sıra günlük hayatta tüketilen bal arısı ürünlerinden propolis ve ballarda da (kestane, ıhlamur vs.) yüksek KYNA içeriği olduğu bildirilmiştir. KYNA'nın biyokimyası ve biyolojik fonksiyonları göz önüne alındığında bal arısı ürünleri başta olmak üzere bazı doğal ürünlerdeki miktarının tespit edilmesi önem arz etmektedir. KYNA'nın tespitine yönelik çeşitli yöntemler önerilmiş ve çalışılmıştır. Ancak bu yöntemlerin bazılarında gerek analiz öncesi hazırlığın uzun olması gerekse analiz için gerekli olan ekipmanın kolay bulundurulamaz olması bazı zorlukları da yanında getirmektedir. Bu sebeple özellikle ballar başta olmak üzere doğal ürünlerdeki KYNA içeriğinin belirlenmesi için hızlı ve uygulanabilir bir yöntemin oluşturulması ve validasyonunun yapılması bu çalışmada amaçlanmıştır.

Gereç-Yöntem: Uygulanacak analizde örneklerin hazırlanmasında kullanılacak olan çözücünün belirlenmesi için farklı çözücüler ile analizler yapılmıştır. Aynı zamanda geliştirilecek olan metotta kullanılacak olan dalga boyunun seçimi için KYNA standardının 200-800 nm dalga boyu aralığında spektrum taraması yapılmıştır. Çalışmamız kapsamında çeşitli arı ürünleri ve bitki kökenli örnekler için KYNA içeriğinin belirlenmesine yönelik RP-HPLC-UV sisteminde hızlı ve kolay uygulanabilir metot geliştirmesi yapılmış ve validasyon çalışmaları kapsamında kesinlik, doğruluk, geri kazanım, bağıl hata, LOD ve LOQ gibi parametreler incelenmiştir.

Bulgular: Arı ürünleri ve diğer bazı bitki kökenli örneklerde KYNA içeriğin tespiti için UPW uygun çözücü olarak belirlenmiş ve MeOH, %70 EtOH ve EtOH ile hazırlanan standartların kromatogramlarındaki pikte omuzlanma veya genişleme olduğu gözlenmiştir. Metotta kullanılmak üzere KYNA'ya ait dalga boyunun belirlenmesi için yapılan spektrum taraması neticesinde 330 nm'nin analizde kullanılabileceği görülmüştür. Oluşturulan analiz metoduna ait validasyon parametrelerine bakıldığında aynı gün ve farklı günlerde geri kazanım değerleri sırasıyla %98,254 ve %97,762 olarak bulunmuştur. Standarda ait kalibrasyon grafiğinin R^2 değeri 0,999 ve LOD-LOQ değerleri sırasıyla 0,030-0,092 µg/mL olarak tespit edilmiştir. Analiz edilen örnekler içinden de CH5'in KYNA içeriğinin en yüksek olduğu belirlenmiştir.

Sonuç: Çalışmamız kapsamında KYNA analizinin özellikle balların botanik kökeninin değerlendirilmesine ve ayırt edici bir belirteç olarak kullanılmasına katkı sağlanması amaçlanmıştır. Bu bağlamda uygun maliyetli, kolay uygulanabilir ve minimum numune hazırlama adımları gerektiren bir yöntem geliştirilmiştir. Bu sayede kestane balları da dahil bazı arı ürünleri ile bitki kökenli bazı örneklerde KYNA analizinin karakterizasyonunun literatüre ve paydaşlara fayda sağlayabileceği düşünülmektedir.

INTRODUCTION

Kynurenic acid is a quinoline monocarboxylic acid that is quinoline-2-carboxylic acid substituted with a hydroxy group at C-4. Kynurenic acid (KYNA) is a tryptophan metabolite and has been reported to be present in living systems. Since its discovery in dog urine in 1853 (Liebig 1853), it has been found in insects (Smirnov et al. 2006), mammals (Moroni et al. 1988), plants (Starratt and Caveney 1996), and biological fluids and organs (Kuc et al. 2006, Kuc et al. 2008). Although the main product of kynurenine catabolism is nicotinamide adenosine dinucleotide (NAD+), KYNA is also produced due to the activity of kynurenine aminotransferases (Adams et al. 2012, Schwarcz et al. 2012). While the physiological functions of all TRP metabolites have not yet been elucidated, it is known that certain TRP metabolites, such as kynurenic acid (KYNA) and quinolinic acid (QA), exhibit bioactivity. It has been demonstrated that KYNA is an antagonist of ionotropic glutamate receptors (Mok et al. 2009, Alt et al. 2004) and alpha 7 nicotinic receptors (Hilmas et al. 2001). Since these two receptors are predominantly expressed in the brain, it has been indicated that KYNA is present in the brain (Moroni et al. 1988). In addition, it has been found that KYNA is an agonist of G-protein-coupled GPR35 receptors. It is noted that GPR35 receptors are primarily located in the gastrointestinal

system (Wang et al. 2006). In some studies, KYNA has been suggested to have positive properties in various pathologies of the gastrointestinal system, particularly colitis (Varga et al. 2010), colon obstruction (Walczak et al. 2011), or ulceration (Glavin and Pinsky 1989).

It has been reported that bee products, especially honey, are important in the pathophysiology of the gastrointestinal system and that this effect is due to various components (Coşkun and Coşkun 2022). Essentially, the positive properties in the gastrointestinal system and the ability to reduce hypermotility call for further extensive research on the intake of KYNA from foods (Varga et al. 2010, Kaszaki et al. 2008). For example, both nettle and St. John's Wort have been reported to be rich in KYNA and are frequently used as remedies to alleviate symptoms of digestive system disorders (Turski et al. 2011). In addition, it has been reported that propolis and honey consumed in daily life, including chestnut and linden honey, also have a high KYNA content (Turski et al. 2016, Pavlin et al. 2023).

Considering the biochemistry and biological functions of KYNA, determining its quantity in certain natural products, especially bee products, becomes crucial. Various methods have been proposed and studied for the detection of KYNA. However, some of these methods pose challenges, such as prolonged pre-analysis preparation (Turski et al. 2016) or the difficulty in obtaining the required equipment for analysis (Fukushima et al. 2022). Therefore, this study aims to establish and validate a rapid and applicable method for determining the KYNA content in natural products, especially honey.

MATERIALS AND METHODS

Samples

Honey samples were obtained from experienced beekeepers in different regions of Türkiye. Pollen samples were sourced from experienced beekeepers in Artvin and Trabzon. Chestnut flowers were collected from chestnut trees in the Trabzon province. All samples studied are from the year 2022.

Preparation for Analysis

An absorbance scan of the KYNA standard within the wavelength range of 200-800 nm was conducted to determine the wavelength to be used in the method. Additionally, various solvents are employed for extraction in natural products and bee products. The selection of an appropriate solvent for the analysis is as crucial as the analysis itself. In the new method to be employed for the determination of KYNA in natural products and bee products, different solvents, namely UPW, MeOH, EtOH, and 70% EtOH were used to dissolve standard KYNA (Sigma Aldrich) for analysis, aiming to reveal the effects of these solvents. All samples to be analyzed were extracted in UPW (1/5, w/v) and then filtered through ordinary filter paper (Turski et al. 2016). It was then analyzed by passing through 0.45 µm membranes. Each sample underwent three injections for robust analysis.

RP-HPLC-UV Condition

High-Performance Liquid Chromatography (HPLC) utilizing a UV detector (Elite La Chrom Hitachi, Japan), was employed for analyses. The method involved a reverse phase C18 column (250 mm x 4.6 mm, 5 µm) and utilized acetonitrile and water in an isocratic program. For RP-HPLC-UV analysis, the mobile phase comprised ultrapure water (UPW) and acetonitrile (Carlo Erba, France) in a ratio of 91:9, containing 20 mM ammonium acetate (Isolab, Germany) and 35 mM acetic acid (Merck, Germany). The sample injection volume was set at 20 µL, maintaining a column temperature of 25 °C, a flow rate of 0.7 mL/min, and an analysis duration of 15 minutes. The wavelength for analysis was set to 330 nm.

Method Validation

In the concentration range of 1.563 to 50 µg/mL, calibration curves were constructed, each composed of six data points replicated three times. Repeatability, accuracy, and detection limits were thoroughly investigated to validate the developed method in this study. Evaluation of validation parameters involved the calculation of relative standard deviation through the plotting of calibration curves. Limits of detection (LOD) and limits of quantification (LOQ) were established utilizing LOD = 3.3 × SD / m and LOQ = 10 × SD / m, where 'm' denotes the slope, and 'SD' represents the standard deviation at the lowest level of the calibration curves.

RESULTS

In studies analyzing KYNA in various samples, it is observed that different wavelengths are used (Sousa et al. 2021). Therefore, wavelength scanning was performed in present study, and it was decided that the optimum wavelength for our method is 330 nm (Fig 1). Various trials were conducted using different solvents for kynurenic acid prior to method validation. Optimal results were observed when kynurenic acid was dissolved in ultrapure water (UPW) (Fig 2). The comparative analysis of values obtained under identical conditions using alternative solvents revealed the following order: UPW > 70% Ethanol (70%EtOH) > Methanol (MeOH) > Ethanol (EtOH).

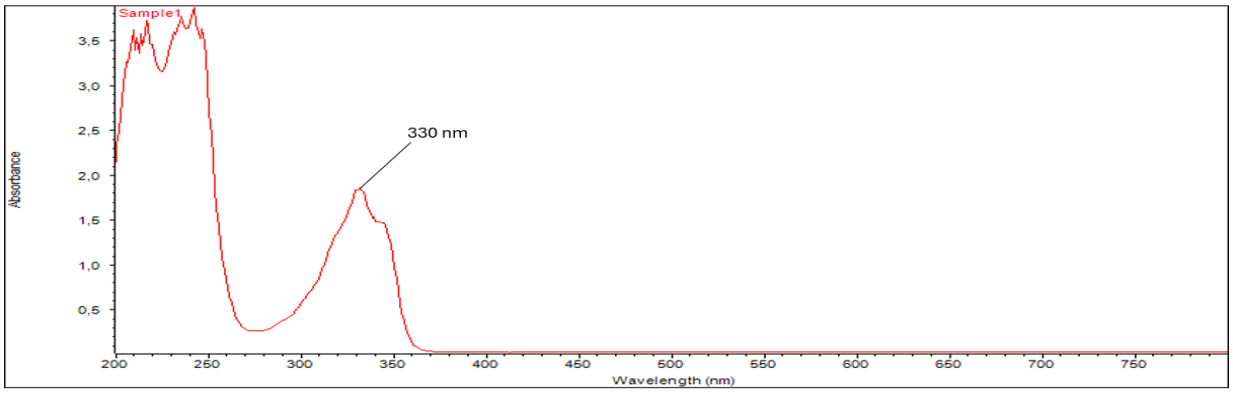

Figure 1. KYNA's wavelength scanning spectrum.

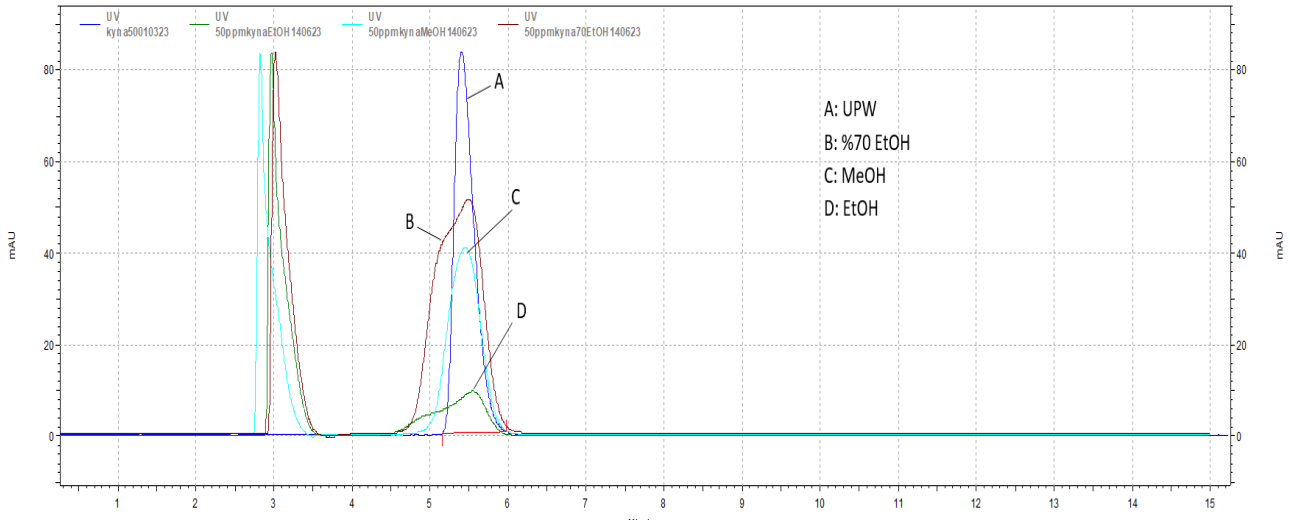

Figure 2. Chromatogram of the kynurenic acid standard in different solvents.

Chromatographic methods are commonly used in separation and purification processes. Considering the literature and our experiments, a UPW:ACN (91:9) solution containing 20 mM ammonium acetate and 35 mM acetic acid was used as the mobile phase in isocratic flow. At the optimized conditions, no interference was detected at the retention time corresponding to KYNA, validating the method's suitability for quantifying KYNA.

KYNA exhibited strong linearity with a correlation coefficient of $R^2 \geq 0.999$, while the recovery values for calibration standards were determined as 97.762% and 98.254% for inter-day and intra-day precision, respectively. Accuracy, reflecting the proximity of results to the true value, was evaluated

through absolute and relative error measurements. Absolute error quantifies the disparity between measured and true values, whereas relative error is calculated by dividing the absolute error by the true value. In present study, the relative error for KYNA was observed to be 0.017. Additionally, LOD and LOQ values for detection and quantification limits were determined as 0.030 µg/mL and 0.092 µg/mL, respectively (Table 1).

Table 1. Validation parameters of kynurenic acid

Kynurenic acid	
Linear range (µg/mL)	1.563-50
R^2	0.999
Relative Error	0.017
Limit of Detection (LOD) (µg/ml)	0.030
Limit of Quantification (LOQ) (µg/ml)	0.092
Recovery (%)	
Intra-day	98.254
Inter-day	97.762

As a result of the KYNA content analysis conducted on various honey, pollen, and flower samples using the developed method within the scope of the study, it was observed that the highest content was in chestnut honey (ChH5: 2688.949±0.257 µg/g). KYNA contents of the samples are given in Table 2. It is seen that the KYNA contents of chestnut honey vary between 192.254 and 2688.949 µg/g.

Table 2. Kynurenic acid content of various honey, pollen, and flowers samples

Sample	**Kynurenic acid (µg/g sample)**
Thyme honey (TH)	40.593±0,125
Parsley honey-1 (PH1)	84.977±0,247
Parsley honey-2 (PH2)	41.140±0.175
Lavender honey (LH)	43.169±0.163
Flower honey (FH)	45.194±0.109
Cedar honey (CeH)	47.463±0.118
Chestnut-oak honey (COH)	151.661±0.198
Chestnut honey-1 (ChH1)	192.254±0.252
Chestnut honey-2 (ChH2)	866.780±0.268
Chestnut honey-3 (ChH3)	1773.919±0.202
Chestnut honey-4 (ChH4)	1377.809±0.295
Chestnut honey-5 (ChH5)	2688.949±0.257
Chestnut honey-6 (ChH6)	930.548±0.226
Thyme honey (TH)	48.554±0.106
Oak honey (OH)	42.275±0.164
Astragalus honey (AH)	2.954±0.105
Chestnut pollen-1 (ChP1)	777.162±0.213
Chestnut pollen-2 (ChP2)	57.951±0.137
Chestnut flower (ChF)	15.245±0.116

The KYNA contents in chestnut pollen were observed to be 57.951 and 777.162 µg/g. Additionally, it has been observed that honey samples derived from various botanical origins also contain KYNA, albeit at relatively lower levels (Fig 3). Within the scope of present study, it was observed that the chestnut flower analyzed had the lowest KYNA content (15.245 µg/g).

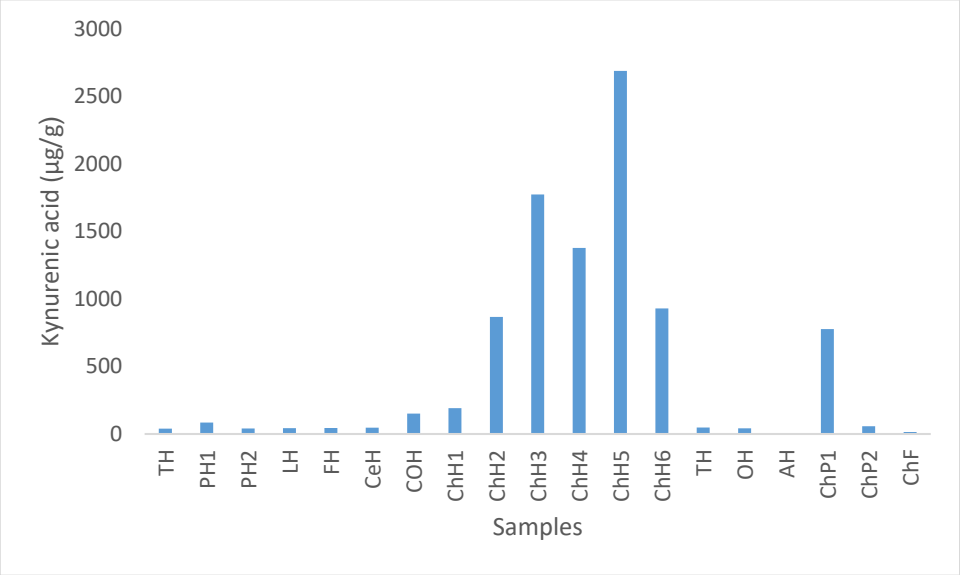

Figure 3. Visualization of the kynurenic acid levels in the samples.

DISCUSSION

Kynurenic acid (KYNA) is a tryptophan metabolite that exhibits a range of positive effects, including anti-inflammatory and antioxidative activities. It has been stated that the levels of tryptophan metabolites are important for assessing the stage of neurological disorders and could be evaluated for clinical diagnosis (Fukushima et al. 2022). It has been reported that KYNA is an agonist of the GPR35 receptors located in the gastrointestinal system (Wang et al. 2006), and it has been stated to have a positive effect on various gastrointestinal system pathophysiologies (Walczak et al. 2011, Wirthgen et al. 2018). Considering the bioactive properties of KYNA, its intake through the diet is also considered significant. According to the literature, some foods rich in KYNA can be listed as follows: nettle, St. John's Wort, certain parts of the chestnut tree, and chestnut honey samples (Turski et al. 2011, Pavlin et al. 2023).

The determination of KYNA content in foods has become important, especially in complementary medicine. In this context, the development of simple and easily applicable methods for determining KYNA content is crucial. Within the scope of our study, a rapid and easily applicable method using RP-HPLC-UV has been developed and validated. The wavelength specific to the analyte was first determined for the analysis conditions of the developed method. For this purpose, a spectrum scan of standard KYNA was performed, and 330 nm was identified as the optimum wavelength. Sousa et al. (2021) and Kim et al. (2022) reported conducting analyses at wavelengths of 344 nm and 240 nm, respectively, in their studies. Meanwhile, Beratta (2009) adjusted the UV-DAD detector to 327 nm in their study to conduct to analyze KYNA.

Subsequently, to determine the ideal solvent for analysis, a KYNA standard prepared in different solvents was analyzed using the established method. However, negative aspects such as shoulder formation or peak broadening were encountered with solvents other than UPW. Therefore, it was decided to use UPW both as the solvent for the standard substance and for the extraction of samples. Kim et al. reported a special extraction method for determining the KYNA content in chestnut honey samples, stating that the KYNA content in samples left to stand for 6 hours in 10% EtOH was higher (Kim et al. 2022). In their KYNA analysis studies, Sousa et al. (2021) and Lan-Gan et al. (2009) reported using distilled water to prepare their standard stock solutions. Although the use of distilled water has been reported, we also identified the potential of different solvents in our study.

In a study, a method was developed using HPLC-UV/FD, and ammonium formate/formic acid was employed as the buffer solution for the mobile phase

(Sousa et al. 2021). In another study, trifluoroacetic acid was used to create the mobile phase (Beretta et al. 2009). Lan-Gan reported using zinc acetate and sodium acetate in the mobile phase containing 6% ACN in their KYNA analysis study conducted using a fluorescence detector (Lan-Gan et al. 2009). In present study, to eliminate interferences other than the analyte and to accurately determine the analyte peak, we used ammonium acetate and acetic acid as buffers in our mobile phase containing 9% ACN.

It has been reported that chromatographic methods employing UV or fluorescence detectors are commonly used for the determination of TRP and its metabolites, especially in serum and plasma fluids (Fukushima et al. 2022). In a study, analysis was conducted using the HPLC-UV/FD method for the determination of TRP and its metabolites, and UV detection in isocratic flow was reported for KYNA analysis (Sousa et al. 2021). In another study, KYNA contents were analyzed using High-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (HPLC-ESI-MS) to determine markers related to the botanical origin of honey (Beretta et al. 2008). Turski et al. (2016) stated that they conducted the analysis of KYNA content in various chestnut honeys and parts of chestnut trees fluorometrically in their study. Pavlin et al. (2023) developed a method for the analysis of KYNA content using HPLC-MS/MS and reported its application in various honeys. In present study, we developed a simple and easily applicable method was developed for analyzing KYNA content in various honeys, chestnut pollen, and chestnut flowers using an HPLC device equipped with a UV detector.

The validation of the method developed in present study was conducted, and the LOD-LOQ values were determined to be 0.030-0.092 µg/mL, respectively. In studies aimed at determining KYNA content, it has been reported that some developed methods have LOD and LOQ values of 0.013-0.050 µg/mL (Sousa et al. 2021) and 0.001-0.010 µg/mL (Pavlin et al. 2023), respectively. Although the LOD and LOQ values may vary depending on the detector used, it was found that the obtained values are not significantly different from those reported in the literature.

KYNA can be obtained from various natural sources, including honey and various parts of certain plants. In present study, it is seen that the flora of the regions where chestnut honey is obtained creates significant differences in the KYNA content. It has also been observed that other honeys contain KYNA but at relatively lower levels. (Table 3). It is considered that the KYNA content of chestnut pollen may be related to its botanical origin. In a study conducted using 20 different botanical sources and 44 commercially available Italian honey samples, it has been suggested that chestnut honey exhibits significantly higher levels of KYNA content and could serve as a biomarker for chestnut honey (Beretta et al. 2008). In a study where a combination of HPLC-DAD-ESI MS and NMR techniques was used to analyze KYNA and some derivatives in various commercial honeys, it was emphasized that KYNA and its derivatives could be significant markers in chestnut honeys. Furthermore, in this study, it was suggested that KYNA and its derivatives may possess antinociceptive activity (Beretta et al. 2009).

In Slovenia, KYNA analysis was conducted using HPLC-MS/MS in 129 honey samples obtained from local beekeepers and commercially sourced. It was reported that chestnut and linden honey samples contained high levels of KYNA. Additionally, it was stated that the sample preparation process for this analysis was kept to a minimum, and they avoided complex extractions (Pavlin et al. 2023). It has been reported that KYNA analysis was conducted using HPLC-UV in chestnut honey samples collected from nine different regions of Korea. Additionally, it was stated that the most suitable method for obtaining high KYNA content in honey samples was to extract with 10% EtOH at a ratio of 1:20 for 6 hours (Kim et al. 2022).

Various honey samples collected from different Mediterranean countries and certain parts of the chestnut tree were analyzed for KYNA using HPLC with fluorometric detection. They reported that chestnut honey had the highest KYNA content, followed by the male flowers of the chestnut tree. Additionally, it was noted that the KYNA content was much lower in female chestnut flowers. Based on their findings, they observed that chestnut honey samples from Mediterranean countries could be rich in KYNA (Turski et al. 2016).

Conclusion: Within the scope of our study, the analysis of KYNA aimed to contribute particularly to the assessment of the botanical origin of honey samples and its use as a distinctive marker. It was aimed to overcome the complexity of the extraction conditions in previous studies, the lack of a wide range of uses of the method chosen for detection,

and the need for more information than in the current study for its use. In this context, present study aimed to develop a method that is cost-effective, easily applicable, and requires minimal sample preparation steps. This way, it is thought that the characterization of KYNA analysis in chestnut honey samples and some other related samples could provide benefits to the literature and stakeholders.

Author Contribution: Yakup Kara: analysis, investigation, writing–original draft; Sevgi Kolaylı: review & editing, research planning

Data Availability: The data can be found within the manuscript.

Declaration of interest: The authors state that they have no conflicts of interest to disclose.

Ethics: The research did not involve any animals or human.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

INFLUENCE OF ALUMINUM OXIDE NANOPARTICLES ON BIOLOGICAL FEATURES AND HOST HEMOCYTES OF *Galleria mellonella* L. (Lepidoptera: Pyralidae) WITH ITS ENDOPARASITOID *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae)

Alüminyum Oksit Nanopartiküllerinin *Galleria mellonella* L. (Lepidoptera: Pyralidae) ile Endoparazitoiti *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae)'nın Biyolojik Özellikleri ve Konak Hemositleri Üzerine Etkisi

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ABSTRACT

Nanoparticles (NPs) are released directly or indirectly into nature with increased production and consumption, and their effects on insects, which occupy a large place in the ecosystem, are of interest. There is also interest in the potentially toxic effects of NPs applied to hive pests on parasitoids, honey bees, and host-parasitoid relationships. The influence of aluminum oxide (Al_2O_3) NPs on the biological features of the hive pest *Galleria mellonella*, total counts of hemocyte, and hemocyte types; as well as on the biological features of the endoparasitoid *Pimpla turionellae* were investigated. The data obtained revealed that Al_2O_3 NPs caused a decrease in the larval, pupal, and adult development time of *G. mellonella*. The immature developmental time of *P. turionellae* was reduced. It was also demonstrated that Al_2O_3 NPs decreased the total counts of hemocytes in *G. mellonella* larvae; granulocyte, spherulocyte, oenocytoid, and prohemocyte counts decreased at all NP concentrations, while plasmatocyte counts increased. The data showed that Al_2O_3 NPs affected the biological properties of the hive pest model organism *G. mellonella* and indirectly affected its endoparasitoid *P. turionellae*. In addition, Al_2O_3 NPs showed a suppressive effect on cellular immune system responses, decreasing hemocyte counts. Our study results suggest that honey bees, honeycomb pests, and parasitoids may be negatively affected by NPs, which have increased in recent years as environmental pollutants, and that NPs may have insecticidal effects.

Keywords: Aluminium oxide nanoparticle, Biological features, *Galleria mellonella*, Hemocyte, *Pimpla turionellae*

ÖZ

Dünya çapında üretim ve tüketimin artmasıyla birlikte nanopartiküller (NP'ler) doğrudan ya da dolaylı olarak doğaya salınmaktadır ve ekosistemde büyük bir yer kaplayan böceklerde etkileri merak uyandırmaktadır. Ayrıca kovan zararlısına uygulanan NP'lerin parazitoitler üzerinde muhtemel toksik etkileri, diğer bir deyişle bal arıları ve konak-parazitoit ilişkileri ilgi çekmektedir. Bu nedenle alüminyum oksit (Al_2O_3) NP'lerin kovan zararlısı *Galleria mellonella*'nın biyolojik özellikleri, toplam hemosit sayısı

ve hemosit tipleri ile endoparazitoid *Pimpla turionellae*'nın biyolojik özellikleri üzerindeki etkisi araştırıldı. Elde edilen veriler, Al_2O_3 NP'lerin *G. mellonella*'nın larva, pupa ve ergin gelişim sürelerinde azalmaya neden olduğunu ortaya koydu. *P. turionellae*'nın ise olgunlaşma öncesi gelişim süresi kısaldı. Aynı zamanda Al_2O_3 NP'lerin *G. mellonella* larvalarındaki toplam hemosit sayısını azalttığı; granülosit, sferülosit, önositoid ve prohemosit sayılarının tüm NP konsantrasyonlarında azaldığı, plazmatosit sayılarının ise arttığı tespit edildi. Bulgular, Al_2O_3 NP'lerin kovan zararlısı model organizma *G. mellonella*'nın biyolojik özelliklerini etkilediğini ve endoparazitoiti *P. turionellae*'nın dolaylı olarak etkilendiğini gösterdi. Ayrıca Al_2O_3 NP'lerin hücresel bağışıklık sistemi tepkileri arasında yer alan hemosit sayılarının azalması ile sonuçlanarak baskılayıcı etki gösterdiği görüldü. Çalışma sonuçlarımız bal arılarının, petek zararlılarının ve parazitoitlerinin çevresel kirleticiler olarak son yıllarda artan NP'lerden olumsuz etkileneceği ve NP'lerin insektisidal etki gösterebileceği düşüncesini ortaya koymaktadır.

Anahtar Kelimeler: Alüminyum oksit nanopartikülü, Biyolojik özellikler, *Galleria mellonella*, Hemosit, *Pimpla turionellae*

GENİŞLETİLMİŞ ÖZET

Amaç: Ağır metaller ve bu metallerin oksitlenmiş nano yapıları günlük hayatta sıklıkla karşılaşılan toksik maddelerden biridir. Ağır metaller arasında yer alan alüminyum zorlu çevresel ve iklim koşullarına olan dayanıklılığı, hafif ve sünek yapılarından dolayı kolay şekil alabilmesi nedeniyle sıklıkla üretimde tercih edilmektedir. Alüminyumun oksijen ile tepkimesi sonucu oluşan alüminyum oksit (Al_2O_3) nanopartikülleri (NP) fiziksel ve kimyasal özellikleri nedeniyle birçok uygulama alanında diğer NP'lere kıyasla daha fazla ilgi görmektedir. Metallerin özellikle Al_2O_3 NP'ler gibi nanoparçacık formları vücuda beslenme, solunum ve deri yoluyla kolaylıkla alınmaktadırlar. Biyolojik olarak vücuttan atılımları kolay olmayan bu nano yapılı metal oksit türevleri canlı sağlığını tehdit etmektedir. Bununla birlikte son yıllarda Al_2O_3 NP'lerin böceklerde insektisit etkileri merak konusu olmuştur. Bu nedenle böcekler ve insanlar dahil tüm ekolojik sistemler üzerinde oluşturabileceği etkilerin belirlenmesine ihtiyaç duyulmaktadır. Bal arısı, *Apis mellifera* ve *Apis cerana*'nın bir zararlısı olan büyük balmumu güvesi *Galleria mellonella* bal arısı popülasyonlarında azalmaya neden olur ve bu zararlı türlerle mücadele etmek arıcılık endüstrisi için önemli bir sorun haline gelmiştir. Diğer yandan *Pimpla turionellae*, bu zararlıların endoparazitoidi olarak tanımlanır ve biyolojik mücadelede etkilidir. Çevrede artan NP konsantrasyonları direkt veya konak ile etkileşimleri sonucu dolaylı olarak endoparazitoitleri etkileyebilir. Bu nedenle çalışmada farklı konsantrasyonlarda Al_2O_3 NP'lerin konak *Galleria mellonella*'nın ve endoparazitoiti *Pimpla turionellae*'nın biyolojik özelliklerine etkisini incelemek amaçlandı. Aynı zamanda bu NP'lerin konak türün hemosit aracılı immün sistemine etkileri de belirlendi.

Gereç-Yöntem: *Galleria mellonella* larvaları 50, 100, 500 ve 1000 ppm konsantrasyonlarında Al_2O_3 NP içeren solüsyonlar hazırlanarak sentetik besinin su içeriğine eklendi ve ilk evre larvalardan son evre larvalara gelişinceye kadar beslendi. Al_2O_3 NP'ler sadece larval gelişim süresince uygulandı. Al_2O_3 NP'lerin *G. mellonella*'nın larva, pupa ve ergin gelişim süreleri ile ağırlık ve uzunlukları gibi yaşam döngüsü parametrelerine etkisi belirlendi. Pupaların bir kısmı parazitleme için kullanıldı. Parazitlemenin ardından bu NP'lerin endoparazitoit *P. turionellae*'nın olgunlaşma öncesi gelişim süresi ve ergin ömrü gibi yaşam döngüsü parametrelerine etkileri gözlendi. Bununla birlikte *P. turionellae*'nın ağırlık ve uzunluk gibi morfolojik özellikleri de kaydedildi. Konak türün tüm deney gruplarını oluşturan son evre larvalardan alınan hemolenf süspansiyonları Neubauer hemositometresine yüklendi ve total hemosit sayısındaki değişiklikler faz kontrast mikroskobunda gözlemlendi. Son olarak Al_2O_3 NP kaynaklı hemosit tiplerindeki değişiklikler Giemsa boyama yöntemi kullanılarak faz kontrast mikroskobunda belirlendi.

Bulgular ve Sonuç: *G. mellonella*'da Al_2O_3 NP'ler larval, pupal ve ergin gelişim sürelerini (gün) doza bağlı bir şekilde azalttı. *P. turionellae*'nın olgunlaşma öncesi gelişim süresinde doza bağlı bir şekilde azalma düşük dozlardan itibaren görüldü. Diğer taraftan *P. turionellae*'nın ergin ömrü, ağırlığı ve uzunluğunda önemli bir farklılık görülmedi. Çalışmamız metal Al_2O_3 NP'lere kronik olarak maruz kalan konak *G. mellonella*'nın biyolojik kontrol ajanı parazitoit *P. turionellae* ile etkileşimleri sonucu

gelişim sürelerini etkilediğini ortaya koymaktadır. Ancak bu NP'lerin konak ve parazitoitlerin morfolojik özelliklerine önemli etkileri gözlenmedi.

Al_2O_3 NP'lerin tüm deney gruplarında toplam hemosit sayısında azalmaya neden olduğu tespit edildi. Hemosit tiplerinden granülosit, sferülosit, önositoid ve prohemosit sayılarının tüm dozlarda azaldığı, plazmatosit sayılarının ise arttığı belirlendi. Elde edilen sonuçlar ile Al_2O_3 NP'lerin model organizma *G. mellonella*'nın hücre-aracılı bağışıklık sistemi üzerinde baskılayıcı etkileri olduğunu ortaya koymaktadır. Verilerimiz metal türevli Al_2O_3 NP'lerin *G. mellonella*'da insektisidal etki gösterebileceğini ve potansiyel insektisit olabileceğini vurgulamaktadır. Ancak ekosistemde önemli bir biyolojik kontrol ajanı olan *P. turionellae*'nın da NP kaynaklı toksisiteden etkilenebileceği belirlendi. Bu sonuçlar insan beslenmesinin önemli kaynağı olan bal arılarının da nanokirleticilerden etklenebileceğini ortaya koymaktadır. Bu nedenle Al_2O_3 NP'lerin daha iyi anlaşılması ve yönetimine dikkat edilmesi son derece önemli olacaktır.

INTRODUCTION

Since nanomaterials are increasingly used in a wide variety of fields, their toxic effects are a matter of curiosity. Among them, nanoparticles (NPs) are utilized in many industries from biomedicine to engineering (Bankier et al. 2019). They have an exceptional place in the industrial field due to their properties such as their nano size, high reactivity, and physical and chemical features. Metal and metal oxide NPs constitute more than 30% of the entire NP-containing products (Kumar et al. 2018, López-Muñoz et al. 2019). At the same time, metallic nano and microparticles, which can be comprised of both natural periods and anthropogenic factors are among the major causes of environmental pollution (Yanar et al. 2022). Nano-sized NPs may be more toxic to living organisms than their ionic forms (Tunçsoy 2018, Eskin and Bozdoğan 2022) and micro / macro-sized materials (Das et al. 2019). NPs may enter organisms through the respiratory or digestive system and may be carried by circulation to several organs and tissues. They can enter the cell through biological membranes through endocytotic transport processes such as phagocytosis, pinocytosis, or receptor-mediated endocytosis (Ahmad et al. 2019, Assar et al. 2022, Tuncsoy and Mese 2021). After that, they can cause cellular toxicity and become lethal factors (Assar et al. 2022, Tuncsoy and Mese 2021). Both *in vitro* and *in vivo* investigations have shown that metal oxide NPs have genotoxic (Sharma et al. 2009), carcinogenic, and mutagenic (Kumar et al. 2011, Pan et al. 2010) potentials.

Aluminum constitutes nearly 8% of the elements in the Earth's crust (Barabasz et al. 2002, Kara et al. 2020). Aluminum oxidizes spontaneously in the air to form aluminum oxide (Al_2O_3) NP with a prooxidant feature (Fricault 2018). Since metals are prone to hydrolysis in an aqueous environment, they can affect oxidoreduction processes in biological systems. A metal with a redox potential may produce reactive oxygen species by interfering with reactions such as Fenton that occur in living cells (Egorova and Ananikov 2017). Their toxicity on organisms is not adequately understood; research on their acute, chronic, and environmental toxicity is also inadequate (Ismail et al. 2021). Al_2O_3 NPs' impact on biological systems, including insects, has raised concerns. Studies indicate that these nanoparticles can induce oxidative stress, alter enzymatic activities, and affect cellular structures in insects (Kara et al. 2020; Demirtürk et al. 2023). For example, exposure to Al_2O_3 NPs may lead to changes in hemocyte counts and function, which are critical components of the insect immune system. However, some of them have been recommended as possible biopesticides for seed conservation apart from their normal usage (López-Muñoz et al. 2019, Sahayaraj 2017). Therefore, Al_2O_3 NPs may have an essential role in agricultural applications (López-Muñoz et al. 2019, Poborilova et al. 2013, Willhite et al. 2014) and may be used instead of traditional insecticides (Ismail et al. 2021). Such research results may contribute to its use in Integrated Pest Management.

Honey bees have a crucial role in ecosystem function as well as in agricultural production and are considered as essential pollinators. However, they face an increasing number of stressors, especially xenobiotics, directly and indirectly (Hung et al. 2018, O'Connell et al. 2024). The greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), is a natural pest of honey bee (*Apis mellifera* L.) colonies. By inactivating honey bee colonies, they may periodically cause significant losses to the beekeeping industry (O'Connell et al. 2024). On the other hand, NPs of xenobiotic origin can affect the hive pest *G. mellonella* and indirectly honey bees. Additionally, insects are considered bioindicators in studying the toxicity, bioaccumulation, and

biotransfer of metals in the ecosystem and determining their effect on environmental pollution (Banville et al. 2012, Kara et al. 2020, Wu and Yi 2015). The immune system of *G. mellonella*, which is among the larvae of Lepidopteran species, resembles the mammalian immune system both in structure and function (Gwokyalya and Altuntaş 2019, Tunçsoy et al. 2021). *G. mellonella* is hence of interest for physiological, immunological, and toxicological investigations (Altuntaş 2015, Uçkan et al. 2021). Hemocytes of *G. mellonella* recognize foreign substances and phagocytose them, similar to neutrophils in the mammalian immune system (Browne et al. 2013, Tunçsoy et al. 2021). Species of the Lepidoptera generally have particular hemocytes: granulocytes, plasmatocytes, spherulocytes, oenocytoids, prohemocytes, and adipohemocytes (Lavine and Strand 2002, Altuntaş et al. 2012). Nanotoxicological studies with this type of insect may help to detect the potential impacts on human health and the ecosystem (Eskin and Bozdoğan 2022, Zorlu et al. 2018). *Pimpla turionellae* (Hymenoptera: Ichneumonidae) is one of the endoparasitoids of the host *G. mellonella* (Kansu and Uğur 1984, Uçkan et al. 2011). Nanomaterials that affect the host are likely to indirectly affect parasitoids, and these particles could potentially disrupt their host- parasitoids interactions. As hemocytes in *G. mellonella* play an crucial role in defence against parasitoid invasion, NPs may compromise this defence by affecting hemocyte viability and function, making the host more susceptible to parasitism. Conversely, nanoparticles may also affect the parasitoid's ability to successfully parasitise the host by interfering with its biological processes. The introduction of nanoparticles into the environment, including agricultural settings, may pose a risk to non- target organisms like beneficial insects. Therefore, Al_2O_3 NPs were given to *G. mellonella* larvae via diet, and their influence on the life cycle of host and endoparasitoid *P. turionellae*, and total hemocyte counts and hemocyte types of host larvae were investigated. Our results provide information on the influence of Al_2O_3 NPs on biological and hemocyte-mediated immunity in host-parasitoid insects. The study aimed to reveal the effects of Al_2O_3 NPs on the life cycle and immune system of *G. mellonella* due to their environmental toxicity (with nutrition) their impact on host-parasitoid interactions.

MATERIALS AND METHODS

Host and Parasitoid Rearing

The host greater wax moth *G. mellonella* was reared at 25 ± 3 ºC, a humidity of 60 ± 3%, and 24 hours of darkness. A synthetic diet was prepared from honeycomb, bran, honey, glycerin, and distilled water for feeding during larval stages (Bronskill 1961, Sak et al. 2006). The endoparasite *P. turionellae* was reared at a temperature of 25 ± 3 ºC, a humidity of 60 ± 3%, and 12: 12 h (Light: Dark) lighting conditions. They were fed with sterile cotton wool soaked with honey solution (30%, V: V) diluted with distilled water and pupal hemolymph of *G. mellonella* (two pupae for five females) three times a week.

Nanoparticles

Aluminum oxide nanoparticles (nanopowder Al_2O_3 NPs, TEM particle size < 50 nm) were purchased from Sigma Aldrich (Al_2O_3 NPs reference: 544833). For the preparation of NP solutions, a bath-type sonicator was used at 40 °C for 10 min. Data (scanning electron microscope images and Zeta potentials of Al_2O_3 NPs) on the characterization of Al_2O_3 NPs were given in our previous study (Demirtürk et al. 2023).

Bioassays

Determination of Al_2O_3 NP lethal concentrations (LC_{50}, probit analysis), preparation of larvae diets, and application of NPs to the diet were performed as in the previous study (Demirtürk et al. 2023). Al_2O_3 NP solutions prepared at different concentrations were used instead of water content and fed until the larvae grew to the last instar. As a result of Probit analysis, three concentrations below and one concentration above the LC_{50} value were selected and 50, 100, 500 and 1000 ppm Al_2O_3 doses were used (Demirtürk et al. 2023). The time required to complete the larval, pupal, and adult stages was recorded. Adult weights and sizes were measured. In addition, the pupae were parasitized by reproductively mature *P. turionellae* females. The time from the parasitism of the host *G. mellonella* pupae by *P. turionellae* to the formation of adult parasitoids (immature developmental time) and the longevity time of adult parasitoids were regularly observed and recorded. The weight and size data of *P. turionellae* were also measured.

Hemolymph Collection

To determine the total and differential hemocyte counts of host larvae, firstly, the last instars of *G. mellonella* (0.21 ± 0.01 g) from the experimental groups were chosen randomly. Larvae were anesthetized on ice for approximately 4-6 minutes and sterilized with ethanol (70%). The hind leg of the larvae was punctured with a needle and hemolymph was removed with a micropipette (Eppendorf, St. Louis, MO).

Total and Differential Hemocyte Counts

To determine the influence of NPs on circulating total hemocyte counts (THCs), the protocol recommended by Altuntaş et al. (2012) was applied. Hemocytes were counted under 60 × magnification in a phase contrast microscope (Nikon Eclipse Ti-U Phase contrast microscopy). Results expressed as THCs 10^6 cells / mL hemolymph (Altuntaş et al. 2012).

Giemsa staining protocol was used for differential hemocyte counts (DHCs) (Uçkan and Sak 2010). DHCs in hemolymph preparations obtained from larvae were observed under phase contrast microscopy. For DHCs, 500 cells from a single larva were counted on each slide. Hemocyte types were identified using the morphological characters described by Altuntaş et al. (2012).

Statistical Analysis

The means of data were analyzed by using the Independent Samples T-test and One-Way ANOVA in SPSS version 27. Levene's test analyzed concentration-dependent changes in the means for the normality of the data distribution. In One-way variance analysis, Tukey's HSD (Tukey's Honestly Significant Difference) was applied if the means were homogenous and Tamhane's t2 post hoc test was used if the means were not homogenous. For all the statistical tests, the p-value was taken as 0.05.

RESULTS

Biological Features of *Galleria mellonella* and *Pimpla turionellae*

All Al_2O_3 NP concentrations caused shortening of the larval and pupal developmental time of *G. mellonella* (df1, df2 = 4,70, F = 37.15, p = 0.00 < 0.001; df1, df2 = 4,70, F = 17.09, p = 0.00 < 0.001). For both groups, the greatest decrease occurred at the concentration of 1000 ppm. Adult longevity was shortened at concentrations of 50, 100, and 500 ppm Al_2O_3 NPs. Besides, adult longevity did not become different in the 1000 ppm group (df1, df2 = 4,70, F = 26.76, p = 0.00 < 0.001). Adult weights of larvae in the group treated with 500 ppm Al_2O_3 NP concentration increased, while there was no significant change in the other groups (df1, df2 = 4,70, F = 7.70, p = 0.00 < 0.001). In addition, no changes in adult size were observed (df1, df2 = 4,70, F = 1.12, p = 0.35, Table 1).

Table 1. Aluminum oxide nanoparticles (Al_2O_3 NPs)-associated changes in larval, pupal, and adult development time (day), adult weight (mg) - size (mm) of *Galleria mellonella*

Concentrations of Al_2O_3 NPs (ppm)	Larval Developmental Time (day) *	Pupal Developmental Time (day) *	Adult Longevity Time (day) *	Adult Weight (mg) *	Adult Size (mm) *
Control	27.1 ± 0.33^{a}	14.2 ± 0.42^{a}	14.8 ± 0.43^{a}	79.7 ± 0.40^{a}	13.7 ± 0.38^{a}
50	23.6 ± 0.37^{bc}	12.4 ± 0.41^{b}	10.7 ± 0.43^{bc}	81.1 ± 0.42^{a}	14.4 ± 0.32^{a}
100	24.7 ± 0.33^{b}	12.9 ± 0.50^{ab}	9.33 ± 0.34^{c}	80.3 ± 0.34^{a}	13.9 ± 0.35^{a}
500	22.4 ± 0.43^{c}	10.8 ± 0.41^{c}	11.8 ± 0.32^{b}	82.6 ± 0.42^{b}	14.2 ± 0.34^{a}
1000	20.9 ± 0.44^{d}	9.66 ± 0.39^{c}	12.0 ± 0.31^{a}	80.8 ± 0.30^{a}	13.2 ± 0.33^{a}

***** All data for each group are represented as Means ± Standard Errors. In each group, the mean of 15 individuals was given and three replicates were analyzed. Means following the same letter in each column is not substantially different, but particular letters (a-d) are significant (p < 0.001).

The immature developmental time of *P. turionellae* decreased at all concentrations (df1, df2 = 4,70, F = 5.12, p = 0.001 < 0.05). However, on average, the maximum decrease of 12.9% occurred at concentrations of 500 and 1000 ppm. No significant differences were observed in longevity, weight, and size of adult parasitoids (df1, df2 = 4,70, F = 17.09, p = 0.06; df1, df2 = 4,70, F = 26.76, p = 0.92; df1, df2 = 4,70, F = 7.70, p = 0.88, Table 2). Even if there were changes in "Mean ± Standard Errors" values between the groups, they were not statistically significant.

Table 2. Aluminum oxide nanoparticles (Al_2O_3 NPs)-associated changes in immature and adult development time (day), adult weight (mg) - size (mm) of *Pimpla turionellae*

Concentrations of Al_2O_3 NPs (ppm)	Immature Developmental Time (day) *	Adult Longevity Time (day) *	Adult Weight (mg) *	Adult Size (mm) *
Control	20.9 ± 0.33[a]	24.5 ± 0.30[a]	18.3 ± 0.26[a]	11.3 ± 0.23[a]
50	19.6 ± 0.41[ab]	23.0 ± 0.38[a]	17.9 ± 0.23[a]	10.9 ± 0.18[a]
100	19.3 ± 0.36[b]	23.3 ± 0.34[a]	17.6 ± 0.26[a]	10.6 ± 0.37[a]
500	18.4 ± 0.34[b]	23.8 ± 0.22[a]	17.7 ± 0.31[a]	11.0 ± 0.22[a]
1000	18.6 ± 0.31[b]	24.2 ± 0.26[a]	18.0 ± 0.30[a]	10.8 ± 0.26[a]

* All data for each group are represented as Means ± Standard Errors. In each group, the mean of 15 individuals was given and three replicates were analyzed. Means following the same letter in each column is not substantially different, but particular letters (a-d) are significant ($p < 0.05$).

Total and Differential Hemocyte Counts

Hemocyte counts in larvae were decreased at all Al_2O_3 NP concentrations (df1, df2 = 4,70, F = 3.59, p = 0.01 < 0.05). At the lowest concentrations of 50 and 100 ppm NP, THCs decreased by 30.6% and 44.9%, respectively. In the higher concentrations of 500 and 1000 ppm NP groups, THCs decreased by 58.9% and 62.7%, respectively (Figure 1).

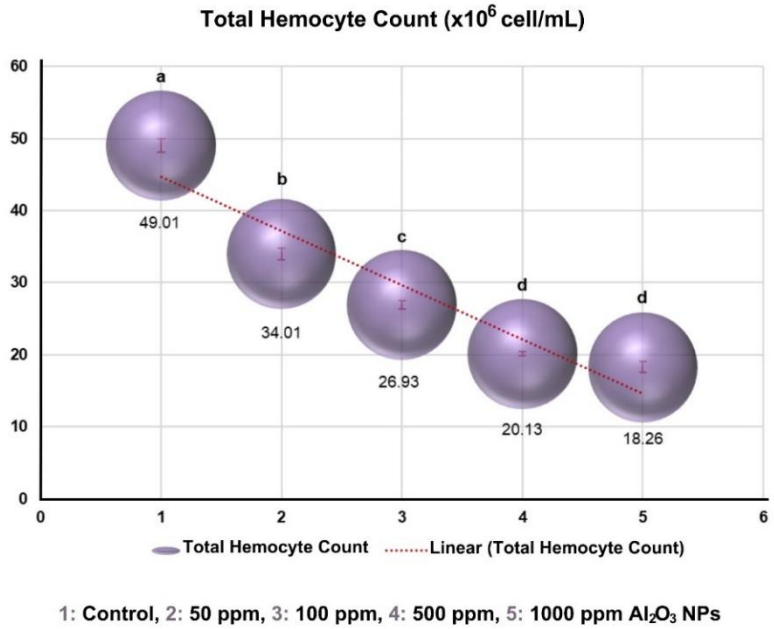

Figure 1. Chronic toxic effects of aluminum oxide nanoparticles (Al_2O_3 NPs) on total hemocyte count (× 10^6 cells / mL) of *Galleria mellonella* Data represent "Mean ± Standart Error" of 15 larvae in total ($p < 0.05$; One-way ANOVA, Tukey's HSD).

The differences in differential hemocyte counts (cells/500) of *G. mellonella* larvae associated with Al_2O_3 NPs are given in Figure 2. Plasmatocyte counts were significantly higher at all concentrations compared to the control group (df1, df2 = 4,70, F = 402.56, p = 0.00 < 0.05). In each treatment group,

important changes were noticed equated to the control (98.75 ± 2.16) and the maximum plasmatocyte increase in the 1000 ppm group was 165.9%. The major hemocyte type in *G. mellonella* larvae was plasmatocyte cells, which constituted 147.03 ± 2.22, 172.23 ± 3.51, 233.11 ± 3.25, and 262.62 ± 4.63 of the total hemocyte population at all concentrations (50, 100, 500 and 1000 ppm), respectively. On the contrary, significant decreases in granulocyte counts were observed in all groups with Al_2O_3 NP concentrations (df1, df2 = 4,70, F = 121.48, p = 0.00 < 0.05). Granulocyte count was 195.03 ± 1.70 in the control group and a 56.5% decrease was observed at the highest (1000 ppm) Al_2O_3 NP concentration. Means ± Standard Errors (Means ± SE) values of granulocyte counts from low to high concentrations were 181.60 ± 2.19, 169.92 ± 2.64, 134.51 ± 2.37, and 124.65 ± 4.21, respectively. Similarly, significant decreases in spherulocyte, oenocytoid, and prohemocyte counts have been observed at all low and high concentrations (df1, df2 = 4,70, F = 14.81, p = 0.00 < 0.05; df1, df2 = 4,70, F = 34.94, p = 0.00 < 0.05; df1, df2 = 4,70, F = 18.58, p = 0.00 < 0.05). Maximum reduction rates (at 1000 ppm concentration) were 73.2% (spherulocyte), 88.8% (oenocytoid), and 85.6% (prohemocyte).

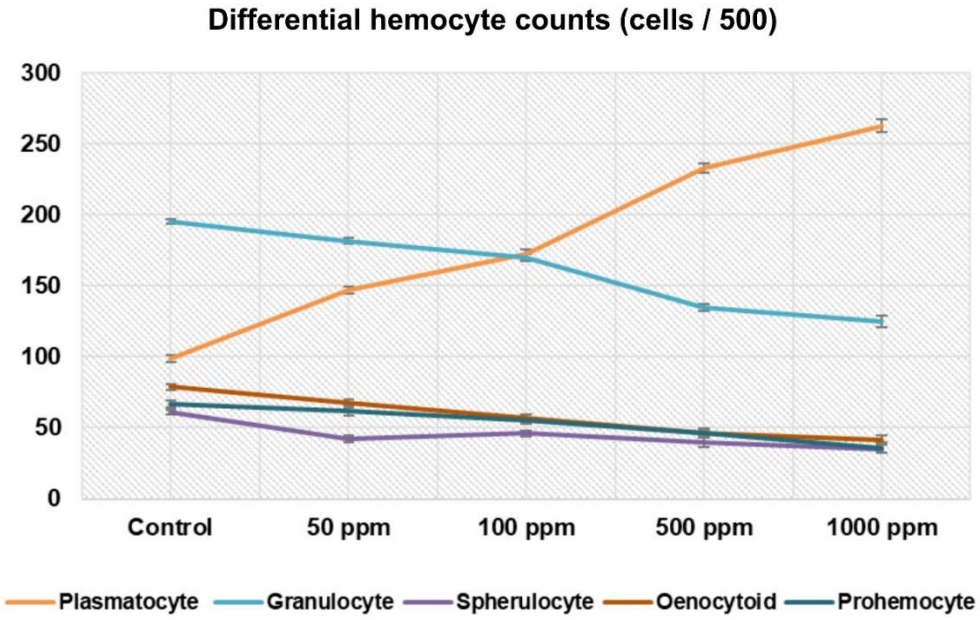

Figure 2. Influence of Aluminum oxide nanoparticles (Al_2O_3 NPs) on plasmatocyte, granulocyte, spherulocyte, oenocytoid, and prohemocyte counts *Galleria mellonella* larvae. Data represent "Mean ± Standart Error" of 15 larvae in total (p < 0.05; One-way ANOVA, Tukey's HSD).

DISCUSSION

The expanding use of NPs in numerous industries and their accumulation in the environment is critical for host-parasitoid relationships, which are the life vests of the ecosystem (Uçkan and Gülel 2002). The effects of NPs on the hive pest *G. mellonella*, which adversely may affect honey bees (pollinators and honey producers), and its endoparasitoid *P. turionellae* species are particularly vital presently. Research on the relation between the biological features of insects and metal oxide NPs is restricted and gives varying results. Especially studies on Al_2O_3 NPs are few (Assar et al. 2022, Kara et al. 2020). In a study conducted on the house fly model "*Musca domestica* L.", silver (Ag), Al_2O_3, and ZnO NPs were administered to larvae with diet for 72 hours, and it was reported that all NPs caused elongation in larval and pupal developmental time (Assar et al. 2022). In *G. mellonella*, it was found that pupal development time and pupal weights were not different from iron oxide NPs (Fe_3O_4) NP application (Eskin et al. 2021). In another study investigating the biological properties of *G. mellonella*, it was reported

that TiO_2 NPs extended the development time of larvae and pupae (Zorlu et al. 2018). The research shows that Al_2O_3 NPs can alter developmental timelines, with larvae and pupae of *G. mellonella* experiencing shortened developmental stages at various concentrations of Al_2O_3 NPs. This contrasts with other studies showing that different metal oxide NPs extend development times. Such findings indicate that Al_2O_3 NPs have a unique impact on insect physiology, potentially by accelerating metabolic processes or through toxicological stress that prompts faster development as a survival response.

Adult longevity is an important parameter in insect biology and studies on nanotoxicity show highly variable results. Eskin et al. (2021) reported that Fe_3O_4 NPs did not differ in adult weights and lifespan of *G. mellonella*. It has been documented that 5000 ppm ZnO NPs extend the adult lifespan of *G. mellonella* (Eskin and Nurullahoğlu 2022). In contrast, it has been observed that different ZnO NPs applied on *Spodoptera frugiperda* shortened female and male adult lifespans in a dose-dependent manner (Pittarate et al. 2021). It has been reported that TiO_2 NPs shorten adult lifespan even at low concentrations in *G. mellonella* (Zorlu et al. 2018). We observed that Al_2O_3 NPs (50, 100, and 500 ppm), like ZnO and TiO_2 NPs mentioned in previous research, also shorten adult longevity. In addition, only the 500 ppm Al_2O_3 NP group showed an increase in adult weight, while no change was observed in adult size. Heavy metals such as copper and zinc have been expressed to decrease the longevity of insects (Coskun et al. 2021, Sang et al. 2018). The reduction in adult longevity for *G. mellonella* exposed to Al_2O_3 NPs, along with an increase in adult weight at higher NP concentrations, suggests that these NPs might be influencing energy allocation and stress responses. The observed shortening of lifespans parallels findings with other NPs, indicating a potential universal stress response across different NP types. According to the study of Uçkan et al. (2015) with the parasitoid *P. turionellae*, treatment with indole-3-acetic acid (IAA) did not affect adult weights, only 5000 ppm IAA decreased female weights. In addition, while the size of adult females did not change, declines in immature developmental time and, adult longevity have been observed at IAA doses ≥ 1000 ppm (Uçkan et al. 2015). Unlike these data, the immature developmental time of *P. turionellae* parasitoids decreased at all Al_2O_3 NP concentrations, while no change was observed in the longevity of adult parasitoids. The unchanged adult longevity and weight of *P. turionellae* despite its host's altered development time and immune responses indicate a complex interaction where the parasitoid might be indirectly affected by the host's exposure to NPs. This relationship is critical for understanding ecosystem dynamics and potential cascading effects within food webs.

Hemocytes have a substantial role in the cellular and humoral immune systems of insects. The functioning of many systems in the organism is related to the immunity and hemocytes of insects (Kaya et al. 2021). As a response to immune defense, the number and morphology of hemocytes may change depending on toxic substances (Coskun et al. 2021, Yucel and Kayis 2019). Kara et al. (2020) have stated that Al_2O_3 NPs applied at different concentrations and for different periods decreased THCs in *G. mellonella* larvae. It was found that ZnO NPs significantly reduced the counts of hemocytes in *G. mellonella* (Nurullahoğlu et al. 2015). Likewise, it has been stated that TiO_2 NPs reduced the counts of hemocytes in the hemolymph of *G. mellonella* (Zorlu et al. 2018). Tuncsoy and Mese (2021) have documented that there were significant decreases in THCs in the groups where the lowest and highest concentrations of TiO_2 NPs were applied. The same researchers have found significant decreases in THCs at high CuO concentrations, another metal oxide NP (Tunçsoy et al. 2021). Our data showed that Al_2O_3 NPs can cause changes in total and differential hemocyte counts in *G. mellonella* hemolymph. Earlier reports showing the interactions between THCs and metal oxide NPs in insects support our study with Al_2O_3 NPs. It was observed that all concentrations of Al_2O_3 NPs were effective and THCs decreased in *G. mellonella* larval hemolymph. These decreases in THCs may be associated with an increase in apoptosis in hemocytes or inhibition of hemocyte production and release by affecting the deterioration of hematopoietic function due to the toxic influence of Al_2O_3.

Although their influence is various in the insect immune system, the roles of hemocyte types are crucial. Metal oxide NPs are effective in THCs, but can also be effective in the number of hemocyte types. Tunçsoy et al. (2021) have observed substantial increases in the counts of granulocytes in LC_{10} group larvae as a result of CuO NP application. Plasmatocyte counts were high in all

CuO NP groups, and the biggest increments were detected in prohemocyte and spherulocyte counts as a result of LC_{10} application and in oenocytoid counts in 1000 mg / L application (Tunçsoy et al. 2021). Administration of ZnO NPs treated with mulberry leaves to *Bombyx mori* for 12 and 24 h increased the number of granulocytes and plasmatocytes, while the population of prohemocytes and spherulocytes decreased (Mir et al. 2020). Similarly, it was shown that the population of DHCs was significantly decreased, while the count of oenocytoid was increased significantly in *B. mori* larvae fed with mulberry leaves treated with ZnO NPs (Belal and Gad 2023). Plasmatocye counts increased at all Al_2O_3 NP concentrations, while granulocyte, spherulocyte, oenocytoid, and prohemocyte counts decreased. These changes in hemocyte counts may be due to increased cell division rate, the release of bound hemocytes, or the attendance of hemocytes in the cellular responses including phagocytosis, encapsulation, and melanization. Since plasmatocyte has an essential function in the formation of these cellular responses. Furthermore, the population of hemocytes is affected as a result of mitotic division of prohemocytes (Er et al. 2011). It is considered that the presence of Al_2O_3 NPs as a threat to the organism may have resulted in the differentiation of prohemocytes into plasmatocytes and the decrease in the prohemocyte population may be related to these conditions. In our previous study, we also investigated encapsulation and melanization data related to cellular immunity. We observed that Al_2O_3 NPs decreased larvae's strong encapsulation and melanization responses at certain times (at 4 and 24 h) in a concentration-dependent (Demirtürk et al. 2023).

Compared with the results, the increase in plasmatocyte counts observed in encapsulation and melanization responses supports this hypothesis. However, the decrease in other hemocyte counts does not confirm this. At that point, decreases in other hemocyte counts may be associated with apoptosis or necrosis. This is also a curiosity about other influences of Al_2O_3 NPs. Al_2O_3 NPs affect the immune system of *G. mellonella* by altering hemocyte counts. Decreases in THCs and changes in specific hemocyte types suggest that these NPs could weaken the insect's immune defense, making them more susceptible to pathogens and parasites. This has broader implications for insect health and survival in environments contaminated with NPs.

Conclusion: Collected data demonstrate that Al_2O_3 NPs cause significant changes in the life cycle of *G. mellonella* and *P. turionellae* and the total, and differential hemocyte means of the host species. Therefore, this study may contribute to accumulating of knowledge about the lifetime and cellular immunity of nano Al_2O_3, which is among the metal oxide NPs. A material that may cause nanotoxicity in a living species in ecosystems may affect the food chain or other living species due to its interaction with other species. It is concluded that it may represent the influence of Al_2O_3 NPs and may be helpful for future research or insight into potential influences on humans. Similar to *G. mellonella*, bees exposed to NPs can experience adverse effects. NPs can accumulate in bee tissues, causing oxidative stress, disrupting metabolism, and impairing immunity. This has significant implications for bee health and hive stability, crucial for pollination and ecosystem balance. Studies on the environmental impact of NPs highlight their potential to contaminate nectar and pollen, which bees collect. This contamination can lead to bioaccumulation and magnification of NP effects within the hive, affecting brood development and overall colony health. Understanding these impacts is essential for developing safer agricultural practices that minimize harm to beneficial insects while leveraging the advantages of nanotechnology.

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Author Contribution: The authors contributed equally.

Conflict of Interest: The authors declare they have no conflict of interest.

Data Availability: Data are available in Manuscript.

Ethical Issue: Not applicable.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

ANALYSIS OF SIMILARITIES AND DIFFERENCES IN BEEKEEPING BETWEEN TÜRKİYE AND EUROPEAN UNION COUNTRIES

Türkiye ve Avrupa Birliği Ülkeleri Arasında Arıcılıktaki Benzerliklerin ve Farklılıkların Analizi

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ABSTRACT

This study aims to determine the similarities and differences between Türkiye and European Union countries in terms of beekeeping and classify similar countries. The main materials of the study consist of the number of beekeepers, the amount of honey produced, and the trade balance values of European Union countries and Türkiye. In this study, multidimensional scaling analysis and cluster analysis were conducted to reveal the similarities and differences between Türkiye and European Union countries regarding beekeeping. The analysis results indicate that Spain and Romania are the most similar countries and Türkiye and Germany significantly differ from other European Union countries regarding beekeeping. Specifically, Türkiye was differentiated from other countries by its high honey production amount. The key characteristics that differentiated Germany from other countries were the number of beekeepers and a high trade deficit. In order to compete effectively with European Union countries in beekeeping, Türkiye should prioritize policies that encourage the export of honey in small, branded packaging.

Keywords: Beekeeping, Multidimensional Scaling Analysis, Cluster Analysis, Türkiye, European Union Countries

ÖZ

Bu çalışmanın amacı Türkiye ile Avrupa Birliği ülkeleri arasında arıcılık açısından benzerlikleri ve farklılıkları belirlemek ve benzer ülkeleri sınıflandırmaktır. Çalışmanın ana materyalini Avrupa Birliği ülkeleri ve Türkiye'nin arıcı sayısı, üretilen bal miktarı ve dış ticaret dengesi değerleri oluşturmaktadır. Bu çalışmada Türkiye ile Avrupa Birliği ülkeleri arasında arıcılık konusundaki benzerlik ve farklılıkları ortaya koymak için çok boyutlu ölçekleme analizi ve kümeleme analizinden yararlanılmıştır. Analiz sonuçları İspanya ve Romanya'nın en benzer ülkeler olduğunu, Türkiye ve Almanya'nın ise arıcılık açısından diğer Avrupa Birliği ülkelerinden belirgin şekilde farklı olduğunu göstermektedir. Türkiye'yi diğer ülkelerden ayıran temel özellik üretilen bal miktarıdır. Almanya'yı diğer ülkelerden ayıran temel özellikler ise arıcı sayısı ve yüksek ticaret açığı olmuştur. Türkiye'nin arıcılıkta Avrupa Birliği ülkeleriyle etkin bir şekilde rekabet edebilmesi için küçük, markalı ambalajlarda bal ihracatını teşvik eden politikalara öncelik vermesi gerekmektedir.

Anahtar Kelimeler: Arıcılık, Çok Boyutlu Ölçekleme Analizi, Kümeleme Analizi, Türkiye, Avrupa Birliği Ülkeleri

GENİŞLETİLMİŞ ÖZET

Amaç: Bu çalışmanın amacı Türkiye ile Avrupa Birliği (AB 27) ülkeleri arasında arıcılık açısından benzerlikleri ve farklılıkları belirlemek ve benzer ülkeleri sınıflandırmaktır. Elde edilen bulgular arıcılığa yönelik yapılacak karşılaştırmalı üstünlük çalışmaları ve bu alanda geliştirilecek politikalar için değerlendirilebilir niteliktedir.

Gereç ve Yöntem: Çalışmanın ana materyalini Avrupa Birliği ülkeleri ve Türkiye'nin arıcı sayısı, üretilen bal miktarı ve dış ticaret dengesi değerlerine ilişkin istatistiki veriler oluşturmaktadır. Veriler FAO (Food and Agriculture Organization of the United Nations), ITC (International Trade Centre) ve TÜİK (Türkiye İstatistik Kurumu) veri tabanlarından ve Avrupa Komisyonu tarafından hazırlanan arıcılık sektör raporundan elde edilmiştir. Bu çalışmada Türkiye ile Avrupa Birliği ülkelerinin arıcılık konusundaki benzerliklerini ve farklılıklarını ortaya koyabilmek için çok boyutlu ölçekleme analizi ve ülkelerin sınıflandırılması için kümeleme analizi yapılmıştır. Çok boyutlu ölçekleme analizi kullanımının amacı nesnelerle ilgili birçok özelliği değerlendirerek birimler arasındaki mesafeleri ve yakınlıkları belirlemek ve böylece ülkelerin arıcılık faaliyetlerindeki benzerliklerini ve farklılıklarını ortaya koymaktır. Çalışmada grupları belirlemek için kullanılan kümeleme analizi ise verileri benzerliklerine göre sınıflandırarak araştırmacıya yorumlanabilir özet bilgiler sağlamaktadır. Sınıflandırma çalışmalarının temelini oluşturan bu analiz yöntemi, bireylerin veya nesnelerin sınıflandırılmasını ayrıntılı bir şekilde açıklayabilmektedir.

Bulgular ve Sonuç: Avrupa Birliği ülkeleri dünya bal üretiminin %12,11'ini, Türkiye ise %5,44'ünü gerçekleştirmektedir. Çalışmada Türkiye ile AB ülkeleri arasındaki farklılıklar ve benzerlikler ortaya konulmuştur. Türkiye, birinci boyutta pozitif yükler açısından diğer ülkelerden en fazla farklılaşan ülke olarak öne çıkarken, Almanya ikinci boyutta en fazla farklılaşan ülke konumundadır. Türkiye'yi diğer ülkelerden ayıran temel özellik üretilen bal miktarıdır. Almanya'yı diğer ülkelerden ayıran temel özellikler ise arıcı sayısı ve yüksek ticaret açığı olmuştur. Almanya 2021 yılı verilerine göre hem bal ithalat değeri (314,76 milyon ABD Doları) hem bal ihracat değeri (148,48 milyon ABD Doları) bakımından AB ülkeleri arasında ilk sırada yer almaktadır. Bu durum Almanya'nın ithal ettiği bala katma değer sağlayıp, bunu daha yüksek bir fiyatla yeniden ihraç etme stratejisiyle açıklanabilir. Türkiye'nin bal ithalat değeri ise 378.000 ABD Doları ve ihracat değeri 31,15 milyon ABD Dolarıdır. Ayrıca Almanya, Türkiye'nin bal ihracatının değer bazında %22,32'sini oluşturmaktadır ve Türkiye'nin AB ülkeleri arasında en çok bal ihraç ettiği ülke konumundadır. Analiz sonuçları İspanya ve Romanya'nın en benzer ülkeler olduğunu, Türkiye ve Almanya'nın ise arıcılık açısından diğer AB ülkelerinden belirgin şekilde farklı olduğunu göstermektedir. Türkiye'nin arıcılıkta AB ülkeleriyle etkin bir şekilde rekabet edebilmesi için küçük, markalı ambalajlarda bal ihracatını teşvik eden politikalara öncelik vermesi gerekmektedir. Bu araştırma bal üretim miktarı, arıcı sayısı ve dış ticaret dengesi değişkenleriyle sınırlı kalmıştır. Gelecek araştırmalar arı sütü, polen, propolis gibi ek arı ürünü üretim verilerini modele dahil ederek genişletilebilir. Bu daha geniş veri seti Türkiye ve AB ülkelerindeki arıcılığı daha kapsamlı bir şekilde değerlendirmeye yardımcı olacaktır.

INTRODUCTION

Honey bees are greatly appreciated worldwide for their importance. They are quite important in terms of honey and beeswax production and are essential for pollinating numerous vital crops (VanEngelsdorp and Meixner 2010). Bee products are extensively utilized in industrial manufacturing, food processing, medicine, and the realm of natural healing (Ghanshyam et al. 2021). Furthermore, in natural areas like mountains and forests where beekeeping is practiced, a novel trend known as apitourism has arisen, accompanied by a health-conscious way of living that captures the community's attention (Šuligoj 2021, Topal et al. 2021). According to FAO data for the year 2021, the global honey production amounted to 1771944 tons. 42.84% (759178 tons) of the produced honey was traded internationally, generating a revenue of 2.70 billion US Dollars from foreign trade (ITC 2023). European Union countries (EU 27) account for 12.11% of global honey production, while 5.44% is produced by Türkiye (FAO 2023). Türkiye's honey production quantity is 96,344 tons, whereas Romania, the leading honey-producing country among the EU countries, produces 30,875 tons. Spain (29,393 tons) and Germany (28,651 tons) follow Romania.

In order to analyze the changes in honey production among countries and to make comparisons between them, the annual relative increase in production

quantity was examined over a 20-year period. In addition, this period was divided into two distinct periods in order to reveal the long-term changes in honey production. When the increase in the average honey production quantities for the periods 2001-2010 and 2011-2020 is examined by country, Croatia (222.43%), Lithuania (115.45%), Latvia (111.24%), and Estonia (70.79%) are the countries with the highest proportional increase in honey production. These countries have the highest average annual relative increase in the 2001-2020 period. The geographical proximity of Lithuania, Latvia, and Estonia as countries suggests that this similarity can be associated with geographical features. Cyprus, Slovenia, and Austria are the countries with the highest negative average annual relative increase rate in honey production in the 2001-2020 period. These countries also rank among the countries that show the highest decrease in honey production when the averages of the two periods are compared. Türkiye increased honey production by 35.20% when the 2001-2010 and 2011-2020 periods are compared, and Türkiye's average annual relative growth rate is 2.88% in the period of 2001-2020 (Table 1).

Table 1. Honey production quantity and average annual relative increase by country[1]

Country	Average honey production quantity (ton, 2001-2010)	Average honey production quantity (ton, 2011-2020)	Change between two periods (%)	Average annual relative increase (2001-2020) (%)
Lithuania	1434	3089	115.45	9.06
Estonia	634	1082	70.79	7.08
Croatia	2349	7573	222.43	6.15
Latvia	783	1654	111.24	5.64
Bulgaria	8689	10376	19.41	4.71
Romania	17745	25068	41.26	3.87
Poland	12169	16815	38.18	3.84
Türkiye	76281	103128	35.20	2.88
Greece	15562	19542	25.57	2.25
Finland	1804	2008	11.31	1.94
France	15748	14985	-4.84	1.68
Slovakia	3945	3759	-4.73	1.61
Portugal	6886	10189	47.97	1.50
Germany	20990	22527	7.32	0.62
Luxembourg	166	124	-25.64	0.61
Ireland	230	258	12.11	0.18
Denmark	1500	1500	0.00	0.00
Sweden	3277	3391	3.49	-0.17
Spain	32381	31907	-1.46	-0.19
Italy	10240	9545	-6.79	-0.32
Hungary	18546	23260	25.42	-0.48
Czechia	7228	8482	17.34	-1.16
Cyprus	675	443	-34.37	-2.02
Austria	6470	4800	-25.81	-3.52
Slovenia	1974	1422	-28.00	-3.57

[1] The data has been calculated by the author using data obtained from the FAO (2023). Data for Belgium, Malta, and the Netherlands could not be accessed, and therefore, they have not been included in the calculation.

The share of the EU (27) in the export value is 32.01%, while Türkiye's share in the global honey export value is 1.15%. When examining the honey trade balance of EU countries, Hungary ranks first with a value of 86.34 million US Dollars, while Türkiye ranks fifth with a value of 30.77 million US Dollars. However, the EU's honey trade balance is -233.09 million US dollars (ITC 2023). Türkiye has 8.18 million beehives, while the countries with the most beehives in the EU are Spain (2.95 million), Romania (2.35 million), Greece (2.18 million), and Poland (2.01 million). There are 89197 beekeepers

in Türkiye, and among EU countries, Germany has the highest number of beekeepers with 129048. Other EU countries with the highest number of beekeepers are Poland (74302), Czechia (61572), Italy (56059), and France (53953) (EC 2023, FAO 2023, TURKSTAT 2023). Within the scope of the national apiculture programs, support is provided to EU countries by the Union, and the total support value in 2021 amounted to 39.44 million Euros. The countries receiving the highest share of this support are Spain (5.64 million Euros), Romania (5.25 million Euros), Poland (3.94 million Euros), and Italy (3.55 million Euros), respectively (EC 2023) (Figure 1) When examining the honey production quantities and the number of beehives by country, it is observed that the amount of support is related to these variables.

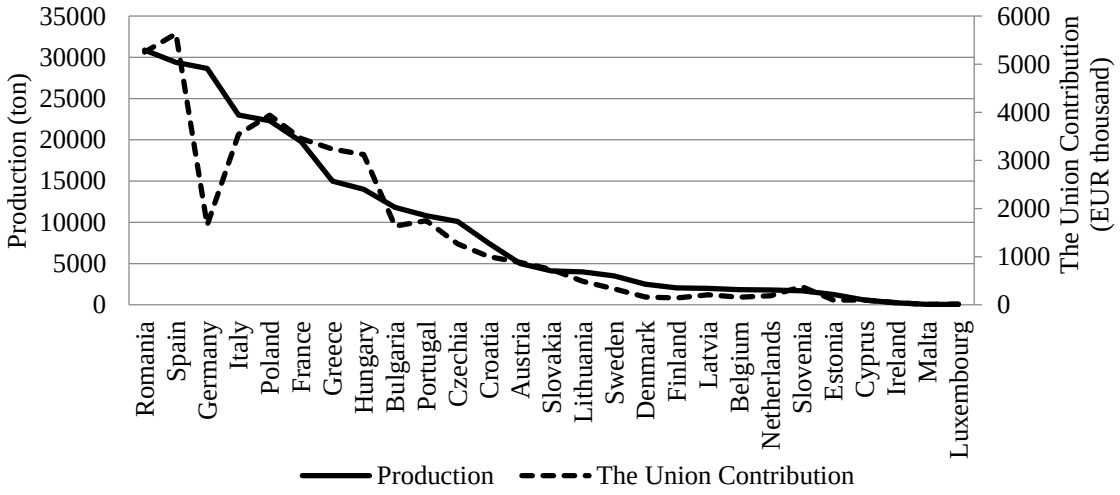

Figure 1. The Union contribution and the honey production quantity of the EU countries

Among the EU countries, Germany has the highest honey yield, with a yield of 29.18 kg per hive in the year 2021. Following Germany are Estonia (25.06 kg/hive), Finland (23.94 kg/hive), the Netherlands (22.98 kg/hive), and Belgium (22.44 kg/hive). The honey yield per hive in Türkiye is 11.78 kg. When looking at per capita honey consumption in 2020, Croatia leads among EU countries with a per capita consumption of 1.86 kg per year. Following Croatia are Greece (1.75 kg/year), Germany (1.05 kg/year), Austria (1.04 kg/year), and Lithuania (1.04 kg/year). Bulgaria and Hungary have the lowest per capita honey consumption among EU countries, at 0.01 kg per year. Türkiye's per capita honey consumption is 1.14 kg per year (FAO 2023).

The production and trade of bee products are directly related to bee populations, and the honey bee population has decreased in many countries in Europe (VanEngelsdorp and Meixner 2010). Climate change also significantly affects beekeeping in Europe (Van Espen et al. 2023). The number of beehives, the number of beekeepers, the amount of honey produced, and the balance in value vary significantly from country to country in beekeeping (EC 2023). However, beekeeping is supported in both EU countries and Türkiye. Determining whether these supports are consistent between countries and whether they are based on rational decisions can provide guidance. Furthermore, revealing the similarities and differences between Türkiye, which ranks first in global honey production, and EU countries can be beneficial for reviewing support policies for beekeeping in terms of Türkiye's competitiveness.

In this study, multidimensional scaling analysis (MDS) and cluster analysis were conducted to reveal the similarities and differences between EU countries and Türkiye in terms of beekeeping. There are studies in the literature that use these methods together in the field of agriculture. Srivastava et al. (2005) studied genetic diversity in silkmoth species, Şahin et al. (2008) analyzed agricultural and environmental characteristics of provinces in the Aegean Region, Ozturk et al. (2009) investigated

honey bee genotypes in 30 provinces in Türkiye, Türkekul et al. (2010) examined the competitiveness of olive oil-exporting countries worldwide, Gevrekçi et al. (2011) analyzed the structure of provinces in Western Anatolia, and Yüksel (2017) studied the sheep farming structure in the Southeastern Anatolia Region. Turgut (2016) focused on the agricultural structure of provinces in the Central Anatolia Region, Gonzalez-Mejia et al. (2018) investigated the extensive and intensive production structures of dairy cattle farms in England and Wales, and Güler (2021) examined the similarities and differences in sericulture among 24 regions in Türkiye, categorizing them into similar groups. Additionally, there are studies related to animal husbandry that solely utilize multidimensional scaling analysis (Çelik 2015, Güler et al. 2018).

The aim of this study is to determine the similarities and differences between EU countries and Türkiye in terms of beekeeping and classify similar countries. The findings are useful for evaluating comparative advantage studies in beekeeping and for developing policies in this field.

MATERIALS AND METHODS

Materials

The main materials of the study consist of the number of beekeepers, the amount of honey produced, and the trade balance values of EU countries and Türkiye. The data was obtained from the FAO, ITC, and TURKSTAT databases, and the beekeeping sector report (EC 2023).

Methods

In this study, multidimensional scaling analysis (MDS) and clustering analysis have been utilized. All data were evaluated by SPSS 20 software.

Multidimensional scaling analysis

The use of MDS aims to determine the distances and proximities between units by evaluating a multitude of features related to objects (Hair et al. 1998). In this method, the primary goal is to represent the structure of objects as closely as possible to the original form using distance values, with as few dimensions as possible (Özdamar 1999, Tatlıdil 2002). Distances in multidimensional scaling are determined by using distance matrices, so appropriate distance matrices need to be calculated depending on the type of data. If the data is obtained at interval or ratio scales, distances are calculated in the form of Euclidean, Squared Euclidean, Chebyshev, Block, or Minkowski distances (Özdamar 1999). The difference between the actual shape and the shape estimated in k-dimensional space in the analysis forms the stress value. This value indicates the goodness of fit for models created for various dimensions. For non-metric scaling, the stress value is given below, and it is desired to be close to zero (Johnson and Wichern 2007).

$$\text{Stress} = \sqrt{\frac{\sum\sum\left(d_{ij} - \hat{d}_{ij}\right)^2}{\sum \hat{d}_{ij}^2}}$$

$\hat{d}_{ij}$ = The data distance between individuals i. and j.

d_{ij} = The configuration distance between individuals i. and j.

The adequacy of the obtained solution is explained with a low stress ratio. A high value represents poor fit. The goodness of fit corresponding to the stress value introduced by Kruskal (1964) are given in Table 2.

Table 2. Goodness of fit relationship by stress

Stress	Goodness of fit
20%	Poor
10%	Fair
5%	Good
2.5%	Excellent
0%	Perfect

Approaching zero for the stress statistic indicates an increase in the degree of fit. In multidimensional scaling analysis, the measure of how well the data fits the obtained model called the 'Fit Index,' is determined by R^2, and values greater than 0.60 are considered suitable (Hair et al. 1998). In this study, the ALSCAL algorithm was used for multidimensional scaling analysis.

Cluster Analysis

Cluster analysis, one of the multivariate statistical analysis methods, was used to determine the groups in the study. The general purpose of cluster analysis is to classify data based on their similarities, providing interpretable summary information to the researcher (Tatlıdil 2002). This analysis method, which forms the basis of classification studies, can

explain in detail the classification of individuals or objects (Erilli 2012). Cluster analysis is divided into two main groups based on the approaches followed in determining groups: hierarchical clustering and non-hierarchical clustering (Blashfield and Aldenderfer 1978). At the initial stage of the data matrix, depending on how many clusters are formed and which criterion is initially selected to determine cluster members, stepwise methods are divided into two main groups. These are agglomerative hierarchical clustering methods and divisive hierarchical clustering methods. The distance criteria used in cluster analysis include Euclidean distance, squared Euclidean distance, Manhattan distance, Pearson distance, Mahalanobis distance, Minkowski distance, squared Pearson distance, Hotelling T^2 distance, and Canberra criterion. The decision on which distance measure to use is made based on whether the variables are discrete or continuous, or whether the variables are nominal, ordinal, interval, or ratio scale (Dinler 2014).

In this study, data related to the number of beekeepers, the amount of honey produced, and the trade balances of countries were evaluated to classify countries using the hierarchical clustering method. In this study, which uses the agglomerative clustering method and the squared Euclidean distance was used as the distance criterion. Furthermore, the dendrogram obtained by the average linkage method. The average linkage method calculates the average distance between all points of the two clusters. This means the distance between the clusters is determined by averaging the distances between all individual points (Yim and Ramdeen 2015). The average linkage method has been chosen because it represents the general relationships and similarities between clusters in a more balanced way and reduces the impact of outliers. The classification process was performed in four stages, ranging from binary groups to five groups, and the results obtained were used for comparisons between countries.

RESULTS

In the research, using multidimensional scaling analysis, the similarities and differences between 28 countries in terms of the amount of honey produced, the number of beekeepers, and trade balance in value have been revealed based on the distances in the perceptual map. Initially, the model included the amount of honey produced, the number of beekeepers, the number of beehives, and the trade balance in value. However, after conducting the VIF (Variance Inflation Factor) test, it was found that the amount of honey produced and the number of beehives were causing multicollinearity problems. Therefore, the number of beehives was removed from the model. Thus, the multicollinearity problem in the model was resolved. However, the number of beehives has been presented in the table to facilitate comparisons between countries. Variable data for the countries are provided in Table 3. Accordingly, Türkiye has the highest amount of honey produced and the number of beehives, while Germany has the highest number of beekeepers and a negative trade balance in value.

As a result of the analysis, for n=28 (number of units), p=3 (number of variables), and k=2 (two-dimensional solution), iterations were continued until the improvement in the stress statistic value was less than 0.001, and at the 8th iteration, an improvement value of 0.00075 was reached, leading to the termination of the iterations (Table 4). The stress statistic value, which is close to zero, indicates that the obtained solution is appropriate.

Table 3. The amount of honey produced, the number of beekeepers, the number of beehives, and trade balance in value by country (2021)[1]

Country	The amount of honey produced (kg)	The number of beekeepers	The number of beehives	Trade balance in value (US Dollar thousand)
Türkiye	96344200	89197	8179085	30770
Romania	30875000	23161	2353000	42442
Spain	29393200	28786	2953000	45222
Germany	28651066	129048	982000	-166278
Italy	23000000	56059	1717000	-70681
Poland	22300000	74302	2013000	-28031
France	19788000	53953	1808000	-86364
Greece	15000000	9266	2183000	13074
Hungary	14000000	22447	1207000	86342
Bulgaria	11807269	12260	838000	34968
Portugal	10800000	11301	758000	-1148
Czechia	10113340	61572	695000	-13399
Croatia	7440000	7283	460000	-3857
Austria	5000000	29745	456000	-17470
Slovakia	4112580	18586	344000	15347
Lithuania	4000000	8950	209000	5577
Sweden	3500000	16000	179000	-17113
Denmark	2500000	7000	140000	-8024
Finland	2059000	3200	86000	-10116
Latvia	1998000	3341	104000	1360
Belgium	1840000	8223	82000	-1773
Netherlands	1792700	9345	78000	-32786
Slovenia	1700000	11349	213000	-1743
Estonia	1252900	5215	50000	-448
Cyprus	584144	676	55000	-2640
Ireland	257000	3300	27040	-12824
Malta	60000	234	6000	-966
Luxembourg	48200	456	3000	-1764

[1] The data was obtained from the FAO, ITC, and TURKSTAT databases, and EC (2023).

Table 4. Young's S-stress statistic results

Iteration	S - stress	Improvement	Iteration	S - stress	Improvement
0	0.47618	-	5	0.19506	0.00448
1	0.31409	-	6	0.19242	0.00264
2	0.22751	0.08658	7	0.19105	0.00137
3	0.20801	0.01950	8	0.19029	0.00075
4	0.19954	0.00847			

The stress value calculated according to Kruskal's formula is 0.149, which, according to the table of stress values and goodness of fit, indicates a fair fit. As a result of the analysis, the R^2 (coefficient of determination) expected to be above 60% has been calculated as 0.952. Therefore, for k=2 dimensions, the stress value explains the data by 95.2%.

The two-dimensional geometric representation of the data has shown compatibility, and a linear relationship between observational distances and disparities has been observed (Figure 2).

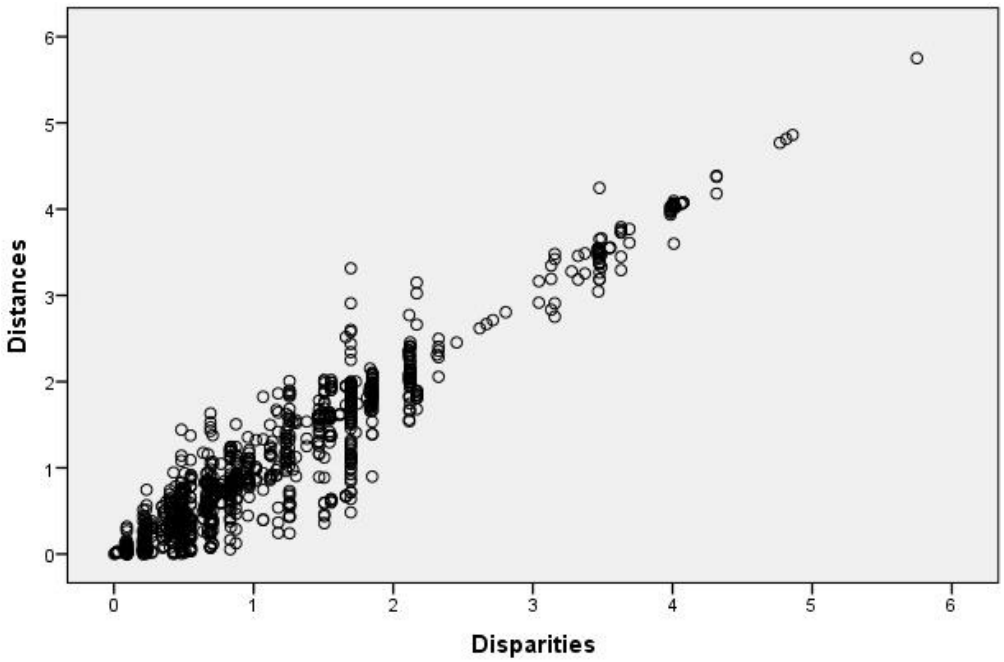

Figure 2. Scatterplot of relationship between distances and disparities

When examining the coordinate values underlying the two-dimensional geometric representation, it was determined that some countries are significantly differentiated from others in terms of beekeeping. In the first dimension, Türkiye (-3.2421) stands out distinctly from other countries, having the most extreme value, while in the second dimension, Germany (-3.8437) differs significantly from others with the most extreme value. Romania and Spain have similar values in both dimensions, indicating that these countries display similar profiles in terms of beekeeping. Hungary, which has the highest value (1.9272) in the second dimension, stands out from the others as the country with the highest trade balance. Furthermore, in the first dimension, Malta (0.8862) and Luxembourg (0.8841) are the countries closest to a positive value of 1. Malta has the fewest beekeepers, and Luxembourg has the lowest honey production, which confirms this result. When the first and second dimensions are evaluated together, the most similar countries to each other are Spain and Romania (Table 5).

In the study, the differences matrix, which shows the proximity and distance between the examined countries, was also evaluated. Countries with values close to zero in the differences matrix are considered to be similar in terms of the examined characteristics, while countries with values above two are considered distant from each other, indicating that these countries are less similar (Gevrekçi et al. 2011). The results indicate that among the examined countries, Türkiye and Germany are the countries with a distance of more than two from the others, and these countries stand out as significantly distinct.

Figure 3 illustrates the relationships between countries in a two-dimensional space. In this coordinate system, countries with similar honey production quantity, number of beekeepers, and trade balance are grouped around the origin, while Türkiye and Germany are located far from the origin. Indeed, Türkiye ranks first among the examined countries in terms of honey production quantity (96344200 kg), while Germany is at the top in terms of the number of beekeepers (129048) and trade balance (166.28 million US Dollars).

Table 5. Coordinates of countries

Country	Dimension 1	Dimension 2
Türkiye	-3.2421	0.3476
Germany	-1.5570	-3.8437
Poland	-1.2276	-0.5763
Czechia	-0.3735	-0.3852
Italy	-1.0439	-1.3781
France	-0.7582	-1.6870
Austria	0.1275	-0.0753
Spain	-1.2777	1.0363
Romania	-1.2831	0.9901
Hungary	-0.2904	1.9272
Slovakia	0.2765	0.5548
Sweden	0.4121	-0.0467
Bulgaria	-0.0112	0.9402
Slovenia	0.7419	0.2147
Portugal	0.1444	0.2616
Greece	-0.0392	0.5435
Lithuania	0.5746	0.2293
Netherlands	0.7699	-0.3588
Belgium	0.7836	0.1984
Croatia	0.4652	0.2212
Denmark	0.7979	0.1582
Estonia	0.8297	0.1967
Ireland	0.8731	-0.1183
Finland	0.8374	-0.1073
Latvia	0.8289	0.1883
Cyprus	0.8709	0.1903
Luxembourg	0.8841	0.1898
Malta	0.8862	0.1886

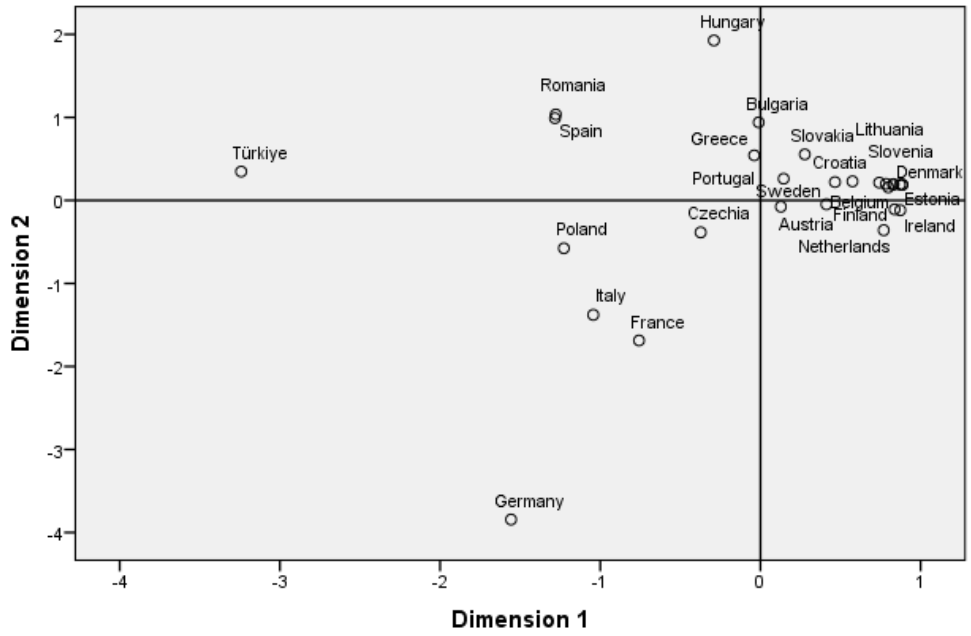

Figure 3. Two-dimensional space representation of countries

The results of the multidimensional scaling analysis are supported by cluster analysis. According to the cluster analysis results, which were evaluated in seven different groups, Germany and Türkiye were placed in a different group in each clustering (Figure 4). The results of the multidimensional scaling analysis are consistent with the cluster analysis.

In the grouping of seven, Germany constitutes the 1st group, Türkiye the 2nd group, Poland, and Czechia the 3rd group, Italy and France the 4th group, Hungary the 5th group, and Spain and Romania the 6th group, while the other EU countries form the 7th group. Poland and the Czechia, both of which are in the 3rd group, are similar in terms of the number of beekeepers and also have a negative trade balance. Within the 4th group, Italy and France are closely matched in terms of honey production quantity and the number of beekeepers. Hungary, which has the highest positive trade balance, forms a separate group within the grouping of seven. Spain and Romania in the 6th group have similar values in terms of the variables considered (honey production, the number of beekeepers, and trade balance).

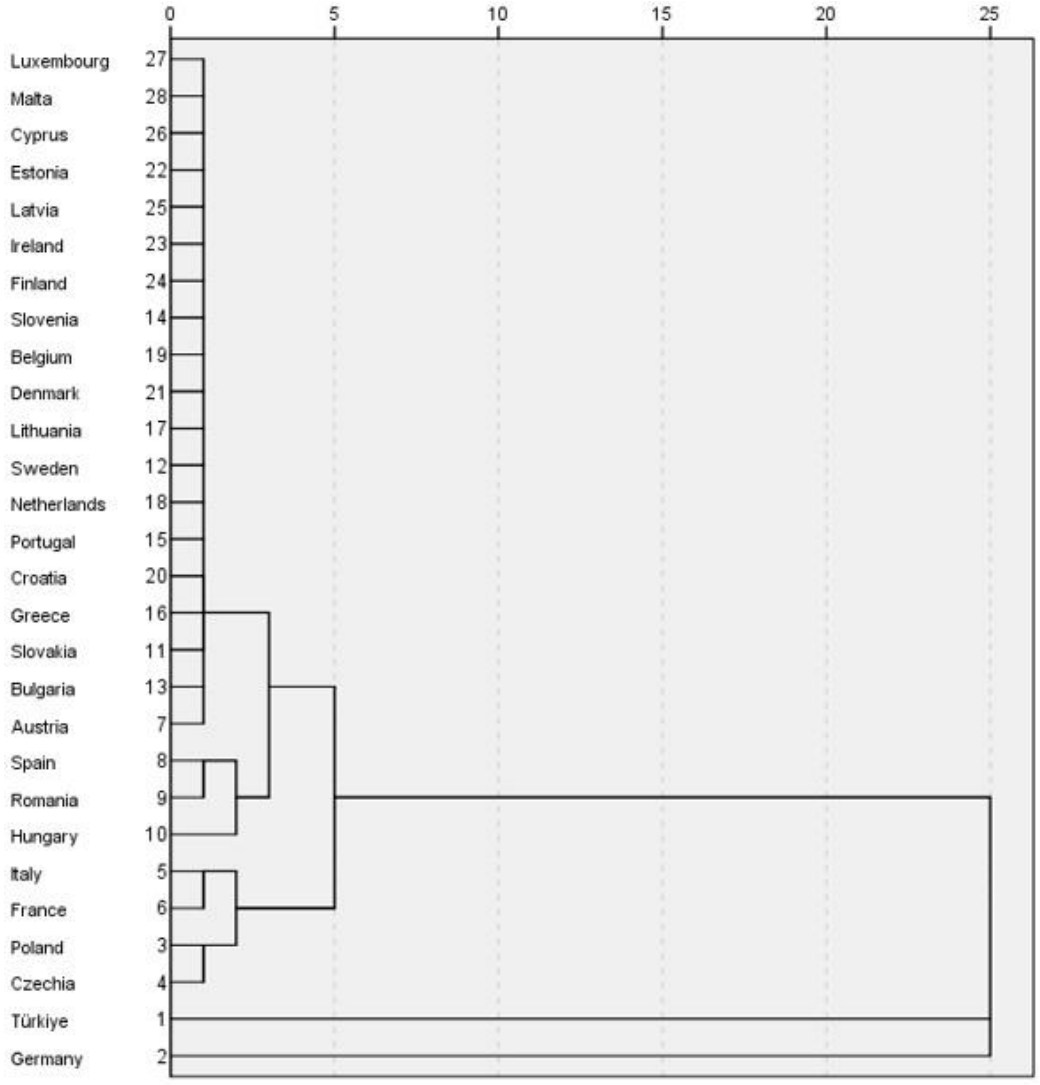

Figure 4. The dendrogram obtained by the average linkage method of beekeeping by country

DISCUSSION

According to the data for the year 2021, Germany ranks first among the EU countries both in terms of honey import value (314.76 million US Dollars) and honey export value (148.48 million US Dollars). This can be explained by Germany's strategy of adding value to imported honey and re-exporting it at a higher price. Türkiye's honey import value is 0.38 million US Dollars, and its export value is 31.15 million US Dollars. Furthermore, Germany accounts for 22.32% of Türkiye's honey exports by value, and Germany is at the top of Türkiye's honey exports among EU countries. However, Germany is the second-highest country in terms of honey import value in the world, following the United States. In Germany's honey imports, Mexico (38.11 million US Dollars), New Zealand (35.06 million US Dollars), and Argentina (30.17 million US Dollars) are at the forefront, while in honey exports, France (20.14 million US Dollars), Switzerland (15.60 million US Dollars), and the Netherlands (13.98 million US Dollars) are among the leading countries (ITC 2023). Germany's honey export strategy differs from both other EU countries and Türkiye. Germany's strategy involves importing honey at a fixed cost and then enhancing its value before exporting it at an elevated price.

The key indicators that distinguish Türkiye from other EU countries are honey production quantity and the number of beehives. From this perspective, Türkiye is in a significantly advantageous position compared to EU countries.

In terms of competitiveness between countries in beekeeping, increasing efficiency in beekeeping is of great importance. In a study conducted by Güler (2021) that examined beekeeping efficiency by provinces in Türkiye, it was found that beekeeping efficiency increased in provinces with large-scale enterprises and high honey yields. Previous studies have also supported this result (Abdul-Malik and Mohammed 2012, Aydın et al. 2020, Ceyhan 2017, Kaya 2020, Makri et al. 2015,). Güler's (2021) study revealed that beekeeping efficiency in Türkiye is low. Indeed, the average honey yield per hive in the EU (27) countries is 17.51 kg, while Türkiye's honey yield is 11.78 kg (FAO 2023). Achieving a high yield in beekeeping depends not only on colony efficiency but also on the diversity and quantity of nectar and pollen sources (Behçet and Yapar 2019). Furthermore, modern beekeeping practices also enhance honey yield and quality (Cabrera et al. 2019). Considering the current vegetation and climate type, it appears possible to increase Türkiye's honey yield average (Onuç et al. 2019).

When associating income from the honey trade with branding, it can be said that the number of registered geographical indications (GIs) for honey in countries is important. According to Güler and Saner's (2018) study, there were 34 types of honey registered as GI by the European Union in 2018, and all of these registrations belong to EU countries. Portugal had the most geographically indicated honey registrations among EU countries with 9 registered honeys. Following Portugal, Spain had 6 registrations, France had 5, Poland had 4, Italy had 3, and Slovenia had 3. Moreover, today there are a total of 51 types of honey across the EU, with 43 registered, 2 published, and 6 in application status. Among these, Türkiye's Bingöl Balı is registered, while Muğla Çam Balı and Sinop Kestane Balı are in the application status (EC 2024).

This study aims to reveal the similarities and differences between Türkiye and EU countries in terms of beekeeping and to classify similar countries. Data on honey production, the number of beekeepers, and the trade balance of each country were evaluated using multidimensional scaling analysis and clustering analysis. The research results showed distinctions among countries. Türkiye emerged as the most important differentiating country in terms of positive loads in the first dimension, while Germany was the most significant differentiating country in the second dimension. Specifically, Türkiye was differentiated from other countries by its high honey production. The key characteristics that differentiating Germany from other countries were the number of beekeepers and a high trade deficit. When the first and second dimensions were considered together, Spain and Romania were found to be the most similar countries.

Conclusion: The quantity and diversity of bee products produced in each country can vary depending on factors such as climate, flora, and production techniques. Additionally, the income generated from bee products in countries depends on factors such as branding, population density, and export quantity. The research results are guiding in evaluating the EU's (27) trade balance for honey. Developing strategic policies for honey exports to Germany, which stands out from other EU countries, is important. However, it is important to focus on

selling packaged honey with added value in both Germany and other countries in honey exports. It is also observed that the support provided by the EU to its member states is proportional to honey production quantities, and the support provided to Germany does not match its production volume. Türkiye also provides support for beekeeping.

This study has been limited to variables such as honey production quantity, the number of beekeepers, and trade balance. Future research can be expanded by incorporating additional bee product production data for countries, such as beeswax, royal jelly, pollen, propolis, etc., into the model. This broader dataset would provide a more comprehensive understanding of beekeeping in Türkiye and EU countries.

Data availability: All data and materials utilized and/or analyzed during the current study are accessible within this manuscript.

Ethical issue: Not applicable because this study does not involve animals or humans.

Source of finance: Not applicable because there is no funding source for this study.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

EFFECTS OF APILARNIL AND QUEEN BEE LARVAE ON LARVAL MORTALITY AND LONGEVITY IN *DROSOPHILA MELANOGASTER*

Apilarnil ve Kraliçe Arı Larvasının *Drosophila melanogaster*'in Larval Mortalitesi ve Uzun Ömürlülüğü Üzerine Etkileri

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ABSTRACT

The basic mechanisms of ageing and longevity are not yet fully understood. More studies are needed to correlate this situation with functional foods and food supplements that have been used frequently recently. Bee products are in the first place in the use of natural products and food supplements. It has been reported that drone larvae (apilarnil) and queen bee larvae, which have become popular in recent years, support the protection of health due to their high nutritional value, but no studies have been conducted on their life-extending efficacy. For this purpose, the efficacy of these two bee products on life span and mortality was investigated in our study. Apilarnil and queen bee larvae lyophilisates added to Drosophila melanogaster medium at different concentrations (0.5; 1.0; 2.5 and 5.0 mg/ml medium) were studied separately in male and female populations for treatment and control groups. As a result, apilarnil at 5 mg/ml concentration showed the best effect in terms of larval mortality compared to the control group, while the most effective group in terms of mean life span was determined as queen bee larvae with 83.1±3.53 days. In general, both bee products increased the life span of flies in parallel with the increase in concentration in both female and male populations. These results were statistically significant at $p<0.05$ level compared to the control group. In our study, it was concluded that apilarnil and queen bee larvae lyophilisates can be used in terms of life-length increasing activity, but the underlying mechanisms should be elucidated by detailed studies.

Keywords: *Drosophila melanogaster*, Apilarnil, Queen bee larvae, Larval mortality, Longevity

ÖZ

Yaşlanma ve uzun ömürlülüğün temel mekanizmaları henüz tam olarak anlaşılamamıştır. Bu durumun son zamanlarda sıkça kullanılan işlevsel gıdalar ve gıda takviyeleri ile ilişkilendirilebilinmesi için daha çok çalışmaya ihtiyaç duyulmaktadır. Arı ürünleri ise, doğal ürünler ve gıda takviyeleri için kullanımda ilk sıralardadır. Son yıllarda popüler hale gelen erkek arı larvası (apilarnil) ve kraliçe (ana arı) arı larvasının da yüksek besin değeri nedeniyle sağlığın korunmasına destek olduğu bildirilmiş ancak ömür uzatıcı etkinliği üzerine çalışmalar yapılmamıştır. Bu amaçla çalışmamızda bu iki arı ürününün ömür uzunluğu ve mortalite üzerine etkinliği araştırılmıştır. *Drosophila melanogaster* besiyerine farklı konsantrasyonlarda (0.5; 1.0; 2.5 ve 5.0 mg/ml besiyeri) eklenen apilarnil ve kraliçe arı larvası liyofilizatları, tedavi ve kontrol grupları için erkek ve dişi popülasyonlarda ayrı ayrı çalışılmıştır. Sonuçta kontrol grubuna göre larval mortalite açısından en iyi etkiyi 5 mg/ml konsantrasyondaki

apilarnil gösterirken, ortalama ömür uzunluğu bakımından ise en etkili grup 83.1±3.53 gün ile kraliçe arı larvası olarak belirlenmiştir. Genel olarak her iki arı ürünü de sineklerin yaşam sürelerini hem dişi hem de erkek popülasyonlarında konsantrasyon artışına paralel olarak artırmıştır. Bu sonuçlar kontrol grubuna göre p<0.05 düzeyinde istatistiksel olarak anlamlı bulunmuştur. Çalışmamız ile apilarnil ve kraliçe arı larvası liyofilizatlarının ömür uzunluğunu artırıcı etkinliği açısından kullanılabileceği ancak altta yatan mekanizmaların ayrıntılı çalışmalarla aydınlatılması gerektiği sonucuna ulaşılmıştır.

Anahtar Kelimeler: *Drosophila melanogaster*, Apilarnil, Kraliçe arı larvası, Larval mortalite, Ömür uzunluğu

GENİŞLETİLMİŞ ÖZET

Amaç: Arılar, dünya yaşamında birçok alanda katkısı bulunan canlılardır. Polinasyondan sonra en önemli katkılarından biri, insan sağlığına sunduğu kendi ürettikleri ürünleridir. Erkek arı larvası (apilarnil) ve kraliçe (ana arı) arı larvası apiterapotik ürünler arasında son yıllarda kullanımı popüler hale gelmiş ürünlerdir. Yüksek besin değeri taşımaları nedeniyle beslenmeye destek olmakta ve bu yüzden çeşitli formları piyasaya sürülmektedir. Bu ürünler hem yumurta hem de larva yapısı nedeniyle yüksek biyolojik aktivite göstermektedir. Bu sebeple tam gıda olarak tanımlanmaktadır. Kraliçe arı larvası ve apilarnilin hem gıda olarak tüketilmesi hem de apiterapide kullanımı için içerik analizlerinin yapılması, kullanım dozlarının belirlenmesi, canlılarda yararlı ya da toksik etkinliklerinin ortaya çıkarılması oldukça önemlidir. Bu çalışmanın amacı da apilarnil ve kraliçe arı larvasının toksisite ya da beslenme araştırmalarında sıkça kullanılan model organizma olan *Drosophila melanogaster* (meyve sineği) üzerinde larval mortalite ve ömür uzunluğunun değerlendirilerek literatürdeki eksiklikleri gidermektir.

Gereç ve Yöntem: Deneysel çalışmamızda gıda takviyesi olarak kullanılan liyofilize apilarnil ve kraliçe arı larvaları Amasya şehrinde faaliyet gösteren bir apiterapi ürünleri işletmesinden temin edildi. Çalışmalarımızda kullandığımız *D. melanogaster*, Amasya Üniversitesi Fen-Edebiyat Fakültesi Biyolojik Araştırma Laboratuvarı'nda yıllardır çoğaltılarak saklanmaktadır. Standart *D. melanogaster* besiyerine farklı konsantrasyonlarda (0.5; 1.0; 2.5 ve 5.0 mg/ml) eklenen apilarnil ve kraliçe arı larvası ekstraktları, uygulama ve kontrol grupları için erkek ve dişi popülasyonlarda ayrı ayrı çalışılmıştır. Öncelikle larval mortalite çalışması yapılmış, daha sonra uygun bulunan konsantrasyonlarda ömür uzunluğu deneyleri yapılmıştır. Her deney seti üç kez tekrar edilmiş ve elde edilen bulguların ortalamaları alınarak istatistiksel değerlendirmeler yapılmıştır.

Bulgular ve tartışma: Her iki cinsiyetteki kraliçe arı larvası uygulama gruplarında tüm konsantrasyonlar karşılaştırıldığında, en uzun maksimum yaşam süresi 5 mg/ml tedavi grubunda ve erkeklerde (92±3.17) gözlendi; en düşük maksimum yaşam süresi kontrol grubunda (66±0.41) gözlendi. Bu sonuçlara göre apilarnil ortamındaki 2.5 mg/ml konsantrasyonu kullandığımız apilarnil ve kraliçe arı larvalarının larval mortalite ve yaşam süreleri üzerindeki etkileri açısından her iki cinsiyet grubunda da etkili olmuştur. Ortalama yaşam süresi açısından gözlenen bu fark her iki cinsiyet grubunda da p<0.05 düzeyinde istatistiksel olarak anlamlıydı.

Sonuç: Çalışmadan elde edilen bulguların değerlendirilmesi ile bireylerin yaşadığı olumsuzluklar ve kullanılan kimyasal ilaçlar ile yapılan tedavi protokollerinin yarattığı güçlü yan etkiler düşünüldüğünde, antioksidan ve antikanserojen etkisi olan ve sağlığı destekleyen gıda takviyelerinin geliştirilmesi önem arz etmektedir. Zengin besin içeriğine sahip olan apilarnilin ve kraliçe arı larvasının *D. melanogaster* ömrü üzerindeki etkinliğinin literatüre önemli katkı sağlayacağı düşünülmektedir.

INTRODUCTION

Many reasons such as the increase in the elderly population in the world, the decrease in the rate of physical activity, the prevalence of harmful habits such as smoking and alcohol, and the change in lifestyles are increasing the rate of global diseases (Onur et al. 2018). These effects can also bring some difficulties in people's quality of life, daily functions and treatment adherence (Duran 2011). Increasing diseases with artificial living conditions, especially drug resistance and side effects of chemical drugs in the body have led individuals to seek alternative solutions (Sorucu 2019). In different

countries of the world, with the need for food that develops due to population growth the solution to the problem in the context of the search for a food source and the search for a food source can be an alternative food for people to utilize the resources. For this purpose, insects and insect products are used as a protein source in many countries of the world. It is consumed as a food product that is a source of food (Hakan et al. 2021).

In order to eliminate nutritional deficiencies, imbalances and protein deficiencies, research is carried out on various functional food sources. Insects have started to be consumed as food in order to use food sources effectively and honeybees are also included in this consumption (Isıdorov et al. 2016). As a result of the research, it has been reported that drone bee larvae (apilarnil) and queen (queen) larvae support health protection due to their high nutritional value. The name Apilarnil was created by Romanian scientist Nicolae Iliesiu, from the Latin name for bees 'api' (*Apis mellifera*), 'lar' from larvae and initials 'nil' (Erdem and Ozkok 2018). Apilarnil is a drone larvae in the 3–7-day larval stage before they pupate. It shows high biological activity due to both egg and larval structure. Apilarnil is defined as "whole food" because it contains all essential amino acids, which are the basic building blocks of our body (Topal et al. 2018).

Queen bee larva is another popular bee larva recently. It is obtained by collecting 3-day-old queen bee larvae from the thimble, which is naturally present in the queen bee cell during the production of royal jelly before the milk harvest (Margaoan et al. 2017). It is estimated that the biggest difference between apilarnil and queen bee larvae is due to the total protein content of the queen bee, as it is fed with pure royal jelly (Keskiner 2021). The chemical composition of apilarnil and queen bee larva homogenate was investigated by GC-MS (Gas Chromatography-Mass Spectrometry). The contents of apilarnil homogenate were 73.75% water, 9.47% total protein, 8.38% lipid, 0.38% fructose and 3.55% glucose. The contents of queen bee larva homogenate were 75.17% water, 12.03% total protein, 10.30% lipid, 1.25% fructose, 2.10% glucose and 0.08% sucrose (Isıdorov et al. 2016).

It is appropriate to collect apilarnil and queen bee larvae in April-May when the best quality food form is preserved. The best process applied to preserve the nutritional values is lyophilization and thus fresh apilarnil and queen bee larvae lyophilized can be safely stored at -15 °C for 1 year (Bruneau 2015).

In the study by Isidorov et al., the biological properties of apilarnil were investigated and some pharmacological chemical substances have been found to show activity. In their studies, apilarnyls with the queen bees in queen larvae. In terms of sugar content, glucose was more dominant in apilarnil larvae, while in queen larvae trehalose was predominant. Amino acid content and essential amino acids of apilarnil homogenates amount was lower than that of queen homogenates. In this study, the chemical composition of apilarnil was determined by GC-MS (Gas Chromatography-Mass Spectrometry) (Isıdorov et al. 2016). Studies have revealed that apilarnil, a bee product, has the potential to shed light on scientific studies.

The recent initiation of studies on apilarnil, the determination of its chemical content and the reduction of its effects on animal subjects on tissue and cellular basis and the results obtained have become promising (Dong et al. 2018, Hakan et al. 2021, Hamamci et al. 2020, Isıdorov et al. 2016). Apilarnil and queen bee larvae are powerful energy providers that stimulate oxidative processes. The accumulation of oxidative damage has been shown to play an important role in some advanced-age diseases and the aging process. Aging and longevity have been the subject of curiosity of people for years. For this reason, it has never lost its topicality in the scientific world. This biological process is quite complex and complicated. Model organisms are materials used to understand and analyze biological processes. The most basic life process character to describe the aging process is lifespan (Coskun 2023).

Longevity is affected by all factors that reduce viability. From this point of view, the effects of apilarnil and queen bee larvae, which have high biological activity, on the life span of adult individuals of *Drosophila melanogaster* were tried to be determined. Basic metabolic molecular pathways are well conserved and approximately 75% of known human disease genes have sequences of interest in *D. melanogaster.*

D. melanogaster has been recognized as a valuable model and has gained interest in nutritional intervention studies. The effects of food on larval mortality and survival were evaluated to investigate food-related pathophysiological mechanisms, including inflammation and stress response.

MATERIAL AND METHODS

Material

Lyophilized apilarnil and queen bee larvae used as food supplements in our experimental study were obtained from an apitherapy products business operating in our region. *D. melanogaster*, which we used in our studies, has been hybridized for years in the Biological Research Laboratory of the Faculty of Science and Letters of Amasya University. The short life cycle (9-10 days), high number of offspring, low rearing conditions and easy observation of possible variations make *D. melanogaster* an ideal experimental organism. In our experimental study, Oregon (R) (wild type) strain of *D. melanogaster* with normal round, red eyes and no mutant characters was used to determine lifespan (Figure 1). The environment of *D. melanogaster* is at 40% - 60% RH, 25±1 °C and under permanent dark conditions.

Figure 1. Images of female and male adult individuals and their developmental periods used in the study

Methods

Lyophilized apilarnil and queen bee larvae were dissolved in distilled water to prepare a 100 ml stock solution at 5mg/ml. 1.5 g of *D. melanogaster* ready-made medium (Instant *D. melanogaster* Medium, purchased from Carolina Biological Supply Company) and apilarnil or queen bee larvae dissolved in 5 ml distilled water were added to 50 ml falcon tubes. In the larval mortality assay, sufficient numbers of male and female individuals of stock *D. melanogaster* were transferred to fresh medium for larval mortality or survival rate tests and kept in an incubator at 25°C and 40-60% relative humidity. humidity for 25 days. The third stage larvae obtained after three days (72±4 h) were transferred to a medium containing different concentrations of ap,larnil and queen bee larvae (0.5-1-2.5 and 5 mg/ml medium). Distilled water was used for the control group. For each experimental group, 100 larvae were used. Test tubes were sterilised. The mouths of the tubes were closed with cotton plugs and placed inside the tubes and the larvae were allowed to mature. During this process, all experimental groups were checked daily and counted for 7 days after the first adult fly was seen. Counts were recorded twice daily, separating males and females. All experiments were repeated 3 times.

In our study, 3rd instar larvae *D. melanogaster* larvae were used to determine larval mortality, and *D. melanogaster* individuals were used to determine lifespan (Figure 1). Males and females of the Oregon R strain were crossbred in culture bottles to create preliminary stocks. Individuals reaching the 3rd larval stage were separated under tap water, and 3rd stage larvae collected with the help of fine-mesh sieves were weighed at the determined concentrations (0.5-1-2.5 and 5 mg/ml) and containing apilarnil or queen bee larvae dissolved in 5 ml of water. *D. melanogaster* was transferred to glass bottles containing ready-made medium. 100 larvae were placed in each bottle and expected to develop into adult flies.

The study was carried out in triplicate. The results obtained were averaged.

The effects of apilarnil and queen bee larvae on lifespan were studied separately in the male and female sexes of *D. melanogaster*. For this purpose,

preliminary stocks were created by crosses in culture bottles containing fresh nutrient medium to obtain same-aged individuals. Unmated female and male flies of the same age (1-3 days old) emerging from the pupa were separated and 100 adult flies were collected for each concentration. Collected adult flies were fed for 10 days in media containing apilarnil or queen bee larvae at different concentrations (0.5-1.0-2.5 and 5 mg/ml). At the end of 10 days, the adult flies, which were taken to the medium-free medium, were transferred to the new medium every three days, while counting was continued and the numbers were noted. All culture bottles were kept in suitable temperature cabinets (25±1 °C). During the experiment, the foods were refreshed every three days. In all control and treatment groups, counting and practice were continued until the last individual died.

Statistical analysis: The analysis of the data we obtained as a result of our study was made with the SPSS version 27.0 (Statistical Package for the Social Sciences) program. For this purpose, the "One-way Analysis of Variance" (One-way ANOVA) method was used. The Duncan test was evaluated at a probability level of 0.05 for data from longevity studies ($p<0.05$). Larval mortality graphs and survival curves of adult individuals were drawn using the Microsoft Windows Office Excel program.

RESULTS

In our experimental study, it was observed that apilarnil and queen bee larvae increased larval mortality and mean lifespan in both female and male individuals in all treatment groups (0.5; 1.0; 2.5 and 5 mg/ml) compared to the control. From the results obtained from the larval mortality studies, it was determined that the highest larval mortality rate was observed in the 1 mg/ml queen bee larvae treatment group (40%) and 0.5 mg/ml queen bee larvae treatment group (20%) (Table 1). The best survival was observed in the 5 mg/ml apilarnil treatment group (95%) and the 5 mg/ml queen bee larvae treatment group (94%) (Table 1).

In the second stage of our study, larvae were collected with a new experimental setup and substance applications were made at determined doses from larva to adulthood. 100 male and female adult individuals obtained from these larvae were fed on standard media and their mortality rates were monitored throughout their lifespan. All studies were repeated 3 times and the averages were taken. Then, the importance controls of the differences between the means obtained as a result of pairwise comparisons of the study groups and control groups were also determined (Table 1-3, Figure 2-4).

Table 1. Survival and mortality rates of larvae chronically fed with different concentrations of Apilarnil (APL) and Queen Bee Larvae (QBL)

Experiment Sets	N	Mortality Rate (%) ± S.E.	Survival Rate (%) ± S.E.
Control	100	5±0.04^{a}	95±1.12^{a}
0.5 mg/ml APL	100	14±1.05^{b}	86±1.04^{b}
1 mg/ml APL	100	18±1.04^{b}	82±2.04^{b}
2.5 mg/ml APL	100	7±0.04^{a}	93±1.14^{a}
5 mg/ml APL	100	5±0.08^{a}	95±1.10^{a}
0.5 mg/ml QBL	100	20±1.11^{b}	80±0.94^{b}
1 mg/ml QBL	100	40±1.14^{c}	60±1.01^{c}
2.5 mg/ml QBL	100	11±0.94^{ab}	89±1.04^{ab}
5 mg/ml QBL	100	6±0.02^{a}	94±1.94^{a}

S.E.: Standard Error, n: number of larvae, QBL: Queen Bee Larvae, APL: Apilarnil, $^{a-d}$Values belonging to experimental groups with different letters in the same column are significant at p<0.05 level.

When the maximum lifespan lengths were analyzed, the longest mean lifespan in apilarnil treatment groups was determined as 103±1.98 and 75±1.04 days in the 5 mg/ml treatment group in males and the lowest maximum lifespan in 0.5 mg/ml treatment group in males, respectively (Table 2 and Figure 2).

When the maximum lifespan lengths were analyzed, the longest mean lifespan in apilarnil treatment

groups was determined as 110±1.49 and 75±1.09 days in 5 mg/ml treatment group in females and the lowest maximum lifespan in 1 mg/ml treatment group in females, respectively (Table 2 and Figure 3).

When all concentrations were compared in apilarnil treatment groups in both sexes, the longest maximum life span was observed in 5 mg/ml treatment group and females (110±1.49); the lowest maximum life span was observed in 0.5 mg/ml treatment group and males (75±1.04) (Tables 2).

Table 2. Lifespan data obtained from larvae treated with Apilarnil (APL)

Experiment Sets	Sex	N	Maximum Lifespan (Days) ± S.E.	Average Lifespan (Days) ± S.E.
Control	Female	100	66±1.04^{a}	65.3±1.06^{a}
	Male	100	66±1.04^{a}	63.4±1.01^{a}
0.5 mg/ml APL	Female	100	78±1.18*	75.8±1.28*
	Male	100	75±1.04^{b}	74.7±1.07^{b}
1 mg/ml APL	Female	100	75±1.12^{b}	72.6±1.29^{ab}
	Male	100	95±2.18^{c}	70.7±1.84^{a}
2.5 mg/ml APL	Female	100	101±2.64^{d}	79.7±1.96^{bc}
	Male	100	98±2.15^{c}	78.6±1.78^{bc}
5 mg/ml APL	Female	100	110±3.49^{d}	72.8±1.24^{ab}
	Male	100	103±2.98^{d}	70.8±1.88^{a}

S.E.: Standard Error, N: Number of larvae, APL: Apilarnil, $^{a-d}$ Values belonging to experimental groups with different letters in the same column are significant at $p<0.05$ level.

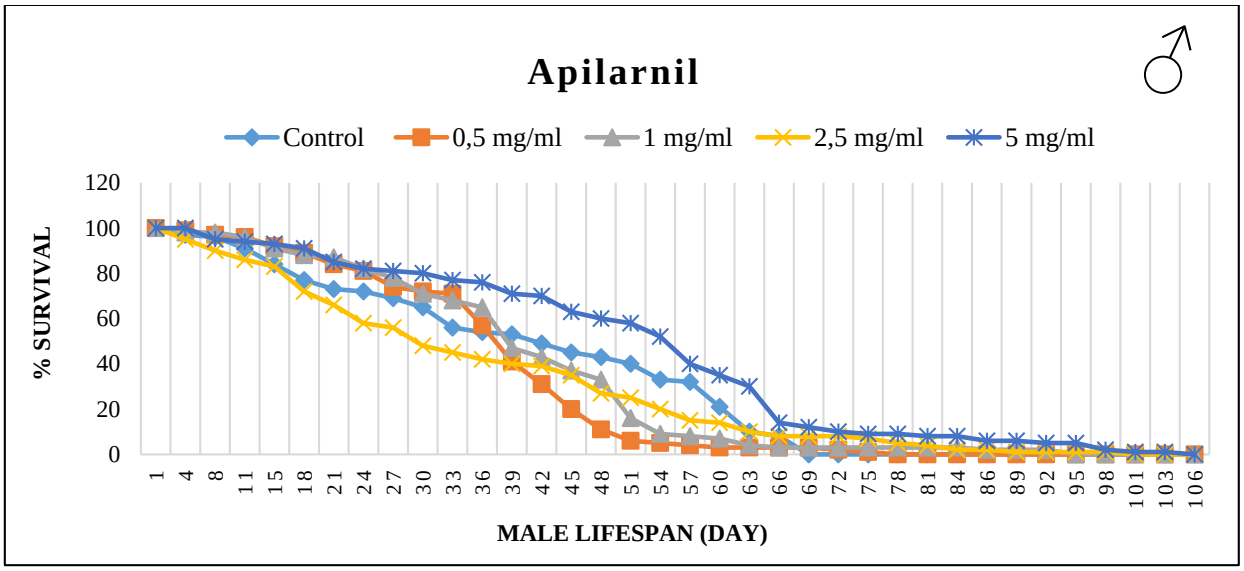

Figure 2. Survival curves of male *D. melanogaster* individuals living on apilarnil medium at different concentrations during their adult lives

When the maximum life span lengths were analyzed, the longest average life span was determined as 92±1.075 and 75±1.052 days in the 5 mg/ml treatment group in males and the lowest maximum life span was determined as 92±1.075 and 75±1.052 days in the 1 mg/ml treatment group in males, respectively (Table 3 and Figure3).

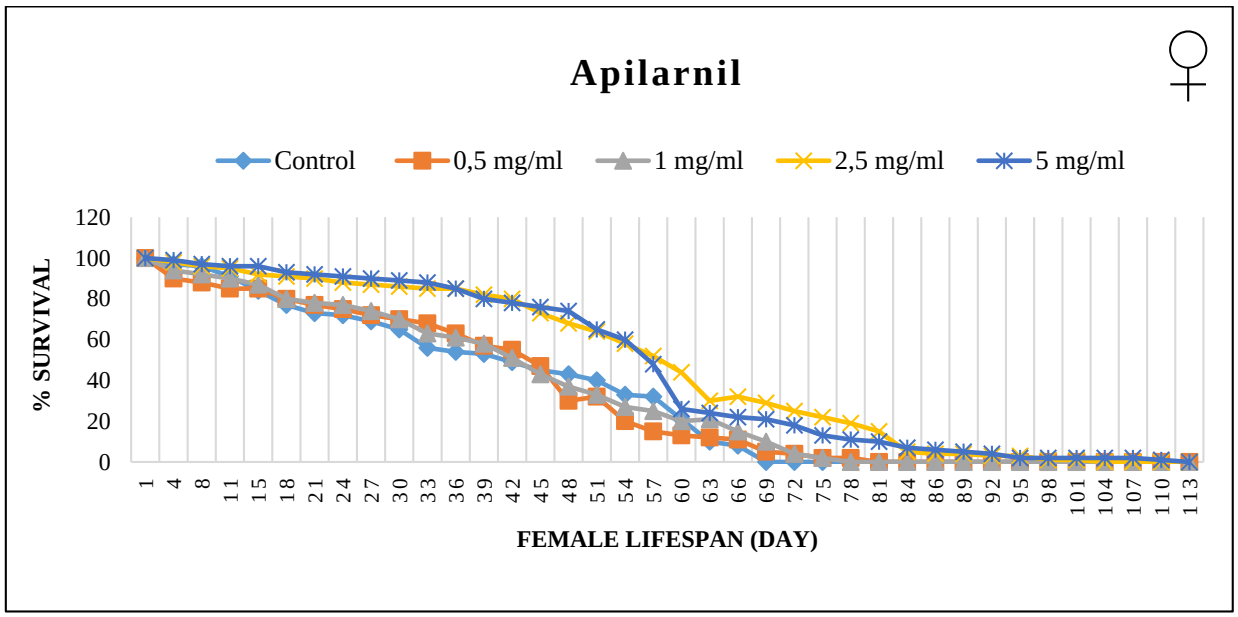

Figure 3. Survival curves of female *D. melanogaster* individuals living on apilarnil medium at different concentrations during their adult lives

Table 3. Lifespan data obtained from larvae treated with queen bee larvae (QBL)

Experiment Sets	Sex	N	Maximum Lifespan (Days) ± S.E.	Average Lifespan (Days) ± S.E.
Control	Female	100	66±0.41[a]	62.4±1.54[b]
	Male	100	66±0.41[a]	60.1±1.18[b]
0.5 mg/ml QBL	Female	100	72±1.48[b]	70,8±1.68[b]
	Male	100	78±2.07[c]	75,7±1.87[c]
1 mg/ml QBL	Female	100	70±1.02[b]	66.6±2.20[c]
	Male	100	75±2.05[c]	68.7±2.52[c]
2.5 mg/ml QBL	Female	100	75±2.09[c]	71.2±3.22[d]
	Male	100	89±3.09[d]	80.7±3.08[d]
5 mg/ml QBL	Female	100	72±1.09[a]	68.8±3.39[d]
	Male	100	92±3.17[d]	83.1±3.53[d]

S.E.: Standard Error, N: Number of larvae, QBL: Queen Bee Larvae, [a-d] Values belonging to experimental groups with different letters in the same column are significant at $p<0.05$ level.

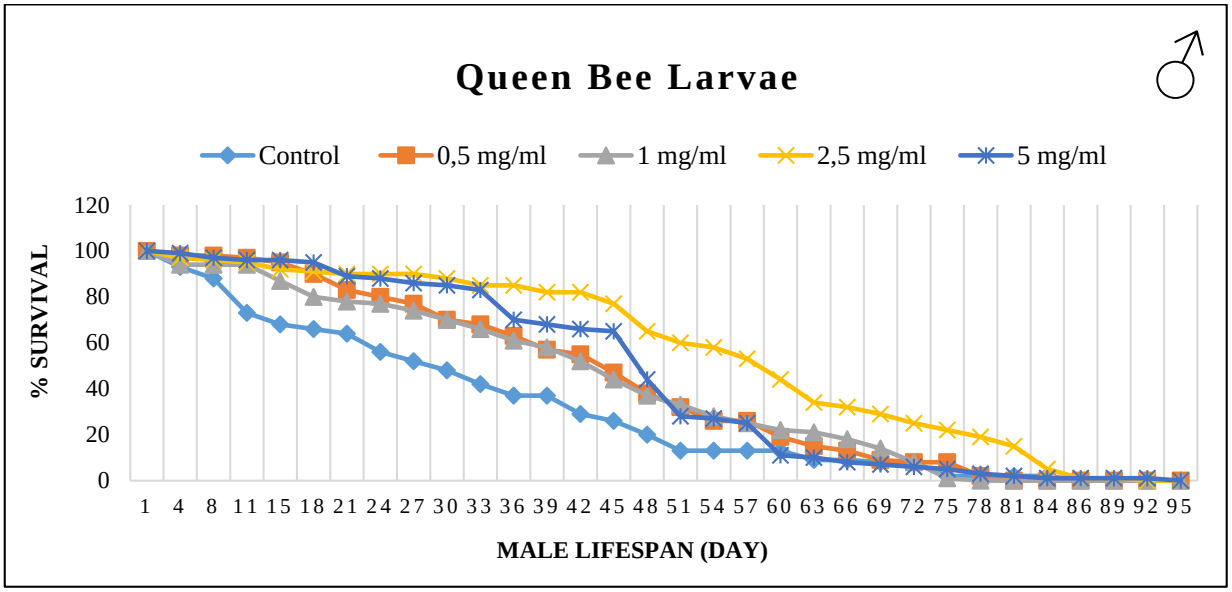

Figure 3. Survival curves of male *D. melanogaster* individuals living in different concentrations of queen bee larvae medium during their adult live

When the maximum life spans were examined in females, the longest maximum life span and mean life span in the queen bee larvae treatment groups were determined as 75±2.09 and 70±1.02 days in the 2.5 mg/ml treatment group, respectively, while the lowest were determined in the control group, respectively (Table 3 and Figure 4).

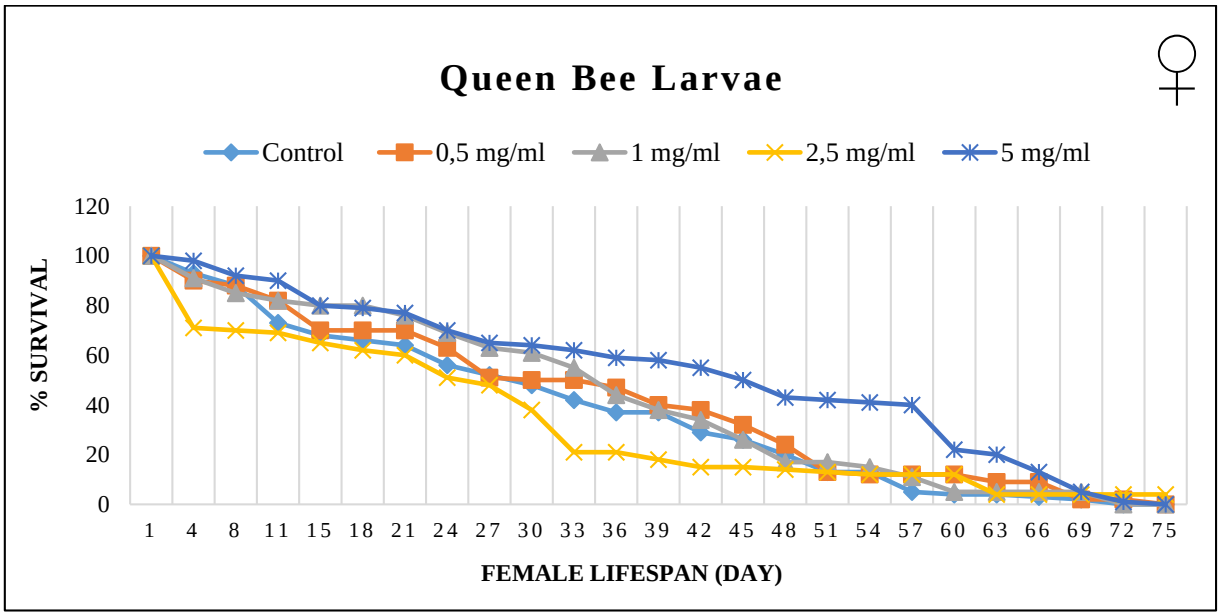

Figure 4. Survival curves of female *D. melanogaster* individuals living in different concentrations of queen bee larvae medium during their adult live

When all concentrations were compared in queen bee larvae treatment groups of both sexes, the longest maximum lifespan was observed in the 5 mg/ml treatment group and males (92±3.17); the lowest maximum lifespan was observed in the control group males and females (66±0.41) (Tables 2 and 3). However, the apilarnil and queen larvae treatment group showed higher lifespan prolonging activity than the distilled water control group in both sexes in both treatment groups.

According to these results, 2.5 mg/ml concentration in apilarnil medium was effective in both sex groups in terms of the effects on apilarnil and queen bee larvae larval mortality and lifespan, which we used in our experiments; 5 mg/ml concentration was effective in queen bee larvae medium. This difference observed in terms of mean lifespan was statistically significant at $p<0.05$ level in both sex groups.

DISCUSSION

Apilarnil and queen bee larvae are powerful energy providers that stimulate oxidative processes. Many bee products within the scope of apitherapy products have been examined and shown to have antioxidant properties due to their powerful energy-providing effects that stimulate oxidative processes. It was determined that the most popular bee products of recent times, apilarnil, which we used in our experiments, and the queen bee larva, adult *D. melanogaster*, reduced mortality.

Today, various functional resources are used due to problems such as the increase in diseases, malnutrition, unbalanced nutrition and insufficient protein intake due to various reasons. Insects have recently been consumed as a food source. Honey bees are also evaluated in this category, and bee products have many positive effects on human health. Apilarnil and queen bee larvae are some of them. It has an important place in medical use with its high nutritional value. In our study, it was observed that the support of apilarnil and queen bee larvae applied to the 3rd stage larvae had a positive effect on the death of adult *Drosophila melanogaster*, thus increasing the lifespan. We think that this positive effect is due to its antioxidant properties. When the literature is examined, there is no study investigating the effects of apilarnil and queen bee larvae on *Drosophila melanogaster*. It has mostly been studied on other organisms, while studies on *Drosophila melanogaster* are other bee products such as honey, propolis, royal jelly and bee venom. Apilarnil has a positive effect on problems such as loss of appetite, hypoproteinemia, premature aging, depression in the elderly, genital diseases, hormone and vitamin deficiency. One of the important reasons why the queen bee resists pathogens is that she is fed with pure royal jelly for life.

Apilarnil has a positive effect on problems such as loss of appetite, hypoproteinemia, premature aging, depression in the elderly, genital diseases, hormone and vitamin deficiency due to various reasons. One of the important reasons why the queen bee resists pathogens is that she is fed with pure royal jelly for life (Yang et al 2017).

As a result of the study of Yucel et al., apilarnilin (drone bee larvae); testosterone levels of 14.80±0.05 ng/g, progesterone levels of 14.40±0.05 ng/g were found to be at high levels, at the same time conjugated linoleic acid was determined as a fatty acid marker with a level of 52.62%. According to the results, it was understood that apilarnil has an important place in terms of bioproperty (Yucel et al. 2019). In a study by Hamamcı et al., apilarnil reduced the decrease in SOD and CAT levels in the brain with sepsis. At the same time, apilarnil decreased the increase in MDA, XOD and testis-1 levels in the septic brain, and as the dose of apilarnil increased, the number of degenerated neurons due to sepsis decreased. Apilarnil reduced the high levels of proinflammatory cytokines (IL-6, TNF-α, IL-1β) induced by sepsis. As a result, apilarnil prevented sepsis-related apoptosis in the brain (Hamamcı et al. 2020). In the 2020 study, it was observed that apilarnil from bee products had a positive effect on oxidative stress, proinflammatory cytokine production and increased apoptosis caused by LPS application. As the doses of Apilarnil increased, the TLR4 / HMGB-1 / NF-kB signaling pathway was inhibited and liver injury decreased. In line with these results, it was thought that apilarnil, which gained importance in terms of biological content, could be an alternative treatment for sepsis (Doganyigit et al. 2020). Another study was conducted on Wistar rats.

As a result of the study, it was observed that apilarnil is a powerful energy source that stimulates oxidative processes with an intense catabolic effect (Kogalniceanu et al. 2010). The most important

markers of antioxidant activity are amino acid sequences and molecular weight.

Dong et al. found a relationship between the hydrophobic amino acid, which is abundant in the content of queen bee larvae, and its high antioxidant activity (Dong et al. 2018). In this study, low (2.5 g/broiler) and high (7.5 g/broiler) doses of apilarnil were administered to male and female broilers between 28 and 55 days. As a result of the study, it was observed that blood sugar and cholesterol levels were lower in the group administered high-dose apilarnil, along with a decrease in fear and stress in animals (Altan et al 2013). In another research paper, it was seen that apilarnil applied to wild boars had a positive effect on sexual dysfunction.

It has been observed to increase the fertility rate by 76.4% in pigs (Bolatovna et al. 2015). Former beekeepers living in Romania consumed queen bee larvae in their entirety in order to prevent disease transmission when they got seasonal flu (Strant 2016). In this paper by Cruz et al., the effect of Brazilian *Pampa biome* honey on adult *Drosophila melanogaster* was investigated. Honey is protected against wing posture error and molecular changes related to mitochondrial pathways induced by hypoxia/reoxygenation. An upright wing posture was observed in some of the flies after reoxygenation, this acquired trait was also associated with death (Cruz et al. 2018).

In a study by Ayikobua et al. on the treatment of parkinsonism in *Drosophila melanogaster,* the effects of propolis and levodopa were compared. Propolis alone had a positive effect on motor activity, antioxidants and life span in *Drosophila melanogaster* compared to PINK1 flies. In combination with levodopa, propolis improved physiological parameters better at lower concentrations in Parkinsonism *Drosophila melanogaster* and showed a positive effect on the side effects of levodopa (Ayikobua et al. 2020).

The contents of this article, *Drosophila melanogaster* was fed with Perga called bee bread and it was observed that this product showed positive effects on vital parameters. While the effect on mortality rates of *Drosophila melanogaster* 3rd instar larvae was 94% in the control group, it was 98% in larvae) fed with Perga (Fidan et al. 2020).

Many experimental studies on bee products have been conducted in the literature. The experimental study of apilarnil and queen bee larvae has only recently begun to enter the literature. As seen in our study, apilarnil and queen bee larvae on *Drosophila melanogaster* showed a positive effect on longevity with their important biokaracteristics and contents. Apitherapy, which is seen as a supporter of medicine, works on many bee products. In this context, apilarnil and queen bee larvae are thought to have an important place in this field. In this way, the use of natural products whose contents have been analysed and biocaracteristics have been determined should be widespread. Since this study is the first study of apilarnil and queen bee larvae, which are the most popular bee products of recent times, on *Drosophila melanogaster*, it is thought that it will make a long-lasting contribution to the literature.

Conclusion: In this study, apilarnil (drone bee larvae) and black widow bee larvae, the most popular bee products of recent times in apitherapy, were used. Their effects on mortality and longevity of Oregon R wild larvae of *D. melanogaster* were examined. For this purpose, the results of different concentrations of selected apitherapy products were compared with the results of the control group. As a result of our study, an increase in the number of maturing larvae was observed for both bee products compared to the control group. It was also found to prolong the lifespan of *D. melanogaster*. Although the basic mechanisms of aging and longevity are not yet fully understood, it is argued that this process can be delayed. Today, studies on humans on this subject are not enough. It is thought that apilarnil, which has a rich nutritional content, and its effectiveness on the lifespan of queen bee larvae will make a significant contribution to the literature.

Author Contributions: AAB and AA conceived and planned the experiments. AAB and AA carried out the experiments. AAB and AA contributed to sample preparation. AAB and AA contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Conflict of Interest: The authors declared that there is no conflict of interest.

Data Availability Statement: The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement: This study does not present any ethical concerns. There is no need to obtain an ethics committee for the this article.

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Citation/Atıf: Ertürk Ö, Sağırkaya D, Algı G, Görgün S. European paper bees, *Polistes dominula* and *Polistes nimpha* (Christ, 1791) (Hymenoptera: Vespidae) pathogens presence and their potential insecticidal effects on honeybees adults of *Apis mellifera caucasia* (Pollmann, 1889). U. Bee J. – U. Arı D. 2024; 24(2): 235-251. DOI: 10.31467/uluaricilik.1519238

ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

EUROPEAN PAPER BEES, *POLISTES DOMINULA* AND *POLISTES NIMPHA* (CHRIST, 1791) (HYMENOPTERA: VESPIDAE) PATHOGENS PRESENCE AND THEIR POTENTIAL INSECTICIDAL EFFECTS ON HONEYBEES ADULTS OF *APIS MELLIFERA CAUCASIA* (POLLMANN, 1889)

Avrupa Kağıt arıları, *Polistes dominula* ve *Polistes nimpha* (Christ, 1791) (Hymenoptera: Vespidae) Patojenlerinin Varlığı ve bu Patojenlerin *Apis mellifera caucasia* (Pollmann, 1889) Bal Arısı Erginleri Üzerindeki Potansiyel Insektisidal Etkileri

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ABSTRACT

Honeybee (*Apis mellifera*) is an important element of biodiversity and terrestrial ecosystems. Any pathogenic infection in this beneficial insect can lead to major undesirable disasters. This study investigated the pathogenic bacteria and fungi from *Polistes dominula* and *Polistes nimpha* wasps and their potential insecticidal effects on *Apis mellifera caucasia*. For this purpose, bacteria and fungi were isolated from dead and diseased bees collected from Terme district of Samsun province in Türkiye in May and June 2020. In the study, *Granulicatella adiacens*, *Staphylococcus xylosus*, *Sphingomonas paucimobilis* bacteria and *Cryptococcus laurentii* and *Candida famata* fungi were obtained from the internal tissues and organs of *Polistes dominula* paper wasp adults. *Staphylococcus xylosus* and *Sphingomonas paucimobilis* were found to be common bacteria in both bee species. *Serratia marcescens* and *Enterococcus faecalis* bacterial species were found to have a very lethal effect on honeybees. Bioassay experiments were performed on the detected fungi, and it was observed that *Cryptococcus laurentii* and *Candida famata* fungi species also had lethal effects on honeybees. It has been revealed that entomopathogenic bacteria, which are known to be very effective in biological control against harmful insects, can cause unwanted infections in honeybees.

Keywords: *Apis mellifera, Polistes dominula*, *Polistes nimpha*, Bacterial flora

ÖZ

Bal arısı (*Apis mellifera*), biyolojik çeşitliliğin ve karasal ekosistemlerin önemli bir unsurudur. Bu faydalı böcekteki herhangi bir patojenik enfeksiyon, büyük istenmeyen felaketlere yol açabilir. Bu çalışmada *Polistes dominula* ve *Polistes nimpha* eşekarısı kaynaklı patojen bakteri ve mantarlar ile bunların *Apis mellifera caucasia* üzerindeki potansiyel böcek öldürücü etkileri araştırılmıştır. Bu

amaçla Mayıs ve Haziran 2020 aylarında Türkiye'de Samsun ili Terme ilçesinden toplanan ölü ve hastalıklı arılardan bakteri ve mantarlar izole edilmiştir. Çalışmada, *Polistes dominula* kağıt yaban arısı erginlerinin iç doku ve organlarından *Granulicatella adiacens*, *Staphylococcus xylosus*, *Sphingomonas paucimobilis* bakterileri ile *Cryptococcus laurentii* ve *Candida famata* mantarları elde edildi. *Staphylococcus xylosus* ve *Sphingomonas paucimobilis*'in her iki arı türünde de görülen yaygın bakteriler olduğu belirlendi. *Serratia marcescens* ve *Enterococcus faecalis* bakteri türlerinin bal arıları üzerinde oldukça öldürücü etkiye sahip olduğu tespit edildi. Tespit edilen mantarlar üzerinde bioassay deneyleri yapıldı ve *Cryptococcus laurentii* ve *Candida famata* mantar türlerinin de bal arıları üzerinde öldürücü etkileri olduğu görüldü. Zararlı böceklere karşı biyolojik mücadelede oldukça etkili olduğu bilinen entomopatojen bakterilerin, bal arılarında istenmeyen enfeksiyonlara neden olabileceği ortaya çıktı.

Anahtar Kelimeler: *Apis mellifera*, *Polistes dominula*, *Polistes nimpha*, Bakteriyel flora

GENİŞLETİLMİŞ ÖZET

Amaç: Arılar, tozlaşma ve bal üretimindeki rolleriyle bilinen eşekarısı ve karıncalarla yakından akraba olan uçan böceklerdir. Arılar, Apoidea ailesi içinde tek tip bir soydur ve bugün Anthophila sınıfı olarak kabul edilmektedir. Yaban arıları, dünya çapında yüz binden fazla tanımlanmış türe sahip olduğu tahmin edilen ve henüz tanımlanacak çok daha fazlasının olduğu düşünülen çeşitli bir gruptur. Yaban arılarının büyük çoğunluğu tozlaşmada etkili değildir, bazı türler polen taşıyabilir ve çeşitli bitki türlerinin tozlaşmasını sağlayabilir. Avrupa kağıt eşek arısı (*Polistes dominula*), Polistes cinsindeki en yaygın ve iyi bilinen sosyal eşek arısı türlerinden biridir özellikle Avrupa'da geniş yayılıma sahiptir. *Polistes nimpha* ise Avrupa genelinde Türkiye, Finlandiya, Estonya ve Letonya'da bulunmaktadır. Bu arılar, ayrıca Kuzey Afrika, Pakistan, İran, Hindistan (özellikle Jammu, Keşmir ve Himaşal Pradeş'in kuzey eyaletlerinde), Kazakistan, Moğolistan ve Çin'de de görülmektedir.

Bal arıları (şu anda sekiz türü tanınan *Apis* cinsi) oldukça sosyaldir ve en bilinen böcekler arasındadır. Bu türlerden biri olan *Apis mellifera*'nın Avrupa, Ortadoğu ve Afrika'ya özgü 31 alt türü bulunmaktadır. *Apis mellifera*'nın en önemli faydalarından biri tarımsal üretimde verimi arttırmak için çiçekli bitkilerin tozlaşmasını sağlamak ve değerli bir ürün olan bal üretimidir. Böcekler de diğer hayvanlar ve bitkiler gibi hastalığa neden olan mikroorganizmalar tarafından enfekte edilir. Böceklerin hastalık yapmasına ve ölümüne neden olan bu mikroorganizmalara genel olarak entomopatojenler adı verilmektedir. Ayrıca Bal arılarında hastalığa neden olan mikroorganizmaların araştırılması, sağlıklı arı kolonilerinin elde edilmesi, kovan başına bal veriminin arttırılması, daha aktif ve etkili işçi arıların sağlanması açısından büyük önem taşımaktadır. Zararlı böceklere karşı biyolojik mücadelede oldukça etkili olduğu bilinen entomopatojenler, bal arılarında istenmeyen enfeksiyonlara neden olabilmektedir. Bu çalışmanın amacı, eşekarısı populasyonun taşıdıkları bakteri, mantar patojenleri ve protistlerin varlığını tespit etmektir. Çalışmanın bu alanda entomopatojenlerin tespitinde ve yeni entomopatojen türlerinin tanımlanmasına önemli katkılar sağlayacağı düşünülmektedir.

Gereç ve Yöntem: Çalışmalarda kullanılan *Polistes dominula* ve *Polistes nimpha* örnekleri Mayıs ve Haziran 2020 aylarında Türkiye'nin Samsun ili Terme ilçesinden toplanmıştır. İnsektisidal çalışmalarda kullanılan sağlıklı *Apis mellifera* örnekleri ise Ordu Arıcılık Enstitüsü tarafından toplanarak temin edildi. Entomopatojenlerin türlerinin belirlenmesi amacıyla Samsun Eğitim ve Araştırma Hastanesi Mikrobiyoloji Laboratuvarı'nda kurulu VITEK® 2 (Biomerieux, Fransa) tam otomatik bakteri tanımlama sistemi kullanıldı, protistler için ise ışık ve elektron mikroskobu kullanıldı. Elde edilen entomopatojenlerin insektisidal etkileri ise biyoassay deney düzenekleri hazırlanarak tespit edildi.

Bulgular ve sonuç: Yapılan çalışmalar sonucunda toplam 18 adet saf kültür elde edildi. Kültür çalışmalarında, 3'ü gram negatif, 3'ü gram pozitif bakteri olmak üzere 6 cinse ait 6 bakteri ve 3 mantar türünden oluşan toplam 9 farklı izolat elde edildi. İzolatlar kodlandı ve numaralandırıldı. *Polistes dominula* ve *Polistes nimpha*'dan izole edilen bakteri ve mantarların morfolojik özellikleri belirlendi. Işık mikroskobu çalışmaları sonrasında microsporidium enfeksiyonunun böceğin bağırsaklarını, malpighi tüplerini ve hemolenfini enfekte ettiği belirlendi. Özel kamera ve resim sistemlerine sahip bir mikroskop kullanılarak, daha önce ışık mikroskobu altında tespit edilen mikrosporların enfekte olduğu dokuların

fotoğrafı çekildi. Işık mikroskobu ve elektron mikroskobu çalışmalarında Coccidia patojeni ilk kez eşekarısı içinde gözlendi. Arı dokusunda görüntülenen bu yapılar hem Işık Mikroskobu hem de SEM altında görüntülendi. VITEK® 2 sonuçları değerlendirilerek insektisit etkisi olduğu düşünülen 9 izolatla ilgili biyoanaliz çalışması yapıldı. Her izolat için 250-300 adet sağlıklı *Apis mellifera* kullanıldı. Biyoanaliz çalışmasının sonuçları, Abbott formülü kullanılarak düzenlendi.

INTRODUCTION

Wasps are winged insects belonging to the Vespidae family, which are neither bees nor ants, from the suborder Apocrita in the order Hymenoptera. Yellow jackets and hornets which most widely known wasps are from the Vespidae family and live together in a nest with an egg-laying queen and non-breeding workers. (Ertürk and Sarıkaya, 2020). Polistinae is the most diverse subfamily of Vespidae and has a fairly wide distribution and spread. *Polistes* Latreille, 1802, are a populous genus and live in North America up to Eurasia (Carpenter 1996, Pekkarinen 1999, Bağrıaçık, 2013, Mielczarek et al. 2021).

European paper wasp, *Polistes dominula* (Christ, 1791) (Hymenoptera: Vespidae), is a species of wasp belonging to the Vespidae family, most common in Europe, and native to the Palearctic Region around the, North Africa, the Middle East, Mediterranean, Southern Europe, and eastern China. It originated in the Near Polar Region of North America in the 1970s and 1980s (Madden 2010). Even though originally found on the east coast of the United States, then they spread to Midwest and currently to the Western and Southwestern United States. *Polistes dominula* was introduced in the 1990s to Canada, including Ontario, Nova Scotia, and British Columbia, as well as to Chile and Argentina in the Neotropical Region, and Western Australia in the Australian Region (Cervo and Turillazzi 1985). *Polistes dominula* habitats are temperate and terrestrial habitats, including forest and grassland ecosystems. They also live in urban and agricultural areas. Because they nest in human structures, they tend to live closely with humans. They also live in forests, on plants where they can feed and build nests (Cervo and Turilazzi 1985). *Polistes nimpha* is all social research found throughout Europe, especially in Türkiye, Finland, Estonia, and Latvia (Pekkarinen 1999, Mielczarek et al. 2021). It is also seen in China, Mongolia, Kazakhstan and Iran, Pakistan, Northern Africa, India (especially in the northern states of Jammu, Kashmir, and Himachal Pradesh). These regions with steppe vegetation and summers are usually hot climate and dry, in winter is relatively cold and snowy (Carpenter 1996).

Bees, closely related to wasps and ants, are flying insects that play an important part in pollination and honey production, and are one of the most valuable factors for agricultural productivity and are also very important in terms of the terrestrial ecosystem and sustainability. Bee pollination is important both ecologically and commercially, and the decrease in wild bees is increasing the value of pollinating hives of commercially controlled honey bees (Canale and Benelli 2021). Unlike honeybees, the main function of the majority of wasps is not to carry pollen, but some wasp species can carry pollen and contribute to the pollination of various plants (Sühs et al. 2009). It is a uniform lineage within the Apoidea family and today they are considered to be the class of Anthophila. There are more than 16,000 known bee species belong to the seven families (Michener 2000, Danforth et al. 2006, Grosch et al. 2021). Wasps are a diverse group estimated to have more than one hundred thousand described species worldwide and likely many more that are undescribed. Stings from these bees can cause both local and systemic allergic reactions and even life-threatening anaphylaxis in humans (Sahiner and Durham 2019).

Honeybees, the genus *Apis* Linnaeus, 1758, of which eight species are now recognized, are highly social andare among the best-known insects. One of these species, *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae), has 31 subspecies native to Europe, the Middle East, Asia, and Africa. The most important benefits of *Apis mellifera* are the pollination of flowering plants in agricultural production and the production of honey. With rapid population growth, people's demands for food are increasing rapidly. To meet theserequirements, bees are of high importance in terms of increasing production and ensuring continuity (McGregor 1976).

Insects, like other animals and plants, are infected by many microorganisms. These microorganisms, which cause disease and death of insects, are generally called entomopathogens. Entomopathogens that cause disease or direct death in insects are viruses, bacteria, rickettsias, protists, fungi, and nematodes, and can act on insects by reducing their feeding and growth rates. They can also act by slowing, inhibiting, or killing their reproduction. Areas of ecological overlap of wasps and honeybees, such as orchards

and hives can enable harmful pathogens to be transferred between species. These organisms cause diseases by multiplying and spreading naturally in the insect population under certain conditions and when the insect density is high (Sharma 2020).

Coccidia (Coccidiasina) is a subclass belonging to the apicomplexan class, Conoidasida, are single-celled, microscopic, spore-forming and obligate intracellular parasites (Urquhart 1996a). Since they are obligate intracellular parasites, their reproduction and survival depend on living host cells. Coccidia parasites are the largest group of apicomplexan protozoa and can infect mammals, birds, fish, reptiles and some amphibians, and infect the intestinal tract. Additionally, most Coccidia species are host selective, meaning they are specific to their host (Yatoo 2013).

Microsporidia are obligate intracellular parasites that form spores and infect a wide range of vertebrates and invertebrates (Pan et al. 2018). Microspores, which prefer a single host in nature, do not cause the death of the pest when they infect but cause a shortened life span, loss of appetite, weight loss, and reduced reproductive availability. They are pathogens suitable foruse in biological control because they only affect the target organism and are specific to only one host. As a result of studies on microsporidia, these parasites have been isolated from mammals, reptiles, fish, amphibians, and insects (Tamim et al. 2020).

Studying microorganisms that cause disease in honeybees is of great importance in terms of obtaining healthy bee colonies, increasing honey yield per hive, and providing more active and effective worker bees (McGregor 1976, Sharma 2020). Entomopathogenic bacteria are the most valuable and effective organisms known in biological control against harmful insects, but they can cause undesirable infections in honeybees. In this study, we determined bacterial flora and presence fungal pathogens of *Polistes dominula* and *Polistes nimpha*. It is thought that this study will make significant contributions to the identification of new entomopathogenic bacterial and fungal species on European paper wasps.

MATERIAL AND METHODS

Obtaining the bees

The *Polistes dominula* and *Polistes nimpha* samples used in the studies were collected from the Terme district of Samsun province in Türkiye in May and June 2020. Healthy *Apis mellifera* samples used in insecticidal studies were collected and provided by Ordu Apiculture Institute.

Bacteria and fungi isolation from bee

Two methods were used for bacterial isolation. While it was made by taking samples directly from the hemolymph from bee samples with intact internal tissues, the samples were crushed in a sterile environment for bees with damaged internal tissues. To isolate bacteria from the collected wasp samples, 20 adult insects from each species were selected and placed in separate sterile tubes. Surface sterilization of wasps placed in tubes was done with 70% alcohol (Poinarand Thomas, 1978). After this procedure, the samples were washed three times with sterile distilled water under aseptic conditions. In the first method, some liquid was taken from the cuticle of the bee to reach the hemolymph with a fine-tipped injector, diluted, and spread directly on Nutrient Agar (Merck) medium. In the second method, 10 mL of sterile distilled water was added to the tubes and the samples were crushed in distilled water until they became homogeneous. After this process, 100 µL was added to Nutrient Agar, and spread plate cultivation was performed.

Determination of insecticidal efficacy of isolated bacteria and fungi

To determine the effect of the isolates obtained in this study on honeybees, 250-300 healthy honey bees were selected for each isolate from Ordu Apiculture Research Institute and an experimental group was formed. Rectangular queen bee breeding hives with a side length of 40 cm and a width of 20 cm were used for the experimental groups. In this stage, glucose-water solution (1:1) was prepared for feeding the experimental groups placed in the hives. 5 mL of the prepared solution was taken and the density was adjusted to 0.5 Mcfarland separately for each of the bacterial isolates. Small 25 mL spray cups were used to contaminate the bacteria (Fig. 3, 4). Five mL of the adjusted bacteria-solution mixture was sprayed into the feeding cups of the bees, allowing the bees to take the bacteria through food. Organisms whose density was adjusted with Mcfarland were added to the prepared solution under aseptic conditions and observed for 7 days. The test was completed after 7 days, paying attention to the number of dead and surviving honey bees (Kämpfer 1995). Experimental groups created during the test were checked daily. Insects that died during the control period were removed from the containers with forceps (Ombui et al. 1996, Swiecicka 2001). Average mortality rates were

determined by determining the number of insects that died each day. This application was repeated three times for each isolate. Control groups were also used for the application.

To detect microspore and Coccidian pathogens, which are the subject of the study, wasp adults were dissected in the prepared Ringer solution. Ringer's solution was obtained by dissolving 8.0 g Sodium chloride (NaCl), 0.25 g Calcium chloride ($CaCl_2$), 0.25 g Potassium chloride (KCl), and 0.25 g Sodium bicarbonate ($NaHCO_3$) in 1000 ml distilled water for bee tissues. It isused in dissection processes in terms of creating the most ideal isotonic environment. Dissection was performed by carefully removing all tissues and organs from the abdomen and thorax to determine in which tissues the pathogen was active. The preparation was examined under a light microscope (Olympus CX41) with magnifications from 40x to 1000x. Infection-detected preparations were reexamined with a DP-25 digital camera and an Olympus BX51 microscope equipped with a DP2-BSW picture system, photographs of the pathogen were taken and necessary measurements were made for its characterization.

Then, to provide further imaging, bacterial cell suspensions of M17 broth and RSM were centrifuged at 3000 rpm for 1 min for scanning electron microscopy for more detailed imaging. To stabilize the protein, they were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 2 hours, followed by 15 min intervals in 0.1M phosphate buffer several times. The samples were exposed to serial ethyl alcohol solutions of 25%, 50%, 75%, 95% and 100% for 5 minutes each and dried. Critical Point Dryer (Polaron, Waterford, England) mounted on aluminum SEM studs, sputter coated with gold (Spi module spray coater, spi structure probe section materials). The samples were examined by SEM at 10-25 KV. Since the reliability of the measurements is based on the accuracy of the method, great care was taken.

Determining the contact of *Polistes dominula* and *Polistes nimpha* wasps with honeybees *Apis mellifera* in their natural habitats

This experiment was designed under natural conditions. A beehive was made by hollowing out the inside of a 30 cm diameter pine tree trunk. There were 5 honeycombs left in this hive, containing approximately 50-60 worker bees, including 1 queen. We specifically placed our hive where there were fruit trees. We placed the beehive 2-3 meters away from a fig tree where fig fruits are abundant. We observed it for approximately 15 days.

RESULTS

Bacteria and fungi isolation

A total of 18 pure cultures were obtained in the studies carried out.A total of 9 different isolates of 6 bacteria and 3 fungal species were obtained from 6 genera, 3 of which were gram-negative and 3 of which were gram-positive bacteria. All isolates obtained were numbered and coded (Tables 1, 2) and no spore-forming bacteria were detected. VITEK® 2 fully automatic bacterial identification system was used to determine the species of the isolates in Samsun Training and Research Hospital Microbiology Laboratory. According to the VITEK® 2 result, 9 of the isolates were identified at the species level. In the study, bacteria from the inner tissues and organs of diseased and dead *Polistes dominula* paper wasp adults *Serratia marcescens* (no.1), *Enterococcus faecalis* (no.6), *Staphylococcus xylosus* (no.9), *Sphingomonas paucimobilis* (no.11), *Staphylococcus lentus* (no.13) and *Candida ciferrii* (M2) fungus was isolated. From the internal tissues and organs of *Polistes dominula* paper wasp adults, the bacteria *Granulicatella adiacens* (No.2), *Staphylococcus xylosus* (No.10), *Sphingomonas paucimobilis* (No.15), and *Cryptococcus laurentii* (M1) and *Candida famata* (M3) (as *Debaryomyces*, *Hansensis,* and *Torulopsis candida* also known) mushrooms were obtained. It was observed that *Staphylococcus xylosus* and *Sphingomonas paucimobilis* were common bacteria in both bee species. The isolates, test codes, percentages and content obtained from *Polistes dominula* and *Polistes nimpha* in the study are given in Table 1. The morphological characteristics of bacteria and fungi isolated from *Polistes dominula* and *Polistes nimpha* are shown in Table 2.

Table 1. The isolates, test codes and percentages obtained from *Polistes dominula* and *Polistes nimpha*.

Wasp species	Isolate no	isolates	Percentage rates
Polistes dominula	1	*Serretia marcescens*	99%
	6	*Enterococcus faecalis*	99%
	9	*Staphylococcus xylosus*	86%
	11	*Sphingomonas paucimobilis*	97%
	13	*Staphylococcus lentus*	88%
	M2	*Candida ciferrii*	95%
Polistes nimpha	2	*Granulicatella adiacens*	98%
	10	*Staphylococcus xylosus*	89%
	15	*Sphingomonas paucimobilis*	93%
	M1	*Cryptococcus laurentii*	89%
	M4	*Candida famata*	98%

Table 2. Morphological characteristics of bacteria and fungi isolated from *Polistes dominula* and *Polistes nimpha*.

Species Name	Locality	Gram	Spor	Bacteria shape	Colony shape	Colony color
Staphylococcus lentus	feces	+	-	Coccus	Round	**White**
Sphingomonas paucimobilis	feces	-	-	Bacil	Convex	**Yellow**
Enterococcus faecalis	wasp internal tissue	-	-	Coccus	Round	**Off-white**
Serratia marcescens	wasp internal tissue	-	-	Rod	Round	**Red**
Staphylococcus xylosus	wasp internal tissue	+	-	Coccus	İrregular structure	**Yellowish**
Granulicatella adiacens	feces	+	-	Coccus	İrregular structure	**Off-white**
Cryptococcus laurentii	feces					
Candida ciferrii	wasp internal					
	tissue					
Candida famata	**feces**					

Bioassay studies for determination of insecticidal effects

VITEK® 2 results were evaluated, and a bioassay study was conducted with 9 isolates thought to have an insecticidal effect. For each isolate, 250-300 healthy *Apis mellifera* were used. Comparisons of death rates of bees in terms of isolated microorganism species was shown in Table 3. The results of the bioassay study were arranged using the Abbott formula (Table 4). The death rates of bacteria were shown in Fig. 1 and the mortality rates of fungi were shown in Fig. 2. At the end of the calculation, it was observed that the bacteria *Serratia marcescens* and *Enterococcus faecalis* and the fungal isolates of *Cryptococcus laurentii* and *Candida famata* showed the highest insecticidal effect. The pathogenicity of the isolated and identified bacteria and fungi on *Apis mellifera* was also tested. For this, the insecticidal activity of isolates at doses of 1.8 × 10 bacteria/mL within 7 days of application to healthy *Apis mellifera* adults was tested in several bioassay experiments. Especially *Serratia marcescens* and *Enterococcus faecalis* of our isolates caused mortality in 81.01% and 89.1% of *Apis mellifera* with large percentage. Most of the dead bees had diarrhea. The insecticidal activity of our other bacteria, which is the study

material, was not very high and the values were close to each other. *Granulicatella adiacens*, *Sphingomonas paucimobilis*, *Staphylococcus xylosus,* and *Staphylococcus lentus* have a mortality rate of 10.18%, 18.9%, 24.32%, and 27%, respectively (Table 4). Some of these bacteria were previously isolated from *Apis mellifera*. In our study, fungi isolated from *Polistes dominula* and *Polistes nimpha* caused more and more effective mass deaths than bacteria. Almost all of the dead bees died within 5-7 days, and they were piled up in one place and their bodies became very soft and they had diarrhea. As a result of the calculations, mortality rates were found as M1 numbered *Cryptococcus laurentii* 83.78%, M2 number *Candida ciferrii* 41.51%, and M3 number *Candida famata* 94.59% (Fig. 1, 2). These fungi, especially *Cryptococcus laurentii* and *Candida famata*,caused mass mortality in honey bees with a high percentage of 83.78% and 94.59%. Fungi had created a higher rate against bacteria and great disinformation of the dead bees both physiologically and morphologically. In the observations we made in the beehives, it was determined that the bees were dying together in clusters.

Table 3. Comparisons of mortality rates of bees in terms of isolated microorganism species.

		Mortality		**Survive**		**Total**	p
		n	%	n	%	n	
n		940	100,0	860	100,0	1800	
Staphylococcus lentus							**<0.001**
	Yes	54	27,0	**146**	**73,0**	200	
	Others	886	55,4	714	44,6	1600	
Sphingomonas paucimobilis							**<0.001**
	Yes	38	19,0	**162**	**81,0**	200	
	Others	902	56,4	698	43,6	1600	
Enterococcus faecalis							**<0.001**
	Yes	**178**	**89,0**	22	11,0	200	
	Others	762	47,6	838	52,4	1600	
Serratia marcescens							**<0.001**
	Yes	**162**	**81,0**	38	19,0	200	
	Others	778	48,6	822	51,4	1600	
Staphylococcus xylosus							**<0.001**
	Yes	49	24,5	**151**	**75,5**	200	
	Others	891	55,7	709	44,3	1600	
Granulicatella adiacens							**<0.001**
	Yes	20	10,0	**180**	**90,0**	200	
	Others	920	57,5	680	42,5	1600	
Cryptococcus laurentii							**<0.001**
	Yes	**167**	**83,5**	33	16,5	200	
	Others	773	48,3	827	51,7	1600	
Candida ciferrii							**0.001**
	Yes	83	41,5	**117**	**58,5**	200	
	Others	857	53,6	743	46,4	1600	
Candida famata							**<0.001**
	Yes	**189**	**94,5**	11	5,5	200	
	Others	751	46,9	849	53,1	1600	

Those marked with a dark color indicate cells with statistically significant high levels compared to other cells. The survival rate in bees from which *Staphylococcus lentus* was isolated was found to be significantly higher than in bees from which other microorganisms were isolated (73.0% vs. 44.6%; p<0.001) (Table 3). The mortality rate in bees from which *Enterococcus faecalis* was isolated was significantly higher than in bees from which other microorganisms were isolated (89.0% vs. 47.6%; p<0.001). In other words, while the mortality rate in bees from which some microorganisms were isolated

was significantly higher than in other isolate types, it was found to be significantly lower for some species. This finding shows that the microorganism types with significant high levels are directly related to mortality in these bees (Table 3).

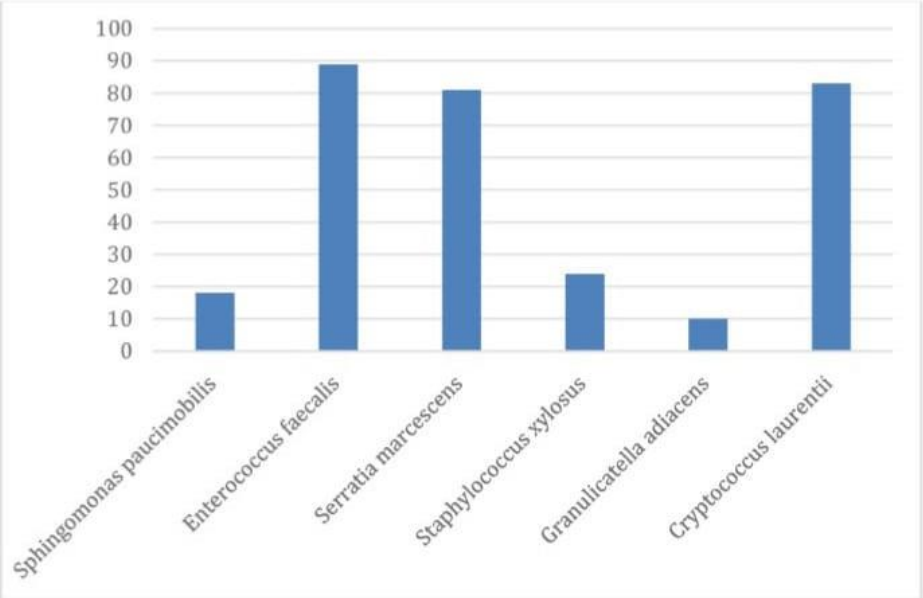

Figure 1. Graphic showing the mortality rates of bacteria.

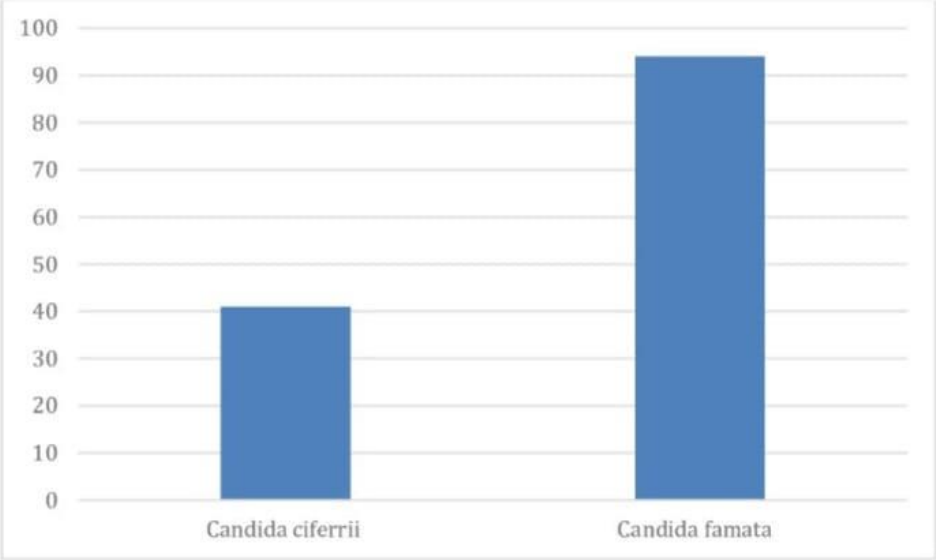

Figure 2. Graphic showing the mortality rates of fungi.

Table 4. Abbott's analysis results of insecticidal effects of bacteria and fungi isolated from *Polistes dominula* and *Polistes nimpha* on *Apis mellifera*.

Microorganisms	Bee Numbers	Mortality Percentage(Abbott)
Staphylococcus lentus	200±10	**%27±1.76**
Sphingomonas paucimobilis	200±10	**%18.9±2.753**
Enterococcus faecalis	200±10	**%89.1±3.33**
Serratia marcescens	200±10	**%81.01±4.64**
Staphylococcus xylosus	200±10	**%24.32±349**
Granulicatella adiacens	200±10	**%10.18±5.32**
Cryptococcus laurentii	200±10	**%83.78±5.53**
Candida ciferrii	200±10	**%41.51±2.11**
Candida famata	**200±10**	**%94.59±3.85**

Microscope studies

Important life stages of the microsporidium pathogen were tried to be determined carefully in wasp samples dissected for examination in light microscopy studies. During the direct examination of fresh tissues, the presence of infection was determined by comparing the morphological differences in the tissues with microsporidium infection with those of normal tissues. Damage to the tissues of the host was observed in fresh preparations. After light microscopy studies, it was determined that microsporidium infection infects the intestine, Malpighi tubes, and hemolymph of the insect. Using a microscope with a special camera and picture systems, the tissues infected by the microspores previously detected under the light microscope were photographed (Fig. 3). In light microscopy and electron microscopy studies, the Coccidia pathogen was observed for the first time in wasps (Fig. 3, 4). These structures, which were visualized in the bee tissue, were visualized both under the Light Microscope and SEM (Fig. 5). Some biochemical test results for gram positive and gram negative bacteria obtained from both types of paper bees are shown in (Table 5). As a result of the study, coccidian, and microsporidia pathogens, which we detected from *Polistes dominula* and *Polistes nimpha* wasps, could not be reproduced, so no bioassay study was performed on *Apis mellifera*. In addition, coccidian and microsporidia pathogens, which we detected from *Polistes dominula* and *Polistes nimpha* wasps, could not be reproduced, so no bioassay study was performed on *Apis mellifera*.

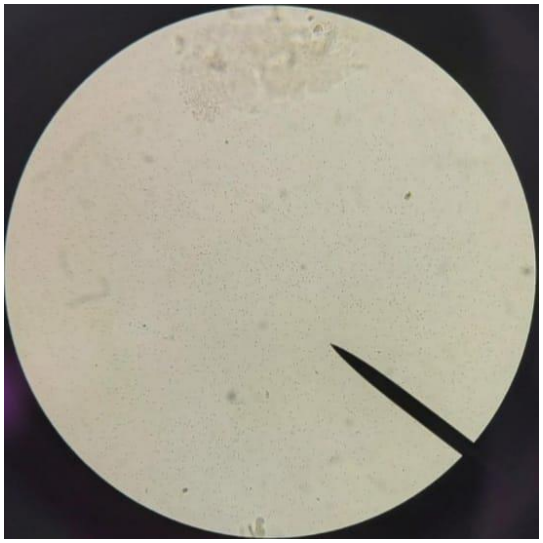

Figure 3. The appearance of microspores in the light microscope.

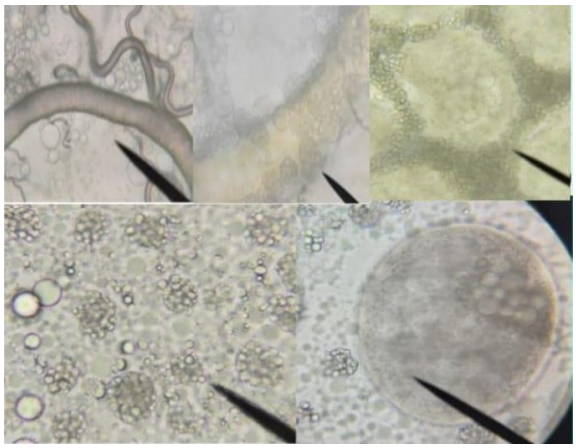

Figure 4. The coccidian pathogen in the light microscope.

Table 5. Physiological and biochemical properties of bacteria isolated from *Polistes dominula* and *Polistes nimpha*.

EXPERİMENTS	*Serratia marcescens*	*Sphingomonas paucimobilis*	*Enterococcus faecalis*	*Staphylococcus xylosus*	*Staphylococcus lentus*	*Granulicatella adiacens*
Gram stain	**-**	**-**	**+**	**+**	**+**	**+**
Growth at pH 3	-	-	-	-	-	-
Growth at pH 5	+	+-	+	-	+	+
Growth at pH 7	+++	+++	+	+	++	+
Growth at pH 9	+	+	+++	++	++	-
Growth at pH 10	+	+	+	-	-	-
Control (NB)	++	++	+++	+++	+++	+++
NB+ growth at 2% NaCl	+++	++	+	+	+	+
NB+ growth at 3% NaCl	++	++	+	+	+	+
NB+ growth at 4% NaCl	+	+	+	++	++	+
NB+ growth at 5% NaCl	+	+-	+++	+	+	+
NB+ growth at 7% NaCl	+	-	+	+	-	-
NB+ growth at 10% NaCl	-	-	+	+	-	-
Growth at 25˚C	+	++	+	+	+	
Growth at 36 ˚C	++	++	+++	++	++	++
Growth at 40˚C	-	Dormant at 37°C.	+++	+++	+++	+++
Morphology	Rod	Bacil	Cocci	Cocci	Cocci	Cocci
Gas	+	+	-	-	-	-
Indole	-	+	-	-	-	-
Methyl Red	-	+	+	+	+	+
Voges-Proskauer	+	-	+	-	+	+
Citrate	+	+	-	-	-	-
Glucose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Lactose	-	-	+	+	+	-
H_2S	-	+	+		+	-
Galactose	+	+	+	+	-	-
Urease	-	+	-		-	-
MacConkey growth morphology	++	++	-	-	-	-
Length (µm)	0.8 ± 23 µm	1.4 ± 08 µm	1.48 ± 0.31 µm	09 ± 04 µm	08 ± 05 µm	09 ± 02 µm
Diameter (µm)	0.5 ± 45 µm	0,13 ± 07 µm	1.38 ± 0.31 µm	09 ± 04 µm	07 ± 02 µm	06 ± 06 µm

Observation of the contact between honeybee and wasps

As a result of our observation, we noticed that bees especially attacked the sugar of figs and all three types of bees settled on the same fruit. These wild paper bees attack fruit sugars a lot in nature, and as a result, they come into contact with honeybees, which may be the reason for the transmission of microorganisms between these species. In addition, many studies have found that there are common microorganisms in all three species. However, because of this study, bee farms should not be placed too close to orchards both for wintering and for making honey. In addition, we observed that the hornets started to fight with the honeybees and eventually most of the honeybees died and some of them left the hive.

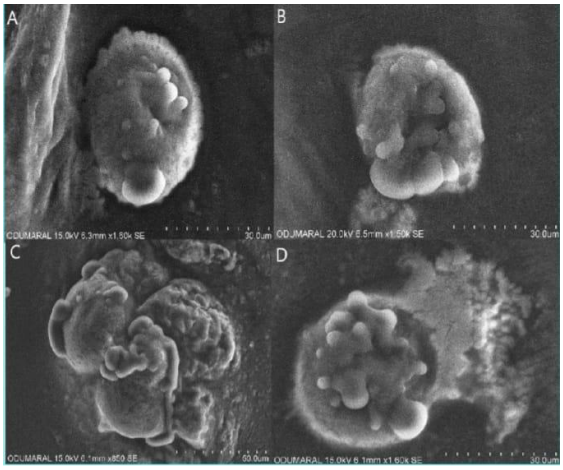

Figure 5. Coccidian pathogen in scanning electron microscopy (SEM).

DISCUSSION

European honeybees have been the most researched insects in Türkiye and the world due to their important role in agriculture and the ecosystem and their high economic value. In addition to producing honey, propolis and beeswax, which are the most valuable roles of honeybees, they are also important for agriculture and sustainability. A highly adaptable species, the bees' habitat extends throughout Asia and Africa, from the southern parts of Scandinavia to the Central (Sheppard and Meixner 2003, Avci et al. 2022).

Many various microorganisms found in bees are associated with honey bees. In recent years, studies on bees by scientists have reported serious bee losses from beehives and a decrease in bee populations (Stathers 2016, Potts 2020). Bees are now making a great contribution to the food chain, directly or indirectly, on earth. Honeybees have been faced with mass deaths in some places in recent years. There are different causes of these deaths. One of them is microorganisms. Our aim in this study was to seek answers to the questions of whether bacteria and fungi that we will isolate from wasps have negative infections on honeybees or do they cause mass deaths if any.

Wasps encounter honeybees directly or indirectly at some times of their lives, sometimes in theform of a flower, sometimes fruit, sometimes a nest raid, or a direct encounter with the honey bee itself. Individuals of these wasp populations can infect honeybees with some bacterial and fungal species they carry in their bodies.

When aerobic and facultative anaerobic bacteria isolated from the intestines of worker bees were identified according to biochemical and 16S rDNA sequence analyses; *Firmicutes*, *Proteobacteria* and *Actinobacteria* were detected at higher rates. Additionally, opportunistic commensal bacteria such as members of *Staphylococcus haemolyticus* and *Sphingomonas paucimobilis* were also identified in the hive environment of these bees (Anjum et al. 2018). The eastern honey bee bird feeds mainly on bees and wasps. The gut microbiome of this bird of prey was found to be rich in *Firmicutes* and *Bacteroidetes*. This information provides clues that bee populations carry common bacterial species (Nagai et al. 2018).

Some bacterial species isolated in our study were similar to the species detected in a different study on the bacterial flora of honeybees previously (Przybyłek and Karpiński 2019, Bog et al. 2020). As an example, *Staphylococcus lentus* and *Sphingomonas paucimobilis* bacterial species were detected in the study conducted in honeybees collected from Ordu and 9 districts (Bog et al. 2020), while the same bacterial species were found in wasps collected from the Terme district of Samsun in our study. This similarity may suggest that the bacteria are common to both species or that they were formed after an infection.

As a result of this study, *Serratia marcescens* bacteria, one of the bacteria obtained from the digestive system (midgut and hindgut) of *Polistes dominula* and *Polistes nimpha* wasps, is a great potential source of Gram-negative bacterial entomopathogens, new toxins, and metabolites that can be used in insect control programs. However, this bacterium is a member developed as a biocontrol product (Ferreira et al. 2021). *S. marcescens* infects both invertebrates and vertebrates but is considered an important pathogen of insects, most commonly causing bacteremia (presence of bacteria in the bloodstream) and rapid insecticide (Grimont and Grimont 2006, Ishii et al. 2014).

In a study, the pathogenicity of *S. marcescens* isolates against these pests, which was isolated and characterized using bioassays from the larvae of *Plodia interpunctella* and *Ephestia kuehniella*, was evaluated. These bacterial cells were injected into the hemolymph or added to the insect's diets. Compared to the control group, the survival rates of insects, especially larvae, exposed to different *S. marcescens*

concentrations were significantly reduced. The results of our study showed that this *S. marcescens* isolate has valuable potential for use in biological control of harmful insects. In addition, these results can be considered as a source of bioactive molecules and genes useful in the development of insect-resistant plants, biological control and biotechnology applications. (Bidari et al. 2018). In this study, we obtained, *Granulicatella adiacens*, compared to other streptococci, is rarely involved in infections because of isolation but part of the oral, gastrointestinal and urogenital commensal flora. These have been reported to cause mostly bacteremia and endocarditis and, less frequently, device-related infections such as meningitis, breast implant infections, and peritoneal dialysis-associated peritonitis (Christensen and Facklam 2001).

Another isolate of our study, *Sphingomonas paucimobilis*, has been isolated from insects and bees in many previous studies. In a study conducted in Ordu, bacteria were obtained from sick and dead honey bees collected from 9 regions. Eighteen of the pathogenic bacteria were isolated from non-spore-forming bacteria. One of these isolates is *Sphingomonas paucimobilis* (Bog et al. 2020). However, *Sphingomonas paucimobilis* has been found to be an opportunistic pathogen in honey bees that have been found to multiply in the hive environment (Anjum et al. 2018). In another study, these bacteria, which are frequently seen among bacteria isolated from some Tabanidae species that fall into insect traps, are also bacteria found in foods, various water sources, wastes, feces, and everywhere (Fukui et al. 1999, Köseoğlu et al. 2019). In addition, some strains of this bacterium were isolated and identified from the guttation fluid of anthuriums by Fukui et al. (Fukui et al. 1999). In our study, it is thought that *S. paucimobilis* probably infect honeybees when they collect nectar from flowers.

Staphylococcus xylosus, which is present in honeybees and domestic waste water, was obtained by characterizing and identifying proteolytic bacteria in the gut of the velvet bean caterpillar (*Anticarsia gemmatalis*) (Visotto et al. 2009). However, this isolate was also isolated in the study with bees (Christensen and Facklam 2001, Essa and El-Gayar 2018) characterized bacteria isolated from two domestic waste water treatment plants in Jazan, KSA. Domestic wastewater can be considered a suitable environment for the survival of a wide variety of microorganisms. In the identification and diagnosis of bacterial isolates, researchers were use morphological and biochemical tests. As a result of the study, both Gram-positive and Gram-negative bacterial strains were isolated, including the *Staphylococcus xylosus* species. In many studies, the bacteria we obtained from wasps were also isolated from honeybees, which shows us that there is microorganism transmission between these species. In addition, many studies have found that there are common microorganisms in all three species (Anjum et al. 2018, Essa and El-Gayar 2018).

Enterococcus faecalis is an important bacterial species among the isolates we obtained in our study. This isolate has been isolated from different insects by many researchers. In a study, *Enterococcus faecalis* was isolated from different insect guts. Microbial colonies with different criteria were selected for identification based on their size, shape, color and other visual characteristics and investigated using physical and biochemical methods for their identification. Fifteen isolates representing twelve different genera were identified from the 19 colonies obtained. Among the isolates obtained from *Ocymyrmex velox*, *Enterococcus faecalis* and *Serratia marcescens* bacteria are also present. In spite of different bacterial species were detected in the guts of different insect species in this study, this does not stated that they contain common bacteria. (Shil et al. 2014). Another study found a bacterial disease in the beet caterpillar *Spodoptera exigua* (Hübner). Epizootic disease has been observed in the blackened body of infected larvae, especially in intersegmental areas. It was identified as *Enterococcus faecalis*, a gram-positive bacteria isolated from the hemolymph of infected 5th instar larvae (Park et al. 2002).

Fungi are widespread in nature and have been isolated from plant and animal surfaces, air, food, and sugary substrates such as flower nectar and fruit juices. (Ingram 1955, Last and Price 1969). The relationship fungi with insect guts is well known. Just as flower nectar is a rich source of fungi, pollinator bees, contain fungi, especially in their nectar sacs. The connection between some fungi, flowers and insects is that and also transfer fungi to flower nectars during their visits to entomophilous flowers (Buchner 1965, Batra et al. 1973).

In this study, species belonging to *Candida*, *Debaryomyces*, *Hansenula,* and *Torulopsis* fungal genera obtained from wasps were determined. In other studies, similar species and species of fungi have been identified from the honey stomachs of pollinating bees. *Cryptococcus laurentii* and *Candida ciferrii* fungi were obtained in our study. These two

fungi were isolated from the surface of healthy apple fruits collected from organic production orchards in the south of Uruguay with biocontrol agents (Vero et al. 2022).

In another study, a small amount of *Candida famata* was detected in isolates obtained from mastitic Anatolian buffalo district milk samples. In another study, biological control of *Candida famata* isolates obtained from fig leaves on orange fruits was reported in Sardinia. In terms of host-antagonist-pathogen interaction, the fungus, alone or artificially inoculated, has been found to induce the fruit to produce associated phytoalexins (scoparone and scopoletin) in significantly varying concentrations (Arras 1996). In this study, microsporidium was detected in dissection samples from wasps. In the study, the spore stage, which is the most basic stage in the detection of the pathogen under the microscope, was observed in many dissections. Spores with the characteristic features of the microsporidium pathogen are distinguished from other tissues of the host in that they refract light differently and have the same size and shape.

Coccidia pathogen was observed for the first time in wasps used in our thesis study. In this way, it has the distinction of being the first. Coccidia is a subclass of Apicomplexan class and microscopic, unicellular, spore-forming, obligate intracellular parasites belonging to the Conoidasida (Urquhart et al. 2001). This infection with parasites is known as Coccidiosis. Depending on the Coccidia species, infection in bees can cause nervous system effects, vomiting, fever,muscle pain, diarrhea, behavioral changes and death. Diagnosis of Coccidiosis is made by finding oocysts in stool smears. In the early stages of the disease, very few oocysts may be shed and a negative test does not exclude the disease. Treatment of coccidiosis is usually with Coccidiostats, a group of medications that block the growth of Coccidia. The most administered Coccidiostat in dogs and cats are sulfate-based antibiotics. Once reproduction has stopped, recovery can occur on its own, a process that depends on several factors, including the animal's immune system and the severity of the infection, and can take several weeks (Chapman 2013).

In conclusion, the fact that similar bacteria and fungi obtained from honeybees in previous studies with the wasps in our study are common in both species may indicate that this is a result of contamination between bees. It is thought that bumblebees and honeybees sometimes meet indirectly or directly when they visit flowers (collect nectar), sometimes at fruits, and sometimes when raiding nests. This may indicate that bumble bee communities can transmit some types of bacteria and fungi that they carry in their bodies to honeybees, causing infections in bee colonies and negatively affecting them. In this case, it can be thought that some of the bacteria we obtain from wasps can be used as biocontrol products, in microbial control of insect pests, in insect control by developing insect-resistant plants, and in biotechnology applications. Honey bees are economically very valuable as they pollinate a significant portion of the world's crops. Additionally, honeybees' hive products, such as residues, are used to feed cattle. The amount of pollination worldwide is €153 billion, representing 9.5% of world human food agricultural production in 2005 (Gallai et al. 2009). However, in recent years, increasing colony losses have been reported in the USA, Europe and elsewhere (Gallai et al. 2009, Biesmeijer et. al. 2006, Ghazoul 2005).

Losses of honeybees have been particularly evident in Colony Collapse, a serious disease that caused the loss of 50-90% of colonies in beekeeping operations across the United States in 2007. An opportunistic pathogen, *Serratia marcescens* has a lethal effect not only on animals and plants but also on honey bees and poses a threat to the generation of bees. (Raymann et al. 2018). In another study conducted in Sudan, twenty-three strains of *Serratia marcescens* were isolated in pure culture from diseased honeybee larvae (El Sanousi et al. 1987).

Honeybees are major pollinators of crops and flowers worldwide, and pathogens and parasites that threaten the health of bees are of utmost importance. To determine the presence of bacteria, fungi and different entomopathogenic organisms in the bodies of two wild bees, *Polistes dominula* and *Polistes nimpha*, in the honeybee *Apis mellifera* is important. Another important issue is the determination of the occurrence and prevalence of these pathogens and parasites and their infections on honeybees, especially in regions where beekeeping is at the forefront in Türkiye. We conducted a nationwide survey. In our study, we found evidence of very high rates of infections caused by certain bacteria and fungi isolated from two species of wasps, *Polistes dominula and Polistes nimpha;* We found these to be linked to declines in the population of honeybees. Wild bees are bees that do not make honey but feed on ready-made food. In our observations, we observed that the bees doing this were found in hives with honeybees, especially those located close to orchards. For the purpose of testing,

we put worker bees in a hive in an environment with fig fruit, and they landed on the same fruit, especially the sugary liquid that the fig fruit leaks, and the fruits both on the tree and on the ground were invaded by these two different bees. Approximately 20-25 days later, wild bees entered the hive containing honeybees and killed and dispersed the honeybees in the hive. The honeybees struggling in this hive may have definitely been contaminated with parasites or microorganisms hosted by wild bees and may have met with other honeybees on some occasion. This situation may turn into an undesirable mass death of bees. An infection similar to the Covid-19 pandemic that our world has experienced in recent years is in honeybee populations. It has been reported that Trophallaxis (food exchange) increases in young adult honey bees infected with *Nosema ceranae* microsporodian (Lecocq et al, 2016).

This change in behavior provides evidence that the spread of infection may occur more frequently in honeybee and wasp populations that share common feeding grounds. Beekeepers in areas where *Polistes dominula* and *Polistes nimpha* are common may install alternative water sources and dummy hives to limit competition and interaction between bee populations. As the coccidian and microsporidia pathogens that we detected because of the study could not be reproduced, it could not be determined whether they have insecticidal effects on honeybees.

Our results show that *Polistes dominula* and *Polistes nimpha* are microorganisms isolated from wasp, bacterial and fungal infections occurring in a region throughout Türkiye, and that wild honey growers come from different regions of Türkiye in spring and summer to meet with other honey growers and share their experiences. This current study highlights a number of processes that are potentially of interest in terms of pathogen-host interaction and biological control. Although the results of this study provide a foundation, further research, including empirical studies, is needed to better understand the effects and outcomes of these processes. These processes will have significant impacts on the health of the native honey bee Apis mellifera and the continuity of native bee species in beekeeping in Türkiye.

Conclusion: In this study, the fact that both species carry common pathogens may indicate that it is a result of contamination between bees. It is thought that wasps and honeybees sometimes meet indirectly or directly when they visit flowers (collect nectar), sometimes at fruits, and sometimes when raiding nests. This may indicate that wasp communities may cause infections in bee colonies and negatively affect them by infecting honey bees with some types of bacteria and fungi that they carry in their bodies. In this case, it can be thought that some of the bacteria we obtained from wasps can be used as a biocontrol product, in the microbial control of insect pests, in insect control and biotechnology applications by developing insect-resistant plants.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

INVESTIGATION OF ANTIMICROBIAL AND ANTIVIRAL EFFECTS OF TÜRKIYE PROPOLIS WATER EXTRACTS: AN *IN VITRO* STUDY

Türkiye Propolis Su Ekstraktlarının Antimikrobiyal ve Antiviral Etkilerinin Araştırılması: *İn Vitro* Çalışma

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ABSTRACT

Propolis is a natural bee product used as a therapeutic agent for centuries. Propolis extracts are natural resources that attract the attention of scientists looking for new components due to the insufficiency of existing drugs. In current study, antiviral and antimicrobial activity of propolis water extracts prepared from three different raw propolis samples collected from Northeast of Türkiye (Ardahan, Rize, and Trabzon) were investigated. The total flavonoid contents (TFC) and total phenolic content (TPC) of the extracts were measured. It was determined that TPC and TFC ranged from 5.87±0.36 to 20.47±1.46 mg GAE g^{-1}, and 0.48±0.04 to 2.10±0.22 mg QUE g^{-1}, respectively. The antimicrobial activity of the extracts against 14 microorganisms (*Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Mycobacterium smegmatis* ATCC 607, *Klebsiella pneumoniae* ATCC 13883, *Chromobacterium violaceum* ATCC 12472, *Candida parapsilosis* ATCC 22019, and *Candida albicans* ATCC 10231) and their effect against the *Pseudomonas aeruginosa* biofilm were investigated. Additionally, anti-quorum sensing and anti-swarming activities of the extracts were tested. The antiviral activity of the extracts was examined against Herpes simplex virus type 1 (HSV-1) by MTT and qRT-PCR methods. The water extracts of propolis samples did not show antimicrobial, anti-swarming, anti-quorum sensing, and anti-viral activities. However, extracts were found to have strong anti-biofilm activities. The results show that aquatic propolis extracts can be evaluated in the treatment of biofilms.

Keywords: Antimicrobial, Biofilm, Phenolic, Propolis, RT-PCR

ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

ÖZ

Yüzyıllardır tedavi edici ajan olarak kullanılan ve doğal bir arı ürünü olan propolis, mevcut ilaçların yetersiz kalması sonucu yeni bileşenler arayan bilim adamlarının ilgisini çeken doğal kaynaklardan biridir. Bu çalışmada Türkiye'nin kuzeydoğusundan (Ardahan, Rize, Trabzon) toplanan üç farklı propolis örneğinden su ile hazırlanan propolis ekstraktlarının antimikrobiyal ve antiviral aktivitesi araştırıldı. Ekstraktların toplam fenolik içeriği (TPC) ve toplam flavonoid içeriği (TFC) ölçüldü. TPC ve TFC'nin sırasıyla 5.87±0.36 ila 20.47±1.46 mg GAE g^{-1} ve 0.48±0.04 ila 2.10±0.22 mg QUE g^{-1} arasında değiştiği belirlendi. Ekstraktların 14 mikroorganizmaya (*Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Mycobacterium smegmatis* ATCC 607, *Klebsiella pneumoniae* ATCC 13883, *Chromobacterium violaceum* ATCC 12472, *Candida parapsilosis* ATCC 22019 ve *Candida albicans* ATCC 10231) karşı antimikrobiyal aktivitesi ve *Pseudomonas aeruginosa* biyofilmine etkinliği, ayrıca anti-quorum sensing ve anti-swarming aktiviteleri araştırıldı. Ekstraktların Herpes simpleks virüs tip 1 (HSV-1)'e karşı antiviral aktivitesi, MTT ve qRT-PCR yöntemleriyle test edildi. Çalışmada kullanılan propolis su ekstraktlarının antimikrobiyal, anti-swarming, anti-quorum sensing ve anti-viral aktivite göstermediği, bununla birlikte ekstraktların güçlü anti-biyofilm aktivitesine sahip olduğu tespit edildi. Sonuçlar, propolis su ekstraktlarının bakteri biyofilmi tedavisinde değerlendirilebileceğini göstermektedir.

Anahtar Kelimeler: Antimikrobiyal, Biyofilm, Fenolik, Propolis, RT-PCR

GENİŞLETİLMİŞ ÖZET

Amaç: Yüzyıllardır tedavi edici ajan olarak kullanılan ve doğal bir arı ürünü olan propolis, mevcut ilaçların yetersiz kalması sonucu yeni bileşenler arayan bilim adamlarının ilgisini çeken doğal kaynaklardan biridir. Propolisin içerisindeki biyoaktif molekül profili propolisin toplandığı botanik ve coğrafik kaynak, arı türü, toplandığı mevsim ve çevresel faktörler gibi çeşitli parametlere bağlı olarak değişmektedir. Buna bağlı olarak propolislerin terapötik etkileri değişkenlik göstereceğinden her propolis örneğinin kimyasal ve biyolojik analizinin gerçekleştirilmesi gerekmektedir. Bu çalışmada Türkiye'nin kuzeydoğusundan (Ardahan, Rize, Trabzon) toplanan üç farklı propolis örneğinden su ile hazırlanan propolis ekstraktlarının antimikrobiyal ve antiviral aktivitesi araştırıldı.

Gereç ve Yöntem: Propolislerin su ekstraktı hazırlanarak ekstraktların toplam fenolik içeriği (TPC) Folin-Ciocalteau yöntemi ile, ekstraktların toplam flavonoid içeriği (TFC) Fukumoto ve Mazza yöntemi kullanılarak ölçüldü. TPC ve TFC'nin sırasıyla 5.87±0.36 ila 20.47 ± 1.46 mg GAE g^{-1} ve 0.48 ± 0.04 ila 2.10±0.22 mg QUE g^{-1} arasında değiştiği belirlendi. Hazırlanan ekstraktlarıdaki su liyofilizasyon ile ortamdan uzaklaştırıldı. Kuru madde dimetilsülfoksit (DMSO) kullanılarak çözülerek antimikrobiyal ve antiviral aktivite çalışmalarında kullanıldı. Deneylerde ekstraktların DMSO oranı mikroorganizmaları, virüs partiküllerini ve hücre hatlarını etkilemeyecek konsantrasyon olan %1'in altında tutuldu. Ekstraktların 14 mikroorganizmaya (*Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Mycobacterium smegmatis* ATCC 607, *Klebsiella pneumoniae* ATCC 13883, *Chromobacterium violaceum* ATCC 12472, *Candida parapsilosis* ATCC 22019 ve *Candida albicans* ATCC 10231) karşı antimikrobiyal aktivitesi agar kuyucuk difüzyon yöntemi ile belirlendi. Ekstraktların *Pseudomonas aeruginosa* biyofilmine etkinliği, ayrıca anti-quorum sensing ve anti-swarming aktiviteleri araştırıldı. Ekstraktların Afrika yeşil maymun böbrek epitel hücresi (Vero) hücrelerine sitotoksik etkisi tripan mavisi boyama ve 3-(4,5-dimetiltiyazol-2-yl)-2,5-difeniltetrazolyum-bromür (MTT) yöntemleri kullanılarak araştırıldı. Antiviral aktivite testlerinde kullanılacak Herpes simpleks virüs tip 1 (HSV-1)'in titresi doku kültürü enfeksiyöz dozunun yüzde 50'si ($TCID_{50}$) testi ile belirlendi. Ekstraktların HSV-1'e karşı antiviral aktivitesi, ekstraktların Vero hücrelerine sitotoksik olmayan konsantrasyonları kullanılarak MTT ve qRT-PCR yöntemleri ile test edildi. Elde edilen verilerin istatistiksel analizi

yapılarak sonuçlar değerlendirildi.

Bulgular ve Sonuç: Çalışmada kullanılan propolis su ekstraktlarının antimikrobiyal, anti-swarming, anti-quorum sensing ve anti-viral aktivite göstermediği görüldü. Bununla birlikte Pazar ve Uzungöl ekstraktlarının güçlü anti-biyofilm aktivitesine sahip olduğu tespit edildi. Ardahan propolis ekstraktının da diğer ekstraktlar kadar güçlü olmasa da anti- biyofilm aktivitesinin olduğu görüldü. Pazar ve Uzungöl propolis ekstraktlarının gösterdiği anti-biyofilm aktiviteleri istatistiksel olarak anlamlı bulundu (p=0.001). Sonuçlar, propolis su ekstraktlarının HSV-1 enfeksiyonlarının tedavisinde yetersiz kalabileceğini, bununla birlikte propolis su ekstraktlarının bakteri biyofilmi tedavisinde değerlendirilebileceğini göstermektedir.

INTRODUCTION

Propolis is a natural mixture produced by honeybees (*Apis mellifera*). Honey bees produce propolis from saps, resins, and mucilages collected from various parts of the plants, then mix them with bee enzymes and beeswax. Honeybees use propolis to fix damage in the hive, refine internal walls, and maintain the humidity and temperature of the hive. Propolis also protects the colony against pathogenic microorganisms (Forma & Bryś, 2021). The use of propolis by humans dates back to ancient times. Propolis was used in antiquity and the Middle Ages for different purposes such as the treatment of wounds and burns, and preparation of cosmetic products. At present propolis continues to be used in alternative medicine and as a food supplement (Kocot et al. 2018).

The bioactive molecular profile of crude propolis varies according to the botanical and geographical origin, genetics of bees, season, and environmental factors. The quantity and quality of propolis collected depend on plant variety and availability, source and duration of collection, techniques and practices of beekeepers, and environmental health (Šuran et al. 2021). Raw propolis collected from hives cannot be used directly in treatment or scientific studies. To be used, the active ingredients in propolis must be extracted using various solvents. The solvents often used in the preparation of extracts are ethanol, olive oil, water, dichloromethane, and chloroform. The biological activity of the prepared extract varies according to the type and amount of active ingredients in it (Przybyłek& Karpiński, 2019; Kolayli, 2023).

Herpes simplex virus type 1 (HSV-1) is a human pathogen that replicates in peripheral tissues and then invades the nervous system and establishes latent infection (Ahmad & Wilson, 2020). Although it establishes latency, HSV-1 may later reactivate as a response to a stimuli, or spontaneously. In addition to lesions, HSV-1 can cause serious pathologies such as keratoconjunctivitis or encephalitis (Bello-Morales et al. 2021). It is estimated that 3.7 billion people under the age of 50 are infected with HSV-1 worldwide (WHO, 2015). Although there have been advances in the treatment of HSV infections with nucleoside analogs, there is a need for developed therapeutics with alternative mechanisms of action (Whitley & Baines, 2018).

The bioactive component in propolis is subject to variation depending on a number of factors. The biological, chemical and therapeutic properties of propolis exhibit regional differences. The solvents used in the preparation of the extract also ensure that different bioactive components are obtained at different rates. As a result, it can cause different pharmacological properties to be seen in extracts prepared with different solvents. For all these reasons, in the current study, it was aimed to evaluate the antimicrobial effects of water extracts of propolis samples acquired from different regions of Türkiye. It was also aimed to research the in vitro antiviral effect of propolis extracts against HSV-1.

MATERIALS and METHODS

Study Design

Propolis samples were collected from three regions on the coasts of the Eastern Black Sea in Türkiye. Biofilm activity laboratory experiments were run in duplicate (n=6, for each group). The study was completed with a total of 24 samples, including the positive sample group. Data that were inconsistent with the data obtained in the study were excluded from the evaluation.

Standard Drug, Cell Culture, and Virus

The African green monkey kidney cell line (Vero) was obtained from the University of Erciyes and was cultivated in Dulbecco's Modified Eagle's medium supplemented with antibiotics and fetal bovine serum.

HSV-1 Wal strain was first acquired from the University of Sheffield (UK). The antimicrobial activity of propolis extracts was researched against *Bacillus cereus* ATCC14579, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Enterobacter aerogenes* ATCC 13048, *Mycobacterium smegmatis* ATCC 607, *Klebsiella pneumoniae* ATCC 13883, *Chromobacterium violaceum* ATCC 12472, *Candida parapsilosis* ATCC 22019, and *Candida albicans* ATCC 10231.

Acyclovir (5 mg mL^{-1} dissolved in sterile dimethyl sulfoxide (DMSO) was used as positive control in antiviral activity assays. Ciprofloxacin, Gentamicin, Amphotericin B, and Ampicillin were positive controls in anti-bacterial and anti-fungal experiments.

Propolis Extract Preparation Protocol

Three propolis samples were collected from the Ardahan City, Uzungöl district of Trabzon City, and Pazar district of Rize City, Türkiye, and were grounded mechanically into small pieces. 5 mL of glycerol and 40 mL of distilled water were added to a 5 g powdered propolis sample in a glass bottle. The bottles were stirred in an ultrasonic bath for 2 hours with 99 amplitudes and then shaken in a magnetic stirrer at 500-600 rpm for 24 hours at 45-50˚C. After the mixture was filtered, the final volume was made up to 40 mL with distilled water. Half of the extract was reserved for chemical analysis. 20 mL of the mixture was lyophilized, and water was removed. The empty and full bottles were weighed, and the grams of sediment were calculated. The residues were dissolved with DMSO, and sheltered to 4˚C until use. In the experiments DMSO rate of the extracts was kept under 1%, which is the concentration that does not affect the microorganisms, virus particles, and Vero cells.

Determination of Total Phenolic Content (TPC) and Total Flavanoid Content (TFC) of the Extracts

The Folin-Ciocalteau procedure was applied to evaluate the TPC of the propolis extracts (Singleton & Rossi, 1965). In the procedure various concentrations of gallic acid (from 0.015 to 0.5 mg/mL) in a total volume of 20 µL and propolis samples were put in a tube. 680 µL distilled water, and 400 µL 0.2 N Folin-Ciocalteu's phenol reagent were added to the tube. After vortexing, the tubes were incubated for 3 min. Then 400 µL Na_2CO_3 (10%) was added to the mixture and the absorbance of the mixtures was measured against a blank at 760 nm.

The method of Fukumoto and Mazza was applied to evaluate the TFC of the propolis extracts (Fukumoto & Mazza, 2000). Firstly, 0.30 µL of the extract was added 50 µL of 10% $Al(NO_3)_3$ and 50 µL of 1 M ($NH_4.CH_3COO$). The mixture was then diluted to 3 mL with ethanol (99%) and incubated at room temperature. After 40 min incubation, the absorbance was then measured against a blank at 415 nm.

The standard graph was arranged with different concentrations of gallic acid, and quercetin to calculate the concentration of TPC, and TFC in the extracts, respectively. The concentration of TPC was calculated as milligrams of gallic acid equivalent (GAE) per gram (mg GAE g^{-1}), the concentration of TFC was calculated as mg quercetin equivalent (QUE) g^{-1} sample (Kolayli et al. 2022).

Anti-bacterial Activity Assay: Agar Well Diffusion Method

The agar well method published by Denev et al. was modified and used in the current study (Denev et al. 2014). *Candida* species were incubated in Mueller Hinton Agar (MHA) with 2% glucose at 35°C for two days, *M. smegmatis* in Brain-Heart Infusion Agar (BHIA) at 37°C for three days, and other bacteria in MHA at 37°C for one day. Suspensions of bacteria at a density of 0.5 McFarland and of *Candida* species at a density of 1 McFarland were prepared in Phosphate Buffered Saline (PBS). *M. smegmatis* suspension was prepared in Brain-Heart Infusion Broth (BHIB) at a concentration of 0.5 Mc Farland. The prepared bacterial suspensions were spread on MHA, the *M. smegmatis* suspension on BHIA, and the *Candida* suspensions on MHA containing 2% glucose (Woods et al. 2003; CLSI, 2009). Then, 6 mm wide wells were opened on the medium. 50 µL of the 10 mg mL^{-1} propolis extracts, positive and negative controls were placed in the wells. DMSO was used as a negative control. Ciprofloxacin, gentamicin, amphotericin b, and ampicillin were used as positive controls for *M. smegmatis*, Gram-negative bacteria, *Candida* species, and Gram-positive bacteria, respectively. Bacterial cultures were incubated for one day, *Candida* species for two days, and *M. smegmatis* for three days. Zone

diameters of <6 mm were considered ineffective, 6-14 mm were considered moderately effective, and 15 mm and above were considered as high activity (Balouri et al. 2016).

Anti-Quorum Sensing Assay

Sub-MIC concentrations of the extracts were used in the experiment. The overnight culture of the *C. violaceum* strain (50 µL) was added to 5 mL LB soft agar (0.3% w/v) prepared with LB broth (Condalab, Spain) and agar (Sigma-Aldrich, USA) and poured onto LB agar frozen in the petri dish. After the soft agar was frozen, wells were opened in the medium and 50 µL of the extracts were placed in the wells. At the end of one-night of incubation, extracts with growth around the well but no pigment formation was considered positive (Ureyen Esertas et al. 2022).

Anti-Swarming Assay

The agar was allowed to solidify by adding the final concentration of 100 µg mL^{-1} from the extracts into 5 mL of autoclaved but not solidified LB medium. A colony from the overnight culture of *P. aeruginosa* PAO1 strain was placed to the middle of the medium with a sterile toothpick and incubated for 16-18 h at 37˚C. The spread of bacteria from center to periphery was evaluated by comparing it with the *P. aeruginosa* PAO1 strain without extracts (Rashid & Kornberg, 2000).

Anti-Biofilm Assay

To identify the inhibition of the extracts on biofilm development, the *P. aeruginosa* PAO1 strain at a 0.5 McFarland density in LB medium, was diluted by 1% and used in the assay. Extract (40 µL), LB medium (125 µL), and *P. aeruginosa* PAO1 culture (35 µL) were added to each well of the 96-well plate. Wells containing only bacteria was used as control. The plates were washed three times with distilled water after 24 h of incubation at 37˚C. 0.3% crystal violet dissolved in water was added to the wells. After 15 min, the plates were washed three times with distilled water and kept in 95% ethanol for 15 minutes. The colors were measured in a spectrophotometer at 570 nm. The experiment was repeated twice and the results were averaged to create a graph (Fazli et al 2014; Ureyen Esertas et al. 2022).

Cytotoxicity Assays

Trypan blue assay: $1x10^5$ Vero cells were placed in each well of the 24-well plate and incubated until the cells adhered to the plate. Different concentrations of extracts (25-6000 µg mL-1) were placed on the plate Three wells were used for each concentration. The same amount of untreated cells was used as a negative control. Four different plates were prepared to observe the cytotoxic effect of propolis extracts on Vero cells at 24, 48, 72, and 96 hours. At the end of the designated time, the cells were trypsinized. Cells stained with trypan blue were counted using a hemocytometer. The results were evaluated concerning the number of cells in the control wells (Yildirim et al. 2016).

MTT Assay

MTT assay was carried out as described in Cora et al. 2023 (Cora et al, 2023). Briefly, Vero cells were incubated with different concentrations of propolis extracts (25-6000 µg mL^{-1}) for 24, 48, 72, and 96 hours. At the end of the time, the MTT assay was performed. The results were evaluated regarding the control wells (Mosmann, 1983). The experiment was repeated twice.

Determination of Tissue Culture Infectious Dose 50 (TCID50)

The virus was serially diluted across the 96 well plate including confluent Vero cells. The plate was incubated for 3 days at 37°C with 5% CO_2. After the incubation, the wells that were positive for cytopathic effect were counted for each dilution. The 50% infectious dose was determined by performing Spearman-Karber method (Ramakrishnan, 2016).

Antiviral Activity Assays

MTT and quantitative real-time polymerase chain reaction (qRT-PCR) methods were performed to investigate the antiviral activity of the extracts.

MTT assay

Different concentrations of the virus (1, 10, and 100 $TCID_{50}$) were used to infect $1x10^4$ Vero cells in 96-well plates. Infected cells were incubated with different concentrations of propolis extracts (800-12.5 µg mL^{-1}) for three days. Wells containing infected but untreated cells, acyclovir, and wells that contained just Vero cells were used as a negative control, positive control, and reproductive control, respectively. Then, MTT assay was carried out as previously described (Cora et al. 2023). The experiment was repeated twice, and the rate of cell viability was calculated by comparing them with control wells.

qRT-PCR assay

The qRT-PCR assay was achieved as previously described (Cora et al. 2023). After the Vero cells were infected with virus, different concentrations of extracts (12.5-800 µg mL^{-1}) were added to the wells. Acyclovir was used as a positive control, the wells that included infected cells were used as a negative control. After 3 days of incubation viral DNA was isolated from the wells by using a nucleic acid isolation device (Bioneer ExiPrep 16 Plus, South Korea) and isolation cartridges (ExiPrepTMPlus Viral DNA/RNA Kit, South Korea) according to the manufacturer's instructions.

HSV-1 DNA in the wells was quantified with qRT-PCR using LightCycler® 480 System (Roche Molecular Systems, USA) and the results were analyzed using the software of the device. The master mix, primer, probe, and reaction conditions previously mentioned were used in the qRT-PCR assay (Cora et al. 2023).

Statistical Analysis

All experiments were carried out in triplicates and repeated two times. Descriptive statistics were presented as mean ± standard deviation and categorical data were number (n) and percentage (%).

The suitability of the data for normal distribution was evaluated with the Kolmogorov-Smirnov Test and skewness and kurtosis coefficients. There are studies in the literature stating that variables with skewness and kurtosis coefficients between -3 and +3 meet the assumption of normality (Shao, 2002; George & Mallery, 2010; Hair et al. 2013; Tabachnick & Fidell, 2013).

In this study, besides the Kolmogorov-Smirnov test, variables with skewness and kurtosis coefficients between -1.5 and +1.5 were analyzed as having a normal distribution (Tabachnick & Fidell, 2013). The statistical significance of the anti-biofilm activity of propolis on *P. aeruginosa* PAO1 strain was determined using a one-way analysis of variance (ANOVA) test followed by Tukey's post hoc test for multiple comparisons. The *p* values were evaluated at α= 0.05 significance level, two-tailed, and 95% confidence interval.

All figures were visualized in the Microsoft Excel program and statistical analyses were performed in the IBM SPSS 23.0 (IBM SPSS Corp.; version number: 8.5.0.0021; Karadeniz Technical University, Türkiye) version (George & Mallery, 2016). Experimental (post hoc; retrospective; posterior) power analysis was performed in the study to justify the sample size. With alpha (the probability of a Type I error) = 0.05 significance level and high-level effect size (f=0.6), the power of the study (1 − β) was calculated as 0.80. The post hoc achieved power of the study was calculated in the G*Power 3.0.10 program environment.

Furthermore, The percentage of viability (%V) was determined according to the following mathematical equation (Queiroga et al. 2023):

$$\%V = ((Abs(Sample) - Abs(Blank))/(Abs(Control) - Abs(Blank))) \times 100$$

RESULTS

TPC and TFC of Extracts

The highest amount of TPC and TFC was found in Uzungöl propolis water extract with 20.47± 1.46 mg GAE g^{-1} sample, and 2.10±0.22 mg QUE g^{-1} sample, respectively. The TFC and TPC of all studied propolis extracts were summarized in Table 1.

Table 1. Total phenolic content and total flavonoid content of propolis extracts.
Tablo 1. Propolis ekstraktlarının toplam fenolik ve toplam flavonoid içerikleri

Sample	TPC	TFC
	(mg GAE g^{-1})	(mg QUE g^{-1})
Pazar Propolis	5.87± 0.36	0.48±0.04
Ardahan Propolis	6.08± 0.53	0.68±0.03
Uzungöl Propolis	20.47± 1.46	2.10±0.22

Mean ± Standard Deviation

Biological Activity Assay Results of Propolis Extracts

In the agar well diffusion method, the suppressive activity of water extracts of propolis against any of the tested microorganisms was not detected. It was also observed that propolis extracts did not have anti-swarming and anti-quorum sensing effects. However, in the anti-biofilm test performed with the *P. aeruginosa* PAO1 strain, it was determined that the Pazar and Uzungöl of the extracts had strong anti-biofilm activity and was found statistically significant (F=37.08, *p*=0.001). It was determined that Ardahan propolis had anti-biofilm activity but did not show as strong activity as other extracts. Anti-biofilm activity of extracts was shown in Figure 1. One-way ANOVA for anti-biofilm activity between groups was shown in Table 2.

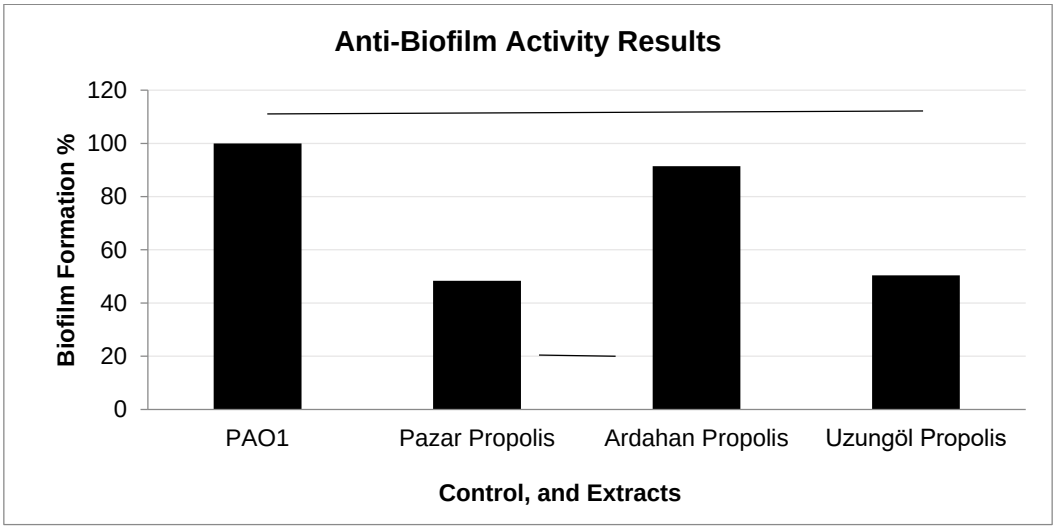

Figure 1. Anti-biofilm activity of water extracts of propolis samples. PAO1; positive control. *Asterisks indicate statistically significant differences (one-way ANOVA) between each concentration of biofilms (*p<0.05, ** p < 0.001).*

Şekil 1. Propolis su ekstraktlarının anti-biyofilm aktivitesi. PAO1; pozitif kontrol. Yıldız işaretleri, her biyofilm konsantrasyonu arasındaki istatistiksel olarak anlamlı farklılıkları (tek yönlü ANOVA) gösterir (*p<0,05, ** p <0,001).

Table 2. One-way ANOVA for anti-biofilm activity between groups.

Tablo 2. Gruplar arası biyofilm aktivitesinin One-way ANOVA sonuçları.

	n	Mean±SE	Lower Bound	Upper Bound	*p*
P. aeruginosa PAO1	6	100±1.40	96.39	103.62	0.001
Pazar propolis	6	47.75±6.03	32.26	63.24	
Ardahan propolis	6	92.54±5.54	78.31	106.78	
Uzungöl propolis	6	49.81±3.64	40.46	59.16	
Total	24	72.53±26.52	61.33	83.72	

SE: Standard Error

For multiple comparisons, Tamhane's test of significance revealed a highly statistically significant difference between group PAO1 and Pazar and between group PAO1 and group Uzungöl (P<0.05) as depicted by Table 3.

Table 3. Multiple comparisons for anti-biofilm activity between groups.

Tablo 3. Anti-biyofilm aktivitesi için gruplar arasındaki çoklu karşılaştırmalar.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	*p*
P. aeruginosa PAO1	Pazar propolis	52.25*	6.19	0.001
	Ardahan propolis	7.46	5.71	0.811
	Uzungöl propolis	50.19*	3.90	0.001
Pazar propolis	*P. aeruginosa* PAO1	-52.25*	6.19	0.001
	Ardahan propolis	-44.79*	8.18	0.002
	Uzungöl propolis	-2.06	7.04	1
Ardahan propolis	*P. aeruginosa* PAO1	-7.46	5.71	0.811
	Pazar propolis	44.79*	8.18	0.002
	Uzungöl propolis	42.73*	6.62	0.001
Uzungöl propolis	*P. aeruginosa* PAO1	-50.19*	3.90	0.001
	Pazar propolis	2.06	7.04	1
	Ardahan propolis	-42.73*	6.62	0.001

* The mean difference is significant at the 0.05 level.

Cytotoxicity Assay Results

Trypan blue assay results

Since there were three wells from each dilution, the number of cells in the wells was counted at the end of 24, 48, 72, and 96 h and averaged. It was observed that water extracts of propolis at concentrations of 800 µg mL^{-1} and below contained similar numbers of cells to the control. Therefore, it was determined that these concentrations did not have cytotoxic effects on Vero cells. The evaluation of the number of cells in the trypan blue test was given in Figure 2 (a, b, c).

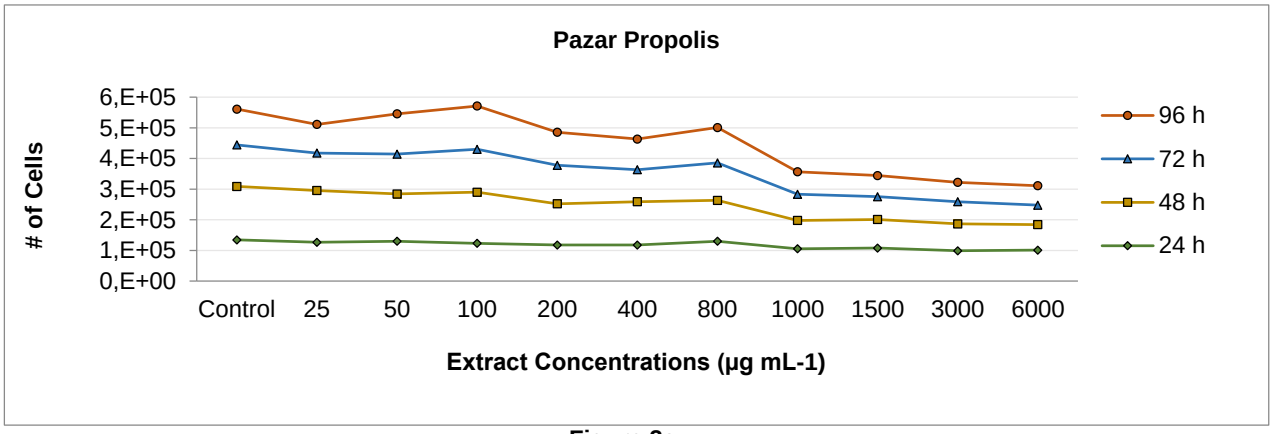

Figure 2a.

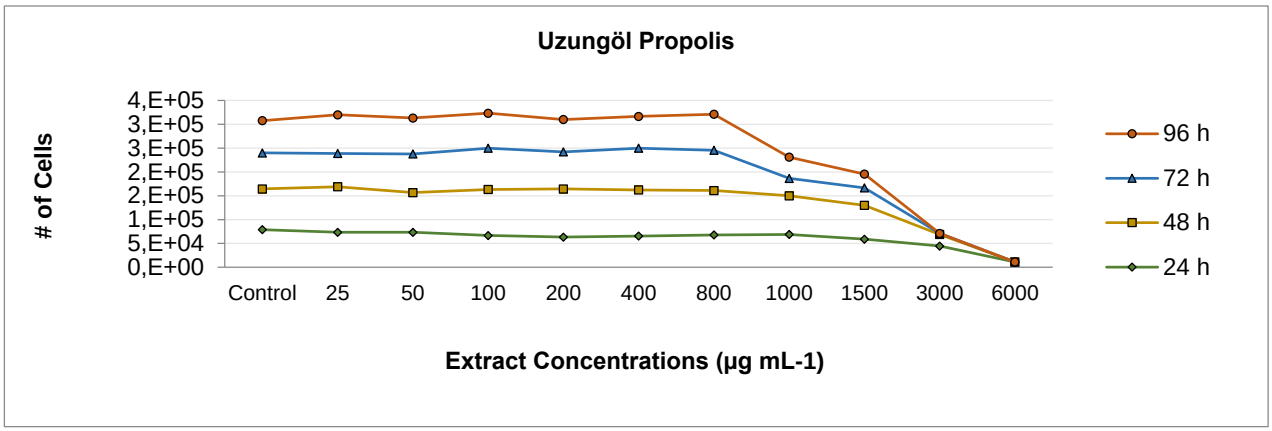

Figure 2b.

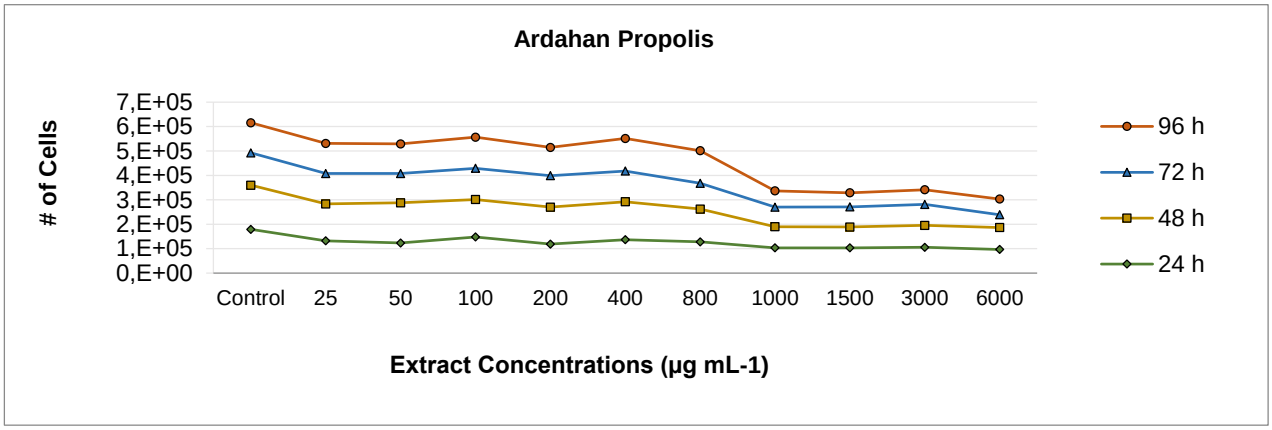

Figure 2c.

Figure 2a, b, c. The cytotoxic effect of Pazar (2a), Uzungöl (2b), and Ardahan (2c) propolis water extracts on Vero cells by trypan blue staining method.

Şekil 2a, b, c. Pazar (2a), Uzungöl (2b) ve Ardahan (2c) propolisi su ekstraktının tripan mavisi boyama yöntemi ile Vero hücreleri üzerine sitotoksik etkisi.

MTT assay results

The viability of Vero cells incubated with dilutions of propolis was evaluated at the end of 24, 48, 72, and 96 h. Cell viability in the wells containing the propolis dilutions was calculated as % by evaluating compared to the control. Since there were three wells from each dilution and control, the average of the viability rate in the wells was calculated. It was determined that no cytotoxic effect was observed in the water extracts of Pazar and Ardahan propolis at concentrations of 800 µg mL^{-1} and below. However, it was observed that Uzungöl propolis had no cytotoxic effect at 1500 and below concentrations. The results were summarized in Figure 3 (a, b, c).

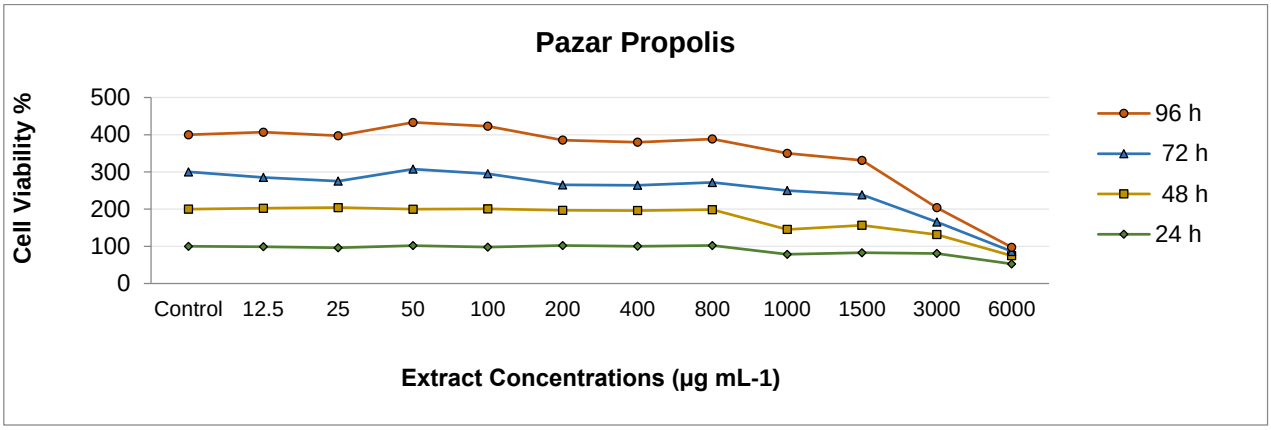

Figure 3a.

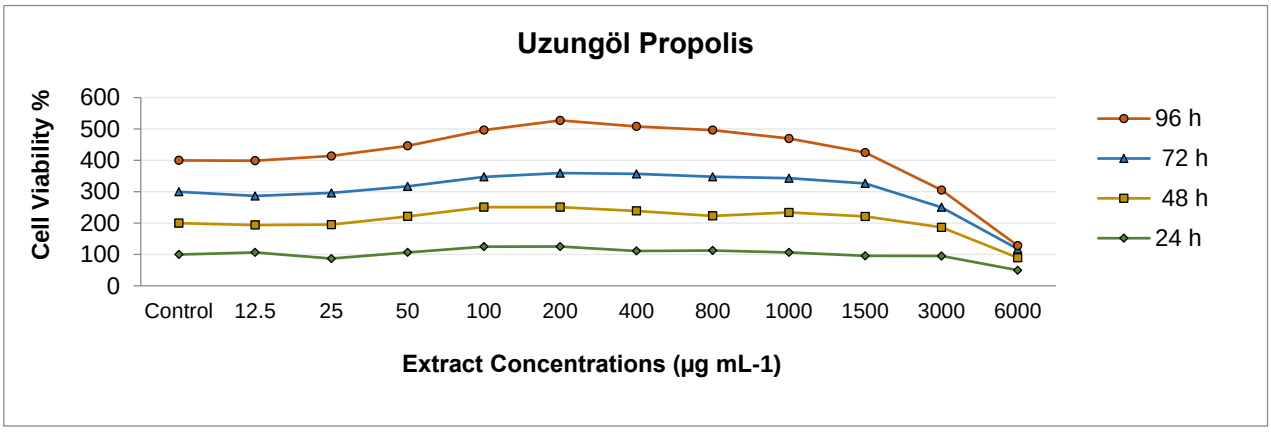

Figure 3b.

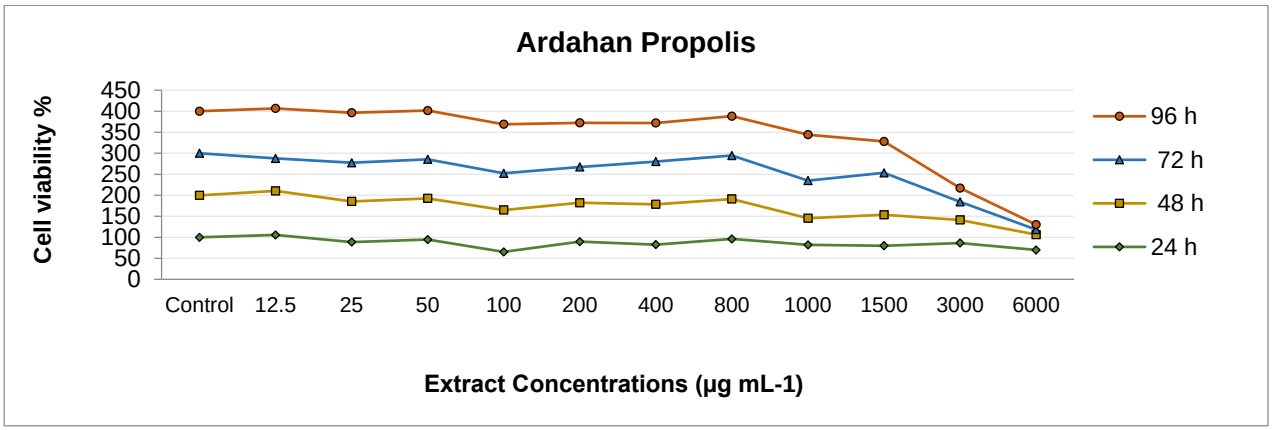

Figure 3c.

Figure 3 a, b, c. The cytotoxic effect of Pazar (3a), Uzungöl (3b), and Ardahan (3c) propolis water extract on Vero cells by MTT method.

Şekil 3 a, b, c. Pazar (3a), Uzungöl (3b) ve Ardahan (3c) propolis su ekstraktının MTT yöntemi ile Vero hücrelerine sitotoksik etkisi.

Antiviral Activity Assay Results

MTT assay results

When HSV-1 is produced in Vero cells, it creates a lytic infection and causes the cells to lyse. However, if acyclovir is added to the breeding medium, the reproduction of the virus is prevented, the existing cells in the medium are not destroyed, and continue to grow. It was noticed that the viability rate of cells in wells containing only viruses decreased as the number of viruses increased. The viability rate of the cells was calculated as 4.8%, 8.2%, and 7% in the wells containing the virus at 1 $TCID_{50}$, 10 $TCID_{50}$, and 100 $TCID_{50}$, respectively. The viability rate in the wells containing acyclovir at 25 µg mL^{-1} concentration was 92.8%, 72.8%, and 36.8% in the wells containing 1 $TCID_{50}$, 10 $TCID_{50}$, and 100 $TCID_{50}$, respectively. Because the viability rate of the cells in the water extracts was similar to the viability rate in the cells containing only the virus, it was understood that the water extracts did not have an antiviral effect on HSV-1. MTT assay results of antiviral activities of water extracts of propolis was shown in Figure 4.

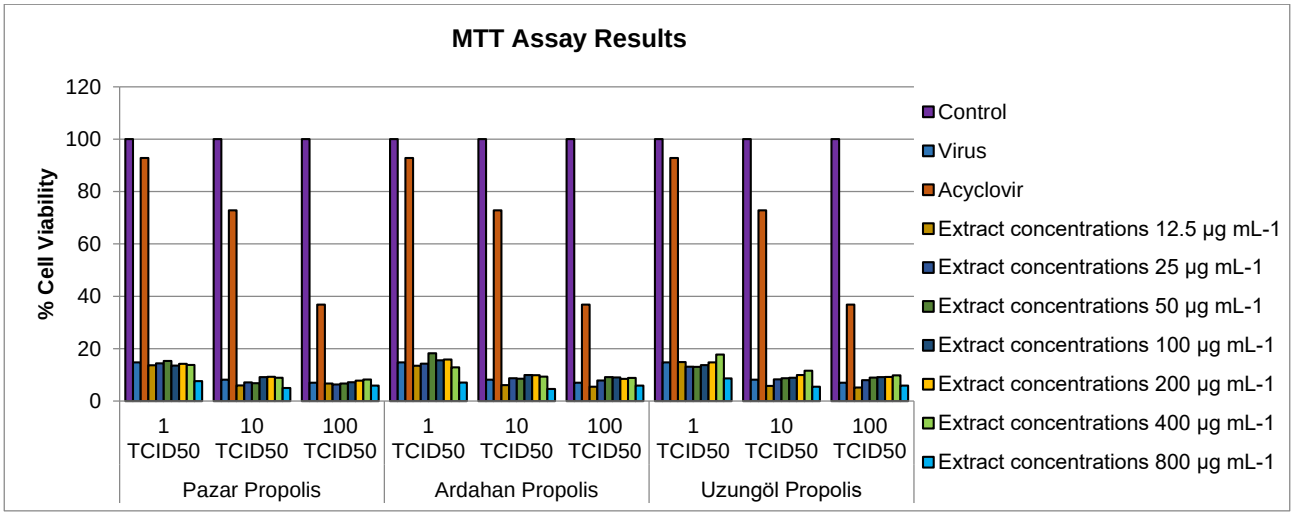

Figure 4. The antiviral activity of propolis samples against HSV-1 with MTT assay. Control; Wells that contain only Vero cells used as reproductivity control, Virus; wells that Vero cells infected with virus used as negative control, Acyclovir; positive control.

Şekil 4. Propolislerin HSV-1 üzerine antiviral etkisinin MTT yöntemi ile arştırılması. Konrol; sadece Vero hücresi içeren kuyucuklar üreme kontrolü olarak kullanıldı, Virüs; virüs ile enfekte edilmiş Vero hücreleri negatif kontrol olarak kullanıldı, Asiklovir; pozitif kontrol.

qRT-PCR assay results

The qRT-PCR method was used to evaluate the effect of propolis extracts on the reproduction of the virus. In this method, the virus was added to the wells of the plate containing confluent Vero cells. Only virus-infected cells were used as negative control and acyclovir was used as positive control. The amount of viral DNA in the samples was determined using standards that included a known amount of virus. It was observed that there was no difference between the water extracts of propolis and the negative control, therefore the water extracts did not affect the reproduction of the virus. qRT-PCR assay results of antiviral activities of water extracts of propolis were shown in Figure 5.

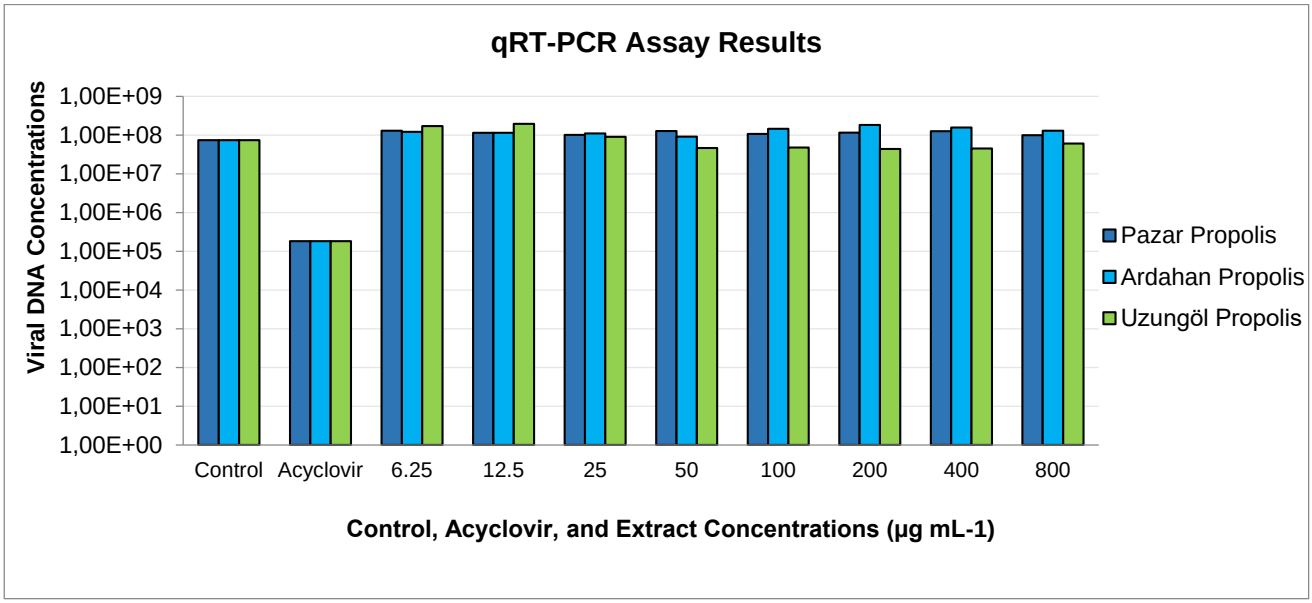

Figure 5. The antiviral activity of propolis samples against HSV-1 with qRT-PCR assay.

Şekil 5. Propolis örneklerinin qRT-PCR testi ile HSV-1 üzerine antiviral etkisi.

DISCUSSION

TFC and TPC values of propolis vary depending on many parameters, therefore these values should be revealed in every study. The TPC and TFC values of propolis samples In Egyptian brown propolis water extract, the total phenolic content was found as 210.33 mg GAE g-1 (Ibrahim & Alqurashi, 2022). Salleh et al. found the TPC as 7.60-13.21 mg GAE mL-1 in three different Malaysian stingless bee propolis water extracts (Salleh et al. 2021). In a study on several Indonesian stingless bee propolis, TPC was found to range between 10 and 28.65 mg GAE mL-1 (Fikri et al 2019). Kubiliene et al. demonstrated that the TPC of Lithuania propolis water extract was 1.2 mg GAE mL-1 (Kubiliene et al. 2018). Abogharip et al. state that the TPC of Egyptian propolis water extract was 5.23 mg GAE g-1 (Abogharip et al 2023). In a study conducted by Omer et al., the TPC value of water propolis extract from the West Blacksea region of Türkiye was found to range between 9.90 and 13.99 mg GAE g-1 (Omer et al. 2023). In the current study, the TPC values of water extract of propolis samples were investigated and it was found as 5.87, 6.08, and 20.47 mg GAE g-1 in Pazar, Ardahan,and Uzungöl propolis, respectively. The TPC values were found to be compatible with most of the studies in the literature.

TFC in three different Malaysian stingless bee propolis water extracts was investigated using rutin as the standard reference. TFC in the samples was found between 34.17-34.53 mg rutin equivalent (RE) mL-1 (Salleh et al. 2021). The TFC in several Indonesian stingless bee propolis was found to range between 1.42 and 1.80 mg QUE g-1 (Fikri et al. 2019). The TFC of Egyptian propolis water extract was found as 5.55 mg QUE g-1 (Abogharip et al. 2023). Omer et al. stated that the TPC value of water propolis extract from the West Blacksea region of Türkiye was found to range between 2.25 and 3.09 mg QUE g-1 (Omer et al. 2023). In the current study, the TFC values of water extract of propolis samples were detected as 0.48, 0.68, and 2.10 mg QUE g-1 in Pazar, Ardahan, and Uzungöl propolis, respectively.

The biological and pharmacological properties of propolis have been revealed in many studies (Milojkovic, 2018). Omer et al. state that in antibacterial studies water extract from the West Black Sea region of Türkiye had activity against *P. aeruginosa*, *E. faecalis*, *S. enterica*, *L. monocytogenes*, and *B. cereus* (Omer et al.2023). In a study, it was demonstrated that Romanian propolis water extract has weak antimicrobial activity against *C. albicans*, *B. subtilis*, and *E. coli* (Nichioti et al, 2023). While Elgin et al. suggest that Turkish propolis water extract inhibits the growth of *E. coli*

BW25113 by affecting nucleic acid metabolism, Campos et al. state that the antimicrobial activity of Brazilian propolis water extract was not observed against *E.coli* and *S. aureus* (Elgin et al.2023; Campos et al. 2020). In the current study antimicrobial activity of water extract of propolis samples was investigated against 14 microorganisms. However, no activity was found against any of the microorganisms studied.

The biofilm is an aggregation of bacteria covered with a self-generated matrix. This is a strategy for bacteria to survive during unsuitable living conditions. The biofilm allows bacteria to escape from the immune system and makes bacteria 1000 times more resistant to antibiotics. *P. aeruginosa* is an opportunistic Gram-negative bacterium that forms biofilm. It is known that the biofilms of *P. aeruginosa* are responsible for 90% of wound infections and complicate the healing of wounds. Therefore, it is important to develop new therapeutic strategies and alternative methods against *P. aeruginosa* biofilms (Thi et al 2020). Among the three propolis water extracts Pazar and Uzungöl propolis extracts significantly inhibited *P. aeruginosa* biofilm formation, while Ardahan propolis inhibited biofilm formation less than others. One of the limitations of our study is the small sample size, which is insufficient to determine the average biofilm activity across propolis levels. Therefore, these results cannot be used to generalize the results to the entire population. Further research with a larger sample size may contribute to our knowledge as an indicator of changes in the anti-biofilm activity of propolis.

In the current study, antiviral activity against HSV-1 was not observed in any of the extracts prepared with water. Similarly, antimicrobial activities of the extracts were not found. However, two of the extracts have been shown to have strong antibiofilm activity. It is thought that the reason for this may be the fact that the components found in propolis are seen in the extract in different solvents at different rates. Kara et al. stated that some phenolic acids (such as gallic acid and protocatechuic) present in propolis can completely pass into aqueous solutions but may not be present in ethanolic extracts. They also showed that these two phenolic acids were mostly obtained through aqueous extracts and were not detected in 70% ethanol by the HPLC–PDA assay (Kara et al. 2022).

Conclusion: In this study, it has been shown that the water extracts of studied propolis samples cannot play a significant role in the development of antibacterial and antiviral agents or in increasing their effectiveness, however, they can be evaluated in the treatment of *P. aeruginosa* biofilms, which prolong the healing process by forming a biofilm in wounds. As a future study, a more detailed investigation can be carried out with different extraction methods and increased number of samples to futher reveal the therapeutic effects of propolis.

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Author Contribution Statement : The authors declare that they have contributed equally to the article.

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Ethics: This study was approved by the local Ethics Committee of Karadeniz Technical University School of Medicine (Protocol/plan code of the research: 2017-166).

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

EXPLORING BEE VENOM VOL ATILES: A PROMISING AVENUE FOR CYSTIC FIBROSIS

Arı Zehri Uçucu Maddelerini Keşfetmek: Kistik Fibrozis İçin Umut Veren Bir Çözüm

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ABSTRACT

Bee venom, a complex mixture of bioactive compounds, has demonstrated anti-inflammatory, antimicrobial, and immunomodulatory properties. Notably, the volatiles released by bee venom components have garnered attention for their potential in respiratory-related disease conditions. Cystic fibrosis (CF) is a challenging disorder, characterized by a genetic mutation affecting the CFTR protein, leading to the production of thick and sticky mucus in various organs, particularly the lungs and digestive system, and necessitating innovative therapeutic approaches. This research explored both bee venom volatiles' chemical composition and the effects on airway inflammation and mucus viscosity in CF patients by *in silico* methods. GC/MS analyses with various SPME fibers have conducted the identification of 67 distinct components in volatile compounds of bee venom. For CW/DVB, CAR-PDMS, and DVB-PDMS fibers, the compounds identified in the highest amounts were perilla alcohol (42.21%), tetradecane (11.48%), and 1,2-benzenedicarboxylic acid, 1,2-bis(2-methylpropyl) ester (39.98%), respectively. *In silico* analyses subsequently indicated that these components exhibit anti-inflammatory effects by modulating key cytokines and reducing inflammatory markers in CF airways. This research highlights the potential of bee venom volatiles as a novel therapeutic avenue for managing CF symptoms. Harnessing the unique properties of bee venom may offer new perspectives in the development of targeted therapies for individuals affected by cystic fibrosis.

Keywords: Bee venom volatiles, Cystic fibrosis, SPME fibers, *In silico*

ÖZ

Biyoaktif bileşiklerin karmaşık bir karışımı olan arı zehri, anti-inflamatuar, antimikrobiyal ve immünomodülatör özellikler göstermektedir. Özellikle arı zehri bileşenleri tarafından salınan uçucu bileşenler, solunum hastalıklarındaki potansiyelleri nedeniyle dikkat çekmektedir. Kistik fibroz (CF), CFTR proteinini etkileyen genetik bir mutasyonla karakterize, başta akciğerler ve sindirim sistemi olmak üzere çeşitli organlarda kalın ve yapışkan mukus üretimine yol açan ve yenilikçi tedavi yaklaşımları gerektiren zorlu bir hastalıktır. Bu araştırma, hem arı zehri uçucularının kimyasal bileşimini hem de CF hastalarında hava yolu inflamasyonu ve mukus viskozitesi üzerindeki etkilerini kimyasal ve

***in silico* yöntemlerle araştırmıştır. Çeşitli SPME fiberleri ile yapılan GC/MS analizleri, arı zehrinin uçucu bileşiklerinde 67 farklı bileşenin tanımlanmasını sağlamıştır. CW/DVB, CAR-PDMS ve DVB-PDMS fiberleri için sırasıyla perilla alcohol (%42.21), tetradecane (%11.48) ve 1,2-benzenedicarboxylic acid, 1,2-bis(2-methylpropyl) ester (39.98) bileşenleri en yüksek miktarda belirlenmiştir. Daha sonra yapılan *in silico* analizler, bu bileşenlerin, temel sitokinleri modüle ederek ve CF hastalarının hava yollarındaki inflamatuar belirteçleri azaltarak anti-inflamatuar etkiler sergilediğini gösterdi. Bu araştırma, arı zehri uçucularının CF semptomlarını yönetmek için yeni bir terapötik yol olarak potansiyelini vurgulamaktadır. Arı zehrinin benzersiz özelliklerinden yararlanmak, kistik fibrozdan etkilenen bireylere yönelik hedefe yönelik tedavilerin geliştirilmesinde yeni bakış açıları sunabilir.**

Anahtar Kelimeler: Arı zehri uçucu maddeleri, Kistik fibroz, SPME fiberi, *In silico*

GENİŞLETİLMİŞ ÖZET

Amaç: Bal arısı (*Apis mellifera*) zehri, arıların karın boşluklarında bulunan zehir bezleri tarafından üretilen ve zararlılara karşı koruma amaçlı iğneleri aracılı zerk ettikleri yüksek protein yapılı bir karışımdır. Arı zehri yüzyıllardır integratif tıp uygulamalarında medikal ve kozmetik olarak kullanılmakta olup son yıllarda da ilaç geliştirme çalışmalarında ve ticari ürünlerin üretiminde de yaygın olarak kullanılmaktadır. Arı zehrinin büyük biyoterapötik potansiyel barındıran uçucu bileşenleri kovan havası solunmasına benzer olarak solunabilir mi sorusunun cevaplanabilmesi ve arı zehrinin barındırdığı potansiyelin keşfedilmesi amacıyla bu çalışma gerçekleştirilmiştir.

Gereç ve Yöntem: Çalışma kapsamında uçucu bileşenlerin analiz edilebilmesi amacıyla bir kovan düzeneği kurulmuş ve zehir elektrik stimülasyonu yöntemiyle toplanmıştır. Kovan düzeneğinin üst kısmında yer alan boşluğa 3 farklı SPME (Katı Faz Mikro Ekstraksiyon) fiberinin (CW/DVB, CAR-PDMS, DVB-PDMS farklı polarite ve yapıda adsorbanlar içerikli 3 fiber) yerleştirilmesi ile uçucu bileşenler fibere adsorplanmış ve GC/MS cihazı aracılığıyla analizler gerçekleştirilmiştir. Sonrasında elde edilen kimyasal bileşenler tek tek kistik fibrozda önemli biyobelirteçler olan ve inhibisyon mekanizmaları çalışılan CFTR, MUC5AC, IL-13 ile moleküler yerleştirme analizlerine tabii tutulmuş ve diğer farmakolojik özellikleri (ADMET, mutajenite ve toksisite) *in silico* olarak incelenmiştir.

Bulgular: Elde edilen arı zehri uçucu bileşen analiz sonuçları 3 farklı fiber için toplam 67 uçucu bileşen ortaya koymuştur. Biyobelirteçlerin bağlanması ve inhibisyonu noktasında izobutil ftalat en aktif bileşen olmuş ve -7.4 bağlanma afinitesi skoru vermiştir. 67 bileşen için gerçekleştirilen toksisite analizi sonrasında bileşenlerin toksik olmadığı belirlenmiştir. Arı zehirinin uçucu bileşikleri üzerine yaptığımız araştırma, kistik fibrozis semptomlarında umut verici potansiyel sergileyen bir dizi biyoaktif bileşeni ortaya çıkarıyor. SPME-GC/MS analizi aracılığıyla, arı zehirinin uçucu kimyasal profilinin ayrıntılı bir şekilde anlaşılması için temel oluşturan 67 farklı uçucu bileşik belirlenmiştir. Burada sunulan bulgular, bu keşifleri uygulanabilir klinik uygulamalara dönüştürmeyi amaçlayan gelecekteki araştırma girişimleri için bir temel oluşturmaktadır. Bu çalışma, solunum yolu hastalıkları yönetiminin geleceğini şekillendirmede arı zehiri uçucularının potansiyelinin daha derinlemesine araştırılmasına zemin hazırlamaktadır.

Sonuç: Arı kovanları, baldan propolise ve arı feromonlarına kadar çok çeşitli uçucu bileşenleri kapsar ve toplu olarak sinerjik bir etki gösterir. Bu çalışmanın bulguları doğrultusunda, önerilen arı zehiri uçucularının inhalasyonu, benzer şekilde, arı zehiri toplama işlemi sırasında kovan aparatından çıkarılan bir inhaler yoluyla uygulamayı içermektedir. Aradaki fark, ortamda yalnızca arı feromonlarının ve arı zehiri uçucu maddelerinin bulunmasında yatmaktadır. Araştırma kistik fibroz perspektifini benimserken, benzer kategorideki solunum bozukluklarında genel rahatlama ve olumlu etkiler potansiyeli barındırmaktadır. Elde edilen sonuçlar arı zehri uçucu bileşenlerin toksik olmadığını ve soluma yoluyla kistik fibroz hastalarında kullanılması durumunda genel şikayetlerde ve semptomlarda rahatlama sağlanabileceğini vurgulamaktadır.

INTRODUCTION

Apitherapy is a complementary medical technique rooted in the historical use of bee products to address various diseases worldwide (Weis et al., 2022). While it is not employed as a standalone treatment, apitherapeutic applications play a crucial role in multidisciplinary treatment approaches

(Fratellone et al., 2016). The integration of complementary therapies with innovative methods holds significant importance in understanding disease mechanisms and exploring novel drugs and treatment modalities. Bee products include a variety of substances derived from bees and hives, such as bee venom, honey, propolis, royal jelly, bee bread, and pollen (Habryka et al., 2016). Bee venom, produced by glands in the abdominal cavity of bees, undergoes rapid crystallization upon contact with air, resulting in a yellow color. Honeybee venom comprises enzymes (such as hyaluronidase and phospholipase), small molecules (including histamine and dopamine), and proteins and peptides, notably melittin, apamin, and mast cell degranulation (Wehbe et al., 2019). Bee venom has historically been associated with traditional applications due to its anti-inflammatory, skin condition-relieving, and joint pain-alleviating biotherapeutic effects. However, since the late 19th century, the neuroprotective, anti-cholesterol, respiratory disease-treating, and antifungal properties of bee venom have been discovered, and these biotherapeutic effects continue to be actively researched today (Ullah et al., 2023). Modern venom research has enabled the discovery of venom components proven to be of pharmacological significance, paving the way for optimizing therapeutic strategies through the use of active compounds like melittin and apamin. Subsequently, the application scope of bee venom has expanded from its traditional antinociceptive effects to addressing degenerative diseases of the nervous system. This is attributed to the natural stability of venom enzymes and peptides as injectable solutes and their efficacy in reaching target tissues (Stela et al., 2024).

Literature reports indicate the positive impact of bee venom and bee venom therapy on respiratory and related diseases (Choi et al., 2018). Cystic fibrosis (CF) is an autosomal recessive disorder stemming from mutations in the gene encoding the cystic fibrosis transmembrane regulator protein (CFTR), a chloride (Cl) channel in the cell membrane (Castellani et al., 2017). Disease incidence varies among ethnic groups, and despite an officially reported incidence of 1/3000 in Türkiye, consanguineous marriages likely contribute to an underestimation (Dogru et al., 2020). The most prevalent mutation in the CFTR gene is F508del, characterized by the deletion of the 508th codon that encodes the amino acid phenylalanine, with a high frequency in Türkiye (Cutting, 2015). The CFTR protein is present in numerous cell types, including airway epithelium, submucosal glands, pancreas, liver, sweat glands, and reproductive organs. Genetic defects leading to CF disrupt ion transport from epithelial surfaces, diminishing the ability of epithelial cells to secrete chloride and absorb sodium in response to c-AMP agonists (Fraser-Pitt and O'Neil, 2015). Consequently, the defective ion transport results in the failure of chloride and fluid secretions in respiratory epithelium, causing mucus to dry, impairing mucociliary clearance, and ultimately contributing to lung disorders observed in CF patients. Patients with CF commonly experience excessive phlegm, persistent coughing, and frequent lung infections due to the obstruction of airways by thick and sticky mucus secretion in both the lungs and upper respiratory system (Naehrig et al., 2017).

Currently, no cure yet discovered for CF that ensures complete recovery. Instead, treatment aims to alleviate symptoms and enhance the overall quality of life (Graeber and Mall, 2023). Current cystic fibrosis treatments typically include mucolytic agents, bronchodilators, and anti-inflammatory medications. Inhaled therapies and mucolytic agents are frequently used to reduce mucus accumulation in patients' airways. Long-term antibiotic therapies play a crucial role in combating chronic infections; however, this can lead to antibiotic resistance. Recently developed CFTR modulators have shown a potential to alleviate disease symptoms by correcting mutation-related protein defects, highlighting the importance of researching targeted bioactive components and uncovering their potential (Graeber and Mall, 2023). Due to the complexity of CF and its varying impact on individual patients, seeking treatment at a specialized center for the disease is highly advantageous. The primary objectives of CF treatment include (i) Prevention and control of lung infections, (ii) Removal of mucus from the lungs, and (iii) Provision of adequate and balanced nutrition, with a focus on prevention and treatment (Allen et al., 2023).

The inhalation of bee venom, as investigated in this study, could serve as a natural therapeutic method, providing synergistic benefits to existing treatment protocols by specifically targeting pathophysiological processes such as chronic inflammation and mucus accumulation. These methods may deliver localized therapeutic effects directly to the lungs while minimizing systemic toxicity. Additionally, natural

components offer high bioavailability and may slow disease progression through the inhibition of pro-inflammatory cytokines and mucolytic effects. Integrating the inhalation of the bee venom volatiles into current treatments is also considered to reduce the risk of pulmonary complications by offering antibacterial protection, especially against resistant bacteria. This study aimed to explore new treatment potentials and discover various biotherapeutic products for investigation. Fresh bee venom samples were analyzed by SPME/GC-MS, volatile composition investigated and the effects on CF were studied by *in silico* methods.

MATERIALS and METHODS

Bee Venom Harvesting

For bee venom sampling, bees of the *Apis mellifera anatolica* race, selected from a specific hive from the Ankara Yıldırım Beyazıt University GETAT Center research apiary, were studied. A special hive system was created to collect bee venom (Figure 1). This system consists of different compartments; a glass front part has been provided for observing the bees, and a gap has been created at the top for the SPME fiber to collect volatile bioactive compounds. A glass plate was placed under the hive to collect bee venom, and bee venom secretion was ensured through Electrical impulses applied to the hive at certain intervals by the standard electrical stimulation method (de Graaf et al., 2021). Meanwhile, volatile components were adsorbed onto the SPME fiber conditioned and placed in the gap on the hive. Electrical impulse was generated with frequency from 50 to 1000 Hz, duration of 2–3 s, and pauses of 3–6 s. This hive setup method was implemented as an alternative to narrow systems like jars (Hasan et al., 2023), which are commonly used in the literature for bee venom extraction. The aim was to minimize the stress that bees might experience due to environmental conditions and to prevent any potential impact on the volatile component profile.

Figure 1. Special hive system for bee venom volatile analysis

Volatile Analysis of Bee Venom

The bee venom HS-SPME volatile analysis was carried out as described with slight modifications (Abd El-Wahed et al., 2021). The different fibers selected in this study were chosen to determine the chemical profiles of bee venom volatiles through various adsorbent materials, highlighting the importance of using different fibers during volatile component analysis and their impact on the results. SPME fibers of StableFlexTM coated with carbowax/divinylbenzene (CW/DMS, 65 µm), carboxen/polydimethylsiloxane (CAR/PDMS, 75 µm), and divinylbenzene/polydimethylsiloxane (DVB/PDMS, 65 µm) were purchased from Supelco (Oakville, ON, Canada). Bee venom was collected as described and SPME fibers were placed on the beehive. The fibers were subsequently withdrawn into a needle and then sampled into the GC–MS port. GC–MS analysis was performed on a Shimadzu GC-2010 gas chromatogram equipped with Flame Ionization Detector and DB-5 column (30 m, 0.25 mm, 0.25 mm film thickness; Supelco) coupled with a Shimadzu QP2010-Plus mass

spectrometer. The injector and the interface temperatures were both set at 250 °C and for the ion source at 220 °C. A gradient temperature program was applied for volatiles analysis. The oven temperature was initially held at 40 °C for 2 min and then was increased to 200 °C at a rate of 3 °C /min held for 10 min, then ramped to 5 °C/min at 250 °C held for 5 min, and finally ramped to 5 °C/min at 275 °C. The Split injection mode was used for 1/50. The SPME fiber was prepared for the next analysis by placing it in the injection port for 10 min at 220 °C to ensure complete elution of volatiles. Blank runs were performed during the sample analyses. The FID temperature was 300 °C and the quadruple mass spectrometer was operated in EI mode at 70 eV, and the scan range was set at m/z 40–700.

GC–MS Data Processing

The identification of volatile compounds in fresh bee venom involved comparing retention times and spectra with standards. The compounds were identified by comparing their relative retention times to a C8–C32 n-alkanes mixture and mass spectra, utilizing NBS75K, Wiley 7, NIST7MS search 2.0 library data from the GC-MS system, literature data, and standards of the primary components. Additionally, the results were validated by comparing compound elution orders with their relative retention indices on a DB-5 column. All analyses were conducted in triplicate for consistency.

Molecular Docking Analysis

Structures of different target proteins were retrieved from the Protein Data Bank (Burley et al., 2017) and given in Table 1.

Table 1. Proteins and other molecular docking parameters

Structure Name	PDB ID	Target Activity	Grid Box Center Coordinates	Grid Box Size
CFTR	5TF7	Cystic Fibrosis Activity	center_x = -49 center_y = 25 center_z = -10	size_x = 21 size_y = 21 size_z = 21
MUC5AC	5AJN	Mucus Activity	center_x = 38 center_y = 32 center_z = -8	size_x = 35 size_y = 35 size_z = 35
IL-13	4I77	Cytokine Activity	center_x = 2 center_y = 16 center_z = -32	size_x = 22 size_y = 22 size_z = 22

To prepare suitable protein targets, Amber's Antechamber module was utilized with Chimera v1.6 (Pettersen et al., 2004). The docking preparation for the target proteins involved the following steps: (a) conducting energy minimization with 100 steepest descent steps, a .02 Å step size, and an update interval of 10; (b) removal of water molecules and co-crystallized ligands; (c) deletion of solvent and non-complex ions; and (d) addition of polar hydrogen atoms and AM1-BCC charges. Ligand structures corresponding to compounds identified in all four plant species were obtained from the PubChem database (Kim et al., 2016) in (.sdf) format. Furthermore, the energies of all ligand structures were minimized, and Gasteiger charges were added. Subsequently, rigid molecular docking was performed using Chimera v1.6, employing Autodock Vina's scoring function. The top poses with the minimum binding energy for each protein were visualized using Discovery Studio Visualizer (Studio, 2008).

Pharmacophore Modeling

Pharmacophore models were predicted using the LigandScout version 4.5 (Wolber et al., 2005). Molecular structures datasets were inputted into program with .sdf formats, allowing the identification of key pharmacophoric features such as hydrogen bond donors, acceptors, hydrophobic regions, and aromatic rings. Advanced algorithms within the program aligned and superimposed the ligands, extracting consensus pharmacophore hypotheses representing common structural elements essential for biological activity. Validation of the models involved assessing their ability to reproduce known bioactivity or predict the activity of new compounds.

ADMET Analysis

An ADME/T (absorption, distribution, metabolism, excretion, and toxicity) analysis was carried out, and

the results of this analysis present promising initial data inputs for subsequent *in vitro* and *in vivo* experiments. These predictions were obtained using both the SwissADME web tool and relevant literature data (Daina et al., 2017).

Toxicity Analysis

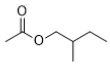
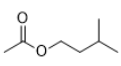
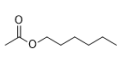
The LAZAR web tool was employed for the *in silico* prediction of the toxicity of volatile compounds presents in the bee venom (Maunz et al., 2013). The LAZAR web tool, an established platform for predictive toxicology assessments, utilizes a range of computational algorithms to evaluate the potential toxicity of chemical compounds. Input data on the bee venom volatile compounds were subjected to the LAZAR webtool's predictive models, allowing for the assessment of acute toxicity levels. The analysis focused on identifying potential toxic effects and elucidating the intricate relationships between the chemical structure of the bee venom volatile compounds and their predicted toxicological outcomes.

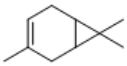
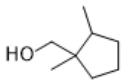
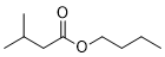
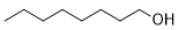
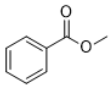
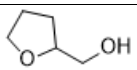
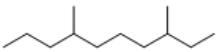
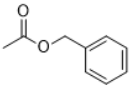
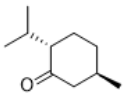
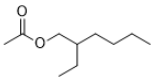
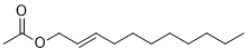
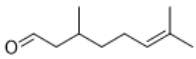
RESULTS

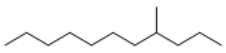
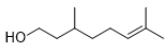
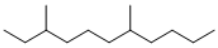
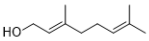
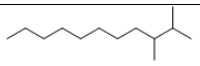
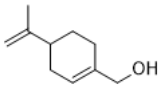
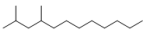
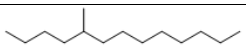
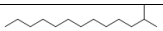
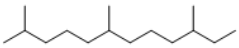
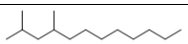
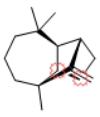
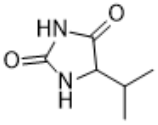
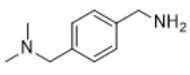
Chemical Composition of Bee Venom Volatile Compounds

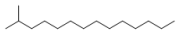
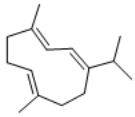
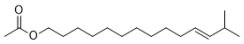
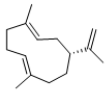
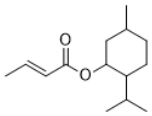
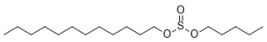
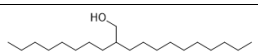
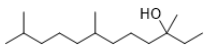
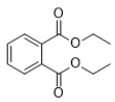
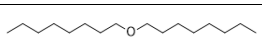
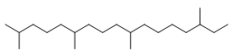
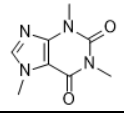
In this study, freshly extracted bee venom volatiles were analyzed by the SPME/GC-MS method, and the results are given in Table 2. Notably, it has been determined that the volatile secretions of fresh bee venom consist of 67 different volatile organic compounds.

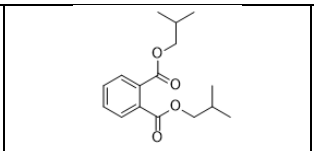
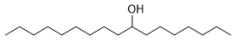
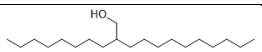
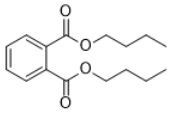
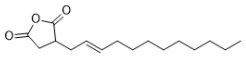
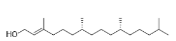
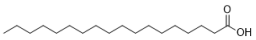
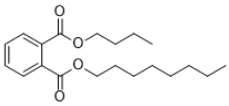
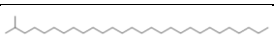
Table 2. Composition of volatiles from fresh honeybee venoms

Volatile Compounds of Bee Venom				**Type of SPME fiber**			
				CW/DVB	**CAR-PDMS**	**DVB-PDMS**	
LRI (cal)	**LRI (lit)**	**Compound**	**Chemical structures of compounds**	**%**	**%**	**%**	**IM**
496	495	Acetaldehyde		0,88±0,05	0,07±0,01	-	a
652	653	Acetic acid		-	0,12±0,01	-	a
667	665	Propanoic acid		0,42±0,02	0,11±0,00	0,98±0,05	a
671	670	2-Propyn-1-ol, acetate		-	0,08±0,01	-	a
870	871	2-Methyl butyl acetate		-	-	0,61±0,07	a
884	885	1-Butanol, 3-methyl-, acetate		-	-	11,13±0,49	a,b
911	912	Cyclobutanol		0,53±0,05	-	-	a
986	988	Acetic acid, hexyl ester		-	-	1,35±0,02	a

992	991	Cyclohexane, 1-isopropyl-1-methyl-		-	2,75±0,08	-	a
998	1001	Octanal		-	0,30±0,05	-	a, b
1007	1008	3-Carene		-	1,45±0,03	-	a
1037	1038	Cyclopentane, 1-hydroxymethyl-1,3-dimethyl-		-	0,21±0,03	-	a
1038	1039	Butanoic acid, 3-methyl-, butyl ester		-	-	1,78±0,22	a
1052	1051	1-Octanol		-	-	0,73±0,08	a,b
1054	1055	2-Hepten-1-ol		-	0,12±0,01	-	a
1077	1076	Methyl benzoate		-	-	0,97±0,07	a
1101	1102	Tetrahydrofurfuryl alcohol		2,44±0,22	-	-	a
1126	1127	Decane, 3,7-Dimethyl		-	0,59±0,04	-	a
1136	1135	Acetic acid, phenylmethyl ester		-	0,06±0,00	0,27±0,02	a
1141	1142	Menthone		-	-	0,29±0,03	a,b
1144	1144	Acetic acid, 2-ethylhexyl ester		-	-	1,84±0,32	a
1149	1151	Acetic acid, undec-2-enyl ester		-	-	0,51±0,04	a
1156	1157	3-Pentanol, 2,4-dimethyl-		-	-	0,41±0,06	a
1158	1158	Citronella		-	-	0,25±0,06	a

1161	1160	4-Methylundecane		-	0,16±0,01	-	a
1170	1171	Menthol		-	-	0,10±0,01	a,b
1208	1208	beta -Citronellol		-	-	0,18±0,01	a
1220	1221	Undecane, 3,7-dimethyl-		-	0,81±0,07		a
1232	1233	trans-Geraniol		-	-	0,43±0,07	a
1251	1250	Undecane, 2,3-dimethyl-		-	0,57±0,07	-	a
1274	1275	Tridecane		-	3,58±0,10	-	a
1296	1297	1-Cyclohexene-1-methanol, 4-(1-methyl ethenyl)-/ Perilla alcohol		42,21±0,12	-	-	a,b
1319	1318	Tetradecane		-	11,48±0,45	-	a,b
1321	1320	2,4-Dimethyldodecane		-	1,12±0,10	-	a
1355	1354	Tridecane, 5-methyl-		-	3,21±0,08	-	a
1362	1363	2-Methyltridecane		-	6,57±0,11	-	
1366	1365	Dodecane, 2,6,10-trimethyl		-	3,46±0,06	0,53±0,02	a
1383	1384	2,4-Dimethyldodecane		-	-	0,31±0,03	a
1426	1427	Junipene		-	-	0,51±0,02	a
1431	1430	5-Isopropyl-2,4-imidazolidinedione		3,45±0,05		-	a
1435	1434	4-Dimethylamino methylbenzylamine		-	0,53±0,04	-	a

1465	1466	Tetradecane, 2-methyl-		-	3,69±0,22		a
1475	1477	Germacrene-C		-	1,87±0,00	-	a
1479	1480	E-11(13-Methyl) tetradecen-1-ol acetate		-	0,97±0,08	-	a
1490	1489	Germacrene-A		-	0,93±0,03	-	a,b
1494	1495	Crotonic acid, menthyl ester		-	1,45±0,11	-	a
1501	1502	Sulfurous acid, dodecyl pentyl ester		-	1,21±0,15	-	a
1534	1535	2-Octyldodecan-1-ol		-	0,33±0,01	-	a
1536	1537	3-Dodecanol, 3,7,11-trimethyl		-	-	0,18±0,00	a
1551	1550	Diethyl Phthalate		-	-	0,23±0,01	a
1620	1620	Hexadecane		-	2,69±0,11	0,43±0,02	a,b
1634	1635	Heptadecane		-	1,88±0,03	-	a,b
1657	1658	Octane, 1,1'-oxybis-		-	0,93±0,04	-	a
1661	1660	Heptadecane, 2,6,10,15-tetramethyl-		-	-	0,17±0,02	a
1745	1746	Dodecane		-	4,75±0,21	-	a,b
1811	1810	Octadecane		-	0,83±0,07	0,25±0,01	a
1839	1840	Caffeine		0,95±0,11	-	-	a,b

1849	1847	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester		-	-	38,98±0,62	a
1872	1873	8-Heptadecanol		-	0,19±0,01	-	a
1884	1885	2-Octyldodecan-1-ol		-	0,11±0,01	-	a
1890	1891	1-Nonadecene		-	-	0,32±0,00	a
1935	1937	1,2-Benzenedicarboxylic acid, dibutyl ester		0,10±0,01	-	0,17±0,01	a
1967	1966	2-Dodecen-1-yl(-)succinic anhydride		-	-	0,67±0,12	a
2121	2122	Phytol		-	1,23±0,07	-	a
2156	2158	Octadecanoic acid		0,25±0,05	-	-	a,b
2315	2317	1,2-Benzenedicarboxylic acid, butyl octyl ester		0,24±0,04	-	-	a
2861	2863	2-methyloctacosane		-	-	0,23±0,01	a

[a] Compounds listed in order of elution from a DB-5 column. b Identification of components based on standard compounds; All values are mean ± standard deviation of triplicates; significant at the $p<0.05$ level

LRI (cal): Linear retention indices (DB-5 column) calculated against n-alkanes. % calculated from FID data with standard LRI (lit): https://pubchem. ncbi.nlm.nih.gov; IM: Identification Method

In Silico Molecular Docking Results of Bee Venom Volatile Compounds

The relevant ligands and analysis results were presented in the heatmap clustering provided in Figure 2. The heatmap was plotted by http://www.bioinformatics.com.cn/srplot, an online platform for data analysis and visualization.

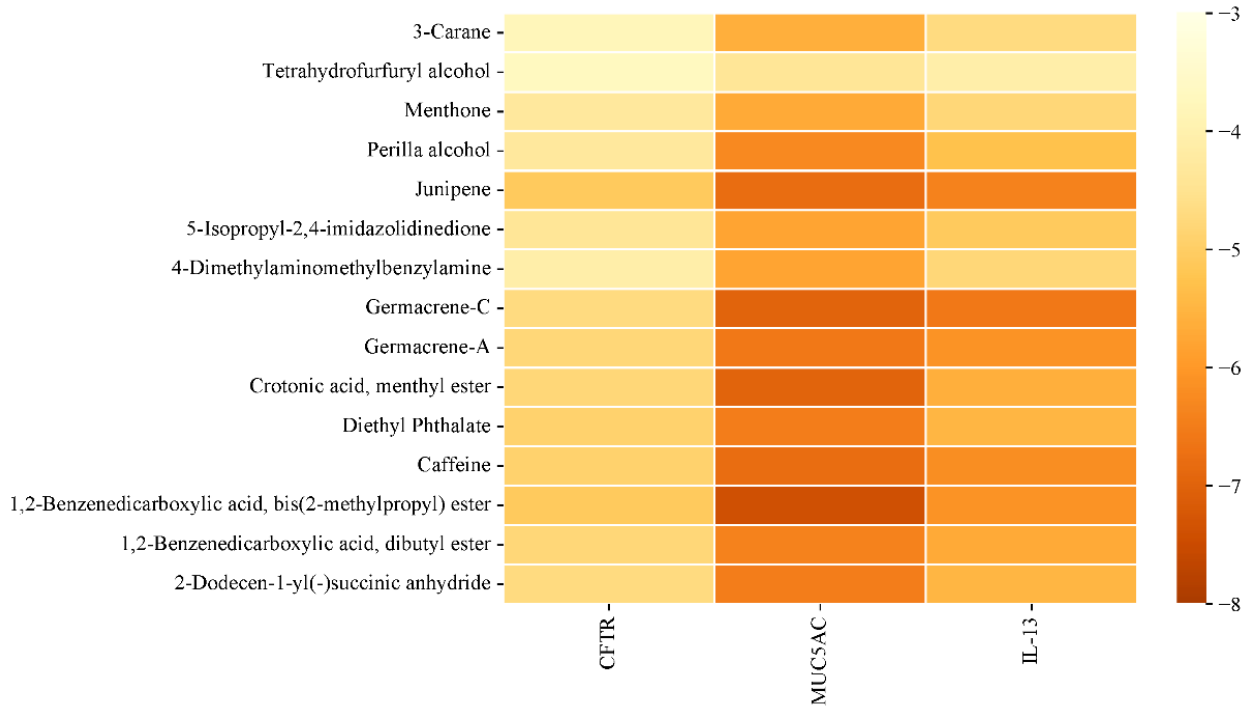


Figure 2. Clustered hierarchical heatmap showing quantified volatiles compounds from bee venom (The binding affinities provided in the chart on the right).

As inferred from the results, volatile components exhibited high inhibitory activity against the MUC5AC structure. The highest binding score was -7.4 for 1,2-Benzenedicarboxylic acid, bis(2-methyl propyl) ester as known as isobutyl phthalate, followed by -7 for crotonic acid, menthyl ester. Subsequently, other volatile components such as Junipene and Germacrene-C have been found to exhibit high IL-13 suppressor activities. Upon examining the pharmacophore analyses of both structures with the highest binding affinities, in addition to hydrophobic binding regions, the H-bond acceptor ends are observed in the isobutyl phthalate binding site (Figure 3a). On the other hand, the same interaction profile was observed with fewer van der Waals interactions for the crotonic acid, menthyl ester (Figure 3b). It is suggested that the direct influence of a high number of Van der Waals interactions contributes to the high binding affinity.

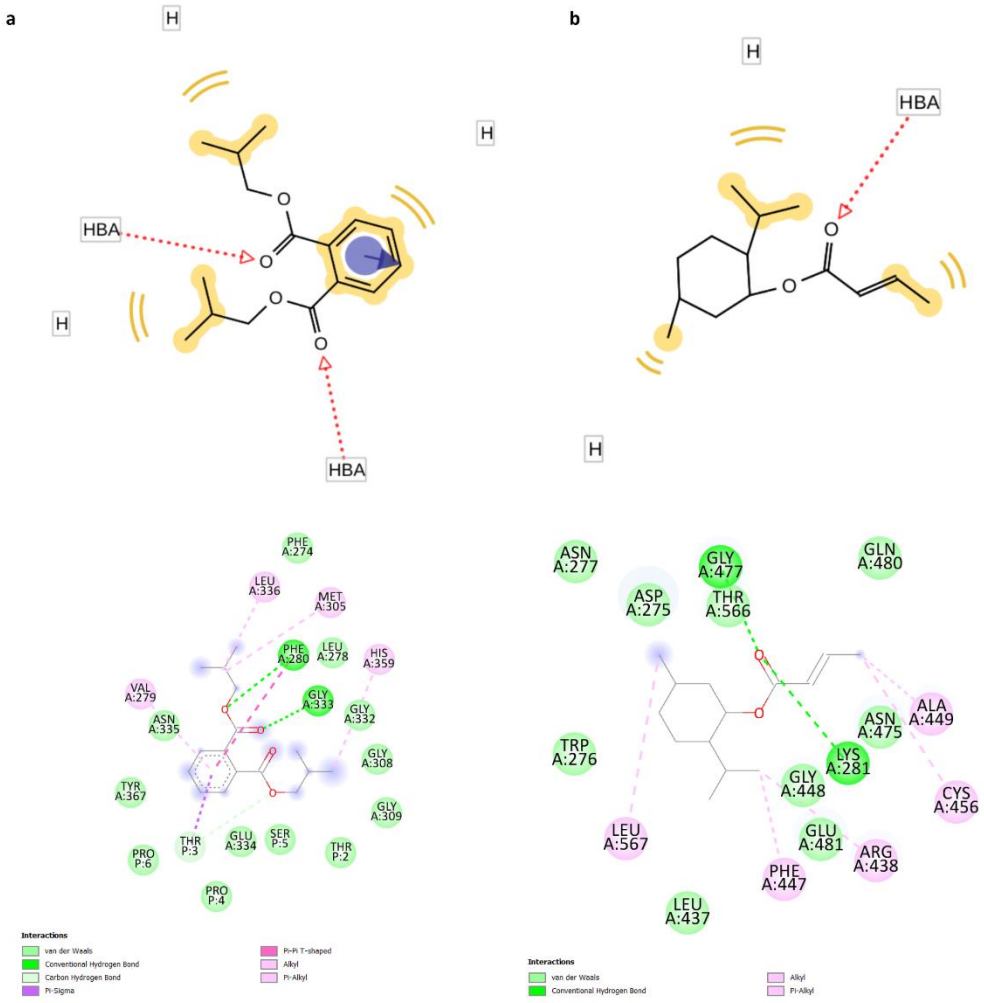


Figure 3. Two-dimensional binding geometry of (a) isobutyl phthalate and (b) crotonic acid, menthyl ester

In silico Toxicity Prediction Results

The bee venom volatile compounds' acute toxicity levels, as analyzed by *in silico* methods, are presented in Table 3.

While components such as junipene and germacrene C exhibit high levels of acute toxicity for both species at minimal concentrations, compounds like tetrahydro furfuryl alcohol and 5-Isopropyl-2,4-imidazolidinedione demonstrate low acute toxic effects even at elevated concentrations. Consequently, it is postulated that the composition of these volatile components exhibits synergistically low or moderate acute toxic effects. Additionally, no mutagenic effects have been identified for any of the volatile components.

ADMET Analysis

SMILES structures were employed for the *in silico* predictions of ADMET properties of certain volatile compounds of bee venom (see Table 4). The radar plots illustrating the general characteristics are presented in Figure 4.

Table 3. I*n silico* toxicity analysis results of some bee venom volatiles

Compounds	Acute toxicity (*Pimephales promelas*) (mmol/L)	Max. tolerated dose (Human) (mmol/kg/day)	Carcinogenicity (Mouse) (mg/L)	Mutagenicity (*Salmonella typhimurium*)
3-Carane	0.212	0.00632	non-carcinogenic	non-mutagenic
Tetrahydrofurfuryl alcohol	8.19	0.217	carcinogenic	non-mutagenic
Menthone	1.2	x	x	non-mutagenic
Perilla alcohol	x	x	non-carcinogenic	non-mutagenic
Junipene	0.00468	0.00803	carcinogenic	non-mutagenic
5-Isopropyl-2,4-imidazolidinedione	10.3	0.0207	x	non-mutagenic
4-Dimethylaminomethylbenzylamine	0.501	0.015	x	non-mutagenic
Germacrene-C	0.0452	x	non-carcinogenic	non-mutagenic
Germacrene-A	0.0458	x	non-carcinogenic	non-mutagenic
Crotonic acid, menthyl ester	0.223	0.0282	non-carcinogenic	non-mutagenic
Diethyl Phthalate	0.143	0.0218	carcinogenic at 0.149	non-mutagenic
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.00705	0.0183	carcinogenic	non-mutagenic
1,2-Benzenedicarboxylic acid, dibutyl ester	0.00359	0.0164	carcinogenic at 0.336	non-mutagenic
2-Dodecen-1-yl (-) succinic anhydride	0.032	0.0203	non-carcinogenic	non-mutagenic

Table 4. SMILES structures of the volatile compounds

Number	SMILES Structure	Compound Name
1	CC1CCC2C(C1)C2(C)C	3-Carane
2	C1CC(OC1)CO	Tetrahydrofurfuryl alcohol
3	CC1CCC(C(=O)C1)C(C)C	Menthone
4	CC(=C)C1CCC(=CC1)CO	Perilla alcohol
5	CC1(CCCC2(C3C1C(C2=C)CC3)C)C	Junipene
6	CC(C)C1C(=O)NC(=O)N1	5-Isopropyl-2,4-imidazolidinedione
7	CN(C)CC1=CC=C(C=C1)CN	4-Dimethylaminomethylbenzylamine
8	CC1=CCCC(=CC=C(CC1)C(C)C)C	Germacrene-C
9	CC1=CCCC(=CCC(CC1)C(=C)C)C	Germacrene-A

Using Diana's approach (Daina et al., 2017), the ADMET properties of certain volatile components have been depicted through radar plots (Figure 4). Most of these components exhibit high lipophilicity and despite a relatively low polarity profile, their structures demonstrate small size and non-flexible

behavior. Given the expected rigid nature of volatile components, such characteristics are within the norm (de Lacy Costello et al., 2014). Furthermore, considering a biopharmaceutical score of 0.55 for all components and their compliance with Lipinski's Rule of five, indicating their drug-like potential, it is plausible to assert that the volatile components of bee venom manifest a favorable ADMET profile.

Figure 4. Radars to assess the general properties and bioavailability of some bee venom volatile compounds.

DISCUSSION

CF is a multifaceted disease characterized by 4 different signaling pathways, for which a definitive treatment has not yet been found. Supportive products related to the disease are often associated with facilitating the dispersion of dense mucus. Mucins are the primary components of mucus produced in the airway epithelium. These are essentially classified as transmembrane or membrane-bound mucins and secreted/gel-forming mucins. As MUC5AC plays a significant role in the pathogenesis of CF, inhibiting the secretion and production of mucin could have a strong impact on the disease (Lillehoj et al., 2013; Samsuzzaman et

al., 2019). On the other hand, Interleukin-13 (IL-13) has been included in this study due to its association with allergic airway inflammation in the context of CF (Nakamura et al., 2017). The target proteins used in the *in silico* analyses were selected within the scope of this study as important markers for revealing their inhibition potential related to the disease. On the other hand, the fact that these ligands selected for computational analyses are bee venom volatiles carries the potential to introduce a new approach to apitherapy.

The analysis of volatile components in bee venom plays a significant role in determining the systematic mechanism of apitherapy applications and elucidating the processes involved. Within the setup established in the study, it is known that the samples obtained from the hive environment contain not only bee venom but also the volatile components of stimulated bees. The intricate social behavior of honeybees involves collective nest protection, executed through an effective system of chemical communication mediated by volatile compounds. Worker bees release some of the volatiles from the Koschevnikov gland during alarm behavior (Noël et al., 2023). The exploration of these volatiles has a longstanding history, initially revealing 3-methylbutyl acetate (isoamyl acetate) as the primary component inducing aggressive behavior (sting pheromone) in bees (Boch et al., 1962).

Subsequent investigations into the chemical composition of bee venoms revealed different components (Simone-Finstrom et al., 2023). In an analysis conducted on hexane extracts of Koschevnikov glands, 22 organic compounds within the C6–C36 range were identified (Camargos et al., 2020). While this study does not directly delve into the analysis of volatile components in bee venom, the shared constituents suggest the synergistic presence of compounds from bee venom, bee pheromones, and other volatile elements in the surrounding environment. Isidorov et al. utilized the same SPME/GC-MS methodology to investigate volatiles in both dry and fresh bee venom (Isidorov et al., 2023). Notably, there are compositional differences in the profiles of volatile components when compared to our results. This is believed to be attributed to factors such as geographical location, bee species, and the harvesting season. Another potential contributing factor is the presence of bee pheromones and bee-derived contaminations. In a study analyzing the volatiles of beehive air, profiles similar to those in our analysis were identified, consisting of aldehyde, acid, and hydrocarbon structures (Abd El-Wahed et al., 2021). Since the samples of bee venom were collected alongside the entire beehive air, it is possible to assert that the content of the hive's air, from which the samples were collected, is parallel to the samples.

On the other hand, when focusing on the *in silico* biotherapeutic effects of these components it has been determined that the volatile components of bee venom exhibit low binding affinities against CFTR targets, and no noteworthy inhibitor activity is demonstrated. It is hypothesized that the thermal behaviors of the protein binding region or the weak profile of volatile components for inhibitor behavior may account for this (Wang et al., 2018; Vega et al., 2016). However, it is possible to state that there is an *in silico* activity against respiratory problems for some of the bee venom volatiles synergistically. The notion that the sample collection environment does not only contain volatile components of bee venom parallels with the content analysis of studies on bee pheromones found in the literature (Li et al., 2014). The compound 1-octanol, reported as an alarm pheromone for *Apis mellifera* species (Wang and Tan, 2019), has been identified in the volatile chemical components of a DVB-PDMS type SPME fiber. This is directly associated with the electrification applied during bee venom collection, inducing bees into an alarmed state and, consequently, leading to venom secretion. The semiochemicals released by bees during their usual activities, compounds such as Geraniol and 1-nonadecene were also found in our study. Similarly, the descriptive fiber for both compounds was DVB-PDMS (Schmitt et al., 2007).

Additionally, crotonic acid, menthyl ester demonstrates high biotherapeutic activity among volatile components, as this observation is consistent with various *in vitro* literature studies (Yassin et al., 2020). When focusing on the potential toxicities of volatile components, no substances exhibiting toxic properties were found, and it is known that the toxic effect of bee venom mainly stems from its enzyme structures and melittin. However, this is specific to bees, and the volatile profiles of venoms from other organisms may exhibit toxicity. For example, phenol-2,4-bis (1,1 dimethyl ethyl) an ant venom volatile compound, display high acute toxicity (Nikbakhtzadeh et al., 2009), emphasizing the significance of approaching bee venom as an allergen despite the low toxicity of its primary volatile components. The recommendation

for the medical use of carcinogenic gases is not a new concept, besides nitric oxide, a well-known carcinogenic gas, is employed in various medical applications, including the treatment of pulmonary hypertension in newborns (Barnes et al., 2020). Fundamental considerations in the utilization of gases and volatile components revolve around concentration and dosage studies. When compared to Table 2, it is observed that the volatile compounds characterized as carcinogenic constitute a relatively small proportion (total 2-3%). Given the consistent exposure of individuals, particularly beekeepers, to similar profiles of compounds during the collection of bee venom, a crucial point emerges: the volatile components of bee venom do not exhibit carcinogenic effects synergistically, as inferred from their chemical composition. This observation is particularly pertinent when considering the continuous inhalation of these compounds during bee venom collection activities (Matysiak et al., 2016).

The utilization of volatile compounds associated with bees in respiratory processes is integratively acknowledged and currently implemented as a complementary method in clinical practices across various regions globally (Abd El-Wahed et al., 2021; Topal et al., 2021). The processes involve inhaling a pre-determined dosage of air by physicians, through an inhaler device emanating from a beehive. Beehives encompass a diverse array of volatile components, ranging from honey to propolis and bee pheromones, demonstrating a synergistic effect collectively. In line with the findings of the present study, the suggested inhalation of bee venom volatiles similarly involves administration through an inhaler extracted from the hive apparatus during the bee venom collection process. The distinction lies in the ambient presence solely of bee pheromones and bee venom volatiles. While the research adopts a cystic fibrosis perspective, it harbors the potential for general relief and positive effects in respiratory disorders of a similar category.

In conclusion, our investigation into the volatile compounds of bee venom unveils a spectrum of bioactive components that exhibit promising potential in cystic fibrosis symptoms. Through SPME-GC/MS analysis, we identified 67 distinct volatile compounds, laying the foundation for a nuanced understanding of the volatile chemical profile of bee venom. The findings presented herein provide a basis for future research initiatives aimed at translating these discoveries into viable clinical applications. This study is setting the stage for deeper exploration and the potential of bee venom volatiles in shaping the future of respiratory diseases management.

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Author contribution: Nilüfer VURAL: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing – original draft; Writing – review & editing. Sibel KAYMAK: Conceptualization; Formal analysis; Software; Validation; Visualization; original draft; Writing – review & editing. Oğuz YÜCE: Conceptualization; Methodology; Funding acquisition; Supervision

Conflict of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical statement: No experimentation on human or animal subjects was involved in this study. At the stage of bee venom sampling from honeybees, ethical permission is not required for insects.

Data availability: Data will be made available on request.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

SOLITARY BEES (HYMENOPTERA: APOIDEA) DIVERSITY AND PALYNOLOGICAL ANALYSIS OF THEIR ASSOCIATED FLORAL RESOURCES IN WESTERN EGYPT

Batı Mısır'daki Bireysel Arıların (Hymenoptera: apoidea) Çeşitliliği ve Bunlarla İlişkili Çiçek Kaynaklarının Palinolojik Analizi

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ABSTRACT

Egypt is one of the important countries in terms of bee diversity in Northern Africa. The Eastern part of the country has been the subject of several studies over the last decade, especially in comparison to the Western part. In this work, we explore the diversity of solitary bees in the Alexandria Governorate, specifically in the Al Hawaria region (30°57'13" N, 29°40'27" E), based on two years of research conducted in 2021 and 2022. A total of 51 species were identified in the region: 25 species of Apidae, 16 of Megachilidae, 5 of Andrenidae, 4 of Halictidae, and one species of Colletidae. Pollen grain identification from plants and some solitary bee species revealed the creation of reference slides for pollen grains from 32 flowering plant species (both crops and wildflowers) across 19 plant families. The plant family preferences were varied among different bee genera. The most commonly visited plant families were Asteraceae, Brassicaceae, and Fabaceae, which accounted for 58% of the total bee-attracting flora. The pollen spectrum from the *Xylocopa aestuans* (L. 1758) nest consisted of two pollen types, while the nest of *Osmia* sp. contained a single pollen type.

Keywords: Apoidea, Bees, Pollination, Pollen grains, Palynology

ÖZ

Mısır, Kuzey Afrika'nın arı çeşitliliği açısından önemli ülkelerinden biridir. Ülkenin doğu kısmı son on yılda batı kısmına kıyasla birçok eserde incelenmiştir. Burada 2021 ve 2022 yıllarında iki yıllık çalışmalar sırasında İskenderiye Valiliği'nin Al Hawaria bölgesindeki 30 57'13" N 29 40'27" E'deki yalnız arıların çeşitliliğini ele alıyoruz. Bölgede bulunan toplam tür sayısı 51 tür (25) idi. Apidae'den 16'sı, Megachilidae'den 5'i, Andrenidae'den 5'i, Halictidae'den 4'ü ve Colletidae'den 1 tür). Bitkilerden toplanan bazı polen tanelerinin ve bazı yalnız arı türlerinin tanımlanması, bu çalışmada 19 bitki familyasına ait 32 çiçekli bitki türünden (mahsuller ve kır çiçekleri) polen tanelerinin referans slaytlarının yapıldığını ortaya çıkardı. Bitki familyalarının tercihi farklı arı cinsleri arasında farklılık gösteriyordu. Arıların ziyaret ettiği en çok temsil edilen familyalar Asteraceae, Brassicaceae ve Fabaceae idi ve bunlar toplam arı florasının %58'ini oluşturdular. *Xylocopa aestuans*'ın çalışma yuvasındaki polen spektrumu toplam iki polen türünden oluşurken, Osmia sp'nin yuvası bir polen türünden oluşmuştur.

Anahtar kelimeler: Apoidae, Arılar, Polinasyon, Polen taneleri, Palinoloji

GENİŞLETİLMİŞ ÖZET

Çalışmanın amacı: Al Hawaria bölgesindeki *Apis* dışı arıların tür kompozisyonunu ve alanın çiçek kaynakları ile etkileşimlerini ve toplanan bireysel arıların (yaklaşık 9 türün vücutlarında polen görülmüştür) vücut setaları üzerinde bulunan farklı polen tanelerini (polen taksonları) tanımlamaktır.

Gereç ve Yöntem: Çalışma alanı, Mısır'ın İskenderiye kentinin batısındaki Burj Al Arab şehrinde bulunan Al-Hawaria bölgesidir (30°57'13“ N, 29°40'27” E). Bu alan şehirleşme ve diğer antropojenik değişikliklerden uzaktır. Yalnız arı türlerinin araştırılması 2021 ve 2022 yıllarında bazı ekilmemiş ve ekili bitkiler üzerinde gerçekleştirilmiştir.

Bireysel arılar iki yıl boyunca, hava durumuna göre belirlenen aralıklarla, aylık veya haftalık olarak bir süpürme ağı kullanılarak toplanmıştır. Arılar siyanür kavanozları kullanılarak öldürüldü ve İskenderiye Üniversitesi Ziraat Fakültesi Entomoloji ve Zooloji Bölümü'ndeki laboratuvara nakledilmeden önce kağıt mendil içinde korunarak iğnelenip etiketlendikten sonra ahşap kutularda saklanmıştır. Etiketler saat, tarih, yer ve toplayıcının adı gibi toplama bilgilerini içermekte oup veriler çeşitli türlere ait bireysel arılar için kaydedilmiştir.

Arıların tanımlanması: Ain Shams Üniversitesi Fen Fakültesi Entomoloji Bölümü ve Süveyş Kanalı Üniversitesi Ziraat Fakültesi Bitki Koruma Bölümü referans koleksiyonları kullanılarak gerçekleştirilmiştir. Bazı türler, özellikle Osminii ve Anthidini kabilelerinden olmak üzere Avrupalı taksonomistlere gönderilmiştir. Bazı türlerin kimliğini doğrulamak için çevrimiçi veri tabanları ve Hymenoptera Atlası da kullanılmıştır. Arıların farklı taksonomik özelliklerini incelemek için stereoskopik mikroskop kullanılmıştır.

Arılar ve bitkiler arasındaki etkileşim, çeşitli bitkilerden, arılardan ve arı yuvalarından toplanan polen tanelerinin tanımlanmasıyla incelenmiştir. Polen analizi, İskenderiye Üniversitesi Ziraat Fakültesi Uygulamalı Entomoloji ve Zooloji Bölümü'nde Westrich & Schmidt (1986), Westrich (1990), Sawyer (1981), Tellería (2000) ve Esmaeil (2016) yöntemleri izlenerek gerçekleştirilmiştir.

Bulgular: 2021 ve 2022 yıllarında Al Hawaria bölgesinde 13 bitki türünün çiçeklenme dönemlerinde toplam 820 bireysel arı örneği toplanmıştır. Arılar beş familya (Apidae, Megachilidae, Halictidae, Colletidae ve Andrenidae) tarafından temsil edilip Melittidae familyası bu çalışmada yer almamıştır. Toplanan arılar 24 cinsi temsil etmekte olup (Apidae'den 9, Megachilidae'den 7, Halictidae'den 5, Colletidae'den 2 ve Andrenidae'den bir) bölgede bulunan toplam tür sayısı 51 tür olarak belirlenmiştir (25 Apidae, 16 Megachilidae, 5 Andrenidae, 4 Halictidae ve bir Colletidae türü). **Aile: Apidae:** Bölgede Anthophora, Amegilla ve Eucera'nın çeşitli arı türleri yaşamakta olup bu üç cinsin ortaya çıkışı, keptoparazitik arı türlerinin (*Thyreus* sp, *Nomada* sp, *Melecta* sp ve *Epeoles* sp) ortaya çıkışıyla aynı zamana denk gelmiştir. 25 Apidae türü Asteraceae, Solanaceae, Brassicaceae, Aizoaceae, Labiatae, Tamaricaceae, Malvaceae, Oxalidaceae, Pedaliaceae ve Fabaceae familyalarından çeşitli bitkileri ziyaret etmiştir. **Aile: Megachilidae:** Megachilidae familyasından 16 tür Asteraceae, Brassicaceae, Aizoaceae, Lamiaceae, Tamaricaceae, Malvaceae, Papaveraceae ve Fabaceae familyalarının florasından toplanmış olup Megachile, Osmia ve Hoplitis cinslerine ait bazı türler tür düzeyinde teşhis edilirken, diğerleri tespit edilememiştir. **Aile: Andrenidae:** Andrenidae, Şubat-Haziran ayları arasında aktif olan ve Asteraceae, Aizoaceae, Fabaceae ve Brassicaceae florasından kaydedilen Andrena cinsinden beş tür ile temsil edilmiştir. **Aile: Halictidae:** Halictidae familyasından *Halictus, Lasioglossum, Pseudapis, Ceylalictus* ve bir cleptoparasitic cins olan *Sphecodes* dahil olmak üzere çeşitli cinsler toplanmıştır. Dört tür tespit edilmiştir: *Ceylalictus variegatus* Olivier, 1789, *Pseudapis nilotica* Smith, 1857, *Lasioglossum vagans* Smith, 1857 ve *Halictus quadricinctus* Fabricius, 1776. Tüm türler Asteraceae, Solanaceae, Brassicaceae, Aizoaceae, Lamiaceae, Tamaricaceae, Pedaliaceae ve Fabaceae'den çeşitli floraları ziyaret etmiştir. **Aile: Colletida:** Colletidae, *Colletes lacunatus* Dours, 1872 baskın tür olmak üzere iki cins (*Colletes* ve *Hylaeus*) ile temsil edilmiştir. Örnekler Brassicaceae ve Aizoaceae familyalarının florasından toplanmıştır. Colletidae, tür zenginliği ve bolluğu açısından diğer familyalara kıyasla en az kaydedilen familyadır.

Palinolojik Analiz: Bu çalışmada 19 bitki familyasına ait 32 çiçekli bitki türünden (ekinler ve kır çiçekleri) polen tanelerinin referans slaytları hazırlanmış ve İskenderiye Üniversitesi, Ziraat Fakültesi, Uygulamalı Entomoloji ve Zooloji Bölümü'nde depolanmıştır. Çalışılan polen tanelerinin mikrofotoğrafları ve ölçümleri bu çalışmaya dahil edilmiştir.

Sonuç: Bu örneklerle, araştırma bölgesinde bal arıları ve diğer yabani arı türleri tarafından ziyaret edilen yem bitkilerinin bir listesini derlenmesi sağlanırken çeşitli botanik familyalardan polen taksonlarının varlığı, toplanan bitkilerin referans slaytları ile karşılaştırılarak tespit edilmiştir. Arıların vücut kıllarının (vücut, abdominal ve tibial setalar) mikroskobik analizi, 10 bitki familyasına ait 17 bitki cinsinin (tarla ve yem bitkileri, yabani otlar, süs bitkileri ve sebze bitkileri) arı poleni yem kaynağı olarak kaydedildiği ortaya konulmuştur. En çok temsil edilen familyalar, toplam arı florasının %58,8'ine katkıda bulunan Asteraceae (4 cins), Brassicaceae (3 cins) ve Fabaceae (3 cins) olmuş, Cucurbitaceae, Apiaceae, Aizoaceae, Tamaricaceae, Solanaceae, Boraginaceae ve Oleaceae gibi familyalar birer cinse sahip olduğu belirlenmiştir.

Apidae familyası diğer dört familyaya kıyasla en yüksek tür bolluğuna sahip olup bitki familyalarının tercihi farklı arı cinsleri arasında değişiklik gösterdiği belirlenmiştir. Arılar tarafından en çok ziyaret edilen bitki familyaları Aizoaceae, Lamiaceae, Asteraceae, Brassicaceae ve Tamaricaceae olarak tespğit edilmiştir. Diğer familyalar dört veya daha az cins tarafından ziyaret edilmiş, Lamiaceae, Asteraceae ve Brassicaceae familyalarının zaten bitkilerin bireysel arılar için en çok tercih edilen polen kaynakları arasında olduğu teyid edilmiştir.

INTRODUCTION

Bees are one of the most diverse and important groups of insects. They are rich in species diversity, sociality and nesting biology (Michener, 2007). Very few bee species produce honey, but the majority provide a valuable pollination service to ecosystems (Osman & Shebl, 2020). There are 20,000 known bee species worldwide (Michener, 2007), but very little information is known for Egypt in terms of diversity, nesting biology and sociality (Shebl et al., 2013). Most of the previous studies were conducted during the last century in several areas of the country (Shebl et al., 2013; Shebl et al., 2021). Several genera have been extensively studied such as *Anthophora* (Priesner, 1957), *Halictus* (Blüthgen, 1933 and 1934), *Andrena* (Moustafa et al., 1979), *Osmia* (Moustafa & El Berry, 1976), *Sphecodes* (El Akkad & Kamel, 2002) and *Nomia* (Shoukry et al., 2004) and there is an urgent need to update the checklist and clarify the status of diversity with the current climatic and environmental changes (Okely et al., 2024; El-Naggar et al., 2022). Two species have recently been recorded from the Al-Hawaria region (western part of Egypt) of genus *Hoplitis* (Shebl et al., 2023).

The development of beekeeping may be hindered by a lack of knowledge about the forage plants that are good for domesticated honeybees and wild bee pollinators, including the names of the plants, when they flower and their potential benefits for bees. To date, wild and honey bee plants have not been well studied throughout Egypt (Abou-Shaara, 2015). The purpose of this research was to list the solitary bees present in the Al-Hawaria region (western part of Egypt). In addition, to identify different types of pollen grains (pollen taxa) present on the body setae of solitary bees. Using these samples, we would be able to compile a list of the forage plants visited by honey bees and other wild bee species in the study area.

MATERIAL AND METHODS

Study Area and Specimen Collection

This survey was conducted in the Al-Hawaria region of Alexandria (30°57’13” N, 29°40’27” E). Several plant species were sampled in the field during the winter, spring, summer, and autumn seasons of 2021 and 2022. The following plants were studied: *Lycium shawi*, *Enarthrocarpus lyratus*, *Diplotaxis harra*, *Papaver rhoeas*, *Malva parviflora*, *Centaurea alexandrina*, *Centaurea glomerata*, *Oxalis pes-caprae* L., *Mesembryanthemum crystallinum*, *Tamarix aphylla*, *Sesamum indicum*, *Trifolium alexandrinum*, and *Ocimum basilicum*.

Solitary bees were collected using a sweep net at intervals over two years, in either monthly or weekly sessions depending on weather conditions. Bees were killed in cyanide jars and placed in tissues before being transferred to the Entomology and Zoology Laboratory at the Faculty of Agriculture, Alexandria University, for pinning, labeling, and storage in wooden boxes. The labels included the collection time, date, location, and collector’s name. Data were recorded for solitary bees of different species.

Identification

Bees were identified using reference collections from the Department of Entomology, Ain Shams University, and the Department of Plant Protection, Suez Canal University. Additionally, some species

were sent to European taxonomists, particularly from the tribes Osmiini and Anthidini. Online databases and the *Atlas of Hymenoptera* were also used to confirm species identities. A stereoscopic microscope was employed to examine the taxonomic features of the bees.

Identification of Pollen Grains from Plants and Bees

Pollen analysis from wild bees was conducted in the Applied Entomology and Zoology Laboratory at the Faculty of Agriculture, Alexandria University, using the methods outlined by Westrich & Schmidt (1986) and Westrich (1990).

Reference Slide Preparation

Accurate identification of pollen collected by wild bees often requires a reference collection. Therefore, reference slides of pollen grains from available flowering plant species (crops and wildflowers) within the study area were prepared. All potential pollen sources were collected and identified, noting their frequency, distribution, flower density, and flowering period. If possible, a flower or single stamen was kept in water indoors for a few days to ensure a supply of ripe pollen. The pollens were shaken onto a microscope slide, or an anther was removed with forceps and placed on the slide. A drop of ether was added to disperse the pollen, and any visible particles larger than the pollen were removed. Pollens were also obtained from pressed field specimens.

Degreasing (Fat Removal)

Drops of ether were carefully applied to the pollen using a rod or glass tube, dissolving fats or oils, which were then absorbed with tissue paper.

Staining and Mounting

A drop of warmed, stained jelly (or two small drops of different densities of stain) was applied to the pollen with a glass rod. A cover slip was positioned carefully, with one edge lowered first to avoid air bubbles. The slide was placed on a warm plate for about 10 minutes. The jelly should be just sufficient to fill the space under the cover slip. Once set, any surplus was cleaned off with cold water. The cover slip edges were sealed with clear nail varnish to preserve the sample for years (Sawyer, 1981).

Preparation of Pollen from Scopa Setae

Pollen was removed under a stereoscopic microscope using an appropriately sized insect pin. All equipment (needles, forceps, microslides) was kept clean. To minimize pollen loss, the cleaned microslide and cover slip were placed on a piece of paper (~12 x 12 cm). If pollen grains fell off the slide, they were returned using an insect pin. It was crucial to avoid contamination between slide preparations. Degreasing was only necessary for oily pollens (e.g., from Asteraceae and Fabaceae). Each pollen sample was placed on a slide and degreased with ether. A drop of stained glycerin jelly, melted in a water bath, was applied to the cover slip, which was then placed over the pollen layer. The preparation was sealed with diluted Canada balsam (mixed with xylene) and examined under a stereomicroscope (SM) using a micrometer eyepiece and slide at 400x or 1000x magnification. Pollen identification was based on literature (Esmaeil, 2016) and the reference collection of local flora.

Preparation of Pollen Provision from Brood Cells contents (nests).

Pollen provision analysis offers a more complete description of solitary bee diets than direct observation. A total of two pollen mass samples were collected from nests of two genera (*Xylocopa* and *Osmia*). The pollen masses were stored in open vials to avoid fungal development and refrigerated until processed. Each mass was dissolved in 100 ml of distilled water at 80–90°C, stirred for 15–20 minutes with a glass rod and then a magnetic stirrer. Five milliliters of the solution were centrifuged, and the residue analyzed (Tellería, 2000**).** Microscopic samples were mounted with glycerin jelly as previously described.

Pollen Grain Identification

Pollen grain identification was done by comparing samples to a reference pollen collection from local flora and consulting appropriate literature. Identification was generally made to the plant genus level, and sometimes to the species level.

RESULTS

A total of 820 solitary bees were collected during the flowering season of 13 plant species in the Al Hawaria region during the two years 2021 and 2022. The bees were represented by five families (Apidae, Megachilidae, Halictidae, Colletidae and Andrenidae). The family Melittidae was absent in this study and in the two previous studies. The collected bees represented 24 genera (9 of Apidae, 7 of

Megachilidae, 5 of Halictidae, 2 of Colletidae and one of Andrenidae). The total number of species found in the region was 51 (25 of Apidae, 16 of Megachilidae, 5 of Andrenidae, 4 of Halictidae and one of Colletidae) (Tables 1-3).

Family: Apidae: The area was inhabited by various bee species of *Anthophora*, *Amegilla* and *Eucera*. The appearance of these three genera was coincided with the appearance of their cleptoparasitic bees (*Thyreus* sp, *Nomada* spp, *Melecta* sp and *Epeoles* sp). 25 species of Apidae were visited several plants of the families Asteraceae, Solanaceae, Brassicaceae, Aizoaceae, Labiatae, Tamaricaceae, Malvaceae, Oxalidaceae, Pedaliaceae and Fabaceae (Table 1).

Family: Megachilidae: Sixteen species of Megachilidae were recorded from several flora of the families Asteraceae, Brassicaceae, Aizoaceae, Labiatae, Tamaricaceae, Malvaceae, Papaveraceae and Fabaceae. Some species of the genera *Megachile*, *Osmia* and *Hoplitis* were identified to species level, but some others could not be determined **(Table 2).**

Family: Andrenidae: Andrenidae, represented by five species of the genus *Andrena*, were active from February to June, were recorded from the flora of several families, Asteraceae, Aizoaceae, Fabaceae and Brassicaceae (Table 3).

Family: Halictidae: Several genera of *Halictus*, *Lasioglossum*, *Pseudapis*, *Ceylalictus* and a genus of celptoparasitic bee of *Sphecodes* were collected. Four species were identified: *Ceylalictus variegatus* Olivier, 1789, *Pseudapis nilotica* Smith, 1857, *Lasioglossum vagans* Smith, 1857 and *Halictus quadricinctus* Fabricius, 1776. All species were visited a diverse group of flora from Asteraceae, Solanaceae, Brassicaceae, Aizoaceae, Labiatae, Tamaricaceae, Pedaliaceae and Fabaceae.

Family: Colletidae: Colletidae, was represented by two genera (*Colletes* and *Hylaeus*), with *Colletes lacunatus* Dours, 1872) as the dominant species. The specimens were collected from the flora of the Brassicaceae and Aizoaceae families. Colletidae was the least represented family in terms of species richness and abundance compared to the other families.

Palynological analysis: A reference slide of pollen grains from 32 flowering plant species (cultivated and wild), belonging to 19 plant families, was made in this work at the laboratory of the Department of Applied Entomology and Zoology, Faculty of Agriculture, Alexandria University (Table, 4). Microphotographs and measurements of the studied pollen grains were included in this study.

The presence of pollen taxa from different botanical families was found and identified by comparison with the reference slides of the collected plants. The results of microscopic analysis of body hairs of solitary bees (body, abdominal and tibial setae) are summarised in (Table, 5, Figs. 1 and 2). The data showed that 17 genera (field and forage crops, weeds, ornamentals and vegetables) belonging to 10 plant families were recorded as sources of bee pollen forage plants. The most represented families were Asteraceae (4 genera), Brassicaceae (3 genera) and Fabaceae (3 genera), they contributed with **58.8%** of the total bee flora, while the families of Cucurbitaceae, Apiaceae, Aizoaceae, Tamaricaceae, Solanaceae, Boraginaceae and Oleaceae had one genus each.

Table 1. List of activity period, and floral resources of the collected species of the family: Apidae.

Species	Activity period	Floral resources
Anthophora aegyptiaca Dalla Torre & Friese.	January	*Lycium shawi*
Anthophora albosignata (Friese,1886).	March	*Enarthrocarpus lyratus*
Anthophora angolensis Dalla Torre,1896.	April	*Centaurea glomerata,*
Anthophora (*Heliophila*) *concinna* (Klug, 1845)	April – May	*Centaurea glomerata, Enarthrocarpus lyratus, Mesembryanthemum crystallinum*
Anthophora dispar Lepeletier, 1841.	January-February	*Centaurea glomerata, Lycium shawi.*
Anthophora fascialoides Brooks, 1988.	January-February	*Lycium shawi, Enarthrocarpus lyratus*
Anthophora hispanica (Fabricius, 1787)	February	*Lycium shawi.*
Anthophora moricei Friese,1899.	January-July	*Centaurea glomerata, Centaurea alexandrina, Lycium shawi, Enarthrocarpus lyratus, Mesembryanthemum crystallinum, Ocimum basilicum*
Anthophora senescens Lepeletier,1841.	January- June	*Lycium shawi, Enarthrocarpus lyratus, Ocimum basilicum*
Anthophora (*Heliophila*) *tenella* Klug,1845.	April -June	*Mesembryanthemum crystallinum, Ocimum basilicum*
Anthophora wegelini Friese,1914.	March -June	*Enarthrocarpus lyratus, Ocimum basilicum*
Amegilla albigena (Lepeletier,1841).	April – December	*Centaurea glomerata, Mesembryanthemum crystallinum, Ocimum basilicum*
Amegilla andresi (Friese, 1914).	June- December	*Lycium shawi, Mesembryanthemum crystallinum, Ocimum basilicum, Trifolium alexandrinum*
Amegilla byssina (Klug, 1845).	June-December	*Lycium shawi, Mesembryanthemum crystallinum, Sesamum indicum*
Amegilla quadrifaciata (de Villers,1789)	February-October	*Enarthrocarpus lyratus, Mesembryanthemum crystallinum, Ocimum basilicum, Trifolium alexandrinum*
Eucera biskrensis (Alfken,1933)	February	*Centaurea glomerata, Enarthrocarpus lyratus*
Eucera cuniculina Klug,1845.	January-April	*Centaurea glomerata, Centaurea alexandrina, Enarthrocarpus lyratus, Mesembryanthemum crystallinum, Malva parviflora*
Eucera dimidiate Brulle,1832	January-February	*Lycium shawi, Enarthrocarpus lyratus*
Eucera eucnemidae Dours, 1873.	February	*Enarthrocarpus lyratus, Malva parviflora*
Eucera thoracica Smith,1854.	January	*Oxalis pes- caprael*
Xylocopa aestuans (L. 1758)	April- July	*Centaurea glomerata, Enarthrocarpus lyratus, Mesembryanthemum crystallinum, Ocimum basilicum, Tamarix aphylla, sesamum indicum, Trifolium alexandrinum*
Ceratina tarsata Morawitz,1870.	May- June	*Ocimum basilicum*
Thyreus hyalintus (Vachal,1903).	April- May	*Centaurea glomerata, Mesembryanthemum crystallinum, Ocimum basilicum*
Nomada mauritanica Lepeletier, 1841.	February- March	*Enarthrocarpus lyratus*
Nomada rhenana Morawitz, 1872.	February	*Enarthrocarpus lyratus*
Epeoles sp.	April	*Centaurea glomerata*
Melecta sp.	February	*Enarthrocarpus lyratus*

Table 2. List of activity period, and floral resources of the collected species of the family: Megachilidae.

Species	Activity period	Floral resources
Megachile flavipes Spinola,1838.	April – June	*Mesembryanthemum crystallinum., Trifolium alexandrina., Ocimum basilicum.*
Megachile minutissima Radoszkowski,1876	April - August	*Mesembryanthemum crystallinum., Ocimum basilicum., Papaver rhoeas., Tamarix aphylla*
Megachile nigripes Spinola,1838.	April -June	*Mesembryanthemum crystallinum., Ocimum basilicum., Trifolium alexandrinum*
Megachile patellimana Spinola,1838.	May -October	*Mesembryanthemum crystallinum., Ocimum basilicum., Tamarix aphylla.*
Megachile submucida Alfken 1926.	June - October	*Tamarix aphylla., Trifolium alexandrinum*
Osmia (*Hoplosmia*) *bidentata* Morawitz, 1876	May	*Ocimum basilicum.,*
Osmia ferruginea Latreille,1811	February- April	*Centaurea glomerata., Centaurea alexandrina., Enarthrocarpus lyratus.*
Osmia submicans Morawitz,1870	February - May	*Enarthrocarpus lyratus., Ocimum basilicum., Malva parviflora.*
Osmia (*Helicosmia*) *latreillei* Spinola,1806	April	*Enarthrocarpus lyratus., Mesembryanthemum crystallinum.,*
Hoplitis (*Hoplitis*) *zonalis* (Perez,1895).	April	*Enarthrocarpus lyratus.*
Hoplitis (*Pentadentosmia*) *moricei* (Friese,1899)	June	*Ocimum basilicum.*
Chalicodoma siculum (Rossi, 1792)	March	*Enarthrocarpus lyratus.*
Pseudoanthidium stigmaticorne Dours,1873.	June	*Centaurea alexandrina., Mesembryanthemum crystallinum.*
Stelis murina Perez,1883	April	*Centaurea glomerata.*
Coelioxys coturnix Perez,1884.	May- July	*Ocimum basilicum., Tamarix aphylla.*
Coelioxys decipiens Spinola,1838.	May	*Ocimum basilicum.*

Table 3. List of activity period, and floral resources of the collected species of the family: Andrenidae

Species	Activity period	Floral resources
Andrena fuscosa Erichson, 1835.	February- April	*Centaurea glomerata.* *Diplotaxis harra.* *Enarthrocarpus lyratus*
Andrena flavipes Panzer 1799	April- June	*Centaurea glomerat* *Centaurea alexandrina* *Mesembryanthemum crystallinum* *Trifolium alexandrinum*
Andrena ovatula (Kirby, 1802).	June	*Trifolium alexandrinum.*
Andrena mariana Warncke,1968	March- June	*Enarthrocarpus lyratus* *Trifolium alexandrinum*
Andrena vetula Lepeletier, 1841	February – March	*Enarthrocarpus lyratus*

Table 4. List of various flowering plants (crops & weeds) within study area in Alexandria at Al-Hawaria region showing its habitats, vegetation type, flowering period and pollen size.

Family	Scientific name	Plant habitats & Vegetation Type	Flowering Period	* Pollen size
Asteraceae	*Centaurea alexandrina*	Perennial herb	April- June	M
	Centaurea glomerata	Annual herb	February -April	M
	Chrysanthemum spp.	Perennial herb	March - September	M
	Sonchus oleraceus	Annual herb	May - September	M
	Senecio vulgaris	Annual herb	June - October	M
	Artemisia Judaica	Annual / biennial herb	June - September	M
Brassicaceae	*Enarthrocarpus lyratus*	Annual herb	January - April	S
	Eruca vesicaria ssp. sativa	Annual herb	May -August	S
	Diplotaxis harra	Annual herb	January- April	S
	Brassica nigra	Annual herb	June – August	S
	Sisymbrium irio	Annual herb	December - April	S
Fabaceae	*Vicia faba*	Annual herb	January - March	M
	Melilotus officinalis	Annual / Biennial herb	June – September	M
	Trifolium alexandrinum	Annual herb	April- May	S
Aizoaceae	*Mesembryanthemum crestallinum*	Perennial herb	April -July	S
	Mesembryanthemum nodiflorum	Perennial herb	April -July	S
Polygonaceae	*Rumex vesicarius*	Annual herb	March - May	S
	Rumex spinosus	Annual herb	March - June	S
Malvaceae	*Malva parviflora*	Annual herb	February - March	L
Apiaceae	*Ammi majus*	Annual / Biennial herb	June - December	S
Papaveraceae	*Papaver rhoeas*	Annual herb	February- April	M
Pedaliaceae	*Sesamum indicum*	Annual herb	July- August	M
Cucurbitaceae	*Cucurbita pepo*	Annual creeping	April- May	VL
Rosaceae	*Malus domestica*	Perennial tree	March- April	M
Boraginaceae	*Echium horridum*	Annual herb	March- May	S
Commelinaceae	*Commelina erecta*	Perennial herb	April - July	M
Convolvulaceae	*Convolvulus arvensis*	Perennial herb	June - September	L
Labiatae	*Ocimum basilicum*	Annual / Perennial herb	All year	L
Solanaceae	*Lycium shawii*	Shrub	December-March	S
Oxalidaceae	*Oxalis pes-caprae*	Perennial herb	January- April	M
Chenopodiaceae	*Chenopodium murale*	Annual herb	March- May	S
Tamaricaceae	*Tamarix aphylla*	Perennial tree	July- October	S

* Pollen size: S: Small (10-25 µm); M: Medium (26-50 µm); L: Large (51-100 µm); VL: Very Large (>100 µm) (Vossler, 2015)

Table 5. Various floral source of pollen grains found on abdominal and tibial scopal hairs of some collected solitary bee samples

Bee Family	Bee species	* Samples Type	Pollen Type (Floral Host)	Plant family
Apidae	*Xylocopa aestuans*	*TS*	*Cucurbita pepo*	Cucurbitaceae
	Eucera caniculina pumila	*TS-BS*	*Centaurea* sp.	Asteraceae
	Anthophora hispanica	*TS-BS*	*Vicia faba*	Fabaceae
			Sisymbrium irio	Brassicaceae
	Anthophora angolensis.	*TS-BS*	*Echium horridum*	Boraginaceae
			Ammi majus	Apiaceae
Megachilidae	*Megachile* sp.	*AS*	*Mesembryanthemum crestallinum*	Aizoaceae
			Chrysanthemum sp.	Asteraceae
			Melilotus officinalis	Fabaceae
			Tamarix aphylla	Tamaricaceae
	Hoplitis sp.	*AS*	*Lycium shawii*	Solanaceae
			Olea europaea	Oleaceae
			Centaurea sp. *Sonchus* sp.	Asteraceae
Andrenidae	*Andrena fuscosa*	*TS-BS*	*Enarthrocarpus lyratus*	Brassicaceae
	Andrena ovatula	*TS*	*Trifolium alexandrinum*	Fabaceae
			Diplotaxis harra	Brassicaceae
Colletidae	*Colletes lacunatus*	*TS*	*Enarthrocarpus lyratus*	Brassicaceae
			Artemisia Judaica	Asteraceae

Samples type: TS: tabial scopal setae; AS: Abdominal scopal setae; BS: body setae

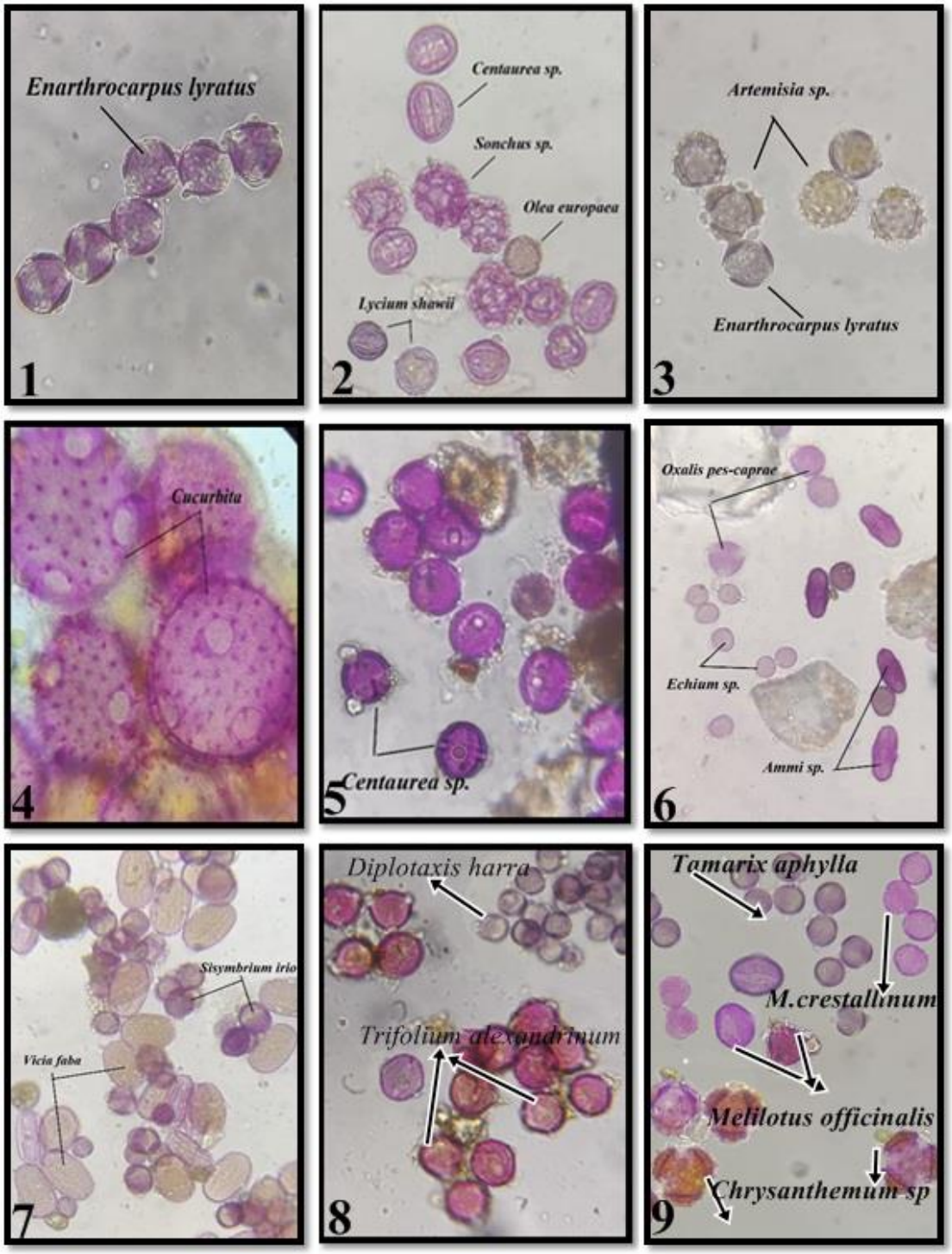

Figure 1. Stereo microscope photographs of some pollen grains found on specialized hairs located in the hind legs or ventral metasoma of solitary bee samples.1: *Andrena fuscosa*, **2**: *Hoplitis* sp., **3**: *Colletes lacunatus*, **4**: *Xylocopa aestuans*,**5**: *Eucera caniculia pumila*, **6**: *Anthophora angolensis*., **7**: *Anthophora hispanica*, **8**: *Andrena ovatula*, **9**: *Megachile* sp.

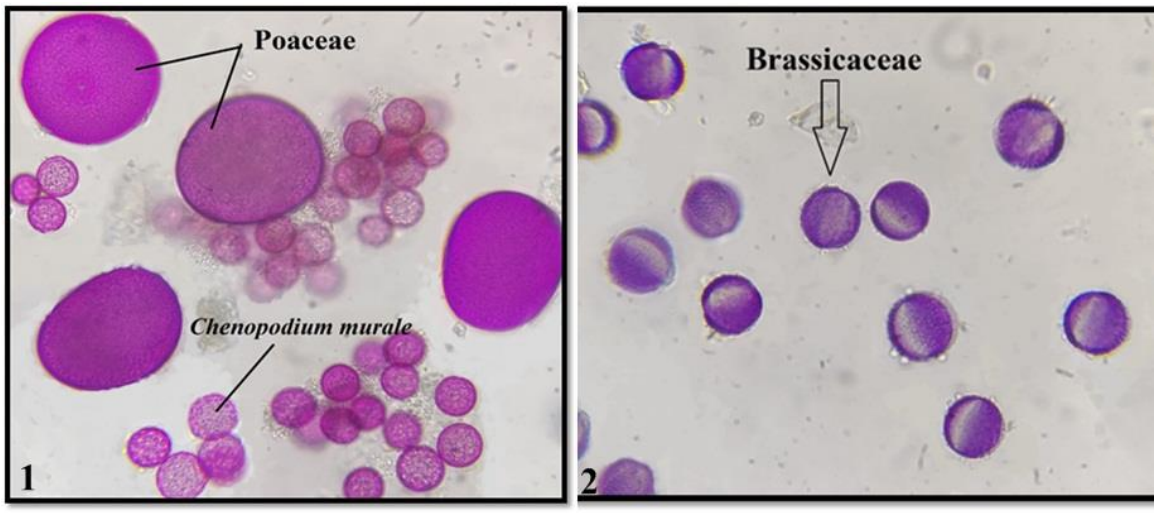

Figure 2. Pollen provision types present in nests of solitary bees 1: *Xylocopa aestuans* (L. 1758) nest and 2: *Osmia* sp. nest

DISCUSSION

Several field expeditions are essential to investigate species composition across Egypt. Some areas have been ignored or poorly addressed in previous works for several reasons (Norfolk and Dathe, 2019). Some areas of North Africa have recently been extensively studied in Lebanon (Boustani, et al., 2021) and Morocco (Lhomme, et al., 2020). In this context, more efforts are need to be made in Egypt and some other countries in Northern Africa to prioritise pollinator research if we hope to quantify and address the ongoing pollinator decline in the region (Shebl, et al., 2021). The current research was conducted to fill the gap in the western part of Egypt, but on a small scale in a small locality with low beekeeping activities. All the collected species were found in other regions such as the canal region in the eastern part of Egypt with some differences in the collected species due to the climatic, vegetation and topographic variations of the area (Shebl et al., 2013; El Aaser, 2013 & Shebl et al.,2015; Shebl et al.,2016). All previous genera were mentioned in (Shebl et al., 2013; Salem & El-Azab, 2017) except *Hoplitis* and *Pseudoanthidium* were not recorded. Two species of the genus *Hoplitis* were recorded for the first time recently: *H. zonalis* (Perez, 1895) appeared in April 2021 on *Enarthrocarpus lyratus* (Brassicaceae) and *H. moricei* (Friese, 1899) was recorded in June 2021 on *Osimum basilicum* (Labiatae) as mentioned in (Shebl et al., 2023). The area hosted a huge diversity of long tongued bees with 16 species of Megachilidae and 25 species of Apidae in addition to some other undetermined species. In contrast, short tongued bees were less diverse with no records of Melittidae. Some species were present in high numbers, such as *Andrena flavipes* Panzer 1799 and *Andrena ovatula* (Kirby, 1802). This high diversity of species composition suggests that there is still much to be done on the bee fauna across Egypt in other areas such as the southern plateau of the country, which occurs in two different biogeographic zones.

Bee communities are influenced by the plant community, plant diversity, canopy cover, land use and nesting suitability (Grundel et al., 2010). Therefore, a complete list of the area's flora was compiled with available pollen resources and palynological analysis of pollen grains in bee scopal hairs and a few nests. The most attractive plant families for bees were Asteraceae, Brassicaceae and Fabaceae, as found in other studies in the Mediterranean area (Zoratti et al. 1995). The most abundant families were Asteraceae, Rosaceae, Labiatae, Fabaceae, Brassicaceae and Poaceae. Plants of Asteraceae taxa were the predominant forage flora for bees, followed by Poaceae, Labiatae and Fabaceae and 11 minor sources (Garg 1996). In the case of oligolectic bees, which were recorded in several specimens in this study, Asteraceae and Fabaceae, followed by Brassicaceae and Lamiaceae, were the most important hosts (Zurbuchen and Müller 2012). The most represented

botanical families visited by honeybee workers in Egypt were Fabaceae, Asteraceae, Brassicaceae, Malvaceae, Cucurbitaceae and Convolvulaceae (Esmaeil 2016; Abou-Shaara, 2015). Similar findings were also observed in European bees, where 73% of foraging visits by solitary bees were to Asteraceae (Centaurea sp.) and Apiaceae, which were occurred naturally on farmland (Wood et al. 2015). Most monolectic and oligolectic species were also attracted to the plant family Asteraceae, followed by Fabaceae, Brassicaceae and Campanulaceae (Bogusch et al. 2020.). Many Asteraceae taxa were highly attractive hosts (66.7% of visits) for oligolectic wild bees, followed by Brassicaceae, Lamiaceae, Fabaceae and *Echium vulgare* (Boraginaceae) (Kuppler et al. 2022).

The pollen spectrum present in the nests of two solitary bee genera (*Xylocopa aestuans* (L. 1758) and *Osmia* sp.) was determined by studying the pollen load residues found on the larval cells of the nests. From the data obtained, it was found that the pollen spectrum in the studied nest of *X*. *aestuans* L. was composed of a total of two pollen types, of which *Chenopodium murale* (Chenopodiaceae) showed the highest frequency of occurrence, followed by pollen grains of *Phragmites* sp. (Poaceae). On the other hand, the second nest was composed of one type of pollen grain (Brassicaceae), present in the nest of *Osmia* sp. The same results were found that two species of *Xylocopa* (*X. latipes* Drury and *X. pubescens* Spinol) were collecting nectar and pollen from several plant species belonging to different families, which were considered as polylectic bees (Raju and Rao, 2006). *Osmia submicans* Morawitz, 1870, which was also collected from the area, is known to be a polylectic bee (Amiet et al., 2004). In this context, the use of field strips and the maintenance of wild flowers around cultivated crops could increase bee species richness and abundance (Mohamed et al., 2024; Owayss, et al., 2020). In this perspective, the conservation of pollinators is mandatory and through this approach their ecological services in the ecosystem could be enhanced and secured for better pollination services.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

PHYSICOCHEMICAL, BIOCHEMICAL AND SENSORY CHARACTERIZATION OF BEE BREAD FROM BURSA (TÜRKİYE) REGION

Türkiye'de Bursa bölgesinde Arı Ekmeğinin Fizikokimyasal, Biyokimyasal ve Duyusal Karakterizasyonu

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ABSTRACT

Bee bread, also known as perga, is a product created through anaerobic lactic fermentation, meticulously crafted by bees. Worker bees mix collected pollen with nectar and their specialized enzymes, then pack and store this nutrient-dense substance in honeycomb cells. Bee bread is highly esteemed as a valuable food source due to its rich protein content, antioxidants, phenolic compounds, vitamins, and minerals. Its health benefits have been increasingly recognized in recent years. This study aims to investigate the physical and chemical properties, as well as the aroma constituents, of bee bread samples sourced from Bursa and its surrounding areas. The analysis includes measurements of moisture content (17.89%), ash (2.53%), crude fat (9.16%), and crude protein (19.06%). Additionally, total phenolic content was determined 9.91 mg gallic acid equivalent per gram (mg GA/g), total flavonoid content at 0.32 mg quercetin equivalent per gram (mg QE/g), CUPRAC activity at 12.97 mg Trolox equivalent per gram (mg Trolox/g), and TEAC activity at 0.55 mM Trolox per milliliter (mg Trolox/mL). Aromatic compounds were identified and their percentage ratios determined using Solid Phase Microextraction (SPME) combined with Gas Chromatography-Mass Spectrometry (GC-MS). These findings align with previous research in the field, although significant variations among parameters are noted due to factors such as geographic location, climate, vegetation, collection time, and sample collection methodology.

Keywords: **Bee Bread, Physicochemical Characterization, Bioactivity, Volatile Compounds, Sensory Properties**

ÖZ

Arılar tarafından toplanan polen, çeşitli enzimler ve bal ile karıştırılarak petek gözlerinde depolanır ve burada fermantasyona uğrar. Bu süreç sonunda anaerobik laktik fermantasyon ürünü olan arı ekmeği oluşur. Sonuçta arı ekmeği; işçi arıların topladıkları poleni, salgıladıkları nektar ve özel enzimlerle karıştırıp, petek hücrelerinde paketleyip depoladıkları değerli bir besin maddesidir. Arı ekmeği, yüksek

protein içeriği, antioksidanlar, fenolik bileşikler, vitamin ve mineraller ile sağlığa birçok faydalı etkisi olan ve son yıllarda değeri keşfedilmeye başlanan önemli bir arı ürünüdür. Bu çalışmanın amacı, Bursa ve çevresinden temin edilen arı ekmeği örneğinin fiziksel, kimyasal özellikleri ve aroma maddelerinin incelenmesidir. Bu çalışmada kül, nem, yağ, protein, antioksidan aktivite, toplam fenolik madde, toplam flavonoid madde ve aroma analizleri gerçekleştirilmiştir. Aroma bileşenlerinin tanımlanması ve yüzde oranlarının belirlenmesi, SPME tekniği kullanılarak GC-MS cihazında yapılmıştır. Arı ekmeğinin nem içeriği %17.89, kül içeriği %2.53, ham yağ içeriği %9.16, ham protein içeriği ise %19.06 olarak tespit edilmiştir. Toplam fenolik madde miktarı 9.91 mg GAE/g, toplam flavonoid madde miktarı 0.32 mg QE/g, CUPRAC aktivitesi 12.97 mg Trolox/g ve TEAC aktivitesi 0.55 mM Trolox/mL olarak belirlenmiştir. Sonuçlar literatürdeki diğer çalışmaları desteklemektedir. Parametreler arasındaki anlamlı farklılığın, arı ekmeğinin elde edildiği yer, iklim, bitki örtüsü, toplama zamanı ve toplama yöntemi gibi faktörlerden kaynaklandığı düşünülmektedir.

Anahtar Kelimeler: **Arı Ekmeği, Fizikokimyasal Karakterizasyon, Biyoaktivite, Uçucu Bileşikler, Tanımlayıcı Duyusal Analiz**

GENİŞLETİLMİŞ ÖZET

Amaç: Arı ekmeği, polenin arı vücut salgıları ile karıştırılıp petek gözlerinde depolanması ve burada fermantasyona uğraması sonucu oluşan doğal bir üründür. Arı ekmeği polenin fermente halidir ve karbohidrat, protein, yağ, vitamin ve mineral içeriği bakımından zengin bir üründür. Çalışmanın amacı, Bursa yöresinden elde edilen arı ekmeği örneğinin biyokimyasal karakterizasyonunun, antioksidan özelliklerinin, uçucu bileşenlerinin, tanımlayıcı duyusal özelliklerinin belirlenmesidir.

Gereç ve Yöntem: Çalışmada kullanılan arı ekmeği örneği 2021 yılında Bursa ilinden temin edilmiş olup analize kadar -18°C' de depolanmıştır. AOAC metodu kullanılarak, arı ekmeğinin nem içeriği, konveksiyonlu bir fırın içerisinde 105°C'de kurutularak sabit ağırlıkta gravimetrik olarak belirlenmiştir (AOAC, 2005). Ham protein değeri, Kjeldahl metodu kullanılarak tayin edilmiştir. Azot yüzdesini ham protein yüzdesine dönüştürmek için 6.25'lik bir dönüşüm faktörü kullanılmıştır. Ham yağ, AOAC yöntemine (AOAC, 1984) göre bir soxhlet cihazı ve *n*-hekzan kullanılarak ekstrakte edilmiştir. Numunelerin kül içeriği gravimetrik olarak belirlenmiştir (AOAC, 2005). Numunenin toplam fenolik içeriği Singleton ve Rossi (1965) tarafından tanımlanan Folin-Ciocalteau yöntemine göre belirlenmiştir. Toplam fenolik içerik, gallik asit ile hazırlanan standart eğrinin denkleminden hesaplanmıştır. Numunedeki toplam fenolik bileşik miktarı “mg GAE/ g numune” olarak ifade edilir. Numunenin toplam flavonoid içeriği Chang ve arkadaşları (2002) tarafından geliştirilen yöntemle belirlenmiştir. Standart olarak Kuersetin kullanılmıştır. Toplam flavonoid içeriği standart eğri denklemi yardımıyla hesaplanmıştır. Sonuçlar “mg QE (kuersetin eşdeğeri)/g” cinsinden ifade edilir. ABTS. analizi Re ve arkadaşlarının (1999) geliştirdiği yöntemle belirlenmiştir. Sonuçlar “mg TEAC troloks eşdeğer antioksidan kapasitesi)/g” cinsinden ifade edilir. CUPRIC-ION Arı ekmeğinin Antioksidan Kapasitesini Düşürücü etkisi Apak ve arkadaşları (2004) tarafından geliştirilen yöntemle bulunmuştur. Neocuproin (Nc) ve Cu(II) tarafından oluşturulan Cu(II)-Nc kompleks materyalinin spektrofotometrede 450 nm'de absorbe olan Cu(I)-Nc şelatına indirgenmesine dayanmaktadır (Thermo Scientific, Multiskan™ GO Microplate Spectrophotometer, USA). Uçucu Bileşik Analizleri Uçucu bileşiklerin tanımlanması ve miktarının belirlenmesi için Katı faz mikro ekstraksiyon tekniği (SPME) Gaz Kromatografisi-Kütle Spektrometresi (GC-MS) (GC 6890, MS 6890N, Agilent Technologies, Wilmington, DE, ABD) kullanılmıştır. Arı ekmeği örneğinin tanımlayıcı duyusal analizi Spectrum™ yöntemi kullanarak yapılmıştır. Yaşları 25-55 arasında değişen 6 eğitimli panelist (2 erkek ve 4 kadın) tarafından yürütülmüştür (Meilgaard ve diğerleri, 1999). Elde edilen sonuçlar örümcek ağı diyagramı ile gösterilmiştir.

Bulgular: Bursa Bölgesinden temin edilen arı ekmeğinin nem içeriği %17.89, kül içeriği %2.53, ham yağ oranı %9.16 ve ham protein oranı %19.06 olarak tespit edilmiştir. Toplam fenolik madde miktarı 9.91 mg GA/g, toplam flavonoid madde miktarı 0.32 mg QE/g, CUPRAC aktivitesi 12.97 mg Trolox/g ve TEAC aktivitesi 0.55 mM Trolox/ml olarak belirlenmiştir. GC MS analizi sonucunda numunede aldehitler, ketonlar, yağ asitleri, hidrokarbonlar ve karboksilik asitlerin yaygın olduğu tespit edilmiştir. Tanımlayıcı duyusal analiz sonucunda, arı ekmeği

için sekiz parametre belirlenmiştir. Bunlar; meyveli/kiraz, çam/reçine, ıhlamur çiçeği, kuru kayısı/erik, oksitlenmiş/balık yağı, ransit, odunsu, buruk. Panelistler tarafından arı ekmeği parametrelerine verilen puanlar sırasıyla; 2.83 meyveli/kiraz, 2.83 çam/reçine, 2.33 ıhlamur çiçeği, 2 kuru kayısı/erik, 1.5 oksitlenmiş/balık yağı, 4.25 ransit, 2 odunsu, 3.33 keskin olarak belirlenmiştir.

Sonuç: Bursa Bölgesine ait arı ekmeği örneğinin nem içeriği, kül içeriği, ham yağ içeriği, ham protein içeriği, antioksidan aktivite, toplam fenolik madde, toplam flavonoid madde ve aroma analizleri gerçekleştirilmiştir. Yapılan analizler doğrultusunda elde edilen sonuçlar literatürdeki diğer çalışmaları desteklemektedir. Arı ekmeğinin bileşimi iklim, coğrafi koşullar, bitki örtüsü, botanik orjin, arının türü, toplama zamanı, toplama yöntemi, depolama koşulları ve ekstrakte ediliş şekli gibi birçok faktörden etkilenmekte buna bağlı olarak parametreler arasında anlamlı farklılığa sebep olduğu düşünülmektedir.

INTRODUCTION

Bee products have gained significant traction as functional foods among consumers in recent years, owing to their rich nutritional content and bioactive properties. These products have emerged as compelling candidates for safeguarding and promoting human health. Within the realm of traditional and complementary medicine, apitherapy has garnered attention for its utilization of various bee-derived substances including honey, beeswax, pollen, propolis, apilarnil, royal jelly, bee bread and bee venom (Ekici and Gölgeli, 2021). Bee bread also known as perga, represents a natural nutrient used by bees to nourish offspring. Honey bees collect pollen from plants, mixing it with their digestive enzymes, honey and beeswax before storing it in honeycomb cells, where it undergoes lactic acid fermentation, culminating in the formation of bee bread within approximately two weeks (Gilliam, 1979).

Bee bread is renowned for its comprehensive nutritional profile, comprising essential amino acids, vitamins C, B1, B2, E, H, carotenoids and anthocyanins, saccharase, amylase, phosphatase enzymes and a myriad of minerals (Mutsaers et al, 2005). This nutrient-rich composition renders bee bread a vital protein source rich in essential amino acids, fats minerals, vitamins and flavonoids, serving as the primary sustenance for bees (Karaman et al., 2017). With approximately 20% protein, 3% lipid, 24-35% carbohydrates, 3% vitamins and minerals, bee bread emerges as a functional food owing to its potent ingredients, endowing it with antioxidant, antimicrobial, antiviral, antiarrhythmic, antibiotic, anti-inflammatory and anticancer properties (Khalifa et al.,2020; Ekici and Gölgeli, 2021). Bee bread is similar to bee pollen in terms of its compositional properties, but it has a richer content and contains proteins, amino acids, carbohydrates, lipids, vitamins, minerals, phenolic acids and polyphenols (Aylanc et al. 2021a). Moreover, bee bread, in its fermented state, surpasses pollen in nutritional value and digestibility, making it more readily assimilated by organisms (Habryka et al. 2016).

Bee bread is a natural product like other beekeeping products and has many nutritional, functional and biological properties due to the compounds in its structure. The biochemical characterization and biological activity properties of bee bread (perga) exhibit significant variability, influenced by factors such as geographical region, climate, bee species and plant types (Karataş and Şerbetçi, 2008). This study aims to elucidate the biochemical characterization, biological activity properties and antioxidant capacity of bee bread produced in the Bursa (Türkiye) region. Additionally, we seek to conduct analyses of volatile aroma compounds and descriptive sensory evaluations of bee bread, thereby contributing to a comprehensive understanding of its multi faceted attributes.

MATERIALS AND METHODS

Sample

Bee bread was obtained from honey producer in Bursa region during spring of 2021 and stored at -18°C until further analysis.

Chemicals

All chemicals used in the experiments were of analytical grade. Ethanol, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), sulfuric acid, gallic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman carboxylic acid), quercetin, sodium chloride, 2-methyl valeric acid, 2-methyl-3-heptanone, Folin-ciocalteau solution, sodium carbonate, potassium acetate, aluminum nitrate from Sigma, Kjeldahl tablet, hydrochloric acid, hexane, were obtained from Merck.

Preparation of Extract from Bee Bread

Extraction procedure outlined by Zhou et al. (2015) was followed with some modifications. Briefly 1.5 g of bee bread sample was weighed and coarsely ground using a laboratory mortar. The ground bee bread was transferred a centrifuge tube and 10 ml (95%) ethanol was added. The sample was then subjected to ultrasonic bath treatment for 60 minutes at 40 °C to ensure homogenous disintegration. Subsequently, the tubes were centrifuged at 5000 rpm and 40 °C for 30 minutes. This procedure was repeated twice. The resulting supernatants were collected in a 25 ml beaker. This mixture was completed to 25 ml with 95% ethanol. It was filtered through 45 µm pore-sized micro filters before analysis (Mayda, 2019). The resulting extract was utilized for the determination of total phenolic substance amount, total flavonoid substance amount and ABTS. activity, CUPRAC activity.

Chemical Analysis of Bee Bread Samples

Moisture Content

Using the AOAC method, the moisture content of bee bread was determined by drying it gravimetrically in a convection oven at 105 °C to constant weight (AOAC, 2005). Moisture contents of the samples were measured using the loss on drying technique with the help of a moisture analyzer. (Shimadzu, Japan). Firstly, bee bread sample was ground to achieve homogeneity. Subsequently, 3 g of the sample was weighed and dried in an oven at 105 °C until it reached a constant weigh. After allowing it to cool down, the sample was weighed on a precision balance, and the percentage moisture content was calculated. The results were recorded as g/100g moisture.

Ash Content

The ash content of the bee bread samples was determined following the methods outlined in AOAC (1999). To determine the total ash, a 5 g portion of bee bread sample was weighed into a crucible of known weight and burned in a muffle furnace at 550 °C until a carbon-free white ash was obtained. Subsequently, the crucible was placed in a desiccator to cool and then reweighed. The procedure was repeated twice. The percentage ash content was calculated.

Total Protein Content

The protein content of the bee bread was determined using the Kjeldahl method (AOAC, 1990). A homogenized 1 g sample was weighed into the combustion tube and 1 Kjeldahl tablet (Merck) was added. Then 15 mL of H_2SO_4 (98%) was added, and the combustion process continued gradually until the blue clear color was obtained. Subsequently, the distillation process was applied to the solution in balloons. Upon completion of the, distillation process, the distillate was titrated with 0.1 N HCl (Merck) until a gray-lilac color was formed. The amount of HCl consumed in the titration was substituted into formula to determine the percentage nitrogen. The protein content was then calculated by multiplying this value by factor of 6.25.

Total Crude Fat Content

Fat content was determined using the Velp SER 148 solvent extraction system. Initially, 5 grams of sample was weighed on filter paper and placed in the extraction cartridge. The cartridge containing the sample was then inserted into the extraction system. Hexane was utilized as the solvent for extraction, with the process comprising 90 minutes immersion, 90 minutes washing and 90 minutes recovery processes at 130°C, respectively. Following the extraction procedure, the sample container containing the extracted oil was placed in an oven at 105°C overnight to ensure complete drying. Subsequently the oil content of the sample was calculated by weighing.

Analysis of Total Phenolic Content

The total phenolic content of the sample was determined according to the Folin-Ciocalteau method as recommended by Singleton and Rossi (1965). Gallic acid was used as standard. Total phenolic content was calculated using the equation derived from the standard curve prepared with gallic acid. The total amount of phenolic compounds in the sample is expressed as “mg GAE/ g sample”.

Total Flavonoid Content Analysis

The total flavonoid content of the sample was determined using the method developed by Chang et al. (2002), with quercetin (QE) as the standard. The total flavonoid content was calculated using the standard curve equation and expressed as "mg QE (quercetin equivalent)/g".

ABTS·+ Antioxidant Capacity Determination

The ABTS·+ antioxidant capacity of the bee bread was determined following the method developed by Re et al. (1999). The results were expressed as "mg TEAC (trolox equivalent antioxidant capacity)/g".

CUPRIC-ION Reducing Antioxidant Capacity Determination

The CUPRIC-ION Reducing Antioxidant Capacity of bee bread was assessed using the method developed by Apak et al. (2004). This method is based on the reduction of the Cu(II)-Nc complex to form the Cu(I)-neocuproine chelate, which exhibits absorbance at 450 nm. Spectrophotometric measurements were conducted using a Thermo Scientific Multiskan™ GO Microplate Spectrophotometer (USA).

Determination of Volatile Compounds

Volatile compound analysis was performed using Solid-Phase Microextraction (SPME) combined with Gas Chromatography-Mass Spectrometry (GC-MS) (GC 6890, MS 6890N, Agilent Technologies, Wilmington, DE, USA) for identification and quantification of volatile compounds. A HP-INNOWax column (60 m x 0.250 mm id x 0.25 µm film thickness) was used (J&W Scientific, Folsom, CA, USA). For the analysis, 5 g of bee bread was placed into a 40 mL SPME vial (Supelco, Bellafonte, USA) along with 5 ml of saturated sodium chloride solution and 2.5 µL of internal standard (consisting of 0.1 µL of 2-methyl valeric acid and 0.6 µL of 2-methyl valeric acid in 1 mL of methyl-3-heptanone). The mixture was incubated in a 50°C water bath (GFL, Model 1103, Burgwedel, Germany) for 30 minutes. Subsequently, the SPME fiber (2 cm-50/30 µm DVB/Carboxen/PDMS stable flex, Supelco, Bellafonte, USA) was exposed to the vial in the 50°C water bath for another 30 minutes, then injected into the GC-MS system. The carrier gas flow rate was set at 1.2 mL/min, with a furnace program initiating at 40°C for 5 minutes, followed by a ramp of 10°C/min to 230°C, maintaining the final temperature for 20 minutes. The National Institute of Standards and Technology (NIST, 2008) and Wiley Registry of Mass Spectral Data (Wiley, 2005) libraries were used for identification of volatile components. The amount of volatile components was determined based on their proportional abundance (Avsar et al. 2004).

Descriptive Sensory Assessment

The bee bread sample was stored under refrigerator conditions (4^0 C) until analysis. Before evaluation, the sample was allowed to equilibrate to room temperature for 30 minutes. Descriptive sensory analysis of the products was conducted using the Spectrum™ method; with six trained panelists (2 males and 4 females) aged between 25 and 55 (Meilgaard et al. 1999). The results obtained were presented using a spider web diagram.

RESULTS

Chemical Composition

The results of the nutritional composition, as shown in Table 1, indicated that proteins (19.06 ± 0.45 g/100 g Bee Bread) and fat (9.16 ± 0.06 g/100 g Bee Bread) were the primary macronutrients in bee bread. Minor components included ash (2.53 ± 0.21 g/100 g Bee Bread) and moisture (17.89 ± 0.13 g/100 g Bee Bread).

Table 1. The chemical content of bee bread.

Composition	Amount (g/100g Bee Bread)
Moisture	17.89 ± 0.13
Ash	2.53 ± 0.21
Protein	19.06 ± 0.45
Fat	9.16 ± 0.06

Antioxidant activity

Biochemical activity of bee bread was evaluated as a result of the study. In Table 2 the results of the biochemical activity were shown. Total phenolic content of bee bread was determined as 9.91 ± 0.87 mg GAE/g. The total amount of flavonoid substance was determined as 0.32 ± 0.07 mg QE/g for bee bread. Cu(II) ıon reducing antioxidant capacity was 12.97 ± 1.8 mg Trolox/g for bee bread. ABTS radical scavenging activity of bee bread was calculated as 0.55 ± 0.001 mM Trolox/mL.

Table 2. Antioxidant activity, total phenolic and total flavonoid contents of bee bread.

Total amount of phenolic substance (mg GAE/g)	9.91 ± 0.87
Total amount of flavonoid substance (mg QE/g)	0.32 ± 0.07
ABTS (mM Trolox/mL)	0.55 ± 0.001
CUPRAC (mg Trolox/g)	12.97 ± 1.8

Descriptive Sensory Profile

The results of descriptive sensory evaluation were presented Figure 1. The sensory panel identified eight flavor descriptors for bee bread; fruity/cherry, pine/resin, linden flower, dried apricot/prune, oxidized/fish oil, rancid, woody, acrid. According to the scores provided by the sensory panel for the flavor of bee bread; fruity/cherry with value of 2.83, pine/resin with value of 2.83, linden flower with value of 2.33, dried apricot/prune with value of 2, oxidized/fish oil with value of 1.5, rancid with value of 4.25, woody with value of 2, acrid with value of 3.33.

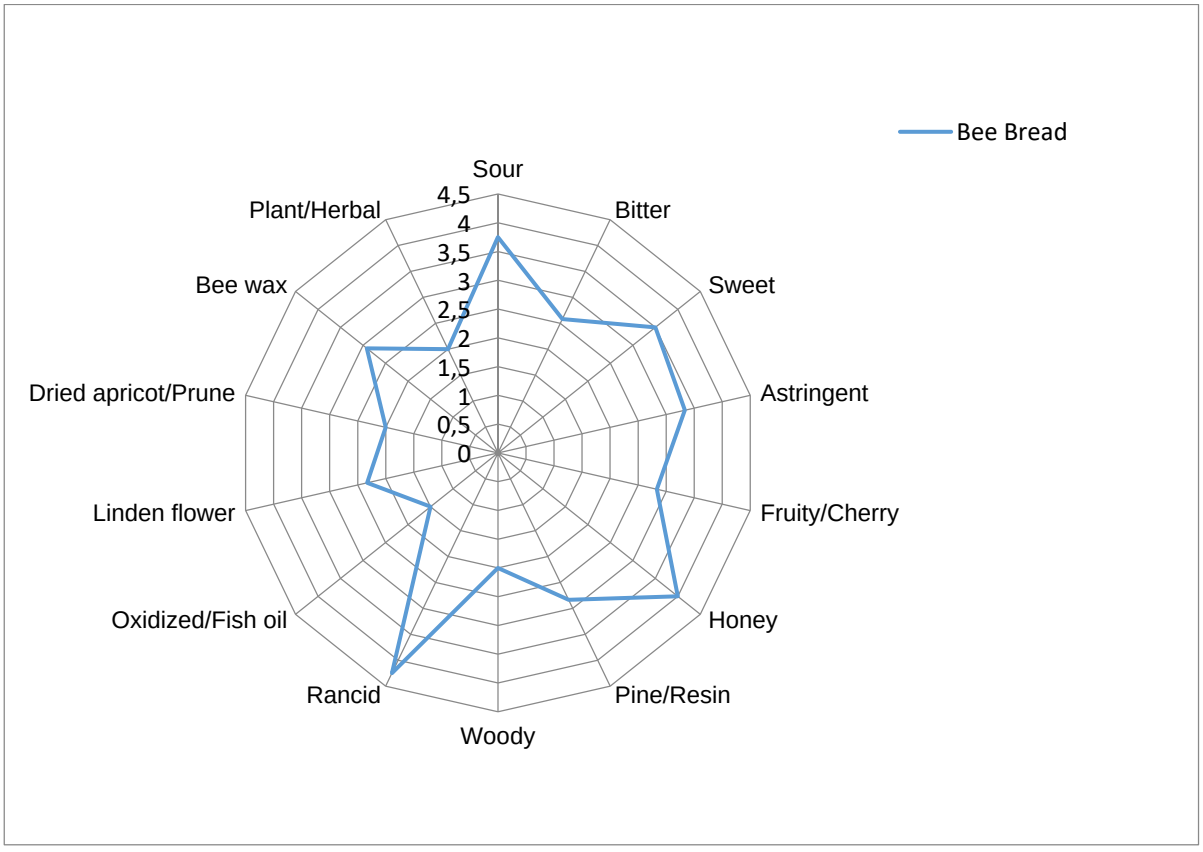

Figure 1. Sensory profile of bee bread.

Volatile Compounds

Volatile compounds determined in bee bread sample were presented in Table 3. As a result of the GC MS analysis, aldehydes, ketones, fatty acids, hydrocarbons and carboxylic acids were common in the sample.

Table 3. Aroma compounds detected in bee bread.

Compound	Odor	**Mean** (µg/100g)
Acetic acid	Sour, pungent, vinegar	16.92±5.73
Acetic acid, hexyl ester	Fruit, herb	0.08±0.11
Benzyl Alcohol	Sweet, floral	0.10±0.01
Borneol	Camphor	0.09±0.03
Butanoic acid	Rancid	7.29±8.24
Decanoic acid, methyl ester	Wine	0.51±0.15
Dodecane	Alkane	0.41±0.12
Dodecanoic acid, ethyl ester	-	0.17±0.04
Dodecanoic acid, methyl ester	-	0.84±0.47
Phenylethyl alcohol	Floral	0.20±0.13
Phenol	Phenolic	0.05±0.04
Furfural	Bready	1.17±0.73
Heptanoic acid	Cheesy	0.35±0.5
Hexanal	Green, fruity, oil	0.04±0.05
Hexanoic acid	Fatty, cheesy, soft	8.02±2.78
Hexanoic acid, ethyl ester	Apple peel, fruit	0.11±0.07
Hexanoic acid, methyl ester	Fruit, fresh, sweet	1.25±0.81
Camphene	Rosemary, volatile oil	0.13±0.05
Camphor	Camphor	0.62±0.48
Limonene	Lemon, citrus	1.47±0.48
Linalool	Floral, sweet, lavender	2.12±1.73
Methyleugenol	Clove, spice	0.34±0.17
Oxime-, methoxy- phenyl-	Honey	0.70±0.33
Nonanal	Waxy, Citrus	1.40±0.55
Nonanoic acid	Waxy, grass, oil	2.96±0.04
Nonanoic acid, ethyl ester	-	0.23±0.04
Octanoic acid	Cheesy	9.77±2.97
Octanoic acid, methyl ester	Orange	1.37±0.65
Octanoic acid, ethyl ester	Fruit, fat	0.50±0.01
Eugenol	Clove, honey	0.40±0.19
Pentanoic acid	Cheesy	0.36±0.05
Tetradecane	Alkane	0.33±0.1
1,8-Cineole	Mint	1.95±2.13
2-Phenylindolizine	-	0.38±0.54
2-Undecanol	Waxy	0.05±0.07
Pyrazine, 2, 6-dimethyl-	Chocolate	0.16±0.23
2-Butanoic acid 3-methyl-	-	3.85±3.03
Pentanoic acid, 3-methyl-	-	3.85±5.45
3-metil-2(5H)-furanone	-	0.07±0.04
2-Cyclohexen-1-one, 3,5,5 trimethyl-	-	0.55±0.47
4-Ketoisophorone	Moldy	0.14±0.09
6-Methyl-5-Hepten-2-One	Green, citrus,	0.78±0.12
ρ-Cymene	Solvent, gasoline, citrus	0.79±0.31
ρ-Cymen-2-ol	Citrus, moldy	0.02±0.03
ρ-Cymen-3-ol	Citrus, moldy	0.05±0.07
Alpha pinene	Pine, turpentine	0.09±0.07
Alpha Terpinolene	Terpenic	0.36±0.52
Beta Myrcene	Spice	0.35±0.41
Beta Thujone	-	0.38±0.36

Odor description source: flavornet.org

DISCUSSION

Current literature suggests that bee pollen and bee bread are excellent sources of PUFAs, which are essential for human nutrition and cannot be synthesized by the body. However, scientific research on bee bread is limited, highlighting the need for further studies (Silici, 2015). This study explores the physical and chemical properties, as well as the aroma constituents, of bee bread sample collected from Bursa (Türkiye) region. The analyses covered moisture, ash, protein, fat, antioxidant activity, total phenolic content, total flavonoid content, and aroma profile. It is well-established that the composition and nutritional value of bee products are influenced by plant species and environmental conditions (Küçük et al., 2024). Studies on bee bread from various regions remain scarce.

Regarding chemical composition, Karlıdağ et al. (2021) found that the protein content of fermented pollen was 18.70%. Mayda et al. (2020) reported that the average protein content of bee bread samples from different regions of Türkiye was 22.2%. Dranca et al. (2020) observed a protein ratio of 18.60% for bee bread sourced from the Iasi region of Romania. A study conducted in Colombia determined that the protein content of bee bread ranged from 19.10% to 27.30 % (Zuluga et al., 2015). In our study, the protein content of bee bread was found to be 19.6%, aligning with previous findings. Bee bread is known for its high protein content and it has been reported that the protein content may vary depending on the botanical origin (Waykar, 2016; Kaplan et al., 2016; Mohammad et al., 2021).

Bakour et al. (2019) reported a lipid content of 1.90 g/100 g in bee bread, while Kaplan et al. (2016) found lipid content ranging from 5.93 g/100 g to 11.55 g/100 g in samples from various regions. Another study reported that the lipid contents of 15 different bee bread samples collected in Colombia ranged between 1.65% and 5.50%. The study suggested that this variability is related to the fatty acid, carotene and vitamin contents of the pollen in the bee bread (Zuluaga et al., 2015). Andjelkovic et al. (2012) reported a range of 4.51% to 4.92% in Serbian bee bread. Our study revealed the fat content of bee bread to be 9.16%. The lipid content of bee bread varies greatly depending on the plant origin of the pollen. Lipids are represented by fatty acids, which contribute to the biological value of this bee product (Urcan et al., 2017).

Previous studies reported moisture content in bee bread as 15.6% (Zuluaga et al., 2015), 11.4% to 15.9% (Kaplan et al., 2016), and 9.85% (Bakour et al., 2017). Mayda et al. (2020) found moisture content in bee bread from various regions in Türkiye to range from 17.7% to 22.3%. In a different study conducted in Türkiye, it was determined that the moisture content of bee bread samples was in the range of 11.54-23.07 g/100g (Kalaycıoğlu, 2022). In our study, the moisture content was 17.89%. Bee bread has a high water content due to the hygroscopic properties of the pollen in its structure. This characteristic, along with the absorption of environmental moisture and the presence of bee secretions and honey, gives bee bread its sticky and moist texture (Mohammad et al., 2021).

The ash content in our study was 2.53%. Literature values for ash content include 2.45% (Zuluaga et al., 2015) and 3.32% (Bakour et al., 2019). A study in Malaysia found the ash content of bee bread produced by stingless bees to be 1.7 g/100 g (Ismail et al., 2018). Hazır and Özer (2019) reported an ash content of 8.14 g/100 g in their bee bread sample from Kayseri. Differences in ash content may be attributed to regional and bee species variations.

Biochemical characterization of bee bread is crucial for understanding its health effects and biological activities. In our study, the total phenolic content was 9.91 mg GAE/g. Karlıdağ et al. (2021) found the phenolic content of fermented bee pollen to be 6.12 mg GAE/g. Bayram et al. (2021) reported an average total phenolic content of 8.26 mg GAE/g in bee bread from various locations. Zuluaga et al. (2015) determined the phenolic content of Colombian bee bread to be 8.9 mg GAE/g. Literature shows significant variation in total phenolic content, influenced by factors such as the collection region, climate, bee breed, and plant species (Bayram et al., 2021).

The total flavonoid content in our study was 0.32 mg QE/g. Karlıdağ et al. (2021) reported 2.73 mg QE/g in fermented pollen sample. Mayda et al. (2020) observed an average content of flavones/flavonols 1.81 mg QE/g in bee bread from different regions in Türkiye. Urcan et al. (2018) noted that flavonoid content in bee bread is typically lower in flavones/flavonols compared to flavanones/dihydroflavonols. Another study reported total flavonoid content of bee bread 13.56 to 18.24 µg QE/g (Ivanišová et al., 2015). Zuluaga et al. (2015)

found flavonoid content to range from 1.9 and 4.5 mg QE/g.

The ABTS radical scavenging activity of bee bread was measured as 0.55 mM Trolox/mL. Mayda (2020) reported ABTS capacity values of bee bread between 0.375 mg TEAC/g and 1.55 ± 0.12 mg TEAC/g. Another study showed ABTS capacity ranging from 4.86 mg TEAC/g to 5.70 mg TEAC/g (Čeksterytė et al., 2016). Zuluaga et al. (2015) reported ABTS capacity between 46.1 µmol TEAC/g and 76.3 µmol TEAC/g, with an average of 61.5 µmol TEAC/g.

The Cu(II) ion reducing antioxidant capacity was 12.97 ± 1.8 mg Trolox/g in our study. Kutlu et al. (2023) reported CUPRAC values ranging from 9.18 ± 0.06 mg Trolox/g to 11.93 ± 0.76 mg Trolox/g. While specific studies on bee bread's CUPRAC antioxidant capacity are limited, Özparlak et al. (2017) reported CUPRAC reducing power activities of pollen extract equivalent to 77.12 mg TE/g. Ulusoy and Kolayli (2014) noted CUPRAC reducing power values between 33.1 µmol TE/g and 91.8 µmol TE/g in their study on Anzer bee pollen. Zuluaga et al. (2015) and Kocot et al. (2018) highlighted that the protein, minerals, and phenolic compounds contribute to the natural antioxidant properties of bee bread.

Information on volatile compound profiles in bee products is limited, with most studies focusing on honey (Starowicz et al., 2021). Kaškonienė et al. (2007) examined volatile compounds in honey and bee bread, noting that while some volatile components were common to both, bee bread had a slightly different profile. Compounds such as dimethyl sulfide, pentannitrile, furfural, benzaldehyde, nonanal, benzylnitrile, and decanal were found in both bee bread and honey, while others like 2-methylbutylnitrile, 3-methyl pentanoic acid, benzeneacetaldehyde, linalool, and octanoic acid were unique to honey.

According to HS-SPME/GC-MS results, Starowicz et al., (2021) identified 20 volatiles in beeswax and honey, 32 in bee bread, and 33 in pollen. Bee bread contained 32 volatile compounds, including 15 alkanes, 4 aldehydes, 5 acids, 2 benzenes, 2 ketones, and other compounds like disulfides, furans, pyrroles, and lactones (Starowicz et al., 2021). In our study, we identified 49 volatile aromatic compounds in bee bread. Kolaylı et al., (2024) reported 119 volatile aromatic compounds in the bee breads from Anatolia by SPME-GC-MS.

Volatile components are key to determining the taste and aroma of bee products. Bee bread's volatile profile differs from that of honey (Mayda, 2019). Common aroma components in bee bread include aldehydes, ketones, acids, alcohols, hydrocarbons, benzene, furan derivatives, and terpenes. Notable compounds in bee bread from monofloral and polyfloral sources include 1-heptadecen and acetic acid (Mărgăoan et al., 2020).

Bee bread is a mixture of honey and bee pollen, and the volatile aromatic compounds in this mixture play an important role in shaping its aroma profile (Kolaylı et al., 2024).

The unique sensory properties of bee products result from the combined and synergistic effects of their volatile compounds. This study identified six odor descriptors for bee bread: honey-like, sweet, acidic, pungent, waxy, and plant-based (Starowicz et al., 2021).

A recent study conducted a sensory analysis of 10 bee bread samples from various Turkish companies with 13 panelists (4 women and 9 men, aged 20-65). The sensory profile included descriptors such as fermented taste, bitterness, saltiness, sourness, caramelized taste, floral aroma, fruity aroma, sour smell, animal feed smell, distinctive smell, floral smell, and fruity smell (Soykan-Çiftçi, 2022). The taste, aroma, and color of bee bread (perga) vary depending on the pollen source plant (National Nutrition Council Bee and Bee Products Scientific Commission Report, 2022).

The composition of bee bread is influenced by factors such as climate, geographical conditions, plant variety, bee species, collection methods, storage conditions, and extraction techniques. The results of this study align with existing literature and suggest that significant differences in parameters are largely due to the location, climate, vegetation, collection time, and storage conditions of bee bread.

Conclusion: Based on the literature review, studies on the composition, phenolic profile and bioactive properties of bee bread produced in Türkiye are limited. Therefore, we believe that this study makes a significant contribution to the existing body of research. In this study, the biochemical characterization, antioxidant properties, volatile components, and descriptive sensory properties of bee bread were examined. The findings indicate that bee bread has a high antioxidant capacity and significant nutritional value from a nutritional

physiology perspective. The results suggest that bee bread could be a valuable source of nutrients and bioactive compounds for food supplements and functional foods. Consequently, it was concluded that bee bread can be consumed as a functional food. However, further studies are needed to develop new functional foods related to bee bread.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

AYÇİÇEĞİ BALI TEMELLİ GÜMÜŞ NANOPARTİKÜLLERİN YEŞİL SENTEZİ, KARAKTERİZASYONU VE BİYOLOJİK AKTİVİTELERİNİN BELİRLENMESİ

Green Synthesis, Characterization and Determination of Biological Activity of Sunflower Honey Based-Silver Nanoparticles

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ÖZ

Tıp, ilaç salınım sistemleri, eczacılık, tarım gibi geniş bir yelpazede uygulama alanı bulan nanoteknolojinin yapı taşları olan altın, gümüş, çinko gibi nanopartiküller yeşil sentez tekniği kullanılarak çevre dostu, ekonomik ve biyouyumlu olarak sentezlenebilmektedir. Gümüş nanopartiküllerin yeşil sentezinde içermiş oldukları biyoaktif bileşenler nedeniyle bitkiler veya bitki temelli ürünler yaygın olarak kullanılmaktadır. Bal içermiş olduğu fenolik bileşenler ve şekerler ile gümüş nanopartiküllerin sentezinde kullanılabilecek önemli doğal ürünlerden biridir. Yapılan bu çalışmada, biyoaktif bileşen yönünden kestane ve meşe balına göre daha zayıf olan ayçiçeği balının gümüş nanopartiküllerin sentezinde kullanım potansiyeli tespit edilmiştir. Sentezlenen ayçiçeği balı temelli gümüş nanopartiküller (SH-AgNPs) karakterize edilmiş ve daha sonra antioksidan aktiviteleri ile yara iyileşmede önemli rolü olan myeloperoksidaz ve kollegenaz enzimleri inhibe etme özellikleri tespit edilmiştir. Sentezlenen nanopartiküllerin 440 nm' de maksimum absorbans verdiği, partikül boyutlarının 33 nm ile 38 nm arasında değiştiği tespit edilmiştir. Sentezlenen nanopartiküllerin DPPH·radikal süpürme aktiviteleri ve FRAP demir indirgeme kapasiteleri sırasıyla % 81±1,42 and % 86±1,24; myleoperksidaz ile kollegenaz enzimlerini inhibe etme özellikleri sırasıyla % 63±1,45 and % 37±1,14 olarak tespit edildi. Elde edilen bulgular ayçiçeği balının nanoteknoloji alanında kullanım potansiyeli olduğunu göstermektedir.

Anahtar Kelimeler: Biyolojik sentez, Gümüş nanopartikül, Nanoteknoloji, Antioksidan aktivite, Enzim inhibisyonu

ABSTRACT

Nanoparticles such as gold, silver, zinc, which are the building blocks of nanotechnology, which have a wide range of applications in medicine, drug release systems, pharmacy, agriculture, can be synthesized in an environmentally friendly, economical and biocompatible way using green synthesis technique. In the green synthesis of silver nanoparticles, plants or plant-based products are widely used due to the bioactive components they contain. Honey is one of the important natural products that can be used in the synthesis of silver nanoparticles with its phenolic components and sugars. In this study, the potential of sunflower honey, which is weaker than chestnut and oak honey in terms of

bioactive components, for the synthesis of silver nanoparticles was determined. The synthesized sunflower honey-based silver nanoparticles (SH-AgNPs) were characterized and then their antioxidant activity and inhibition of myeloperoxidase and collagenase enzymes, which play an important role in wound healing, were determined. It was determined that the synthesized nanoparticles gave maximum absorbance at 440 nm and particle sizes ranged between 33 nm and 38 nm. The DPPH· radical scavenging activities and ferric reducing capacity (FRAP) of the synthesized nanoparticles were determined as 81±1.42% and 86±1.24%, respectively, and the inhibition properties of myleoperoxidase and collagenase enzymes were determined as 63±1.45% and 37±1.14%, respectively. The findings obtained show that sunflower honey has the potential for use in the field of nanotechnology.

Keywords: Biological synthesis, Silver nanoparticles, Nanotechnology, Antioxidant activity, Enzyme inhibition

EXTENDED ABSTRACT

Objective: Since metals such as gold and silver find a wide range of applications in biology, medicine, environment and industry, their use in studies has been increasing day by day. The physicochemical properties of silver nanoparticles are of interest to many researchers. Nanoparticles can be synthesized using chemical or physical methods. However, the so-called green synthesis method using plants, yeasts, bacteria and fungi is important because it enables environmentally friendly, nontoxic and high purity synthesis. Chemical and physical methods can successfully produce pure, well-defined nanoparticles, but these techniques are more expensive, energy consuming and potentially toxic to the environment. Therefore, biological synthesis (green synthesis), which is an environmentally friendly, economical and rapid synthesis method, is of great interest.

Materials and Methods: Sunflower honey-based silver nanoparticles were obtained according to the method described by Keskin (2022). Accordingly, a 20% (w/w) honey solution was prepared from sunflower honey purchased from a local beekeeper in Bilecik city. Then, the formation of nanoparticles was observed by mixing 0.005M $AgNO_3$ solution with sunflower honey solution at a ratio of 1:1 at room temperature with a magnetic stirrer at a constant speed. After the detailed characterization, DPPH·, FRAP, myeloperoxidase and collagenase enzyme inhibition properties were determined.

Results and Discussion: The optical properties of the synthesized nanoparticles were determined using UV-Vis spectrophotometer. It was observed that the obtained nanoparticles gave maximum absorbance at 440 nm. The reduction of silver ion to metallic silver was also observed by the color change. The optimum pH and temperature values required for synthesis were determined. It was observed that the size of the obtained nanoparticles varied between 33 and 38 nm. DPPH· radical scavenging activity and ferric reducing capacity (FRAP) of the obtained nanoparticles were calculated as 81±1.42% and 86±1.24%, respectively. The amount of inhibition of myeloperoxidase and collagenase enzymes of the obtained nanoparticles was calculated as 63±1.45% and 37±1.14%, respectively.

Conclusion: Silver nanoparticles are materials that have the potential to be used in a wide range of fields from medicine to agriculture with their properties. The synthesis of silver nanoparticles using environmentally friendly green synthesis technique is very popular. In this study, sunflower honey, which has lower biological activity than honey such as chestnut and oak, was used in the green synthesis of silver nanoparticles. The synthesised nanoparticles were characterised using different techniques and their biological activities were determined. It was found that the obtained nanoparticles inhibited myeloperoxidase and collagenase enzymes, which have an important role in wound healing, to a good extent and the nanoparticles showed very good antioxidant activity. It is also possible to determine the potential use of the obtained nanoparticles in different areas.

GİRİŞ

Nanoteknoloji, nano ölçekte şekil ve boyutu kontrol ederek yapıların üretilmesi, taşınması ve uygulanması için kullanılan bir terimdir (Wady vd. 2014). Nanoteknoloji alanı, malzeme bilimlerindeki en dinamik araştırma alanıdır ve nanopartiküllerin (NP'ler) sentezi dünya çapında önemli ölçüde artmaktadır. NP'ler, boyut (1-100 nm), şekil ve yapıları ile diğer partiküllere göre daha yeni veya geliştirilmiş özellikler göstermektedir (Wady vd.

2014; Parvekar vd. 2020). Altın ve gümüş gibi soy metaller, biyoloji, tıp, çevre ve endüstri gibi geniş bir yelpazede uygulama alanı bulduğu için yapılan çalışmalarda kullanımları gün geçtikçe artmaktadır (Yokoyama ve Welchons, 2007). Gümüş nanopartiküllerin fizikokimyasal özellikleri birçok araştırmacının ilgisini çekmektedir (Sharma vd. 2009). Nanopartiküller kimyasal veya fiziksel yöntemler kullanılarak sentezlenebilmektedir (Hanžić vd. 2015; Maleki vd. 2012; Okitsu vd. 2001). Ancak bitki, maya, bakteri ve fungilerin kullanıldığı yeşil sentez olarak adlandırılan metot çevre dostu, nontoksik ve yüksek saflıkta sentez yapılmasına olanak sağladığından önemlidir (He vd. 2018; Kumar ve Yadav 2009; Makarov vd. 2014). Gümüş nanopartikül sentezinde kullanılan kimyasal ve fiziksel yöntemler saf, iyi tanımlanmış nanopartikülleri başarılı bir şekilde üretebilir, ancak bu teknikler daha pahalı, enerji tüketen ve çevre için potansiyel olarak toksiktir (Okafor vd. 2013). Bu nedenle çevre dostu, ekonomik ve hızlı sentez yöntemi olan biyolojik sentez (yeşil sentez) oldukça ilgi çekicidir. Biyolojik sentez yöntemlerinde nanopartikül üretimi için mikroorganizma hücreleri veya bitki özütleri elektron vericisi olarak kullanılmaktadır (Okafor vd. 2013; Keskin vd. 2022; Khan vd. 2023; Azwatul vd. 2023; Matar vd 2023; Can ve Keskin 2024).

Yeşil sentez ile üretilen gümüş nanopartiküllerin (AgNPs) antimikrobiyal, antikanser, antiinflamatuvar, antifungal, antiviral vb. birçok özelliği olduğu yapılan çalışmalarda belirtilmiştir (He vd. 2018; Jeyaraj vd. 2013; Monteiro vd. 2012; Wong ve Liu 2010; Zhang vd. 2016), Bu uygulamaların yanı sıra gümüş nanopartiküller boya, deterjan, giyim ve ilaç endüstrilerinde de kullanılmaktadır (Li vd. 2011; Okaforvd. 2013). Bitki ve doğal kaynaklar kullanılarak gümüş nanopartiküllerin eldesi, toksik olmayışları, sentez yöntemlerinin kolaylığı bakımından önem arz etmektedir.

Ayçiçek balı ülkemizde önemli miktarda üretimi yapılan bir tür çiçek balıdır. Ancak ayçiçek balının hızlı kristalize olması ve diğer ballara nispeten daha düşük antioksidan aktiviteye sahip olması nedeniyle daha çok kahvaltıda kullanımı tercih edilmektedir. Apiterapi uygulamalarında ayçiçek balının kullanılabilmesi için fonksiyonelleştirilmesi ve katma değerinin arttırılması gerekmektedir. Bu nedenle ayçiçek balının farklı kullanım alanlarının belirlenmesi, teknolojiye, kozmetik alanına, apiterapi uygulamalarına kazandırılma potansiyelinin belirlenmesi önemlidir. Ayçiçek balı içermiş olduğu fenonik bileşenler, şekerler ve diğer farklı biyoaktif bileşenler ile gümüş nanopartiküllerin yeşil sentezinde elektron vericisi olarak kullanım potansiyeli olan önemli bir arı ürünüdür. Yapılan bu çalışma ile bu potansiyeli tespit etmek amacıyla yeşil sentez ile ayçiçek balı temelli gümüş nanopartiküller sentezlenmiş ve karakterize edilmiştir. Elde edilen nanopartiküllerin antioksidan aktiviteleri ve yara iyileştirmede önemi olan enzimler üzerine inhibisyon etkileri belirlenmiştir.

GEREÇ ve YÖNTEM

Bal temelli gümüş nanopartiküller Keskin (2022)' de bildirilen metoda göre elde edilmiştir. Buna göre Bilecikte üretilmiş ayçiçek balından %20'lik (w/w) bal çözeltisi hazırlanmıştır. Daha sonra analitik saflıkta olan $AgNO_3$' ten (Sigma- Aldrich) 0,005M çözelti hazırlanmış ve 1:1 oranında ayçiçek balı çözeltisi ile oda sıcaklığında manyetik karıştırıcı ile sabit bir hızda karıştırılarak nanopartiküllerin oluşumu gözlemlenmiştir. Oluşan karışım 9000 rpm'de 15 dk boyunca santrifüjlenmiş (DLab, Çin) ve elde edilen nanopartiküller 60 °C' de 2 saat kurutulmuştur (Şekil 1).

Şekil 1. SH-AgNP'lerin sentezi **Figure 1**. Synthesis of SH-AgNPs

Optimum pH Belirlenmesi

Gümüş nanopartikül sentezine pH etkisini incelemek amacıyla bal çözeltileri pH 5,0 için asetat tamponu; 7,0 için fosfat tamponu (Na_2HPO_4-NaH_2PO_4, Sigma-Aldrich); 9,0 için glisin tampon çözeltileri kullanılarak hazırlanmıştır. Her bir çözelti ile ayrı ayrı nanopartikül sentezi yapılmış ve UV spektrofotometre (Hach, DR/4000U) absorbans değerleri karşılaştırılmıştır.

Optimum Ekstrakt Derişimi Belirlenmesi

Gümüş nanopartikül sentezine ekstrakt derişiminin etkisinin belirlenmesi amacıyla 1:1, 2:1 ve 3:1 oranlarında bal çözeltileri ile 0,005M $AgNO_3$ karıştırılarak gümüş nanopartiküller sentezlenmiş ve UV spektrofotometre absorbans değerleri karşılaştırılmıştır.

Optimum Sıcaklık Değerinin Belirlenmesi

Gümüş nanopartikül sentezine sıcaklık etkisini belirlenmek amacıyla reaksiyon ortam sıcaklığı 20 ^{0}C, 40 ^{0}C ve 60 ^{0}C getirilmiş ve UV spektrofotometre absorbans değerleri karşılaştırılmıştır.

Elde Edilen Gümüş Nanopartiküllerin Karakterizasyonu

Optimum koşullarda elde edilen gümüş nanopartiküllerin karakterizasyonu meydana gelen absorbans değişimlerini tespit etmek amacıyla UV spektrofotometre, değişikliğe uğrayan (indirgenen-yükseltgenen) fonksiyonel grupları belirlemek amacıyla FT-IR (Thermo Fisher) ve elde edilen nanopartiküllerin boyutlarını belirleyebilmek amacıyla SEM (ZEISS/Supra 40 VP) cihazı kullanılmıştır.

Antioksidan Kapasitenin Tayini

DPPH• radikal (2,2-difenil-1-pikrilhidrazil) süpürme aktivitesi

DPPH• radikali (2,2-difenil-1-pikrilhidrazil, Sigma-Aldrich) ticari olarak satın alınabilen bir radikal olup denemelerde satın alınan bu radikalin 100 µM'lık metanolik çözeltisi kullanılmıştır. Eşit hacimde (750 µL) DPPH• çözeltisi ve numune çözeltileri (0,25 mg/mL, 0,5mg/mL, 1 mg/mL, 1,25 mg/mL ve 1,5 mg/mL, R^2: 0,998). karıştırılıp oda sıcaklığında 50 dakika bekletilmiştir. Süre sonunda DPPH•'ın maksimum absorbans verdiği 517 nm'de absorbanslar kaydedilmiştir. Bu absorbans değerleri karşılık gelen konsantrasyonlara karşı grafiğe geçirilerek örneklerin % radikal süpürme aktivitesi hesaplanmıştır (Cuendent, 1997).

Demir İndirgeme Kapasitesi (FRAP)

FRAP yöntemi, doğal ürünlerin antioksidan kapasitelerinin tayininde en sık kullanılan yöntem olup, doğal ürünlerde bulunan antioksidan maddelerin Fe(III)- TPTZ kompleksinde bulunan demir (III) iyonunu indirgemesi esasına dayanmaktadır (Benzie ve Strain, 1999). Çözeltide bulunan antioksidan maddeler tarafından indirgenen Fe(III) 593 nm'de absorbans vermektedir. Absorbans değerlerine karşılık gelen konsantrasyonlara karşı grafiğe geçirilerek örneklerin % demir indirgeme kapasiteleri tespit edilmiştir.

Kollegenaz İnhibisyonu

Kollagenaz aktivitesinin inhibisyonu, farklı miktarda nanopartikül ile önceden inkübe edilmiş enzim kullanılarak spesifik kollagenaz substrat FALGPA' nın (Sigma-Aldrich) bölünmesinin izlenmesi ile belirlenmiştir. Kollagenaz enzimi (Sigma-Aldrich) 15 dk boyunca oda sıcaklığında, farklı nanopartikül miktarları kullanılarak, 10 mM $CaCl_2$ ve 400 mM NaCl içeren 50 mM Tris-HCl (pH 7.5) tamponunda inkübe edilmiştir. Daha sonra spesifik kollagenaz substratı FALGPA'nın 0,8 mM'lık çözeltisi nanopartikül karışımı üzerine ilave edilerek 20 dakika boyunca 37 $^{\circ}$C' de inkübe edilmiştir. İlgili süre sonunda tüplerin absorbansları 340 nm'de kaydedilmiştir (Tu ve Tawata, 2015).

Myloperoksidaz İnhibisyonu

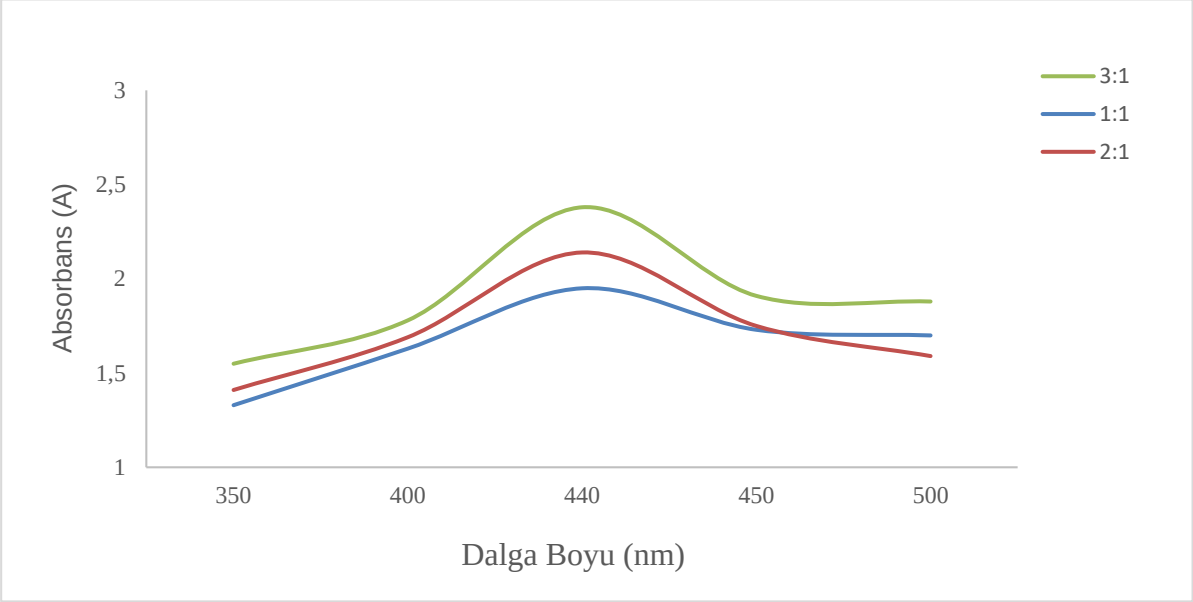
Sentezlenen nanopartiküllerin MPO aktivitesi üzerindeki inhibitör etkisi tespit edilmiştir. Substrat olarak gayakol kullanılmıştır. MPO (2.5 nM, Sigma-Aldrich) enzimi 0,5 mM H_2O_2 içeren 1 mL 50 mM fosfat tamponu (pH 7.4) ve farklı miktarlarda nanopartikül ile 15 dk ön inkübasyona tabi tutulmuştur. Daha sonra reaksiyon tüplerine 1 mM gayakol (Sigma- Aldrich) çözeltisi ilave edilerek 37 ºC'de reaksiyon başlatılmış ve 3 dk' da 470 nm'de absorbans artışı kaydedilmiştir. MPO inhibisyonu;

% İnhibisyon = [1- (inhibitör aktivitesi/Kontrol aktivitesi)]*100

formülüne göre hesaplanmıştır (Khalil vd. 2008).

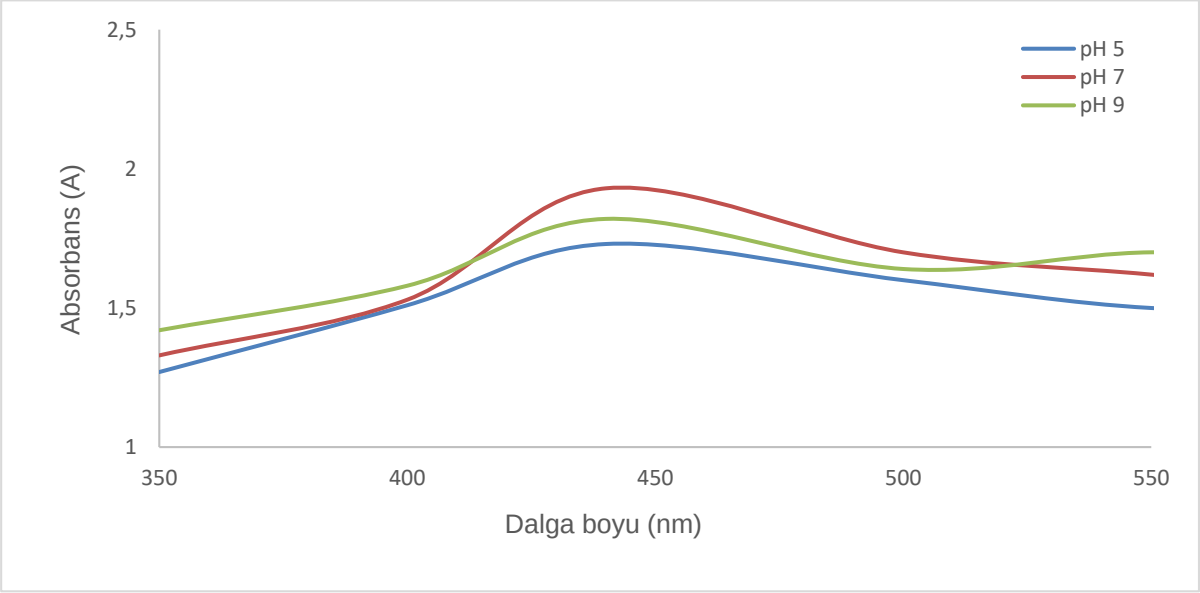
BULGULAR

Sentezlenen nanopartiküllerin optik özellikleri UV-Vis spektrofotometre kullanılarak belirlenmiştir. Elde edilen nanopartiküllerin 440 nm de maksimum absorbans verdiği görülmüştür (Şekil 2).

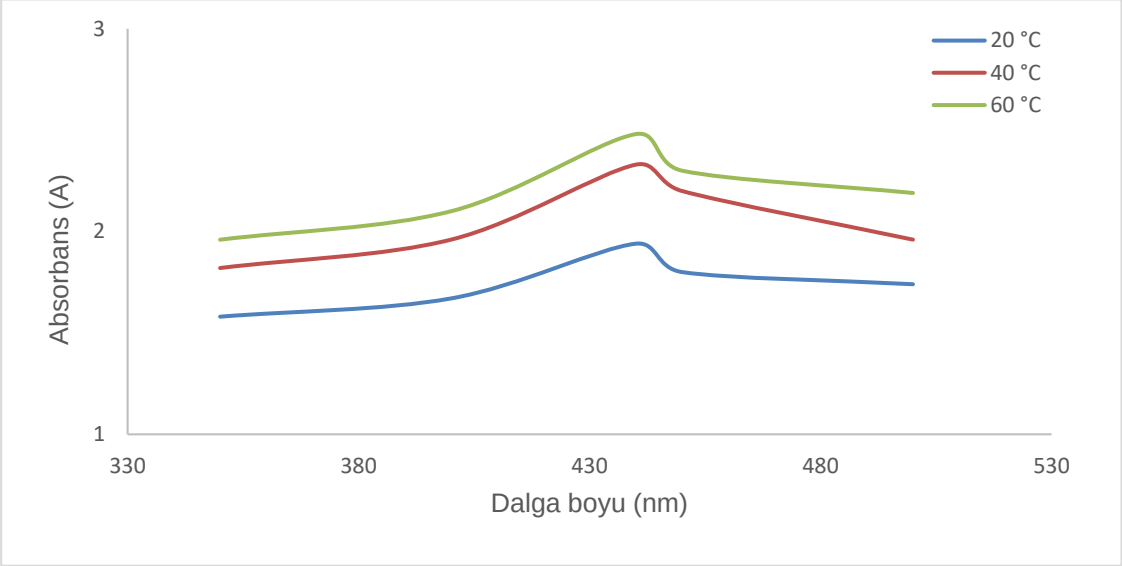


Şekil 2. SH-AgNPs sentezine ekstrakt derişiminin etkisi **Figure 2**. Effect of extract concentration on SH-AgNPs synthesis

Gümüş iyonunun metalik gümüşe indirgenmesi oluşan renk değişimi ile de gözlemlenmiştir. Sentez için gerekli optimum pH ve sıcaklık değerleri ayrı ayrı belirlenmiştir (Şekil 3 ve Şekil 4).

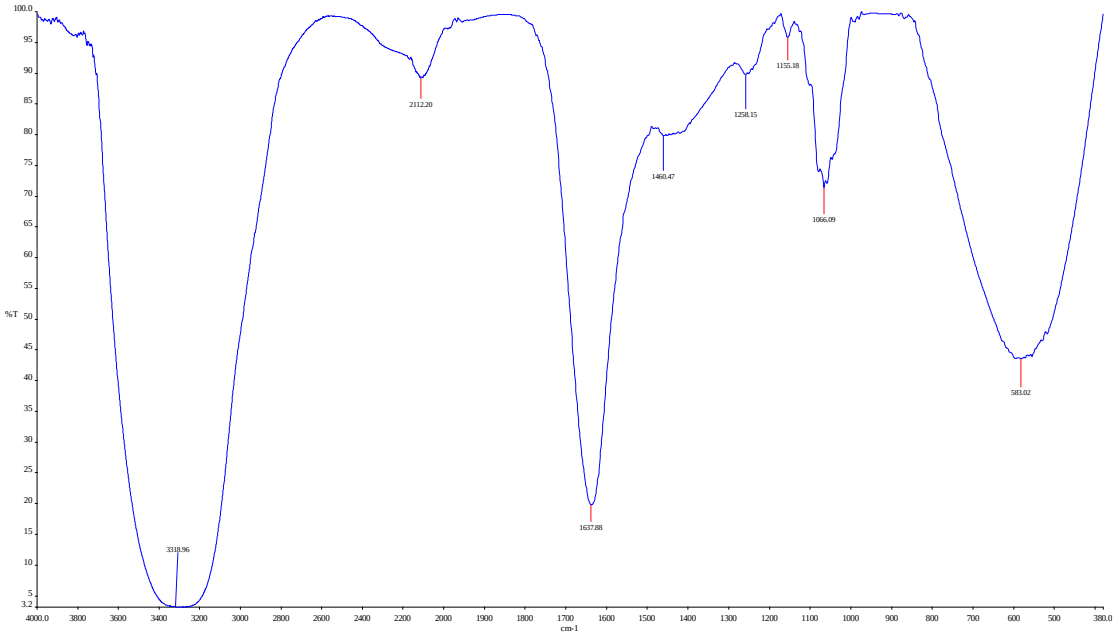


Şekil 3. SH-AgNPs sentezine pH etkisi **Figure 3.** pH effect on SH-AgNPs synthesis

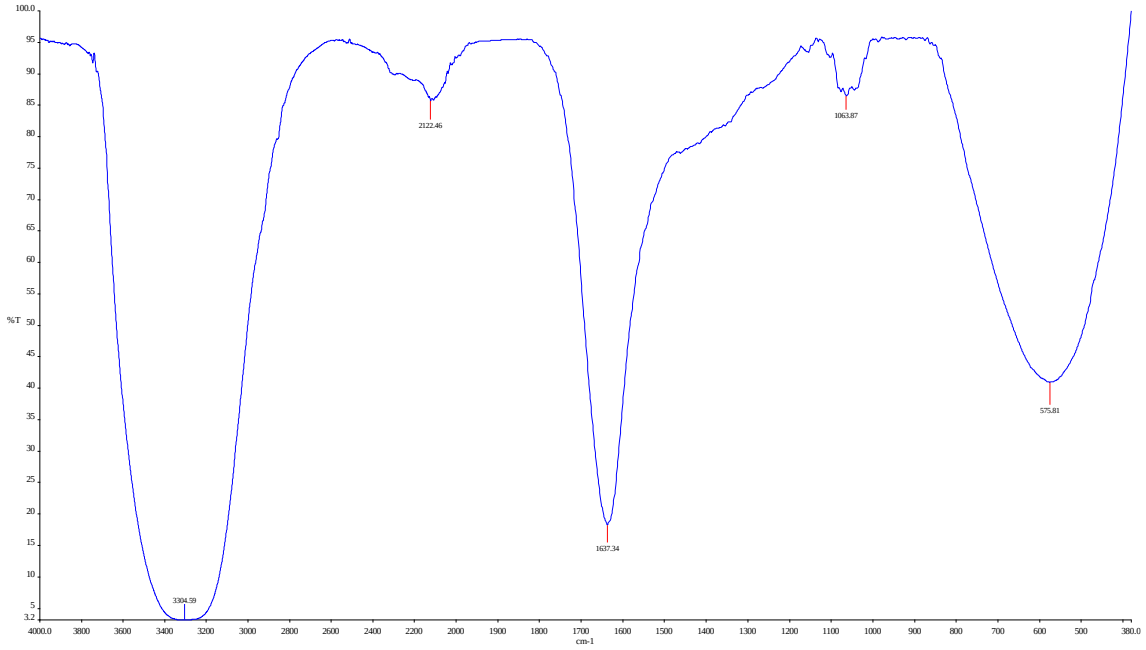


Şekil 4. SH-AgNPs sentezine sıcaklığın etkisi **Figure 4**. Effect of temperature on the synthesis of SH-AgNPs

Ayçiçeği balı ekstresi ve sentezlenen nanopartiküllerin süpernatantının içermiş olduğu fonksiyonel gruplar FT-IR kullanılarak belirlenmiştir. Şekil 5 ve Sekil 6 da örneklerin içermiş olduğu fonksiyonel grupların pikleri görülmektedir. Şekil 5 ve Şekil 6' da görüldüğü gibi piklerin tamamında kayma vardır.

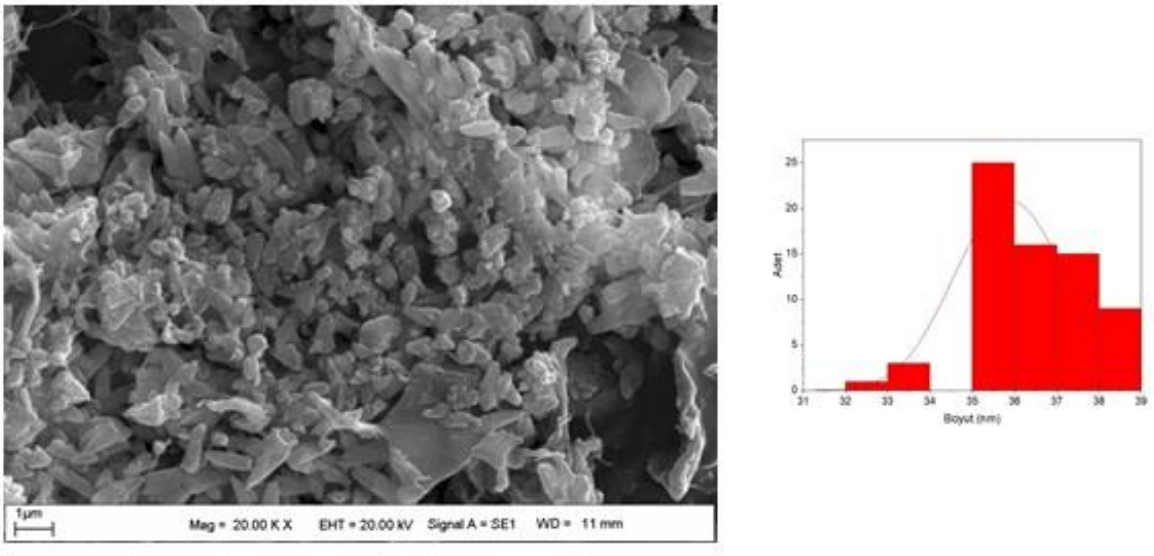


Şekil 5. Ayçiçeği balı ekstraktının FTIR spektrumu **Figure 5.** FTIR spectrum of sunflower honey extract

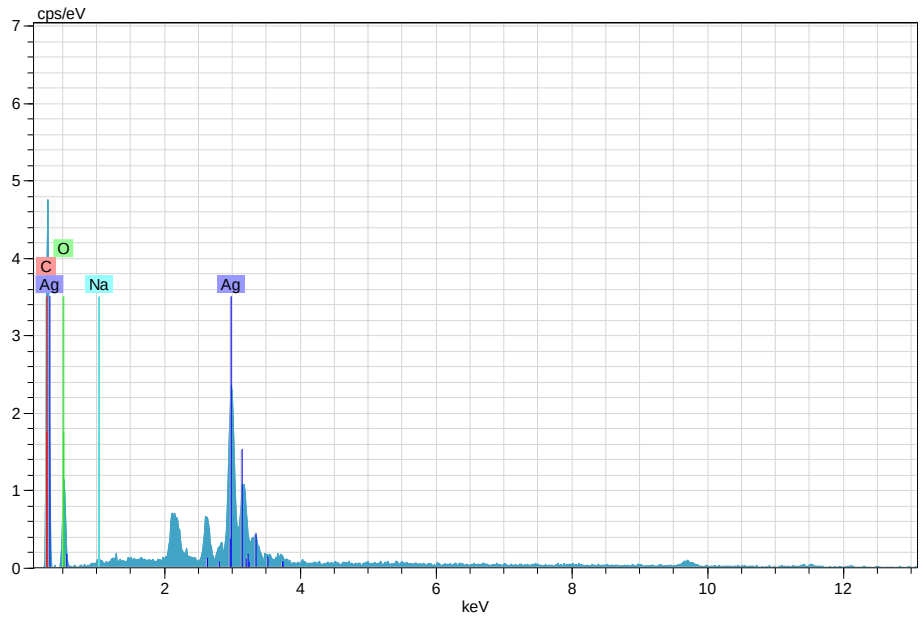


Şekil 6. SH-AgNPs süpernatantının FTIR spektrumu **Figure 6**. FTIR spectrum of SH-AgNPs supernatant

Elde edilen nanopartiküllerin boyutlarının 33 ile 38 nm arasında değiştiği görülmektedir (Şekil 7). Histogram SEM görüntüsü kullanılarak elde edilmiştir. Elde edilen nanopartiküllerin EDX analizi sonucunda 3 keV' da bir pike sahip oldukları görülmektedir (Şekil 8).

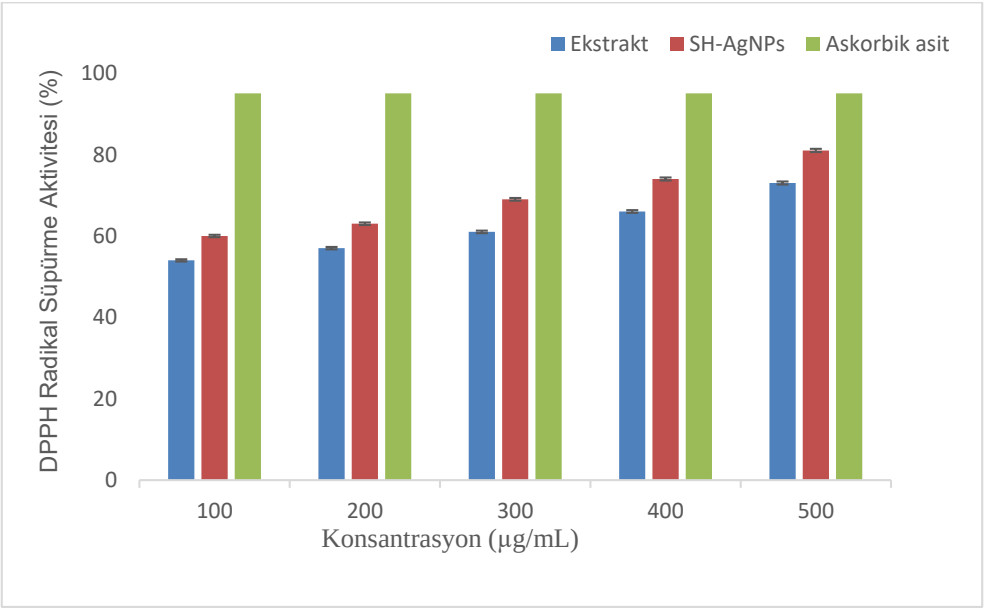


Şekil 7. SH-AgNPs SEM görüntüsü ve histogramı **Figure 7.** SEM image and histogram of SH-AgNPs

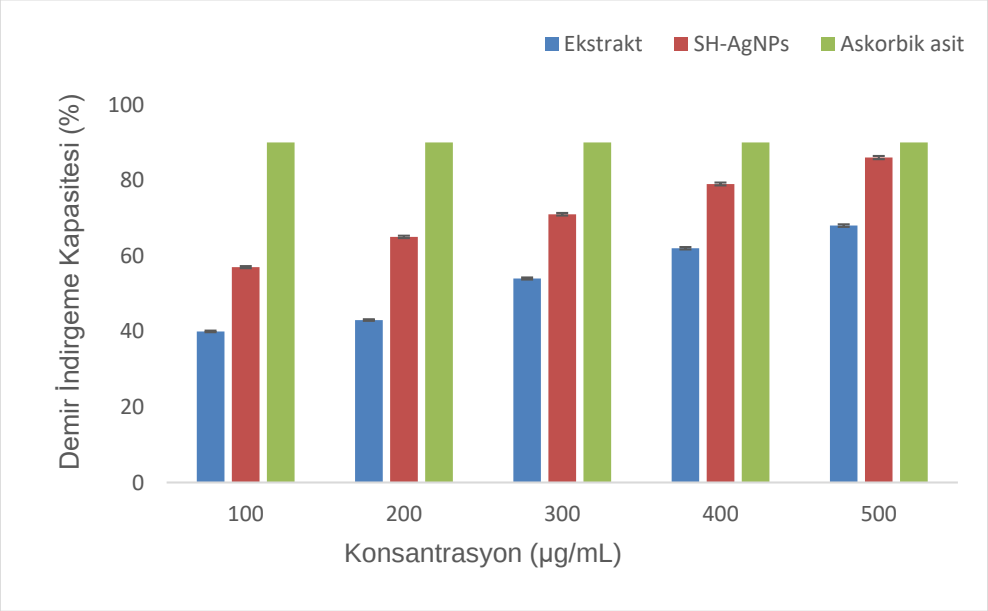


Şekil 8. SH-AgNPs EDX profili **Figure 8.** EDX profile of SH-AgNPs

Elde edilen nanopartiküllerin DPPH radikal aktivite süpürme ve demir indirgeme kapasitesi (FRAP) sırasıyla % 81±1.42 ve % 86±1.24 olarak hesaplanmıştır (Şekil 9 ve Şekil 10).

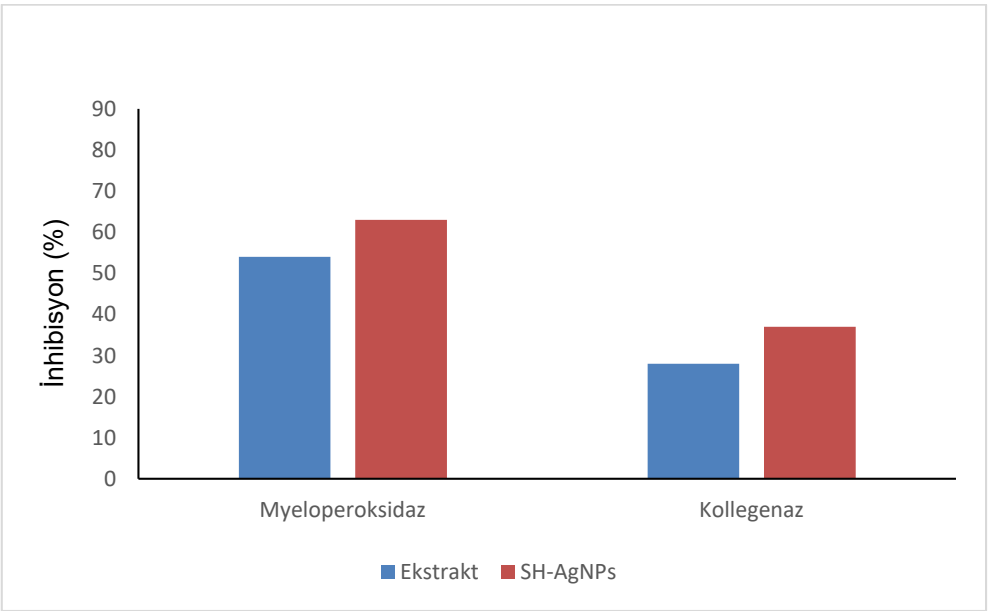


Şekil 9. Ayçiçeği balı ve SH-AgNPs DPPH· radikal süpürme aktiviteleri **Figure 9**. DPPH· radical scavenging activities of sunflower honey and SH-AgNPs



Şekil 10. Ayçiçeği balı ve SH-AgNPs demir indirgeme kapasitesi **Figure 10**. Iron reduction capacity of sunflower honey and SH-AgNPs

Elde edilen nanopartiküllerin myeloperoksidaz ve kollegenaz enzimlerini inhibe etme miktarı ise sırasıyla % 63±1.45 ve % 37±1.14 olarak hesaplanmıştır (Şekil 11).



Şekil 11. Ayçiçeği balı ve SH-AgNPs enzim inhibisyon özellikleri **Figure 11.** Enzyme inhibition properties of sunflower honey and SH-AgNPs

TARTIŞMA

Yeşil sentez tekniği kullanılarak elde edilen nanopartiküllerin UV-spektrofotometre kullanılarak karakterize edilmesi oldukça yaygındır. Genel olarak gümüş nanopartiküllerin geniş bir skalada farklı dalga boylarında 420 nm (Keskin vd., 2023), 417 nm ve 425 nm nm (Okafor vd., 2013), 425 nm (Awwad ve Salem vd., 2012), 440 nm (Al Sufyani vd., 2019), 400nm (Garibo vd., 2020) yüzey plazmon resonansı (SPR) verdiği literatürde ifade edilmektedir. Elde edilen verilerin literatür ile uyumlu olduğu görülmektedir.

3200 cm^{-1} ile 3400 cm^{-1} arasındaki bantlar O-H titreşimlerini ifade etmektedir. Bu piklere ek olarak 2112.20, 1460.40, 1258.15, 1155.18 ve 1066.09 cm^{-1} pikleri bal ekstraktında bulunan; 2122.46, 1637.34 ve 1063.87 cm^{1} pikleri ise AgNPs süpernatantında bulunan piklerdir. C=C, C=O, O-H, C-H, C-N ve -COOH gibi pikler alkan, keton, alken, azotlu moleküllerin titreşimleri ile ilintili olduğu ifade edilebilir. Piklerde gözlenen kaymalar nanopartikül sentezinin başarı ile yapıldığının göstergesi olarak kabul edilebilir (Sadeghi and Gholamhoseinpoor 2015).

Awwad ve Salem (2012) tarafından yapılan çalışmada elde edilen naopartiküllerin boyutlarının 20 ile 40 nm arasında değiştiği**,** Garibo vd. (2023) yapmış oldukları çalışmada elde edilen nanopartiküllerin boyutlarının 1,2 nm ile 62 nm arasında değiştiği ifade edilmektedir. Yapılan başka bir çalışmada ise elde edilen nanopartiküllerin boyutlarının 3 ile 20 nm arasında değiştiği ifade edilmiştir (Mallikarjuna vd 2011). Literatür verilerinden de görüldüğü üzere nanopartiküllerin boyutları geniş bir skalada değişmektedir. Gümüş nanopartiküller için 3 keV' da bir pik karakteristiktir. Yapılan bu çalışmada elde edilen nanopartiküllerin 3 keV' da pik verdiği görülmektedir (Şekil 8).

Kronik (iyileşmeyen) yaralar, çok sayıda karşılıklı nedensel hücresel olayın proteolitik ve nötrofil türevli oksidatif enzimlerin aşırı ekspresyonuna yol açtığı kalıcı bir inflamasyon ile karakterize edilmektedir. Çinko bağımlı bir endopeptidaz ailesi olan matriks metalloproteinazlar (MMP'ler, EC 3.4.24.-), elastin ve kolajen gibi hücre dışı matriks bileşenlerini ve yara bölgesinde oluşan büyüme faktörlerini bozarak iyileşmeyi engeller (Díaz-GonzáLez vd. 2012). Yapılan çalışmalarda ağırlıklı olarak kolajenaz olan toplam matriks metalloproteinaz aktivitesinin, kronik yara sıvılarında akut yara sıvılarına göre 30 kata kadar daha fazla olduğu ifade edilmektedir (Trengove vd. 1999). Bu durum proteaz/antiproteaz dengesizliğine ve hücre dışı matriksin aşırı parçalanmasına neden olmaktadır (Francesko vd. 2013). Dokunun proteolitik hasarı, doğal proteaz inhibitörlerinin ana nötrofil enzimi olan miyeloperoksidaz (MPO) tarafından üretilen hipokloröz asit (HOCl) ile oksidasyonu ile daha da desteklenmektedir (Rojikind vd. 2002). Zararlı kronik yara enzimlerinin sayısının akut yaralarda bulunan seviyelere düşürülmesinin iyileşmenin ilerlemesini sağlayacağına inanılmaktadır (Francesko vd. 2013). Bu nedenle yara iyileşmesinde rolü olan myeloperoksidaz ve kollegenaz enzimlerinin inhibisyonu oldukça önemlidir. Figueiredo vd. (2023) tarafından yapılan bir çalışmada sentezlenen nanopartiküllerin DPPH· radikal süpürme aktivitesinin % 83,6, demir indirgeme kapasitesinin 714.82 µM Trolox Equivalent (TE)/g örnek olduğu, kollegenaz enzimini % 75,33 oranında inhibe ettiği ifade edilmiştir. Keskin vd. (2023)'nin yapmış olduğu çalışmada kestane balı temelli nanopartiküllerin myeloperoksidaz enzimini % 36,4; kollegenaz enzimini ise %74,2 oranında inhibe ettiği ifade edilmiştir. Korkmaz vd. (2024) tarafından yapılan bir çalışmada ise sentezlenen gümüş nanopartiküllerin kollegenaz enzimini inhibe etme potansiyeli olduğu ifade edilmiştir. Elde edilen bulguların literatür ile uyumlu olduğu görülmüştür (Tablo 1).

Tablo 1. Farklı bal kaynakları kullanılarak sentezlenen gümüş nanopartiküllere ait bazı özellikler ve kullanıldıkları alanlar
Table 1. Some properties and applications of silver nanoparticles synthesized using different honey sources

Bal kaynağı	Maksimum UV absorbans değeri (nm)	Partikül boyutu (nm)	Kullanıldığı alan	Kaynak
Marketten satın alınan bal	417	5 ile 25 nm arası	Boya giderimi	Al-Zaban vd. 2021
Malezya orman balı	482	18,98 ile 26,05 nm arası	-	Haiza vd. 2013
Marketten satın alınan bal	411	18,4	-	González Fá vd.2017
Salgı balı	400	42 ile 55 nm arası	Antimikrobiyal aktivite	Czernel vd. 2021
Kaynağı belirtilmemiş	464	11,8	Antimikrobiyal aktivite	Oskuee vd. 2016
Marketten satın alınan bal	400	25 ile 70 nm arası	Antimikrobiyal aktivite	Youssef vd. 2019
Rhododendron balı (deli bal)	456	14,7	Antimikrobiyal aktivite	Matar vd. 2023
Bu çalışma	**440**	**33 ile 38 arası**	**Antioksidan aktivite ve enzim inhibisyonu**	**-**

Sonuç: Gümüş nanopartiküller sahip oldukları özellikler ile tıptan tarıma oldukça geniş bir yelpazede kullanım potansiyeli olan materyallerdir. Gümüş nanopartiküllerin çevre dostu yeşil sentez tekniği kullanılarak sentezlenmesi oldukça popülerdir. Yapılan bu çalışmada biyolojik aktivitesi kestane, meşe gibi ballara nazaran daha düşük olan ayçiçeği balı gümüş nanopartiküllerin yeşil sentezinde kullanılmıştır. Sentezlenen nanopartiküller farklı teknikler kullanılarak karakterize edildikten sonra biyolojik aktiviteleri tespit edilmiştir. Elde edilen nanopartiküllerin yara iyileşmesinde önemli rolü olan myeloperoksidaz ve kollegenaz enzimlerini iyi derece inhibe ettiği ve nanopartiküllerin oldukça iyi antioksidan aktivite gösterdiği tespit edilmiştir. Elde edilen nanopartiküllerin tıp, ilaç taşınım/salınım sistemleri, tekstil, çevre, tarım gibi farklı alanlarda kullanım potansiyellerinin yapılacak olan ileri çalışmalarla belirlenebilmesi de söz konusudur.

Çıkar çatışması: Yazarlar arasında herhangi bir çıkar çatışması bulunmamaktadır.

Etik durumu: Çalışmanın yürütülmesi esnasında hayvan veya insan deneyleri yapılmamıştır.

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Veri kullanılabilirliği: Mevcut çalışmada kullanılan veriler talep edilmesi halinde sorumlu yazardan temin edilebilir

Yazar katkıları: MK**:** Verilerin yorumlanması, makale yazımı ve editi; İU ve GG**:** Verilerin elde edilmesi, projenin yürütülmesi.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

ANTIOXIDANT ACTIVITIES AND PHYTOCHEMICAL COMPOSITION OF STINGLESS BEE *(Heterotrigona itama)* HONEY COLLECTED FROM *Calliandra calothyrsus* PLANTATION IN EAST KALIMANTAN, INDONESIA

Endonezya Doğu Kalimantan'daki *Calliandra calothyrsus* Bahçesinden Toplanan İğnesiz Arı (*Heterotrigona itama*) Balının Antioksidan Aktiviteleri ve Fitokimyasal Bileşimi

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ABSTRACT

Stingless bee honey is one of the most valuable insect products. The increasing popularity of stingless bee honey can be attributed to its composition, which has been linked to medicinal properties. Beekeeping with stingless bees is well-known in Indonesia, with *Heterotrigona itama* is the most popular stingless bee species cultivated in East Kalimantan, Indonesia. Stingless bees utilize various plant species as sustenance sources. Among those plants, *Calliandra calothyrsus* is popular planting in Indonesian bee plantations. This study analyzed the antioxidant (DPPH assay), phytochemical (qualitative method), water, and sugar content of *H. itama* stingless bee honey collected from a *C. calothyrsus* plantation. The results show that the water and sugar contents of the honey in this study were higher than in other research. Meanwhile, antioxidant capacity was also higher than in other studies. The phytochemical contents detected from honey in this study were carotenoids, coumarins, flavonoids, saponins, steroids, tannins, and triterpenoids. Even though the properties of stingless bee honey can differ based on vegetation and geographical origin, *H. itama* stingless bee honey collected from *C. calothyrsus* plantation in East Kalimantan, Indonesia, showed potential antioxidant activity and phytochemical content, which is advantageous to human health.

Keywords: Stingless bee honey, *Heterotrigona itama*, *Calliandra calothyrsus*, DPPH, Phytochemical

ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

ÖZ

İğnesiz arı balı en değerli böcek ürünlerinden biridir. İğnesiz arı balının artan popülaritesi, tıbbi özelliklerle ilişkilendirilen bileşimine bağlanabilir. İğnesiz arılarla arıcılık Endonezya'da iyi bilinmektedir ve *Heterotrigona itama*, Endonezya'nın Doğu Kalimantan bölgesinde yetiştirilen en popüler iğnesiz arı türüdür. İğnesiz arılar, çeşitli bitki türlerini besin kaynağı olarak kullanırlar. Bu bitkiler arasında *Calliandra calothyrsus*, Endonezya arı plantasyonlarında popüler bir ekimdir. Bu çalışmada, *C. calothyrsus* plantasyonundan toplanan *H. itama* iğnesiz arı balının antioksidan (DPPH testi), fitokimyasal (kalitatif yöntem), su ve şeker içeriği analiz edilmiştir. Sonuçlar, bu çalışmadaki balın su ve şeker içeriğinin diğer araştırmalara göre daha yüksek olduğunu göstermektedir. Bu arada, antioksidan kapasitesi de diğer çalışmalara göre daha yüksek. Bu çalışmada baldan tespit edilen fitokimyasal içerikler karotenoidler, kumarinler, flavonoidler, saponinler, steroidler, tanenler ve triterpenoid. İğnesiz arı balının özellikleri bitki örtüsüne ve coğrafi kökene göre farklılık gösterebilse de, Endonezya'nın Doğu Kalimantan bölgesindeki *C. calothyrsus* plantasyonundan toplanan *H. itama* iğnesiz arı balı, insan sağlığı için avantajlı olan potansiyel antioksidan aktivite ve fitokimyasal içerik gösterir.

Anahtar kelimeler: İğnesiz arı balı, *Heterotrigona itama*, *Calliandra calothyrsus*, DPPH, Fitokimyasal

GENİŞLETİLMİŞ ÖZET

Giriş: İğnesiz arı balı en değerli böcek ürünlerinden biridir ve eski insanlar ona tıbbi özellikler atfetmişlerdir. İğnesiz arılar tarafından üretilen balın artan popülaritesi, antiseptik, antimikrobiyal, antikanser, anti-inflamatuar ve yara iyileştirici özelliklerle ilişkilendirilen bileşimine bağlanabilir. Küresel olarak, sıcak ve nemli ormanlar iğnesiz arılara ev sahipliği yapmaktadır. Bu nedenle, Endonezya gibi tropikal ülkelerde iğnesiz arılarla arıcılık daha iyi bilinen bir uygulamadır. Kalimantan'daki arıcılar tarafından birkaç iğnesiz arı türü yetiştirilmektedir ve *Heterotrigona itama* en popüler türdür. İğnesiz arılar, doğal ortamlarındaki bol bitki örtüsünden çiçek nektarları alır ve bunları kimyasal olarak maddeleriyle değiştirir, bunun sonucunda botanik kökeni, coğrafi bölgesi ve çevre koşullarından etkilenen kimyasal bileşim, lezzet ve aromaya sahip benzersiz bir bal elde edilir. Bu bitkiler arasında *Calliandra calothyrsus*, Endonezya arı plantasyonlarında popüler bir ekimdir çünkü bol miktarda çiçekten beslenen çok sayıda koloninin yetiştirilmesi yüksek bal verimine yol açmıştır.

Yöntemler: Bu çalışmada, *C. calothyrsus* plantasyonundan toplanan *H. itama* iğnesiz arı balının antioksidan aktivitesi ve fitokimyasal içeriği analiz edilmiştir. Antioksidan aktivite analizi için DPPH radikal temizleme aktivitesi testi yürütülmüştür. Önemli fitokimyasal içerik için tarama testleri standart nitel prosedürler kullanılarak gerçekleştirilmiştir.

Sonuçlar: Sonuçlar, bu çalışmadaki balın su (%28,91 ± %1,08) ve şeker (%66,68 ± %2,31) içeriğinin diğer araştırmalara göre daha yüksek olduğunu göstermektedir. Bu arada antioksidan kapasitesi de diğer çalışmalara göre daha yüksek olup 12,49 ile 90,85 µg/mL arasında değişmektedir. Bu çalışmada balda tespit edilen fitokimyasal içerikler karotenoidler, kumarinler, flavonoidler, saponinler, steroidler, tanenler ve triterpenoidlerdir. On iki aylık gözlem sırasında *H. itama* balında tespit edilen fitokimyasal içeriklerin esas olarak kumarinler ve flavonoidler olduğu görüldü.

Tartışma: Flavonoidler ve fenolikler balın aroması ve antioksidan aktivitesinden sorumlu bileşiklerdir. Bal örneklerinin flavonoid ve fenolik içeriğindeki farklılıklar arıların tükettiği nektarların çeşitli bitki örtüsüne ve coğrafi kökenlerine atfedilebilir. Flavonoidlerin ve fenolik bileşiklerin antioksidan, antitümör, antimikrobiyal, kardiyoprotektif ajan, antiinflamatuar ajan ve bağışıklık güçlendirici olarak etkili olduğu bildirilmiştir. İğnesiz arı balı, serbest radikalleri nötralize edebilen fenolik ve flavonoid içeriğinden dolayı yüksek bir antioksidan aktiviteye sahiptir. Daha önce belirtildiği gibi, fenolik bileşim bitki örtüsüne ve coğrafi kökene göre farklılık gösterebilir, ancak aynı zamanda her arı türünün bitki örtüsü tercihine göre de değişebilir.

Sonuçlar: İğnesiz arı balının özellikleri bitki örtüsüne ve coğrafi kökene göre farklılık gösterse de, Endonezya'nın Doğu Kalimantan bölgesindeki *C. calothyrsus* plantasyonundan toplanan *H. itama* iğnesiz arı balı, insan sağlığı için avantajlı olan

potansiyel antioksidan aktivite ve fitokimyasal içerik göstermiştir.

INTRODUCTION

Honey is the naturally sweet substance produced by honey bees from the nectar of plants (FAO 2019). Honey consists primarily of carbohydrates and other substances. It is abundant in flavonoids and phenolic acids, which are biologically active and function as natural antioxidants. There are currently two varieties of honey produced and sold globally: traditional honey from bees and honey from stingless bees. Honey produced by stingless bees is known by various names, including *Kelulut* honey, Meliponine honey, and pot honey (Amin et al. 2018).

Honey is one of the most valuable insect products, and ancient peoples attributed it with medicinal properties. The increasing popularity of honey produced by stingless bees can be attributed to its composition, which has been linked to antiseptic, antimicrobial, anticancer, anti-inflammatory, and wound-healing properties (da Silva et al. 2013). In contrast to the population of stingless bees, this honey is less widely distributed than the common honeybee due to the need for more information about this honey, making it less popular. Therefore, stingless bee honey needs further investigation (Abd Jalil et al. 2017).

Globally, warm and humid forests are home to stingless bees. Approximately 500 species of stingless bees and over 60 distinct genera of stingless bees have been identified. Therefore, in tropical countries such as Indonesia, beekeeping with stingless bees is a more well-known practice (Nordin et al. 2018). Ten species of stingless bees are cultivated by beekeepers in Kalimantan, with *Heterotrigona itama* being the most popular species (Syafrizal et al. 2020a). Stingless bee honey breeders are currently cultivating this species due to its larger size, adaptability, and increased honey production (Buchori et al. 2022). Honey produced by *H. itama* contains chemical compositions, including alkaloids, coumarins, flavonoids, saponins, and tannins (Syafrizal et al. 2020b). Stingless bees acquire floral nectars from the abundant vegetation of their native environments and chemically modify them with their substances, resulting in a unique honey with chemical composition, flavor, and aroma which are influenced by its botanical origin, geographic region, and environmental conditions (Avila et al. 2018).

Stingless bees utilize various plant species as sustenance sources (Juliasih et al. 2022). Among those plants, *C. calothyrsus* is popular planting in Indonesia bee plantations because the rearing of numerous colonies that could forage on the abundant blossoms led to a high honey yield (Suliasih et al. 2021; Ustadi et al. 2017). As fast-growing plants, *C. calothyrsus* produces more than 100 L honey per day per hectare (de Luna et al. 2020; Harianja et al. 2023). To our knowledge, no one has investigated the properties of stingless bee honey from the *C. calothyrsus* as the dominant vegetation. This study analyzed the antioxidant activity and phytochemical content of *H. itama* stingless bee honey collected from a *C. calothyrsus* plantation.

MATERIALS AND METHODS

Location of research

The location of the research was a stingless bee honey farm in the Lubuk Sawah Region of Samarinda, East Kalimantan Province, Indonesia, as shown in Figure 1. Samples of honey were collected from vegetation dominated by *C. calothyrsus*, as shown in Figure 2. Samples of honey were extracted using a vacuum device. Honey is packaged and shielded from light and moisture with aluminum sheeting before being placed in a styrofoam box. Before analyzing the honey according to the predetermined parameters, the honey was placed in a refrigerator room at 0° - 5°C temperature.

Figure 1. Lubuk Sawah Region in Samarinda, East Kalimantan, Indonesia as research location

Figure 2. Samples of honey were collected from plantation with *C. calothyrsus* as dominant vegetation in Lubuk Sawah, Samarinda, East Kalimantan, Indonesia

Water and Sugar Contents

The water content was measured using a refractometer (Atago, Japan) according to Indonesian National Standard 8664-2018 for Honey (NSAI 2018). The sugar content was measured using Luff Schoorl methods for reducing sugar according to Indonesian National Standard 01-2892-1992 for Sugar Testing (NSAI 1992).

Antioxidant activity analysis

The antioxidant for radical scavenging activity assay was conducted using DPPH (2,2-diphenyl-1-picrylhydrazyl) according to Sukemi et al. (2021). As working solutions, DPPH and ethanol were used. The positive control for this assay was ascorbic acid. The effect of honey on scavenging free radicals on the measured concentration differed from the test using spectrophotometry. The radical scavenging activity was calculated using the following equation $[(A_c – A_s) / A_s] \times 100$. Where A_c is the absorbance of the control sample, and A_s is the absorbance that contains the test sample. The IC_{50} is the parameter used to express the relative antioxidant capacity. The IC_{50} was calculated by plotting the scavenging percentage against the test sample concentration, using µg/mL units. A linear regression analysis of the inhibition percentage as the honey concentration increased was used to estimate the IC_{50} value (Gulcin & Alwasel 2023).

Phytochemical analysis

Phytochemical assays were conducted to detect alkaloids, carotenoids, coumarins, flavonoids, saponins, steroids, tannins, and triterpenoids. The qualitative screening tests for these phytochemicals were conducted using standardized protocols with specific changes. The alkaloids were identified with HCl and Dragendorff reagents. Stingless bee honey (5 mL) was combined with hydrochloric acid (2 mL) in the tube, followed by Dragendorff reagent (1 mL). The appearance of a yellow substance indicated the alkaloid contents of the honey (Oscar et al. 2020).

Carotenoids were detected by chloroform and sulphuric acid. In a tube, honey (1 mL) was diluted with chloroform (5 mL), agitated briskly, and 85% sulphuric acid (4 drops) was added. The mixtures' blue substance suggested carotenoids (Viji, et al. 2013). Sodium hydroxide and ethanol detected coumarins, with sodium hydroxide (4 drops) and ethanol, added to stingless bee honey (1 mL). The solution's yellow color indicated coumarins (Rao et al. 2023).

Flavonoids were detected with sodium hydroxide and HCl. Honey (1 mL) was treated with 1% sodium hydroxide (5 drops). A colorless solution with 1% HCl turns vivid yellow, indicating flavonoids in honey (Oscar, et al. 2020). The saponins were found in acetone and HCl. Honey (60 mg) was combined with acetone (2 mL) and hot water (3 mL). After cooling, the solution was shaken for 10 seconds. Saponins in honey are indicated by foam bubbles 1-10 cm high for 10 minutes after adding one drop of HCl 2N (Dubale et al. 2023).

Acetic anhydride, sulphuric acid, and acetone identified steroids and triterpenoids. Acetic anhydride (10 drops) and sulphuric acid concentrated (2 drops) were added to acetone-diluted honey (1 mL). The mixture was shaken vigorously. Red-purplish color suggested triterpenoids, while blue-greenish suggested steroids (Rajkumar et al. 2022). The tannins were identified by lead acetate. The honey (1 mL) was combined with three new 1% lead acetate drops. Yellow precipitates indicated tannins (Rao et al. 2023).

RESULTS

The water contents of stingless bee honey from *H. Itama* is 28.91 ± 1.08%, ranging from 26.9 to 30.0%. The sugar content is 66.68 ± 2.31%, ranging from 62.2 to 69.6%. Meanwhile, the antioxidant capacity of stingless bee honey is 47.92 ± 25.06 µg/mL, ranging from 12.49 to 90.85 µg/mL. As shown in Table 1, during the twelve months of observation, the highest water contents were in August, September, and March, and the lowest was in January. The highest sugar content was in January, and the lowest was in September.

Table 1. Water, sugar contents, and antioxidant activity of *H. itama* honey from *C. calothyrsus* plantation during 12 months of observation

The Month of Observation	Water Content (%)	Sugar Content (%)	Antioxidant (IC_{50}, µg/mL)
Apr 2023	28.5	63.3	15.82
May 2023	27.3	68.0	34.26
Jun 2023	28.4	67.6	44.06
Jul 2023	29.9	67.7	43.95
Aug 2023	>30.0	63.5	28.09
Sep 2023	>30.0	62.2	52.56
Oct 2023	29.0	68.1	90.85
Nov 2023	28.5	68.3	83.64
Dec 2023	28.5	67.7	63.71
Jan 2024	26.9	69.6	69.74
Feb 2024	29.9	68.7	35.91
Mar 2024	>30.0	66.5	12.49
Mean ± SD	28.91 ± 1.08	66.68 ± 2.31	47.92 ± 25.06

Meanwhile, the highest antioxidant capacity of stingless bee honey was in March, and the lowest was in October. During twelve months of observation, as shown in Table 2, the phytochemical contents detected from *H. itama* honey were coumarins, flavonoids, tannins, triterpenoids, carotenoids, saponins, and steroids. Coumarins were detected in all months except January. Flavonoids were detected in all months except July and September.

Table 2. Phytochemicals of *H. itama* honey from *C. calothyrsus* plantation during 12 months observation

Month of observation	Stingless Bee Honey Phytochemicals							
	Alkaloid	Carotenoid	Coumarin	Flavonoid	Saponin	Steroid	Tannin	Triterpenoid
Apr 2023	-	-	+	+	-	-	-	-
May 2023	-	-	+	+	-	-	-	+
Jun 2023	-	-	+	+	-	-	-	-
Jul 2023	-	-	+	-	-	-	-	+
Aug 2023	-	-	+	+	-	+	+	-
Sep 2023	-	-	+	-	-	-	+	-
Oct 2023	-	-	+	+	-	-	+	-
Nov 2023	-	-	+	+	-	-	+	-
Dec 2023	-	-	+	+	-	-	+	-
Jan 2024	-	+	_	+	+	-	+	+
Feb 2024	-	-	+	+	-	-	+	-
Mar 2024	-	+	+	+	-	-	+	-

DISCUSSION

The study is the first to analyze stingless bee honey properties from *C. calothyrsus* plantation. Stingless bees utilize various plant species as sustenance sources: those that produce nectar and those that produce resin. The trees frequently planted in Indonesia because their nectar-producing are *Kaliandra* (*C. calothyrsus*), *Kelapa* (*Cocos nucifera*), *Karet* (*Hevea brasiliensis*), *Kapuk* (*Ceiba pentadra*), and *Rambutan* (*Nephelium lappaceum*) (Juliasih, et al. 2022). Among those plants, *C. calothyrsus* is widespread in Indonesian bee plantations because the rearing of numerous colonies that could forage on the abundant blossoms led to a high honey yield (de Luna, et al. 2020). As plants with rapid growth, *C. calothyrsus* produces more than 100 L of honey per day per hectare. During the flowering season, one colony produces more than 3 L of honey over two months (Harianja, et al. 2023).

Research in Java found that *C. calothyrsus* honey showed antioxidant activity higher than *H. brasiliensis* and *C. pentadra* honey. Flavonoid content from *C. calothyrsus* honey was also higher than other honey (Ustadi et al. 2017). The results indicate that *C. calothyrsus* honey has the maximum percentage of DPPH quenched and has the significant antioxidant capacity. These phenolic compositions in *C. calothyrsus* honey indicate that it is an excellent source of natural antioxidants due to its high antioxidant content (Suliasih et al. 2021). Regarding monofloral plantation for beekeeping, research in Turkiye found that lavender (*Lavandula* spp.) honey showed DPPH radical scavenging activity (Kolaylı et al. 2024). Chestnut (*Castania sativa*) honey, another monofloral honey from Turkiye, also showed antioxidant capacity using FRAP assay (Ucurum et al. 2024).

The water contents of the *H. itama* honey in this study (28.91 ± 1.08%) were higher than research results in Malaysia (Omar et al. 2019; Shamsudin, et al. 2019; Souza et al. 2021) and South Kalimantan, Indonesia (Adalina et al. 2020), ranging from 11.09 to 28.43%. Meanwhile, the water contents of honey in this study were lower than study in East Kalimantan, Indonesia (Saputra et al. 2021) and Malaysia (Fatima et al. 2018; Sujanto, et al. 2021), ranged from 30.80 to 33.67%. According to Indonesian National Standard (SNI) 8664-2018 for Honey, the maximum water content for stingless bee honey is 27.5% (NSAI 2018). Meanwhile, the Department of Standards Malaysia also has quality standards for honey from stingless bees. The department said that honey from stingless bees should have no more than 35% water (Department of Standards Malaysia 2017). Water is the second largest component in honey. Moisture is essential because it can change viscosity, specific weight, age, smell, and crystallization. Reports say stingless bee honey has more water than regular honey because the rainfall and humidity in a tropical rainforest (Cardona et al. 2019). Different floral behaviors and sources may also contribute to the variation in moisture content. Due to fermentation during storage, honey with a high moisture content has a shorter expiration life (Shamsudin et al. 2019). The Department of Standards Malaysia also has quality standards for honey from stingless bees. The department said that honey from stingless bees should have no more than 35% water (Department of Standards Malaysia 2017). Water is the second largest component in honey. Moisture is an important part because it can change the viscosity, specific weight, age, smell, and crystallisation. Reports say that stingless bee honey has more water than regular honey because it comes from the rainfall and humidity in a tropical rainforest (Cardona et al. 2019).

The sugar contents of stingless bee honey in this study (66.68 ± 2.31%) were higher than study results in South Kalimantan, Indonesia (62.97%) and Malaysia (47.25 – 55.61%) (Adalina, et al. 2020). According to Indonesian National Standard for Honey, the sugar content of stingless bee honey minimum is 55% b/b (NSAI, 2018). Meanwhile, the Department of Standards Malaysia stated that honey from stingless bees should have a maximum sugar content of 80 g/100g (Department of Standards Malaysia 2017). The composition of nectar is influenced by environmental factors and the floral origin of nectar (Shamsudin et al. 2019). Research shows that *C. calothyrsus* honey has the highest score for physicochemical quality, especially regarding sugar content, taste, and panelist preferences (Triwanto et al. 2021).

This study found that *H. itama* stingless bee honey had higher antioxidant activity than honey from East Kalimantan (Saputra et al. 2021; Saputra & Nurlina 2022), North Kalimantan (Syafrizal et al. 2020b) and Malaysia (Mahmood et al. 2021; Shamsudin et al. 2019), ranged from 105.53 to 59.91 µg/mL. The antioxidant activity in this study was higher than the results of *H. itama* propolis extract in East Kalimantan (Kustiawan et al. 2022). Meanwhile, the antioxidant activity in this study was lower than study in East Kalimantan (Saputra et al. 2021), and Malaysia (Mahmood et al. 2021; Shamsudin et al. 2019; Ya'akob et al. 2019) ranging from 25.0 to 43.54 µg/mL. Honey is often referenced for its potential antioxidant properties, which may benefit human health. The activity of antioxidants is generally ascribed to their capacity to neutralize free radicals (Rozman et al. 2022). Several healing properties, such as anti-inflammation, antibacterial, antitumor, and anti-obesity, are substantially correlated with antioxidant activity, given that antioxidants are well-established and involved in a diverse diseases (Al-Hatamleh et al. 2020). Honey contains numerous compounds with antioxidant properties. It has been reported that honey's antioxidant activity correlates with phenolics and flavonoids content (Abu Bakar et al. 2017). The phenolic compounds in honey are directly related to the nectars supplied to bees; consequently, honey

from various vegetation origins has different bioactive properties (da Silva et al. 2013).

The phytochemical contents detected from *H. itama* honey in this study were carotenoids, coumarins, flavonoids, saponins, steroids, tannins, and triterpenoids. During twelve months of observation, the phytochemical contents detected from *H. itama* honey were mainly coumarins and flavonoids. Coumarins were detected in all months except January, and flavonoids were detected except in July and September. These results were similar to a study in East Kalimantan, which found coumarins and flavonoids as phytochemical compositions (Saputra et al. 2021; Saputra & Nurlina 2022). Another research for *H. itama* honey from East Kalimantan also found alkaloids, carotenoids, coumarins, flavonoids, saponins, tannins, and triterpenoids compositions (Arung et al. 2022; Saputra et al. 2021; Syafrizal et al. 2020b). These results differed from a study in East Kalimantan, which found alkaloids, terpenoids, and tannins as phytochemical constituents from the extract of *H. itama* propolis (Kustiawan et al. 2022). A study in North Kalimantan found alkaloids, coumarins, flavonoids, tannins, and triterpenoids in stingless bee honey, and a study in South Kalimantan found only flavonoids and saponin as phytochemical contents (Adalina et al. 2020; Syafrizal et al. 2020b). Meanwhile, research from Malaysia found flavonoids and carotenoids as chemical compositions from *H. itama* honey (Mahmood et al. 2021; Rozman et al. 2022; Shamsudin et al. 2019; Sujanto et al. 2021).

Stingless bee honey is a natural source of flavonoids, phenolic acids, and their derivatives. Stingless bee honey contains several phenolic acids, such as gallic, syringic, vanillic, *p*-coumaric, cinnamic, and salicylic acids, as well as some flavonoids, including luteolin, naringenin, and taxifolin (Al-Kafaween et al. 2023). Flavonoids are phenolic compounds responsible for the aroma and antioxidant activity of the honey. Variations in the flavonoid content of honey samples may be attributable to the various vegetation and geographical origins of the nectars bees consume (Mwangi et al. 2024; Nasir et al. 2019). Phenolics are a heterogeneous class of compounds produced by the secondary metabolism of plants, and they can be separated into flavonoids and non-flavonoids. Flavonoids are known as phenolic acids. Examples of non-flavonoids are tannins (Biluca, et al. 2020). It has been reported that flavonoids and phenolic compounds are effective as antioxidants, antitumors, antimicrobials, cardioprotective agents, anti-inflammatory agents, and immune boosters (Maringgal et al. 2019). Phenolic compounds were identified in stingless bee honey. Among the phenolic compounds, flavonoids and coumarin with *p*-coumaric acid are among the most prevalent compounds in the studied samples (Biluca et al. 2017). Honey has a high antioxidant activity due to its phenolic and flavonoid content, which can neutralize free radicals. Honey from stingless bees contains more flavonoids than honeybees (Cianciosi et al. 2018). As stated previously, the phenolic composition can differ based on vegetation and geographical origin, but it can also vary based on the vegetation preference of each bee species (Biluca et al. 2016).

Conclusions: The properties of *H. itama* honey from the *C. calothyrsus* plantation show that the water and sugar contents of the stingless bee honey in this study were higher than in other research. Meanwhile, the antioxidant activity of *H. itama* stingless bee honey from *C. calothrysus* was higher than in other studies. The phytochemical contents detected from *H. itama* honey in this study were carotenoids, coumarins, flavonoids, saponins, steroids, tannins, and triterpenoids. During twelve months of observation, the phytochemical contents detected from this stingless bee honey were mainly coumarins and flavonoids. Even though the properties of stingless bee honey can differ based on vegetation and geographical origin, *H. itama* honey collected from *C. calothyrsus* plantation in East Kalimantan, Indonesia, showed potential antioxidant activity and phytochemical contents, which is advantageous to human health.

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Ethics: The research was conducted *in vitro* and not with animals or human.

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ARAŞTIRMA MAKALESI / RESEARCH ARTICLE

HERBAL-INFUSED EGYPTIAN BEE HONEY, A BOON OR A CURSE, ITS IMPACT ON SENSORIAL, PHYSICOCHEMICAL & ANTIBACTERIAL PROPERTIES

Bitkisel İçerikli Mısır Arı Balı, Nimet mi Lanet mi, Duyusal, Fizikokimyasal ve Antibakteriyal Özellikler Üzerindeki Etkisi

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ABSTRACT

Herbal honey mixture remedies are well known for their therapeutic benefits in traditional medicine. This research aspired to assess melissopalynological, sensorial, physicochemical, & antibacterial activity for three types of honey (clover, citrus, and cucurbits) and their mixtures with black seed, date palm pollen, & wheat germ at 1, 2.5, & 5%. The best mixtures were chosen according to overall acceptability. Consumer's preference was given to raw honey. However, some mixtures were as acceptable as raw honey. Melissopalynological analysis and lower glucose level compared to fructose are evidences that honeys are natural. Infusing herbs with different types of honey caused higher electrical conductivity, free acidity, ash, H_2O_2, HMF levels, and lower pH. Type of honey and herb may affect the physicochemical characteristics of honey in different ways. Honey whether used alone or in combination with the three herbs, demonstrated the same significant antibacterial effect for *Staphylococcus aureus* and MRSA. Inhibition zones of honey and its mixtures were lower than the control for *Pseudomonas aeruginosa*. Most undiluted samples created larger bacterial inhibition zones than their 50% diluted counterparts. Depending on the type of honey, the herb and additive concentration, infusing herbs with honey could alter its chemical, physical, and antibacterial qualities.

Keywords: Egyptian bee honey, Herbs, Sensory, Physicochemical & Antimicrobial

ÖZ

Bitkisel bal karışımı ilaçların geleneksel tıpta tedavi edici faydaları olduğu iyi bilinmektedir. Bu araştırma, üç bal türünün (yonca, narenciye ve kabakgiller) ve bunların çörek otu, hurma poleni ve buğday tohumu ile %1, 2,5 ve 5 oranında karışımlarının melissopalynolojik, duyusal, fizikokimyasal ve antibakteriyel aktivitelerini değerlendirmeyi amaçlamıştır. En iyi karışımlar genel kabul edilebilirliğe göre seçilmiştir. Tüketici tercihini ham baldan yana kullanmıştır. Ancak, bazı karışımlar ham bal kadar kabul edilebilirdi.Melissopalynolojik analiz ve fruktoza kıyasla daha düşük glikoz seviyesi balların doğal olduğunun kanıtıdır. Bitkilerin farklı bal türleriyle aşılanması daha yüksek elektrik iletkenliği, serbest asitlik, kül,H_2O_2, HMF seviyeleri ve daha düşük pH'a neden olmuştur. Bal ve bitki türü balın fizikokimyasal özelliklerini farklı şekillerde etkileyebilir. Bal tek başına veya üç bitki ile birlikte kullanıldığında, Staphylococcus aureus ve MRSA için aynı önemli antibakteriyel etkiyi göstermiştir. Bal ve karışımlarının inhibisyon bölgeleri Pseudomonas aeruginosa için kontrolden daha düşüktür. Seyreltilmemiş numunelerin çoğu %50 seyreltilmiş muadillerine göre daha geniş bakteriyel inhibisyon bölgeleri oluşturmuştur. Balın türüne, bitkiye ve katkı maddesi konsantrasyonuna bağlı olarak, bitkilerin bal ile infüze edilmesi kimyasal, fiziksel ve antibakteriyel niteliklerini değiştirebilir.

Anahtar Kelimeler: Mısır arı balı, Bitkiler, Duyusal, Fizikokimyasal ve Antimikrobiyal

GENİŞLETİLMİŞ ÖZET

Amaç: Doğal bir ikili olan bal ve şifalı bitkiler geleneksel olarak yaraları tedavi etmek, astımı hafifletmek, hamile kadınlarda anemiyi önlemek, bağışıklığı artırmak ve sağlıklı bir yaşamı desteklemek için kullanılmaktadır. Bu araştırma, üç çeşit Mısır balı ve bunların bitkisel karışımlarının melissopalynolojik, duyusal, fizikokimyasal ve antibakteriyel aktivitelerini incelemeyi amaçlamıştır.

Gereç ve Yöntem: Üç farklı arı balı örneği, hasat mevsimlerinde Mısır'daki El-Gharbia Valiliği'nde bulunan çeşitli arı kovanlarından elde edilmiştir. Hammaddeler (siyah tohumlar, palmiye polen taneleri ve buğday tohumu) yerel pazardan açıkta satılan bitkiler olarak satın alınmıştır. Elde edilen sonuçlar, melissopalynolojik analizde bu türden gelen polenin baskın olduğunu, bu bitkinin arılar için birincil polen ve nektar tedarikçisi olduğunu ve potansiyel olarak fizikokimyasal ve granülasyon özelliklerini etkilediğini göstermiştir.

Bulgular: Fizikokimyasal özelliklere göre, yonca ve narenciye balı, istatistiksel analizde gösterildiği gibi, Cucurbitaceae balından önemli ölçüde farklı nem seviyelerine sahiptir. Bu oranlar normal aralıktadır (18.00-20.00g/100g). İstatistiksel olarak, glikoz ve fruktoz seviyelerinde bal türleri arasında önemli bir fark bulunmamıştır. Narenciye balı en yüksek şeker azalmasını (63,08±0,43g/100g) sergilemiş, bunu yonca balı (61,08±0,50g/100g) izlemiştir. Buna karşılık, en düşük değer Kabakgiller balında kaydedilmiştir (58.22±0.32g/100g). Kabakgiller balının DN değeri en yüksek (30,80±0,56) ve en düşüktür.

Bitkilerin bal ile karıştırılmasının pH üzerindeki etkisine ilişkin olarak, test edilen bal türleri ve karışımları için değerler 3,98±0,03 (1BS1) ile 5,75±0,11 (Kabakgiller balı) arasında değişmiştir. Yonca balına çörek otu eklenmesi pH'ı düşürürken, buğday tohumu eklenmesi pH'ı etkilememiştir. Bu çalışmada, bala bitki eklenmesi genel olarak serbest asitliği ve hidrojen peroksiti ($_{H2O2}$) artırmıştır. Yonca ve narenciye balına buğday tohumu eklendiğinde elektriksel iletkenlik değerleri artmıştır. Bununla birlikte, kabakgil balına buğday tohumu eklendiğinde istatistiksel olarak anlamlı bir değişiklik olmamıştır. Kül içeriği balın botanik kökeni için bir kalite göstergesi olarak kabul edilmiştir. Yonca ve kabakgil balları bitkilerle birleştirildiğinde kül miktarı artmıştır. HMF, bu ilaveden etkilenmeyen narenciye balı hariç, yonca ve kabakgil ballarının bitkilerle karıştırılmasıyla artmaktadır. Test edilen tüm ballar kabul edilebilir sınırlar içinde kalmıştır. Hurma poleninin en yüksek toplam katı, kül ve karbonhidrat değerlerine (sırasıyla 96.80±0.21, 8.80±0.19 ve 68.11±0.09 g/100g) sahip olduğu tespit edilmiştir. En düşük toplam katı ve kül değerleri buğday tohumunda kaydedilirken (90.50±0.54 ve 3.50±0.06 g/100g), çörek otu karbonhidratlar açısından en düşük değere sahiptir.

Sonuç: Duyusal değerlendirmelerde, tüketiciler genellikle herhangi bir katkı maddesi içermeyen saf bal arısı ürünlerini tercih etmiştir. Bununla birlikte, bazı karışımlar da iyi karşılanmış ve faydalı özellikleri nedeniyle sağlık amacıyla kullanılabilmiştir. Bitkiler içeren veya içermeyen bal *Staphylococcus aureus* ve MRSA (Gram-pozitif bakteriler) üzerinde antibakteriyel etkiye sahip olup *Pseudomonas aeruginosa* (Gram-negatif bakteriler) üzerinde etkiye sahip değildir. Seyreltilmemiş örneklerin çoğunda seyreltilmiş örneklere kıyasla daha geniş bir bakteriyel inhibisyon bölgesi gözlenmiştir.

INTRODUCTION

Antimicrobial resistance (AMR), caused by bacteria evolving to resist antibiotics, is a pressing public health issue in the 21st century. By the year 2050, it is estimated that AMR bacteria could result in the loss of 10 million lives annually. (Kraker et al. 2016, O'Neill 2016). Prestinaci et al. (2015) agreed that the prevalence of antimicrobial resistance was a pressing issue that required a global response and a coordinated action plan to address it. The emergence of antibiotic-resistant bacteria worldwide has led to a lack of effective treatments for several ailments, lengthening treatment times and raising medical expenses (Albaridi, 2019)**.** Ancient cultures recognized honey's therapeutic properties and used it to promote health and treat ailments. The human use of honey since 8,000 years, as reflected in certain Stone Age paintings (Kuropatnicki et al. 2018, Saikaly and Khachemoune 2017). Honey has a long history of use as a natural remedy for common infections. It continues to be a prominent component of many traditional medical regimens for maladies such as cancer, heart disease, cataracts, asthma, and infected wounds (Gündoğdu et al. 2019). Furthermore, the properties and structure of honey are affected by botanical origin, geography, season, environmental conditions, and beekeeper practices (Hossain et al. 2022, Young and Blundell 2023). In recent years, it has been discovered that many drugs

employed in conventional medicine originate from organic sources such as honey and medicinal plants (Khan et al. 2018). Current scientific studies have disclosed that honey is rich in biologically active substances, frequently utilised in folk medicine. It contains antimicrobial, anti-inflammatory, antiproliferative, antimutagenic, anticancer, antidiabetic, antioxidant, antibacterial, and antifungal properties (Küçükaydın et al. 2023).

Honey and herbs, a natural duo, have been traditionally used to treat wounds, alleviate asthma, prevent anemia in pregnant women, boost immunity, and support a healthy life (Kumar et al. 2024). For centuries, *Nigella sativa* seeds and oil have been valued in Ayurvedic, Unani Tibb, and other traditional healing practices for their potential benefits in treating divergent illnesses (Khatoon et al. 2024, Shafodino et al. 2022). It belongs to the Ranunculaceae family (Yarnell and Abascal 2011**).** On the other hand, date palm pollen, or DPP (*Phoenix dactylifera* L.), is a member of the Aceraceae family; it was historically utilized as a medicinal substance by the ancient Chinese and Egyptian cultures. Its widespread utilization is prominent in the Middle East, due to its impressive nutrient profile, comprising proteins, vitamins, minerals, trace elements, carbohydrates, lipids, organic acids, sterols, nucleic acids, enzymes, and cofactors, date palm pollen is a valuable natural dietary supplement. The crucial role of bioactive volatile unsaturated fatty acids, phenolic acids, flavonoids, and other phenolic compounds lies in their powerful antioxidant properties and their anti-breast cancer abilities (El-Kholy et al., 2019).

Wheat germ is a valuable by product of wheat processing, comprising 2-3% of the whole wheat kernel (Yu et al. 2015). It is estimated that its global production reaches 25 million tons per year (Song et al. 2019). Its extract offers a natural and accessible source of antibacterial and antioxidant compounds, making it a potential candidate for use in food supplements or pharmaceutical applications (Mahmoud et al. 2015)**.** This study represents the first attempt to evaluate the effects of combining several herbs with honey. No prior research has been done to determine the impact of combining herbs in this manner; however, studies have only examined the effects of combining black seed oil with honey, not whole black seeds like (Raimi et al. 2024). Even if there are studies on this topic, they focus on the impact of consuming the mixture on patients who have stomach bacteria (Abdullahi, 2023). Thus, understanding how mixing affects the properties, composition, efficacy, and quality of honey is so crucial.

This research sought to examine melissopalynological, sensorial, physicochemical, & antibacterial activity for three types of Egyptian bee honey and their herbal blends.

MATERIALS AND METHODS

Materials

Honey samples: Three different samples of bee honey were obtained from several apiaries located at El-Gharbia Governorate specifically in Tanta region, Egypt during their harvest seasons as follow clover honey (April-May), citrus honey (March) and cucurbitaceae honey (August-September) during 2023. At the laboratory of the apiary yard, Experimental Station, Faculty of Agriculture, Cairo University, all samples (three triplicates per each) were kept at -18ºC until analysis.

Herbs: Raw materials (black seeds, date palm pollen, & wheat germ) were purchased from Harraz Medicinal plant company, the most famous Egyptian herbalist located in Giza Governorate as openly sold herbs. Black seeds were ground before adding them to honey while the other two materials were used directly.

Chemicals: All chemicals used for analysis were purchased from El-Gomhouria Company for Trading Chemicals and Medical Supplies, Cairo, Egypt.

Bacterial Strains: Pathogenic bacteria used as indicators in this assay were obtained from the American Type Culture Collection (ATCC): *Staphylococcus aureus* ATCC 25,923, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43,300, and *Pseudomonas aeruginosa* ATCC 35,032.

METHODS

Pollen analysis: As claimed by Louveaux et al (1978), pollen grains from all evaluated bee honey specimens were examined. A total of ten grams of honey were mixed in twenty millilitres of warm water and centrifuged at 3,500 rpm for ten minutes. After discarding the liquid filtrate, fresh water was added into the tube, and then the centrifuge ran for a further ten minutes. After the silt was completely spread out

over a 20 x 20 mm area and dried slightly at 40 degrees, the glycerine gelatine was added, and a light microscope was used to examine the results. Each sample had a minimum of 100 pollen grains, which were counted and identified via light microscopy.

Physiochemical analysis: The honey samples were subjected to chemical analysis at the food safety and quality control laboratory of Cairo University's Faculty of Agriculture, Giza, Egypt as follow: -

Sugars (Fructose, Glucose, and Sucrose): These were analyzed by HPLC with a Phenomenex Luna NH_2 column (250×4.6 mm). The column temperature was maintained at 30 ºC, and the mobile phase consisted of Acetonitrile: HPLC grade water in an 80:20 (v:v) ratio. Detection was performed using an RI detector, and data integration was carried out using ClarityChrom software.

Hydroxymethylfurfural (HMF): It was determined by spectrophotometer UV/ V, Jenway, England.

Moisture: Water content was determined with a digital refractometer at 20 ºC according to **AOAC, 1990**.

Electrical conductivity: This was conducted via a conductivity meter on a 20% honey weight/volume solution in water at 20 ºC, with the 20% representing the honey's dry matter content. The instrument utilized was a conductivity meter, Five Easy, Mettler-Toledo, Switzerland.

pH: It was measured by pH meter, Boeco, Germany, calibrated with buffers having pH values of 4, 7, and 10.

Ash content: It was determined according to the methods of **AOAC, 1990**.

Free acidity: This was measured by equivalence point titration methods**.**

Hydrogen peroxide assay (H_2O_2): In the presence of peroxidase (HRP), H_2O_2 reacts with 3.5-dichloro-2-hydroxybenzensulphonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore (**Aebi 1984**).

Diastase activity: This was gauged after shade to get the DN (Gothe unit).

Sensory evaluation

Samples preparation: Honey samples were analyzed at room temperature. The honey samples were presented in clear containers to facilitate colour assessment. The samples were prepared one day prior to tasting in order to permit the honey's scent to develop within the headspace of the containers. Honey and additives were blended using wooden spoons. Samples were labeled as follows: 1, 2, and 3 for clover, citrus and cucurbit family honey, respectively, as well as black seed (BS), date palm pollen (DPP) and wheat germ (WG).

The three raw materials were incorporated into each type of bee honey at three discrete percentages (1%, 2.5%, and 5%). This resulted in a total of 27 samples as follows:

- 1BS (1%), 1BS (2.5%), &1BS (5%); 1DPP (1%), 1DPP (2.5%), & 1DPP (5%); 1WG (1%), 1WG (2.5%), & 1WG (5%).

- 2BS (1%), 2BS (2.5%), & 2BS (5%); 2DPP (1%), 2DPP (2.5%), & 2DPP (5%); 2WG (1%), 2WG (2.5%), & 2WG (5%).

- 3BS (1%), 3BS (2.5%), & 3BS (5%); 3DPP (1%), 3DPP (2.5%), & 3DPP (5%); 3WG (1%), 3WG (2.5%), & 3WG (5%).

The sensory attributes (odor, colour, taste, texture, and overall acceptability) of each sample were assessed using a 10-point unstructured descriptive evaluation scale, where 1 indicates strong dislike and 10 represents strong liking (Singh-Ackbarali & Maharaj 2014). Twenty panelists from the Faculty of Agriculture, Cairo University, were asked to rate the sensory appeal of honey samples and their mixtures. The sensory analysis took place in a controlled environment with standard lighting conditions. Instructions were written under sensory sheets as follows:

- Evaluate odor first.
- Mix before taste.
- Before and during the analysis sessions, rinse your mouth with water

Measurement of antimicrobial activity: The antimicrobial properties of honey samples and their mixtures were carried out by the agar-well diffusion assay, following the protocol described by Balouiri et al. (2016). All samples were diluted in absolute ethanol at a 1:1 ratio. In brief, bacterial inoculum was evenly spread across the entire Mueller-Hinton agar plate surface. An aseptic hole with a diameter of 8 mm was then created using a sterile cork borer. Subsequently, 100 µL of the extract solution was

added to the well at the specified concentration. Negative control involved the absolute ethanol alone, while the positive control consisted of novobiocin and polymyxin B against Gram-positive and Gram-negative bacteria, separately. The agar plates underwent a 24-hour incubation period at 37 °C. The inhibition zone diameters (mm) around the wells were measured to evaluate the antimicrobial properties. The assay was conducted in triplicate.

Statistical analysis

Differences between samples were explored through one-way ANOVA, T-tests, and LSD tests at a significance level of $P \leq 0.05$.

RESULTS

Melissopalynological analysis

Figure (1) shows the pollen varieties in examined bee honey; there was a wide variability between bee honey samples according to the melissopalynological analysis. It could be concluded that the highest percentage of pollen grains was for clover (*Trifolium alexandrinum*) (56%) (Fig. 1A). The second place was Family: Umbelliferae (16%) followed by pollen of *Eucalyptus* spp. (12.26%) in clover honey. On the other side, citrus pollen had the highest percentage (30.43%) and followed by pollen of date palm (*Phoenix dactylifera*) (Fig. 1B). The main source of nectar and pollen comes from Cucurbitaceae (52.38%) for the third bee honey sample (Fig. 1C). Otherwise, *Zea mays* pollen grain is the second most frequent grain (19.04%).

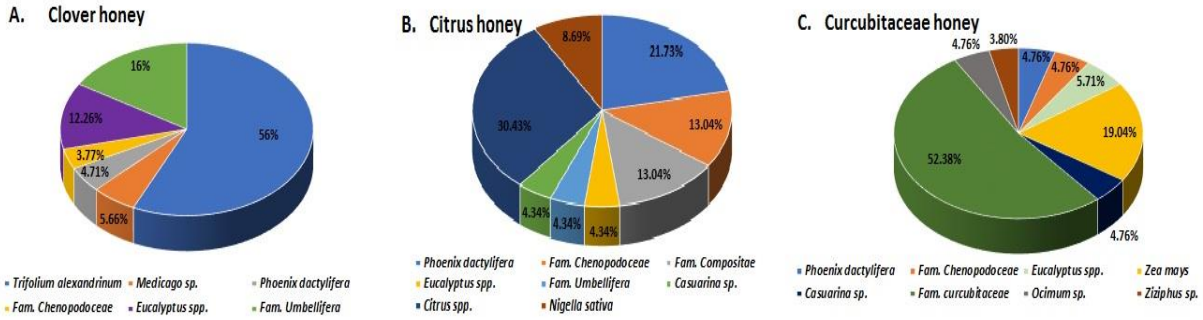


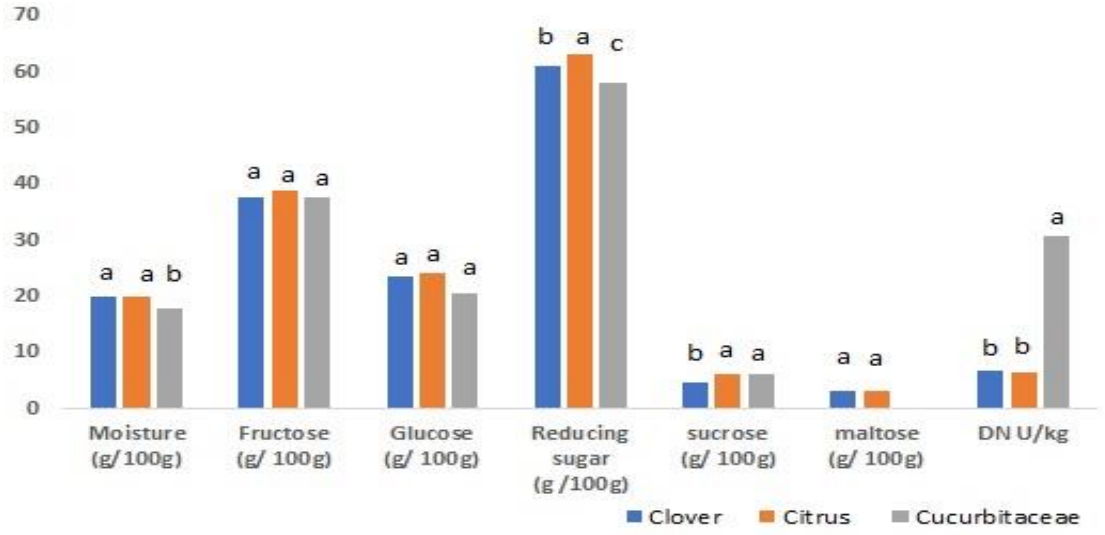
Figure 1. Pollen spectrum of the tested bee honey

Physicochemical characteristics

Bee honey: Clover and citrus honey had significantly distinct moisture levels than Cucurbitaceae honey, as displayed by statistical analysis. These percentages were within the normal range (18.00-20.00g/100g) when compared with Codex & the Egyptian Organization for Standardization and Quality Control (EOSC)**.**

Statistically, no significant differences were found between honey types in glucose and fructose levels (Fig. 2). However, clover honey had significantly lower sucrose levels ($P \leq 0.05$) than the other two types of honey. Sucrose content ranged from 4.70±0.07 to 6.33±0.15 g/100g. Except for clover honey (4.70±0.07g/100g), the examined honey samples did not meet international and national rules, which state that the concentration of sucrose content should not exceed 5g/100g. Furthermore, no significant differences were noticed between clover and citrus honey regarding maltose levels (3.12±0.14 & 3.28±0.19g/100g) in the previous respective order. The obtained data show that disaccharides, such as sucrose and maltose, were present at higher concentrations than those specified honey requirements published by the EOSC. Citrus honey (Fig. 2) exhibited the greatest reducing sugar (63.08±0.43g/100g) followed by clover honey (61.08±0.50g/100g). Conversely, the least value was recorded in Cucurbitaceae honey (58.22±0.32g/100g) this value is not accepted by both of Codex, 2001 and EOSC, 2005**.** The variations of reducing sugars among the three types were substantial ($P \leq 0.05$). DN, one of the honey quality indicators, is adopted to ascertain if honey has been exposed to heating. In Fig. 2, the DN of Cucurbitaceae honey had the highest value (30.80±0.56) and it was acceptable by Codex

Alimentations (2001) (DN>8). In contrast, the least value was recorded by citrus honey (6.50±0.26). Statistically, no significant differences were discovered between clover and citrus honey varieties. However, substantial differences were noted between both of these varieties and the third type.



Each bar represents the mean; means with the same letter are not significantly different ($P \leq 0.05$).

Figure 2. Moisture and sugar analysis of three tested types of honey

Bee honeys and their mixtures: Regarding the effect of mixing herbs with honey on pH, data in Table 1 clarified that pH values for tested types of honey and their mixtures ranged from 3.98±0.03 (1BS1) to 5.75±0.11 (Cucurbitaceae honey). Adding black seeds to clover honey reduced pH, while adding wheat germ did not affect it. No statistical differences were found between citrus honey and its samples. Conversely, mixing cucurbit honey with herbs reduced the pH. The order of the samples was as follows: cucurbit honey, cucurbit honey mixed with wheat germ, and then cucurbit honey mixed with

Table 1. Effect of infusing herbs in types of honey on physicochemical parameters

Parameters	Honeys and their mixtures (mean ±SD)									
	Clover	1BS1	1WG2.5	Citrus	2BS1	2DPP1	2WG2.5	Cucurbitaceae	3BS1	3WG1
pH	4.05±0.09^{B} $_{ab}$	3.98±0.03^{b}	4.23±0.11 $_{a}$	4.12±0.07 $_{B}$	4.31±0.05	4.34±0.20	4.27±0.07	5.75±0.11^{Aa}	5.34±0.15^{c}	5.53±0.06^{b}
Free acidity meq/kg	17.50±0.35^{Ac}	22.00±0.31 $_{b}$	23.60±0.04^{a}	14.00±0.20 $_{Bd}$	16.00±0.17^{c}	18.00±0.35^{b}	23.00±1.00^{a}	18.00±0.20 $_{Ac}$	23.5±0.62^{a}	20.50±0.04 $_{b}$
Ec (ms/cm)	0.241±00.21^{Ac}	0.273±0.0004^{b}	0.322±0.0002^{a}	0.02±0.0017^{Bd}	0.246±0.0002^{c}	0.299±0.0001^{b}	0.304±0.0002^{a}	0.1316±0.001^{Cb}	0.350±0.003^{a}	0.140±0.0002^{b}
Ash	0.03±0.01^{B} $_{b}$	0.12±0.01^{a}	0.13±0.01 $_{a}$	0.06±0.01^{B} $_{b}$	0.15±0.01 $_{a}$	0.15±0.02 $_{a}$	0.04±0.01 $_{b}$	0.79±0.02^{Ac}	0.90±0.03^{b}	1.30±0.01^{a}
H_2O_2 (mM/100 g)	80.00±2.60^{Ac}	217.00±2.00^{a}	189.66±5.03^{b}	67.00±1.50 $_{Bd}$	183.00±1.31^{b}	205.00±2.00^{a}	127.00±2.00^{c}	60.84±1.00 $_{Cc}$	365.00±10.00^{a}	328.00±0.70^{b}
HMF (mg/kg)	16.20±0.47^{Ac}	21.11±0.05 $_{b}$	23.10±0.07^{a}	5.50±0.18^{B} $_{a}$	1.00±0.10 $_{b}$	0.10±0.01 $_{c}$	5.50±0.20 $_{a}$	1.30±0.05^{Cc}	2.20±0.03^{b}	3.00±0.07^{a}

Mean±SD followed by the capital different letters within rows denote significant differences between the three types of honey ($P \leq 0.05$).

Mean±SD followed by the small different letters within rows stand for significant differences between the three types of honey and their mixtures ($P \leq 0.05$).

Bee honeys and their mixtures: Regarding the effect of mixing herbs with honey on pH, data in Table 1 clarified that pH values for tested types of honey and their mixtures ranged from 3.98±0.03 (1BS1) to 5.75±0.11 (Cucurbitaceae honey). Adding black seeds to clover honey reduced pH, while adding wheat germ did not affect it. No statistical differences were found between citrus honey and its samples. Conversely, mixing cucurbit honey with herbs reduced the pH. The order of the samples was as follows: cucurbit honey, cucurbit honey mixed with wheat germ, and then cucurbit honey mixed with black seed. The pH values of three tested types of honey and their mixtures were acidic and within the standard limits of Codex, 2001.

In this study, adding herbs to honey generally increased free acidity and hydrogen peroxide (H_2O_2) levels. All samples did not exceed the total acidity than limit 50 med /kg as required by Codex Alimentations (2001).

For EC, all tested samples ranged from 0.02±0.0017 for citrus honey to 0.350±0.003 for cucurbitaceae honey with black seed. Electrical conductivity values increased when wheat germ was added to clover and citrus honey. Nonetheless, there was no statistically significant change when wheat germ was added to cucurbit honey. The electrical conductivity values increased when honey was combined with palm pollen or black seed at lower rates. The examined Egyptian types of honey and their mixtures were within the standard limits (≤ 0.8 ms/cm) of Codex Alimentations (2001). A honey's ash content can be used to evaluate its mineral content. It is regarded as an indicator of quality for the botanic origin of honey. The amount of ash increased when cucurbit and clover honeys were mixed with herbs. Herbal infused citrus honey had similar trend, except for the wheat germ-infused one. Cucurbit honey and it blends are within the acceptable range (0.6–1.2 g/100 g) as mentioned by Codex Alimentations (2001).

HMF value of tested honeys and their mixture ranged from 0.10± 0.01 (2DPP1) to 23.10±0.07 in (1WG2.5). HMF increases by mixing clover and cucurbit honeys with herbs, except citrus honey, which was different by this addition. All tested honeys fell within the acceptable limits, as mentioned by the EOSC (2005) (not exceeding 80 mg/kg).

Raw materials used in honey mixtures: The outcome of the chemical composition of black seeds, date palm pollen & wheat germ represented in Table 2. It was discovered that date palm pollen possessed the highest total solid, ash, and carbohydrate (96.80±0.21, 8.80±0.19, & 68.11±0.09 g/100 g), respectively. The lowest values of total solid and ash were recorded in the wheat germ (90.50±0.54 & 3.50±0.06 g/100 g) while the black seed had the lowest value regarding carbohydrates. The differences between the three herbs in the previous nutrients were significant ($p \leq 0.05$). Moreover, descendingly substantial variations were noticed between values of fat and fiber of black seed, wheat germ, & palm pollen.

Table 2. Raw materials used in honey mixtures

Raw	Total solid	Ash	Fat	Protein	Fiber	Carbohydrate
Black seed	95.00±0.37^{b}	4.00±0.04^{b}	20.00±0.10^{a}	25.00±0.12^{a}	8.00±0.08^{a}	38.00±0.23^{c}
Date palm pollen	96.80±0.21^{a}	8.80±0.19^{a}	1.70±0.05^{c}	20.74±0.31^{b}	0.65±0.02^{c}	68.11±0.09^{a}
Wheat germ	90.50±0.54^{c}	3.50±0.06^{c}	5.00±0.05^{b}	20.50±0.02^{b}	2.70±0.05^{b}	58.80±0.14^{b}

Mean ±SD followed by the small different letters within the same column denote signify significant differences between the three types of herbs ($P \leq 0.05$).

Sensory evaluation

In order to explore the impact of distinct additives (black seed, date palm pollen, and wheat germ) on the sensory properties of honey, a human sensory analysis was conducted. The best samples were chosen according to the total acceptability of the consumers.

Table (3) illustrates the sensory analysis of clover honey and its mixture samples. Clover honey, clover honey + black seed at 1%, clover honey + wheat germ at 1 & 2.5% are similar in total acceptability.

Table 3. Sensory analysis of clover honey and its mixture samples

Samples	Odour (10)	Colour (10)	Taste (10)	Texture (10)	Total acceptability (10)
Clover honey (1)	7.85±1.59^{a}	8.30±1.45^{a}	8.05±1.20^{a}	8.10±1.61^{a}	8.50±1.24^{a}
1BS (1%)	6.65±1.96^{bc}	5.95±1.75^{e}	7.15±1.86^{abcd}	6.80±2.06^{bc}	8.10±1.14^{ab}
1BS (2.5%)	6.30±2.19^{cd}	4.90±1.37^{f}	6.45±2.04^{d}	6.65±1.93^{bc}	6.80±1.78^{de}
1BS (5%)	6.80±2.42^{abc}	4.70±1.87^{f}	6.20±2.32^{de}	6.10±2.39^{c}	6.20±2.11^{ef}
1DPP (1%)	6.45±2.13^{bcd}	7.00±1.84^{cd}	6.95±2.06^{bcd}	7.05±1.88^{bc}	7.45±1.88^{bcd}
1DPP (2.5%)	6.05±2.40^{cd}	6.10±1.69^{de}	6.20±1.99^{de}	7.15±1.35^{ab}	6.50±2.20^{def}
1DPP (5%)	5.35±2.48^{d}	4.90±2.47^{f}	5.25±2.34^{e}	6.45±1.83^{bc}	5.50±2.20^{f}
1WG (1%)	7.45±1.99^{ab}	8.00±1.18^{ab}	7.55±1.69^{abc}	8.05±1.32^{a}	8.25±1.64^{ab}
1WG (2.5%)	7.05±1.73^{abc}	7.25±1.37^{bc}	7.70±1.49^{ab}	7.35±1.53^{ab}	7.90±1.26^{abc}
1WG (5%)	6.70±1.68^{bc}	6.55±1.86^{cde}	6.55±1.56^{cd}	7.00±2.12^{bc}	6.85±1.56^{de}

Mean ±SD followed by the small different letters within the same column denote significant differences at (P≤0.05) BS: black seed, DPP: date palm pollen, and WG: wheat germ.

Regarding the sensory analysis of citrus honey and its mixtures, Table (4) displays that none of the blends are quite like raw citrus honey in their sensory properties. Next in preference in total acceptability are black seed (1%), date palm pollen (1%), and wheat germ with concentrations of 1 & 2.5%.

Table 4. Sensory analysis of citrus honey and its mixture samples

Samples	Odour (10)	Colour (10)	Taste (10)	Texture (10)	Total acceptability (10)
Citrus honey (2)	8.85±1.01^{a}	9.20±0.81^{a}	8.75±0.98^{a}	8.80±0.98^{a}	9.00±1.00^{a}
2BS (1%)	7.05±1.83^{bc}	6.30±2.15^{de}	7.30±1.73^{b}	6.65±1.71^{bc}	7.05±1.63^{bcd}
2BS (2.5%)	6.60±1.50^{bcd}	6.35±1.90^{de}	6.95±1.77^{bc}	6.60±1.74^{c}	6.75±1.70^{cd}
2BS (5%)	5.80±1.78^{def}	5.15±1.77^{fg}	6.30±2.18^{c}	6.05±2.09^{cd}	5.75±2.12^{e}
2DPP (1%)	6.35±1.80^{cde}	6.60±1.85^{cd}	6.90±1.90^{bc}	6.85±1.49^{bc}	7.10±1.7^{bc}
2DPP (2.5%)	5.65±1.82^{ef}	5.60±1.59^{ef}	6.15±1.10^{c}	6.35±1.74^{c}	6.20±1.86^{de}
2DPP (5%)	4.95±2.01^{f}	4.60±1.77^{g}	5.20±2.08^{d}	5.35±1.96^{d}	5.40±1.91^{e}
2WG (1%)	7.40±1.43^{b}	7.70±1.35^{b}	7.60±1.50^{b}	7.50±1.36^{b}	7.80±1.29^{b}
2WG (2.5%)	7.00±1.73^{bc}	7.25±1.69^{bc}	7.25±1.74^{b}	6.80±2.01^{bc}	7.30±1.55^{bc}
2WG (5%)	6.55±1.80^{bcd}	6.65±2.06^{cd}	6.85±1.80^{bc}	6.55±2.04^{c}	6.75±2.05^{cd}

Mean ±SD followed by the small different letters within the same column denote significant differences at (P≤0.05). BS: black seed, DPP: date palm pollen, and WG: wheat germ.

Table (5) illustrates the sensory analysis of cucurbit family honey and its mixture samples. The most aacceptable samples in table 5 are cucurbit honey, cucurbit honey+ black seed (1%) and wheat germ (1%).

In general, it could be concluded from the three sensory tables that the consumer's preference was given to honey without additives in all sensory characteristics. The most acceptable additive was for both black seed & wheat germ, while the least acceptability was for palm pollen.

Statistical analysis revealed that the most acceptable mixtures were for seven samples (1BS 1%, 2BS 1%, 3BS1%, 2DPP 1%, 1WG2.5%, 2WG2.5%, & 3WG1%). Depending on the obtained results, physicochemical analysis and microbiological tests were performed for previous mixtures.

Table 5. Sensory analysis of cucurbit family honey and its mixture samples

Samples	Odour (10)	Colour (10)	Taste (10)	Texture (10)	Total acceptability (10)
Cucurbit family honey (3)	7.35±1.87^{a}	7.85±1.71^{a}	7.75±1.10^{a}	7.65±1.81^{a}	7.75±1.89^{a}
3BS (1%)	7.15±1.46^{ab}	6.65±1.42^{b}	6.70±1.8^{ab}	6.45±1.47^{b}	6.85±1.93^{abc}
3BS (2.5%)	6.65±1.50^{abc}	6.05±1.57^{bc}	6.50±2.12^{b}	6.15±1.59^{bc}	6.45±1.96^{bc}
3BS (5%)	5.90±1.86^{cd}	4.40±1.31^{e}	5.05±2.06^{de}	5.50±1.82^{bc}	5.15±1.87^{e}
3DPP (1%)	5.80±1.54^{cd}	6.05±1.39^{bc}	5.85±1.50^{bcde}	6.35±0.88^{bc}	6.00±1.30^{bcde}
3DPP (2.5%)	5.65±1.69^{cd}	5.35±1.50^{cd}	5.35±1.69^{cde}	5.90±1.02^{bc}	5.40±1.43^{de}
3DPP (5%)	5.00±1.81^{d}	4.55±1.47^{de}	4.80±1.91^{e}	5.85±1.84^{bc}	5.05±1.90^{e}
3WG (1%)	6.50±2.09^{abc}	6.90±1.21^{b}	6.65±1.84^{b}	6.60±1.14^{b}	7.00±1.49^{ab}
3WG (2.5%)	6.30±1.69^{bc}	6.50±1.79^{b}	6.10±2.22^{bcd}	6.50±1.61^{b}	6.25±1.86^{bcd}
3WG (5%)	6.50±1.70^{abc}	6.20±1.64^{bc}	6.15±2.18^{bc}	6.05±1.79^{bc}	5.85±2.21^{cde}

Mean ±SD followed by the small different letters within the same column denote significant differences at (P≤0.05). BS: black seed, DPP: date palm pollen, and WG: wheat germ.

Antimicrobial activity

The antimicrobial activity of honeys and their mixtures against three different pathogenic bacteria is depicted in Figure 3. Honey, whether used alone or combined with black seeds, had the same effect on *Staphylococcus aureus* as control (novobiocin antibiotic).

The impact of mixtures " honey with wheat germ and palm pollen" was weak, as the inhibition zone ranged from 20.3 to 26.7 mm.

For MRSA bacteria, wheat germ and a mixture of clover honey with wheat germ 2.5% had no significant effect on bacteria. The remaining samples, entailing honey alone and various honey mixtures, exhibited inhibitory effects on MRSA growth. Date palm pollen (b) being the most effective in suppressing its development.

Clear significant differences between the control sample (polymyxin B) and the rest samples (types of honey or mixtures) were noticed; none of them reached the same degree of inhibition of *Pseudomonas aeruginosa* bacterial growth as the control (C).

Figure 4 shows the antimicrobial activity of samples compared with the same diluted samples (50%). For *Staphylococcus aureus*, there are no significant differences between the diluted and nondiluted samples in the microbial growth at clover honey, palm pollen, citrus honey with black seeds 1%, cucurbit honey with black seeds 1% or wheat germ 1%, and clover honey with wheat germ 2.5%. Nonetheless, nondiluted samples (citrus, cucurbit honeys, clover honey+ black seeds 1%, and citrus honey with palm pollen 1% or wheat germ 2.5%) inhibited bacterial growth more than themselves in diluted cases. In contrast, the diluted black seeds & wheat germ had a greater effect than the nondiluted samples, as the inhibitory zone was larger.

The inhibitory zones of clover, citrus, and cucurbit honeys, along with citrus honey containing 1% palm pollen or 2.5% wheat germ, and cucurbit honey with 1% black seeds or 1% wheat germ, disclose statistically greater antibacterial activity against MRSA than the same samples in diluted form. However, there are no notable variances in bacterial proliferation among the samples (black seeds, palm pollen, 1% black seeds in clover honey, and citrus honey with 1% black seeds), regardless of whether they are diluted or not Conversely, the administration of wheat germ and a mixture of clover honey with wheat germ (2.5%) has been observed to have no effect on the activity of MRSA. The tested honeys, black seeds, and cucurbit honey with black seeds 1% or wheat germ 1% showed antibacterial activity against *Pseudomonas aeruginosa* without dilution. The results demonstrated no notable differences in bacterial growth between the diluted and undiluted palm pollen, clover honey with black seeds (1%), citrus with black seeds (1%), and citrus honey with palm pollen samples. Nevertheless, wheat germ powder or its mixture (2.5%) with clover or citrus honeys had no inhibition effect on *Pseudomonas aeruginosa* growth.

From Figure 4, undiluted samples consistently demonstrated significantly greater antibacterial activity than diluted samples against all types of bacteria investigated.

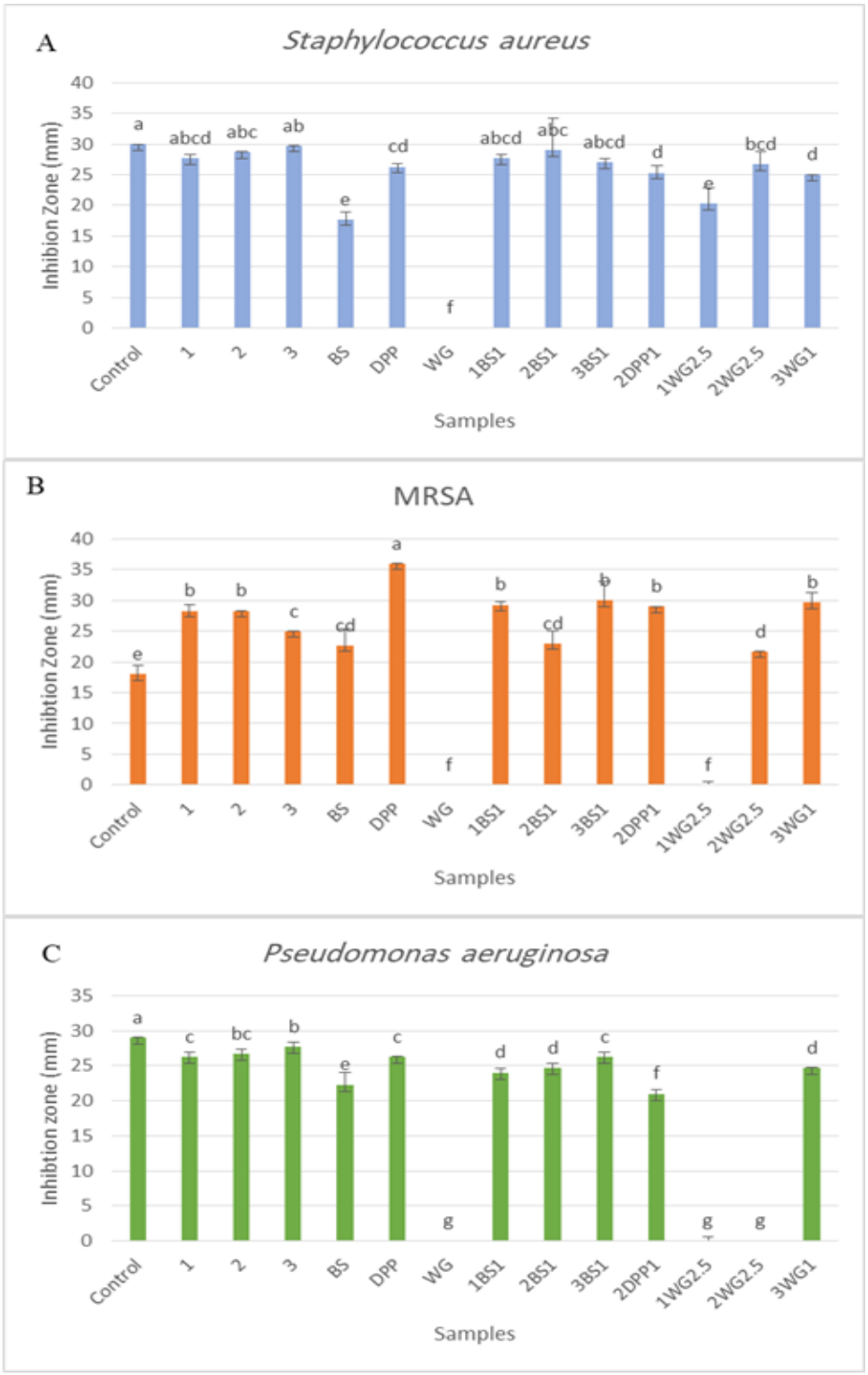


Figure 3. Inhibition zones (mm) of honeys (clover (1), citrus (2), and Cucurbit (3)), black seeds (Bs), Date palm pollen (Dpp), wheat germ (WG), and mixture samples. Nondiluted samples against *Staphylococcus aureus* (A), MRSA, methicillin-resistant *Staphylococcus aureus* (B), and *Pseudomonas aeruginosa* (C). Each bar represents mean ± standard deviation. Means with the same letter are not significantly different ($P < 0.05$).

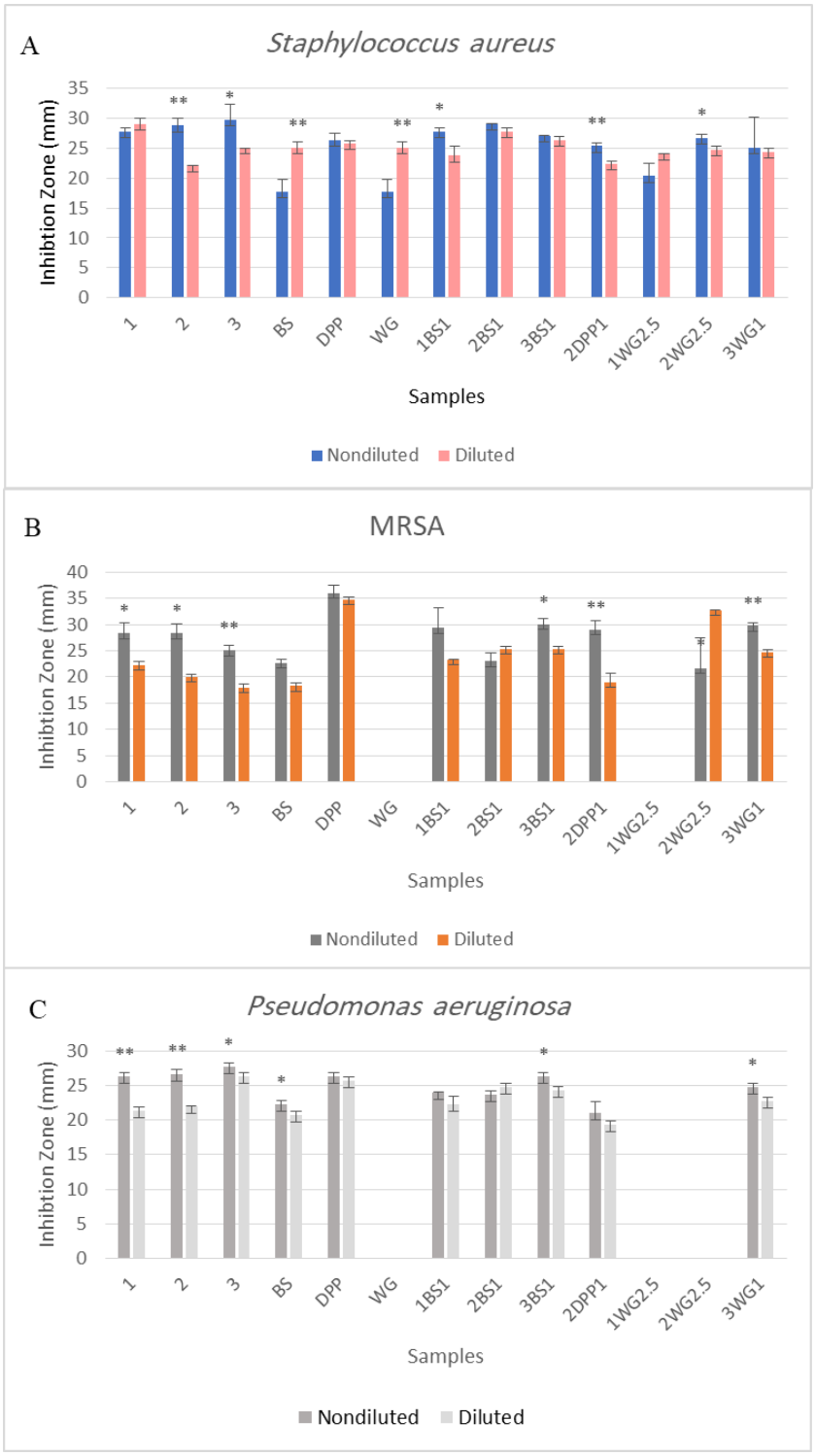


Figure 4. Inhibition zones (mm) of honeys (clover (1), citrus (2), and Cucurbit (3)), black seeds (BS), Date palm pollen (DPP), wheat germ (WG), mixtures, and their dilutions (50%). Samples against *Staphylococcus aureus* (A), MRSA, methicillin-resistant *Staphylococcus aureus* (B), and *Pseudomonas aeruginosa* (C). Each bar represents mean ± standard deviation. Bars with the * are significantly different ($P < 0.05$); bars with ** are significantly different.

DISCUSSION

Public interest in functional foods made from natural ingredients has skyrocketed, driven by a desire for healthier dietary choices (Abd Elmontaleb et al. 2023). There was a wide variability between bee honey samples according to the melissopalynological analysis. Our results are consistent with Louveaux et al. 1978 who explained that dominant pollen is defined as more than 45 percent of the pollen in honey with the exception of citrus honey must include 10%–20% citrus pollen in order to be considered monofloral (Fig 1). Furthermore, findings unveil that the pollens from these species are predominant, making this plant the primary supplier of pollen and nectar for bees, potentially impacting the physicochemical and granulation traits. Our results concur with those attained by Abd El-Dayem et al. (2024) and Seraglio et al. (2021), it was established that melissopalynological analysis represents a fundamental approach for the botanical and/or geographic identification of honey.

Concerning the physical-chemical characteristics, clover and citrus honey had significantly distinct moisture levels than Cucurbitaceae honey, as displayed by statistical analysis (Fig 2). These percentages were within the normal range (18.00-20.00g/100g) when compared with Codex Alimentations (2001) & the Egyptian Organization for Standardization and Quality Control (EOSC) (2005) which state that a honey's moisture content cannot exceed 20%. The moisture composition stands as a vital quality parameter crucial for the longevity, stability, resistance to microbial growth, and overall quality of honey. It can be affected by multiple factors such as harvest timing, seasons, nectar conditions, hive humidity levels, laboratory testing methods, storage and extraction practices (Gela et al. 2021, Singh and Singh 2018).

A higher fructose-to-glucose ratio generally indicates better honey quality and natural bee feeding. Therefore, these findings ascertained the previous studies on different types of Egyptian honey (Abd-Alla & Abd El-Wahab 2019, Abdel-Hameed 2020). The obtained data show that disaccharides, such as sucrose and maltose, were present at higher concentrations than those specified in the 2005 honey requirements published by the Egyptian Organization for Standardization and Quality, (EOSC). Based on the EOSC rules, honey's overall apparent sucrose content (sucrose plus maltose) should not exceed 5%. On the other hand, clover and citrus types of honey were accepted in reducing sugar by both Codex (2001) & EOSC (2005). However, Cucurbitaceae honey reflected lower levels than the standard limits (Not less than 60%). Diastase activity ranged from 6.50±0.26 to 30.80±0.56, which is similar to that reported by El-Metwally (2015) which mentioned that the diastase number ranges from 3.00 to 100.00 U/Kg After evaluating 184 samples of Egyptian honey, the average DN value 18.32 U/Kg. In addition to its floral source, diastase is an essential enzyme that bees use to transform nectar into honey. The activity of the diastase, a measure of honey freshness, is greatly affected by factors such as flower type, climate, inappropriate storage, and heating (Raweh et al. 2023).

Regarding the effect of mixing herbs with honey on pH, values of three tested types of honey and their mixtures were acidic and within the standard limits of (3.40 to 6.10) of Codex Alimentations (2001). Also, all of the mixtures fell within the acceptable range, although their acidity values were higher than those of the pure honey samples. Saeed& Jayashankar (2020) revealed that honey's pH ranged from 3.28 to 5.60. It could be due to the fermentation of the honey's carbohydrates into organic acid or the concentration of minerals (El-Metwally 2015). In this study, adding herbs to honey generally increased free acidity and hydrogen peroxide (H_2O_2) levels. All samples did not exceed the limit of free acidity than 50 meq/kg as required by Codex Alimentations (2001) for acidity. Increased acidity could be an indicator that sugars are fermenting into organic acids (Sancho et al 2013). Honey's sour taste makes it less acceptable, on the other hand, low acidity values indicated freshness (da Silva et al. 2016). Although H_2O_2 is thought to be a significant antibacterial component in diluted honey, some research has shown that its concentration in various types of honey does not match antibacterial action. Additionally, Bucekova et al (2019) and Farkasovska et al (2019) concluded that most significantly the total polyphenol content and the amount of H_2O_2 already present in ripened honeys were connected to the overall antibacterial activity of honeys. Furthermore, the electrical conductivity values increased when honey was combined with palm pollen or black seed at lower rates. The examined Egyptian types of honey and their mixtures were within the standard limits (≤ 0.8 ms/cm) of Codex Alimentations (2001). The

electrical conductivity of honey fluctuates significantly based on the floral origin of the honey, mineral content, overall ash content, salts, organic acids, and protein levels (El-Sohaimy et al. 2015, Leo´n-Ruiz et al. 2011). When clover and cucurbit types of honey were combined with herbs, the amount of ash rose. Herbal infused citrus honey had similar trend, except for the wheat germ-infused one. Multiple factors such as plant type and physiology, soil diversity, weather patterns, and foraging materials collected by bees may affect the mineral (ash) content of honey (Mesele 2021). Concerning hydroxymethylfurfural (HMF), the results showed that the increases by mixing clover and cucurbit types of honey with herbs, except citrus honey, which was not affected by this addition. HMF is a critical component to consider while monitoring beekeeping procedures, honey exposure to high temperatures, and storage circumstances. HMF naturally exists in minimal quantities in fresh honey, but as it is stored and heated longer, it becomes more concentrated (Tafinine et al. 2018).

Pointed to raw materials used, herbs are consumed for their medicinal properties and a wide range of biological activities, such as antiviral, antibacterial, antifungal, anticoccidiosis, anti-parasitic, and antioxidant effects (Idowu et al. 2024). The differences between the three herbs in the previous nutrients were significant ($p \leq 0.05$). Moreover, descendingly substantial variations were noticed between values of fat and fiber of black seed, wheat germ, & palm pollen. These findings were in agreement with El-Rahman and Al-Mulhem (2017) and Salem (2001).

According to sensory evaluation, Piana et al. (2004) stated that the conventional sensory assessment of honey, which was extensively employed in all honey-producing regions of the world, has been shown to be a useful tool for quality improvement and control. Derndorfer et al. (2015) mentioned that Eastern cultures tended to consume honey more frequently than Western ones. The composition and sensory qualities of honey can vary depending on factors like the geographical and plant source of the flora, bee type and behaviour, the extraction methods used, and the storage conditions (Eleazu et al. 2013). In this respect, a study by Ndife et al. (2014) found that the top ratings for general acceptability were observed in honey samples from the northern region of Nigeria. Hashem-Dabaghian *et al.* (2016) discovered that *Nigella sativa* and honey worked well together to eradicate gastric *H. pylori* infections because they both contain anti-*H. pylorus* and anti-dyspeptic properties. Using a combined metabolomic and sensory study, Kang et al. (2023) scrutinized the relationships between the chemical constituents and sensory traits of honey from divergent sources. Analysis of the honey with the senses disclosed that manuka and coffee were comparatively less accepted than sugar-fed, multiflower and acacia honey.

Results in Tables 3, 4 and 5 about mixing honey with the three different herbs, Hassan (2011) stated that palm pollen grains were a promising economic resource, providing essential nutrients that could be used to enhance human nutrition. Also, Altamimi et al. (2020) enhanced the nutritional and taste profile of date palm spathes beverages by adding pollen grains. In an investigation by Dotimas et al. (2024), it was concluded that the wheat germ was packed with beneficial substances that may help improve the health problems associated with obesity. Moreover, the results of an experiment by Tahoon et al. (2024) indicated that honeybee and wheat germ might be beneficial in managing high cholesterol levels in diabetic patients, offering a functional food approach.

There is an urgent need to find substances that have an antimicrobial effect because of the proliferation of resistant harmful bacterial varieties (Kunat-Budzy´nska et al. 2023). Honey, a time-tested remedy, has been valued for its medicinal properties, particularly in treating burns, cataracts, ulcers, and wounds (Alvarez-Suarez et al. 2014). It has diverse health benefits, such as anti-inflammatory, antioxidant, antibacterial, and blood sugar-reducing properties (Erejuwa et al. 2014), make it an ideal candidate for treating gastrointestinal and ophthalmic disorders (Khan et al. 2007). Therefore, honey, with unique properties and botanical additives, increases its benefits, including antimicrobial (Kunat-Budzy´nska et al. 2023) and antioxidant activity (Miłek et al. 2023).

In our study honey, whether used alone or combined with black seeds (Fig.3), had the same effect on *Staphylococcus aureus* as novobiocin antibiotic, due to honey antimicrobial properties, which stated by Kunat-Budzy´nska et al. (2023). In addition, the black seeds (blessing seeds in Arabic countries) obtain aromatic plants (Kolayli et al. 2023), used in healing medicine (Aljawezjjah 2001). Thymoquinone is a key compound in the *N. sativa* seeds. Their extracts contain functional groups, which have

antimicrobial properties (Khatoon et al. 2024, Shafodino et al. 2022,). Due to its antibacterial properties, thymoquinone from *N. sativa* seeds could be explored as a viable treatment for wound infections caused by *S. aureus* (Babu et al. 2023).

Our findings, mixtures of honey with wheat germ and palm pollen had a weak effect as anti-bacterial, agree with those conducted by Ashour et al. (2022) on the varieties cultivated in Libya. Despite palm pollen's abundance of phytochemicals, they discovered that it did not significantly reduce bacterial activity. However, the uniqueness of palm pollen lies in its great effect on the microbial activity of MRSA. It is partly similar to Bentrad et al. (2017) considering its effect on the *S. aureus*, MRSA, and *Listeria monocytogenes* (Tamma et al. 2020). Palm pollen demonstrated a greater efficacy against Gram-positive bacteria than Gram-negative bacteria (Daoud et al. 2019). Given the increasing prevalence of contagious and noncontagious illnesses, *P. dactylifera* should be explored as a promising medicinal plant with preventive potential (Mahomoodally et al. 2023).

In our investigation, wheat germ did not affect bacteria (A), although it was a rich source of nutrients (Brandolini & Hidalgo 2012) and antioxidants, with antimicrobial effects (Hozyen & El-Tohamy 2024), especially for *S. aureus* (Mahmoud et al. 2015).

In general, honey without any additives has an antibacterial effect on *Staphylococcus aureus* and MRSA not *Pseudomonas aeruginosa*. The inhibitory effect of honey on bacteria depends on the following: high viscosity and acidity, high concentration of sugar, low water activity, and the existence of hydrogen peroxide, nonperoxidase components, phenolic acids, flavonoids, proteins, peptides, and nonperoxidase glycopeptides (Luca et al. 2024), in addition to the botanical honey origin, seasonal variations, climate conditions, geographical origin, and applied techniques (Jia et al. 2020, Martinello & Mutinelli 2021).

Samples have an inhibitory effect on *Staphylococcus aureus* and MRSA (Gram-positive bacteria), but not on *Pseudomonas aeruginosa* (Gram-negative bacteria). Resistance can result from any modification of the outer membrane of Gram-negative bacteria, including changes to its hydrophobic abilities and mutations in porins. Therefore, Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria, which lack this important layer (Breijyeh et al. 2020).

Undiluted samples demonstrated significantly greater antibacterial activity than diluted samples against all types of bacteria investigated (figure 4). These results differ from those of previous studies, such as Hegazi research (2011) which used diluted honey at 20.3% and noticed antibacterial activity against *Staphylococcus aureus & Pseudomonas aeruginosa*. Moreover, Basualdo et al. (2007) found that honey at a concentration of 50% and undiluted honey had an inhibitory effect on the growth of *Staphylococcus aureus*. In 2005, the studies of Estrada et al. and Iurlina & Fritz differed on the concentration of honey that could inhibit microbial growth. Estrada et al. reported that the effective concentration was 25%, while Iurlina & Fritz stated that it was 75% or less.

Conclusion: Generally, infusing herbs with different types of honey increased electrical conductivity, free acidity, ash, H_2O_2, and HMF, while reducing pH. The type of honey and herb may affect the physicochemical characteristics of honey in different ways.

In sensory evaluation, consumers generally preferred pure honeybee products without any additives. However, some mixtures were also well received and could be used for health purposes due to their beneficial properties. Bee honey with or without herbs has an antibacterial effect on *Staphylococcus aureus* and MRSA (Gram-positive bacteria), not *Pseudomonas aeruginosa* (Gram-negative bacteria). A larger bacterial inhibition zone was observed in the majority of the undiluted samples than in the diluted samples. Researchers could significantly advance the field by undertaking subsequent studies that evaluate the antioxidant properties and anti-inflammatory effects of honey and herb combinations.

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Author contributions: GME, MIH, and AEA**:** planning, data collection, methodological planning, experimenting, and setting up the study. All authors read and approved the final manuscript.

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DERLEME /REVIEW

A MULTIFACETED BIOACTIVITY OF HONEY: INTERACTIONS BETWEEN BEES, PLANTS AND MICROORGANISMS

Balın Çok Yönlü Biyoaktivitesi: Arılar, Bitkiler ve Mikroorganizmalar Arasındaki Etkileşimler

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ABSTRACT

Honey has been recognized for its medicinal properties for centuries, with well-documented benefits such as antibacterial, anti-inflammatory, and antioxidant activities. However, despite the widespread use of honey for health-related purposes, many of the underlying mechanisms responsible for its bioactivity remain underexplored. This review delves into the complexity of honey's composition, particularly focusing on the active substances and the honey microbiota contribution to its properties. We aim to bridge the gap in understanding how honey's multifaceted bioactivity arises from interactions between bees, plants, and microorganisms. The review sheds light on the key compounds, including hydrogen peroxide, methylglyoxal, polyphenols, and antimicrobial peptides, which play vital roles in honey's health benefits. It also highlights the often-overlooked contributions of the honeybee's gut microbiota and the nectar's microbiota, which together influence the chemical transformation of nectar into honey and enhance its therapeutic efficacy. By examining the current literature, this article emphasizes the need for deeper investigation into how various factors-such as floral origin, bee subspecies, and environmental conditions-affect the medicinal quality of honey. Understanding these mechanisms could lead to optimized use of honey in medical applications and reveal new therapeutic potentials. This article provides a comprehensive review of the intricate processes and components that make honey not only a nutritional food source but also a potent natural medicine.

Keywords: Honey bioactivity, Prebiotic potential, Lactic acid bacteria, Antimicrobial properties, Honey microbiota

ÖZ

Bal, antibakteriyel, anti-enflamatuar ve antioksidan aktiviteler gibi iyi belgelenmiş faydaları ile yüzyıllardır tıbbi özellikleriyle tanınmaktadır. Bununla birlikte, balın sağlıkla ilgili amaçlarla yaygın

kullanımına rağmen, biyoaktivitesinden sorumlu olan altta yatan mekanizmaların çoğu yeterince araştırılmamıştır. Bu derleme, özellikle aktif maddelere ve bal mikrobiyomunun özelliklerine katkısına odaklanarak balın bileşiminin karmaşıklığını incelemektedir. Balın çok yönlü biyoaktivitesinin arılar, bitkiler ve mikroorganizmalar arasındaki etkileşimlerden nasıl kaynaklandığını anlamadaki boşluğu doldurmayı amaçlıyoruz. Bu derleme, balın sağlığa faydalarında hayati rol oynayan hidrojen peroksit, metilglioksal, polifenoller ve antimikrobiyal peptitler gibi temel bileşiklere ışık tutmaktadır. Ayrıca, nektarın bala kimyasal dönüşümünü birlikte etkileyen ve terapötik etkinliğini artıran bal arısının bağırsak mikrobiyomunun ve nektarın mikrobiyotasının genellikle göz ardı edilen katkılarını vurgulamaktadır. Bu makale, mevcut literatürü inceleyerek, çiçek kökeni, arı alt türleri ve çevresel koşullar gibi çeşitli faktörlerin balın tıbbi kalitesini nasıl etkilediğinin daha derinlemesine araştırılması gerektiğini vurgulamaktadır. Bu mekanizmaların anlaşılması, balın tıbbi uygulamalarda en uygun şekilde kullanılmasını sağlayabilir ve yeni terapötik potansiyelleri ortaya çıkarabilir. Bu makale, balı sadece besleyici bir gıda kaynağı değil aynı zamanda güçlü bir doğal ilaç yapan karmaşık süreçler ve bileşenler hakkında kapsamlı bir inceleme sunmaktadır.

Anahtar Kelimeler: Bal biyoaktivitesi, Prebiyotik potansiyel, Laktik asit bakterileri, Antimikrobiyal özellikler, Bal mikrobiyomu

GENİŞLETİLMİŞ ÖZET

Giriş: Bal tarih boyunca tedavi edici özellikleriyle değerlendirilmiş, yaraları iyileştirme, enfeksiyonları yönetme ve bağışıklık sistemini güçlendirme kabiliyetiyle tanınmıştır. Bununla birlikte, bu etkilerin ardındaki mekanizmalar halk veya bilim camiasının çoğu tarafından yaygın olarak anlaşılmamıştır. Arılar, çiçekler ve mikroorganizmalar arasındaki etkileşimler de dahil olmak üzere balın karmaşık bileşimi veya bal mikrobiyomunun rolü hakkında çok az şey bilinmektedir. Bu derleme, mikrobiyomuna özel bir vurgu yaparak balın biyoaktif özelliklerine katkıda bulunan kimyasal ve mikrobiyolojik faktörlere odaklanarak bu boşlukları ele almayı amaçlamaktadır.

Balın prebiyotik potansiyeli: Bal, şekerler, proteinler, lipitler ve mikrobiyal metabolitler dahil olmak üzere 200'den fazla bileşik içeren karmaşık bir üründür. Ayrıca, *Bifidobacterium* ve *Lactobacillus* gibi faydalı bağırsak bakterilerinin büyümesini destekleyen oligosakkaritleri nedeniyle önemli bir prebiyotik potansiyele sahiptir. Oligosakkaritler sindirilmeden alt gastrointestinal sisteme geçer ve burada bu mikroplar için besin görevi görür. Balın prebiyotik etkisi botanik kökenine göre değişir; manuka ve bal özü balları özellikle güçlü etkiler gösterir. Balın mikrobiyomu, prebiyotik yeteneklerini daha da geliştirerek bağırsak sağlığını ve bağışıklık fonksiyonunu artırır.

Bal arısı bağırsağı ve bal mikrobiyomu: Balın tıbbi özellikleri, arılar tarafından nektar toplama ve fermantasyon sırasında ortaya çıkan bakteri, maya ve mantarlardan oluşan mikrobiyomu tarafından şekillendirilir. Bal arısı bağırsak mikrobiyomu, nektar şekerlerinin biyoaktif bileşiklere dönüştürülmesinin ayrılmaz bir parçasıdır. *Lactobacillus*, *Gilliamella* ve *Bifidobacterium* gibi mikroplar balı koruyan ve biyoaktivitesini artıran organik asitler, bakteriyosinler ve enzimler üretir. Bu mikrobiyal süreçler, çiçek kökeni ve arı fizyolojisi ile birleştiğinde, bitki ve mikrobiyal faydaları birleştiren ve balın tedavi edici etkilerine katkıda bulunan benzersiz bir ürün ortaya çıkarır.

Laktik Asit Bakterileri ve Bifidobakteri türevi bileşikler nedeniyle balın prebiyotik potansiyeli

Lactobacillus ve *Bifidobacterium* dahil laktik asit bakterileri (LAB) bal arısı bağırsak mikrobiyomu için kritik öneme sahiptir. Bu bakteriler asidik bir ortam yaratarak patojen büyümesini engelleyen ve balın prebiyotik potansiyelini artıran organik asitler üretir. LAB ayrıca patojenleri hedef alan ve öldüren bakteriyosinler de üretir. Ek olarak, kısa zincirli yağ asitleri gibi LAB tarafından üretilen metabolitler, faydalı bağırsak bakterilerinin büyümesini teşvik eder. Balda ve insan bağırsağında doğal olarak bulunan LAB türlerinin varlığı, balı bağırsak sağlığını ve bağışıklık fonksiyonunu destekleyen etkili bir prebiyotik gıda haline getirmektedir.

Bacillales türevi bileşikler nedeniyle balın prebiyotik potansiyeli: Bal mikrobiyotasında öne çıkan *Bacillus* türleri prebiyotik olarak bilinen bakteriyosinler ve lipopeptitler gibi antimikrobiyal bileşikler üretir. Bu bileşikler, bakteriler öldükten sonra bile biyoaktif kalmaya devam ederek balın uzun vadeli antimikrobiyal etkilerini sağlar. *Bacillus* tarafından üretilen enzimler de karmaşık molekülleri

daha basit formlara dönüştürerek faydalı bağırsak bakterilerini destekler. Sürfaktin ve fengisin gibi lipopeptitler de dahil olmak üzere bu prebiyotik özellikler, balın patojen büyümesini önleme ve bağışıklık fonksiyonunu destekleme yeteneğine katkıda bulunarak onu etkili bir fonksiyonel gıda haline getirir.

Mantar türevi bileşikler nedeniyle balın prebiyotik potansiyeli: *Saccharomyces* ve *Zygosaccharomyces* gibi mayalar balın fermantasyonunda ve prebiyotik özelliklerinde rol oynar. Bu mantarlar balın pH'ını düşüren organik asitler üreterek zararlı patojenleri engellerken yararlı bakteriler için elverişli bir ortam yaratır. Mantar metabolitleri balın stabilitesine ve lezzetine de katkıda bulunur. Bazı mantarlar, balı bozulmaya karşı koruyan mikotoksinler gibi antimikrobiyal bileşikler üretir. Düşük konsantrasyonlarda bulunmasına rağmen, bu bileşikler balın uzun vadeli antimikrobiyal özelliklerini ve sağlık yararlarını artırarak güçlü bir prebiyotik gıda olarak kalmasını sağlar.

Bitki Metabolitlerinin Balın Prebiyotik Özellikleri Üzerindeki Etkisi: Polifenoller, flavonoidler ve fenolik asitler gibi bitki kaynaklı bileşikler balın prebiyotik, antioksidan ve anti-enflamatuar özelliklerine katkıda bulunur. Bu metabolitler serbest radikalleri nötralize ederek oksidatif stresi azaltır ve zararlı patojenleri inhibe ederken faydalı bağırsak bakterilerinin büyümesini teşvik eder. Bu bileşiklerin konsantrasyonu bal türüne göre değişir, daha koyu renkli ballar daha yüksek polifenol seviyeleri ve daha güçlü antioksidan özellikler içerir. Bitki metabolitlerinin bu varlığı balın bağırsak sağlığını, bağışıklık fonksiyonunu ve iltihaplanmayı azaltmayı destekleme kabiliyetini artırarak onu etkili bir fonksiyonel gıda haline getirir.

Sonuç: Bu derleme, balın terapötik özelliklerine katkıda bulunan karmaşık biyolojik mekanizmaları vurgulamaktadır. Balın biyoaktivitesi arılar, çiçekler ve mikroorganizmalar arasındaki etkileşimlerden kaynaklanır ve prebiyotik bileşikler açısından zengin bir ürün ortaya çıkar. Laktik asit bakterileri, Bacillales türleri ve mantarlar da dahil olmak üzere balın mikrobiyomunun rolü, bağırsak sağlığını geliştirme ve patojenlerle mücadele etme yeteneğini şekillendirmede çok önemlidir. Bu süreçleri anlayarak balın fonksiyonel bir gıda olarak potansiyelini daha iyi değerlendirebiliriz. Gelecekteki araştırmalar, balın tüm terapötik potansiyelini ortaya çıkarmak için bu etkileşimleri daha fazla keşfetmelidir.

INTRODUCTION

The honey bee (*Apis mellifera*) produces honey by fermenting flower nectar and enriching it with plant and bee metabolites. Honey comprises over 200 compounds, including sugars, proteins, lipids, vitamins, minerals, polyphenols, enzymes, and microbial metabolites (Kafantaris et al. 2020, Schell et al. 2022). Nectar influences honey's taste, color, and therapeutic properties (Schell et al. 2022). The main carbohydrates are fructose and glucose, constituting up to 80% of total sugars, alongside water, proteins, acids, minerals, plant phytochemicals, and vitamins. Some honeys exhibit increased biological activity due to floral-derived metabolites like oligosaccharides, which are highly effective in vitro (Carter et al. 2016, Miguel et al. 2017, Schell et al. 2022).

Honey is a nutritious prebiotic food with antibacterial, anti-inflammatory, and antioxidant properties, offering therapeutic benefits such as immunomodulatory, antidiabetic, antimutagenic, and anticancer effects (Karabagias 2018, Kafantaris et al. 2020, Seraglio et al. 2019). Honey's properties are influenced by bee subspecies, stomach microbiota fermentation, nectar's botanical and geographical origin, and environmental factors (da Silva et al., 2016; Karabagias, 2018). Historically, honey has been a vital carbohydrate and energy source, reducing bacterial diarrhea in children and preventing organ failure in critically ill patients (Schell et al. 2022). It also protects against viral gastroenteritis and can have a mild laxative effect due to limited fructose absorption (Bogdanov et al. 2008, Schell et al. 2022).

Honey's antimicrobial activity is due to the presence of gluconic acid, which gives the honey an acidic pH of 3–5. This inhibits vegetative bacterial growth due to the low water activity, low pH, and antimicrobial elements like hydrogen peroxide and peptides (Bogdanov et al. 2008, Carter et al. 2016, Olaitan et al. 2007, Schell et al. 2022). Its broad-spectrum antibacterial properties remain effective against multidrug-resistant pathogens. Osmotolerant microorganisms, including spore-forming bacteria and yeasts, are introduced from nectar during pollination (Alvarez-Pérez et al. 2012). Importantly, honey does not induce microbial resistance, attributed to its diverse antimicrobial substances

(Nolan et al. 2019, Maddocks et al. 2013, x Schell et al. 2022).

Honey contains a variety of antimicrobial compounds that effectively inhibit or kill a wide range of microorganisms, including multidrug-resistant pathogens (Combarros-Fuertes et al. 2020, Nolan et al. 2019). It can prevent the growth of harmful bacteia like *Clostridium perfringens*, *Collinsella aerofaciens* in the intestines, and *Listeria monocytogenes* in milk (Shin & Ustunol 2005, Schell et al. 2022). Honey also inhibits various enteropathogenic microorganisms, including multidrug-resistant *Salmonella spp.*, *Shigella spp.*, enteropathogenic *Escherichia coli*, *Enterobacter spp.*, *Yersinia enterocolitica*, *Campylobacter spp.*, and *Clostridioides difficile* (Hammond & Donkor 2013, Schell et al. 2022). It prevents *Salmonella* species from adhering to mucosal epithelial cells, thereby preventing infection (Schell et al. 2022).

Identifying honey's active compounds is crucial for recognizing its antibacterial properties. Key antibacterial compounds in honey include hydrogen peroxide and methylglyoxal, which exhibit a nonspecific mechanism of action. The concentrations of these compounds are found to correlate with the antibacterial activity of honey; typical minimum inhibitory and bactericidal concentrations are observed to fall within the microgram per millilitre range (Brudzynski 2020, Mavric et al. 2008). Hydrogen peroxide causes oxidative damage to bacterial cell components and DNA (Brudzynski et al. 2012). Methylglyoxal, found in manuka honey, irreversibly glycates and cross-links macromolecules, resulting in loss of function (Mavric et al. 2008). Plant-derived secondary metabolites, such as polyphenols, flavonoids, and volatile compounds, also contribute to honey's antimicrobial activity. Although their antimicrobial effect is relatively weak, they add to honey's broad-spectrum activity (Figure 1) (Isah 2019).

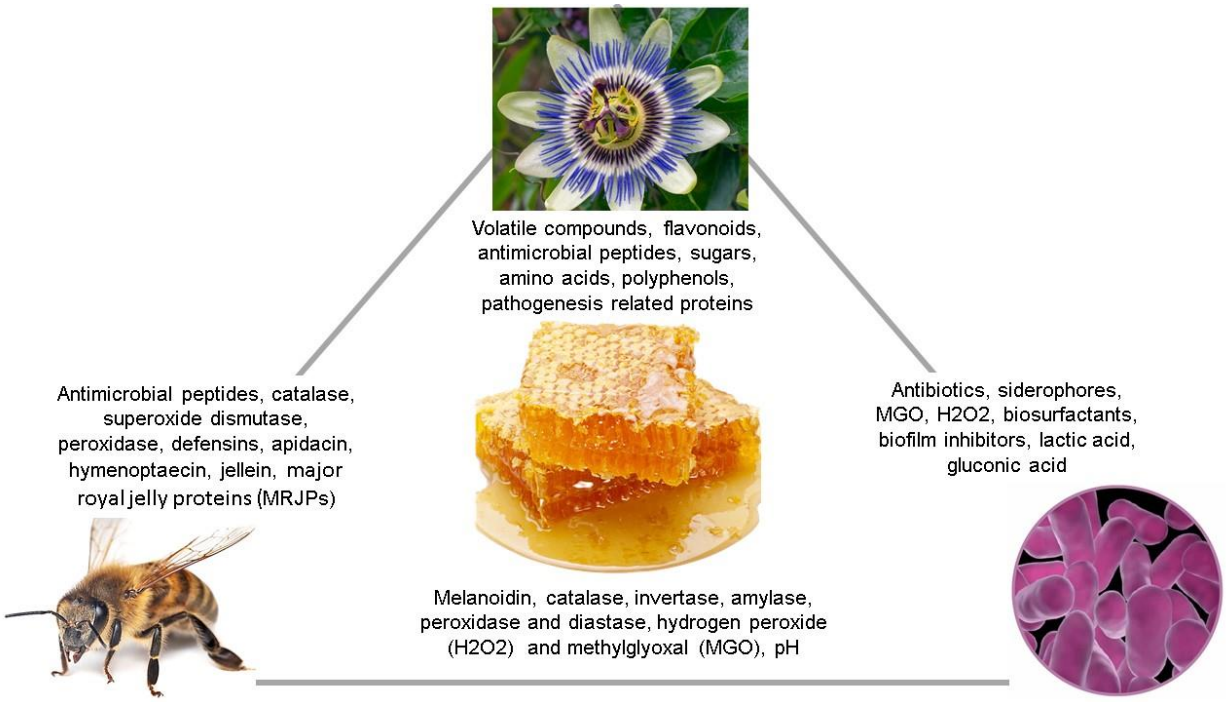

Figure 1. Active metabolites are produced and contribute to the antimicrobial activity of honey through three-way interactions involving microbes, plants, and honey bees.

The nectar-honey bee-honey microbiota axis plays a crucial role in producing various antimicrobial agents, including antimicrobial peptides, bacteriocins, surfactants, siderophores, proteolytic enzymes, and quorum sensing inhibitors (Brudzynski, 2021). Studies suggest honey's effects on bacterial cells include targeted mechanisms not solely explained by hydrogen peroxide or

methylglyoxal, hinting at additional antimicrobial compounds (Henriques et al., 2011; Brudzynski & Sjaarda, 2014). These effects resemble those of β-lactams, antimicrobial peptides, or inhibitors of proton motive force and chemiosmosis. Thus, honey's wide-ranging antibacterial activity, including against multidrug-resistant strains, may involve multiple sources of antimicrobial compounds (Brudzynski 2021).

Phytohormones, or plant hormones, synthesized by plants and some yeasts, significantly impact various life processes in plants and animals even in minute concentrations. Notable phytohormones include auxins, abscisic acid, cytokinins, gibberellins, ethylene, polyamines, brassinolides, jasmonates, salicylic acid, and strigolactones. These hormones influence mammalian physiology and have potential therapeutic applications, such as combating cancer and diabetes and promoting cell growth. For instance, indole-3-acetic acid acts as an antitumor agent, gibberellins promote apoptosis, abscisic acid regulates glucose homeostasis and acts as an antidepressant, and cytokinins serve as anti-aging compounds (Mukherjee et al. 2022).

Pollen remaining after honey bees filter nectar during honey conversion is thought to be the primary source of phytohormones in honey (Herold & Leibold 2000). However, the microflora of the nectar and the bees' foregut might also secrete phytohormones into honey (Ilyasov et al. 2015, Mukherjee et al. 2022). The specific composition of substances released by honey bees during fermentation and ripening of honey remains largely unknown, although honey, nectar, pollen, and beebread have been confirmed to contain phytohormonal substances (Abdulgazina et al. 2015). Auxins and cytokinins primarily originate from nectar, while abscisic acid in honey may also be secreted by honey bees (Abdulgazina et al. 2015, Ilyasov et al. 2015).

Phytohormones impact human health in various ways. For instance, abscisic acid regulates human and animal cells growth and enhances immune responses (Scarfi et al., 2008). Cytokinins promote human and animal cells growth and act as anti-stress agents (Voller et al. 2017). Auxin is recognized as an antitumor agent in human and animal cells, and gibberellin has antioxidant properties (Hamayun et al. 2017, Mukherjee et al. 2022).

Honey contains phytoncides, biologically active substances that kill or inhibit microorganisms. These include benzoic acid, avenacin, juglone, phloridzin, pinosulfan, and tannins. Phytoncides in honey are potent medicines for treating infections and wounds. The botanical composition of honey plants influences the chemical properties and effectiveness of these phytoncides. Honeydew honey from spruce, pine, and fir has strong bactericidal properties, while darker flower honeys like chestnut and buckwheat have moderate bactericidal properties. Light flower honeys such as those from dandelion and white clover exhibit almost no bactericidal effect (Vakhonina 2002).

A comparative analysis of amino acids in pollen and honey of the same botanical origin suggests that additional amino acids in honey come from sources other than pollen, likely due to the bees' physiological activity and the gastric microflora during nectar processing (Vrabie et al. 2019).

The types of plants from which honey bees collect nectar also influence the diastase number, a measure of enzyme activity in honey. Different bee breeds and colony sizes affect the diastase number, with larger colonies producing honey with higher diastase content (Lebedev & Murashova 2004).

Honey's antioxidant effects are attributed to its phenolic components, which protect human blood cells when consumed. Darker honeys generally have higher antioxidant activity due to their higher phenolic content. These phenolics also contribute to honey's anti-inflammatory properties by blocking inflammatory mediators and reducing pro-inflammatory cytokines, making honey valuable in the treatment of wounds and burns (Schramm et al. 2003, Schell et al. 2022, Vallianou et al. 2014; Zhao et al. 2019).

Honey serves as a natural prebiotic, containing metabolites from microbial, plant, and animal sources. Prebiotics are foods containing certain compounds that promote the growth or activity of beneficial microorganisms such as bacteria and fungi. The most common environment affecting human health is the gastrointestinal tract, where prebiotics can alter the composition of microorganisms in the gut microbiota. Probiotics are food containing living microorganisms capable of supporting or improving beneficial bacteria (normal microflora) in gut. Honey, rich in sugars, preserves these biologically active substances, retaining their activity over time. Honey may regulate the immune system, enhance barrier functions, and support microbiota formation. They are considered safe and

can be recommended for early childhood to support microbiota development and for preventing diseases influenced by the gut microbiota, such as inflammatory bowel disease, multiple sclerosis, and Alzheimer's disease. Prebiotic compounds in honey may also aid in preventing and treating SARS-CoV-2 infection (Brudzynski, 2021, Gou 2020).

Despite the widespread recognition of honey's medicinal benefits, the mechanisms responsible for its therapeutic effects remain largely unknown to the general public and even many researchers. Most people are familiar with honey's antibacterial, anti-inflammatory, and antioxidant properties, but few understand the biochemical pathways and interactions that drive these effects. The key active compounds, such as hydrogen peroxide, methylglyoxal, and polyphenols, are often overlooked, as is the significant role of honey's microbiota. The intricate relationship between honeybees, the flowers they pollinate, and the microorganisms involved in honey production is crucial to its bioactivity, yet remains underexplored. This review was written to fill these knowledge gaps and to provide a comprehensive analysis of the underlying mechanisms that contribute to honey's unique properties. Our goal is to offer readers a detailed understanding of how bees, plants, and microorganisms collaborate in shaping honey's composition and bioactivity. By focusing on the lesser-known aspects of honey's microbiota, lactic acid bacteria, and microbial metabolites, we aim to shed light on the factors that elevate honey from a simple natural product to a potent therapeutic agent. This review not only underscores honey's medicinal potential but also highlights the importance of understanding the interactions within the honey ecosystem to fully appreciate its therapeutic benefits.

Prebiotic potential of honey

Honey's primary composition is simple sugars, or monosaccharides, which are readily absorbed in the human intestine (Sanz et al. 2004, Schell et al. 2022). In smaller quantities, it contains di-, tri-, and oligosaccharides. Low-mass oligosaccharides and polysaccharides in honey resist digestion by host enzymes, reaching the lower intestine, where they exert prebiotic effects (Sanz et al. 2005, Schell et al. 2022). The prebiotic effects vary with the botanical origin of the honey, discernible through nuclear magnetic resonance (NMR) spectroscopy combined with chemometric analysis (Karabagias, 2018; Olawode et al., 2018; Kafantaris et al., 2020).

Nuclear magnetic resonance spectroscopy (NMR) spectroscopy and chemometric methods have successfully differentiated honeys by their botanical and geographical origins, including chestnut, acacia, linden, polyfloral types, and those from Greece, Brazil, South Africa, Zambia, and Slovakia (Karabagias 2018, Kafantaris et al. 2020, Olawode et al. 2018). This technology has also classified Vietnamese honey and identified unique compounds in honey samples (Kafantaris et al. 2020, Luong et al. 2019,). Additionally, NMR spectroscopy and ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry have identified chemical indicators in stingless bee honey, revealing unique compounds such as D-fructofuranose in Heterotrigona itama honey, and L-lactic acid in Tetrigona apicalis honey (Kafantaris et al. 2020).

Honey's protein composition includes major royal jelly proteins (MRJPs) and enzymes like glucosidase, amylase, and glucose oxidase, which are markers of authenticity and quality (Chua et al. 2014, Kafantaris et al. 2020; Machado De Melo et al. 2017). Bee-derived proteins such as MRJP 1-5, α-glucosidase, and defensin-1 are prevalent in honey from various floral sources (Di Girolamo et al. 2012, Kafantaris et al. 2020). The antibacterial activity of honey is primarily due to the antimicrobial peptide bee defensin-1 (Kafantaris et al. 2020, Kwakman et al. 2010). Proteomic profiling methods, including SDS-PAGE, native PAGE, MS methods, and 2D electrophoresis with MALDI MS, have identified proteins such as MRJP 1–9, α-glucosidase, and glucose oxidase in honey (Kafantaris et al. 2020, Zhang et al. 2019).

In vitro studies support honey's prebiotic potential, demonstrating its efficacy in promoting the growth of beneficial bacteria like *Bifidobacterium* and *Lactobacillus* species (Jiang et al. 2020, de Melo et al. 2020, Schell et al. 2022). Honey's growth-promoting effects on these bacteria are comparable to oligosaccharide prebiotics such as fructooligosaccharides, galactooligosaccharides, and inulin (Chick et al. 2001; Shin & Ustunol 2005). Moreover, honey enhances bacterial metabolism in the human intestine (Figure 2) (Mohan et al. 2017, Schell et al. 2022).

Table 1. Prebiotic effects of various honeys.

Honey type and plant source	Experimental approach	Prebiotic effects	References
Sundarban, Litchi, Cumin, Eucalyptus and multifloral (India)	Study assessed antioxidant activities (ABTS, DPPH, FRAP, hydroxyl scavenging, lipid peroxidation) in Bifidobacteria from infant feces and Wistar rat intestines.	Enhances *Bifidobacterium* species, *Lactobacillus acidophilus*, and *Lactiplantibacillus plantarum*. Combats *Escherichia coli*, *Klebsiella aerogens*, *Staphylococcus aureus*, *Salmonella typhi*, and *Bacillus cereus*.	Nooh and Nour-Eldien, 2016; Schell et al., 2022
Multifloral (Jordan)	Study used optical density readings to determine colony counts.	Enhances the advantages of intestinal *Bifidobacterium infantis* and *Lactobacillus acidophilus*.	Schell et al., 2022
Multifloral (Indonesia)	Study analyzed intestinal microbiota diversity in honey-fed Pacific white shrimp using DNA sequencing.	Promotes beneficial gut bacteria and improves shrimp survival against *Vibrio parahaemolyticus*.	Weston and Brocklebank, 1999; Fuandila et al., 2019; Schell et al., 2022
Alfalfa and Eucalyptus (Australia)	Study measured bacterial growth using growth media and optical density readings.	Enhances the advantages of *Lactobacillus acidophilus*, *Lactobacillus delbrueckii subsp. bulgaricus*, and *Lactiplantibacillus plantarum*.	Conway et al., 2010
Acacia and Chestnut (Croatia)	Study used agar disk diffusion and colony counting on agar plates for bacterial analysis.	Enhances the efficacy of *Bifidobacterium lactis* Bb-12 and *Bifidobacterium longum* Bb-46 while suppressing *Listeria monocytogenes* FSL N1-017.	Slačanac et al., 2012
Linden honey (Poland)	Study using shake-flask screening method	Activates G-protein-coupled receptors, blocks NMDA and nicotinic receptors, and offers neuroprotective effects by kynurenic acid in honey, from yeasts *Yarrowia lipolytica* and *Candida magnolia*	Beretta et al., 2008; Turski et al., 2009
Honeydew (Spain)	Study employed fecal bacteria fermentation and utilized 16S rDNA sequencing of the V4 region.	Boosts lactobacilli and bifidobacteria, reduces enteric bacteria and *Bacteroides*.	Leeming et al., 2019; Schell et al., 2022
Buckwheat (China)	Study employed fecal bacteria fermentation and utilized 16S rDNA sequencing of the V4 region.	Enhances the favorable effects of *Bifidobacterium* species.	David et al., 2014; Schell et al., 2022
Juazeiro and Jurema-branca (Brazil)	Study used optical density readings to determine colony counts.	Enhances the beneficial effects of *Bifidobacterium lactis* and *Lactobacillus acidophilus*.	Tanes et al., 2021; Schell et al., 2022
Manuka (New Zealand)	Study analyzed microbiota changes with 20 g daily honey intake in a clinical trial using fecal sequencing.	Enhances *Limosilactobacillus reuteri*, *Lacticaseibacillus rhamnosus*, and B. *lactis*; inhibits *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi.*	Gibson and Roberfroid, 1995; Wang et al., 2019; Schell et al., 2022
Clover (New Zealand)	Study assessed the growth of probiotic pure cultures in skim milk with different sweeteners by counting colonies on agar plates for cultural enumeration.	Amplifies the advantageous effects of *Bifidobacterium bifidum* and *Lactobacillus acidophilus*.	Mohan et al., 2017; Schell et al., 2022
Sage, clover, alfalfa, sourwood (United States)	Study employed agar disk diffusion, colony counting on agar plates, and microbroth dilution to assess bacterial growth by measuring optical density.	Enhances *Lactobacillus* and *Bifidobacterium* activity, suppresses pathogenic bacteria, and boosts the effectiveness of prebiotics like fructooligosaccharide, galactooligosaccharide, and inulin.	Popa and Ustunol, 2011; Al-Sheraji et al., 2013; Schell et al., 2022
Acacia and chestnut (Saudi Arabia)	Study used agar disk diffusion and colony counting on agar plates for	Boosts growth *Bifidobacterium* and *Lactobacillus* species, slows their	Raschka and Daniel, 2005;

	bacterial analysis.	replication, and suppresses *Listeria monocytogenes*.	Schell et al., 2022
Acacia and chestnut (Croatia)	Study used agar disk diffusion and colony counting on agar plates for bacterial enumeration.	Enhances the advantages of *Bifidobacterium lactis*.	Nagpal and Kaur, 2011; Schell et al., 2022
Tualang and multifloral (Malaysia)	Study treated honey to remove simple sugars, used the remaining fraction to supplement skim milk, and counted bacterial colonies on agar plates.	Enhances *Bifidobacterium longum* effectiveness by removing simple sugars.	Chick et al., 2001; Schell et al., 2022
Cotton (Egypt)	Study with albino mice focused on collecting cecal contents and enumerating colonic bacteria through viable cell counts on agar plates.	Enhances *Bifidobacterium* and *Lactobacillus* species, increases beneficial bacteria, and suppresses harmful ones, restoring gut microbiota balance.	Roberfroid et al., 2010; Schell et al., 2022
Jarrah (Australia)	Study with 30 BALB/c mice included 16S rRNA sequencing of the V3–V4 region and measured fecal water content by weighing samples before and after drying.	Boosts beneficial bacterial groups and suppresses harmful ones, aiding in gut microbiota balance.	Chauhan and Chorawala, 2014; Schell et al., 2022
Prunella vulgaris (China)	Study involved 24 male Sprague Dawley rats with induced colitis, focusing on colon histology, intestinal mRNA analysis, and gut microbiota profiling via 16S rRNA sequencing of the V3–V4 region.	Boosts Firmicutes and *Lactobacillus*, reduces *Bacteroidetes* and Lachnospiraceae, aiding in ulcerative colitis symptom relief by impacting gut flora.	Haddadin et al., 2007; Schell et al., 2022

Honey also exhibits anti-inflammatory properties, mediated by an increase in anti-inflammatory cytokines (Ranneh et al. 2021, Vallianou et al. 2014). Phytohormones in honey, such as gibberellic acid and abscisic acid, contribute to these effects (Wang et al. 2017). The presence of these phytohormones enhances honey's potential as a functional food with prebiotic benefits (Chanclud & Lacombe 2017, Mohan et al. 2017, Schell et al. 2022).

In vitro studies demonstrate honey's ability to promote the growth of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* species, comparable to other oligosaccharide prebiotics (Rosendale et al. 2008, Schell et al. 2022, Shin & Ustunol 2005). Honey's oligosaccharides selectively support beneficial bacteria while suppressing harmful ones (Sanz et al. 2005, Schell et al. 2022).

Honey's antibacterial properties are attributed to its high sugar content, low pH, hydrogen peroxide, and non-peroxide phytochemicals like methylglyoxal (da Silva et al. 2016, Johnston et al. 2018, Kafantaris et al. 2020). Honey influences bacterial processes, including cell division, motility, and biofilm formation, through gene expression modulation. Studies reveal honey's capability to reduce virulence and biofilm gene expression in pathogens like *E. coli* and *Staphylococcus aureus* (Lee et al., 2011; Kafantaris et al., 2020). RNA sequencing of *Pseudomonas aeruginosa* exposed to manuka honey showed increased expression of growth and adaptation genes, highlighting honey's multifactorial antibacterial effect (Bouzo et al. 2020, Kafantaris et al. 2020).

The anti-inflammatory properties of honey further contribute to its prebiotic potential. Studies show honey increases anti-inflammatory cytokines and decreases pro-inflammatory cytokines in various models, such as rats with induced gastric ulcers or ulcerative colitis (Ranneh et al. 2021, Schell et al. 2022, Vallianou et al. 2014). Honey's polyphenols are responsible for these effects, reducing inflammation and suppressing harmful organisms (Zhao et al. 2019).

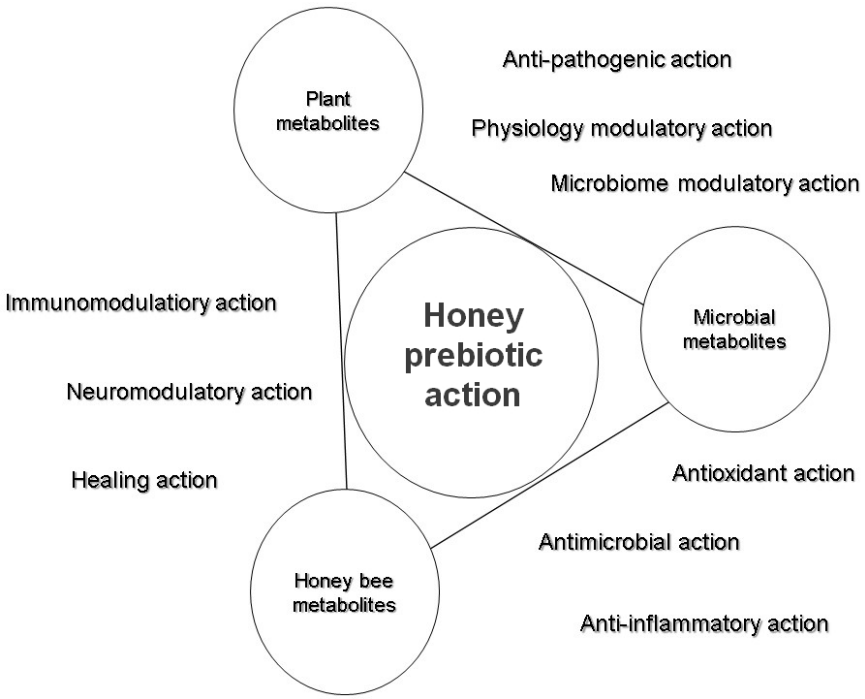

Figure 2. Honey is a complex prebiotic resulting from the interactions between microbiomes, honey bees, and plants.

Honey's oligosaccharide composition, influenced by plant sources, determines its prebiotic properties (Kolayli et al. 2012). New Zealand honey, for example, contains high levels of isomaltose and melezitose, whereas Italian honey is rich in raffinose, leading to variations in prebiotic potential (Kolayli et al. 2012, Schell et al. 2022). Honeydew oligosaccharides increase beneficial bifidobacteria and lactobacilli populations while reducing harmful *bacteroides* and clostridia (Sanz et al., 2005; Schell et al. 2022). These oligosaccharides have prebiotic effects comparable to commercial fructooligosaccharides (Brudzynski 2021).

Human clinical trials have shown that daily honey consumption, including manuka honey, does not significantly alter major gut bacteria families. However, the quality and storage conditions of honey can affect these results (Brudzynski 2021, Schell et al. 2022). In vivo studies on animal models support honey's role as a prebiotic, promoting probiotic bacteria growth and alleviating symptoms of constipation and ulcerative colitis (Li et al. 2020, Schell et al. 2022, Wang et al. 2019). In shrimp, honey stimulates probiotic growth and improves gut health, reducing pathogen load and increasing survival rates when infected with *Vibrio parahaemolyticus* (Table 1) (Fuandila et al. 2019, Hasyimi et al. 2020, Schell et al. 2022).

Honey bee gut and honey microbiota

A. mellifera, the honey bee, serves as an exemplary model organism due to its adaptable microbial community, which, while sharing similarities with mammalian microbiota, is notably simpler (Nowak et al. 2021). Newly emerged honey bees focus on hive maintenance, while worker honey bees undertake various tasks. Routes of infection for newly emerged honey bees include interactions with adult bees, contact with comb, and consumption of bee bread (Dong et al. 2020, Nowak et al. 2021). Gut colonization begins within a day of hatching, with bacteria such as *Gilliamella*, *Frischella*, and *Snodgrassella* establishing early residence. Over time, other bacterial species including *Lactobacillus*, *Bifidobacterium*, and *Commensalibacter* become established, exhibiting dynamic abundance changes (Dong et al. 2020, Nowak et al. 2021).

The honey bee microbiota occupies different gut sections, with *Parasaccharibacter sp.* prominently found in the hypopharyngeal glands of workers (Corby-Harris et al. 2014, Nowak et al. 2021). Adult worker bee intestines harbor specialized microorganisms organized into nine bacterial species clusters, characterized by high resistance to ambient oxygen and transferred through social contacts (Jones et al. 2017, Nowak et al. 2021). Studies using 16S rDNA and metagenomics have

revealed that the honey bee worker's intestine is populated by nine clusters of bacterial species, including *Snodgrasella alvi* and *Gilliamella apicola*, both from the Proteobacteria phylum (Tola et al. 2020, Nowak et al.,2021, Zheng et al. 2017).

Lactobacillus Firm-4 and *Lactobacillus* Firm-5, both Gram-positive species from the Firmicutes phylum, are prevalent in the distal rectum (Jones et al. 2017, Nowak et al. 2021, Tola et al. 2020). Adult workers generally harbor *Bifidobacterium asteroides* in lower amounts (Bleau 2020, Jones et al. 2017, Nowak et al. 2021). A crucial group of microorganisms in bee intestines is the "core bacteria" clusters (Kešnerová et al. 2020, Nowak et al. 2021).

Other bacterial genera commonly found in honey bee digestive systems include *Apibacter*, *Asaia*, and *Acetobacter*. Certain rare bacteria associated with diseases and honey bee mortality, such as *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Serratia*, are also present in the honey bee gut (Raymann et al., 2018). Less common Proteobacteria include *Frischella perrara* (Orbaceae), *Parasaccharibacter apium*, *Bombella favorum*, *Bombella mellum*, *Bombella apis* (Acetobacteraceae), *Commensalibacter sp.* (Alpha 2.1), and *Bartonella apis* (Rhizobiaceae) (Bleau, 2020, Dong et al. 2020, Jones et al. 2017, Kešnerová et al. 2016, Hilgarth et al. 2021, Nowak et al. 2021, Tola et al. 2020). *Apibacter adventoris*, *Apibacter mensalis*, a *Bacteroidetes* members, has also been identified (Nowak et al. 2021).

The honey bee gut comprises 10 known taxa, including four *Lactobacillus sp.*, two *Gilliamella* species, one *Bifidobacterium* species, and one *Snodgrassella* species, considered part of the core gut microbiota. *Frischella* and *Bartonella* taxa may vary by habitat (Nowak et al., 2021). Dominant phyla in the honey bee gut include Proteobacteria (63.2%), Firmicutes (17.6%) with 15.9% *Lactobacillus sp.*, Actinobacteria (4.1%) with 3.34% *Bifidobacterium sp.*, and *Bacteroidetes* (1.7%) with 0.23% *Bacteroides sp.* (Wang et al., 2021; Nowak et al., 2021). The core member *Lactobacillus* firm-4 is detected in 98.4% of examined honey bees (Brudzynski, 2021, Kešnerová et al. 2020, Nowak et al., 2021).

Lactic acid bacteria isolated from honey bee workers, including *Enterococcus faecalis* (HBE1, HBE3, HBE4), *Lactobacillus brevis* (HBE2), and *Lacticaseibacillus* casei (HBE5), show potential as pharmacobiotics for functional dietary foods that promote human health (Elzeini et al. 2021). These bacteria exhibit enhanced antimicrobial, antioxidant, and anti-inflammatory effects and can survive in human and animal gastrointestinal tracts under stressful conditions (Elzeini et al. 2021, Nowak et al. 2021).

Research in Sub-Saharan Africa, particularly in Kenya, highlights significant members of the honey bee gut microbiota, including *Gilliamella*, *Snodgrassella*, *Lactobacillus* (Firm-4 and Firm-5), *Bifidobacterium*, *Frischella*, *Commensalibacter*, *Bombella*, *Apibacter*, and *Bartonella* (Nowak et al. 2021, Tola et al. 2020). Fungal taxa such as *Saccharomyces*, *Zygosaccharomyces*, and *Candida* are also present in honey bee digestive systems (Nowak et al. 2021).

Paenibacillus species, commonly found in honey bee hives and wild solitary bee nests, include pathogenic strains such as *P. alvei, P. apiarius*, and *P. larvae*, which coexist with *Melissococcus plutonius*, the agent of European foulbrood (Grady et al. 2016). *P. larvae* causes American foulbrood disease (Genersch 2010). *Paenibacillus* strains produce antimicrobial compounds like lantibiotics, bacteriocins, lipopeptides, and putative sactipeptides, exhibiting broad-spectrum activity against foodborne pathogens (Grady et al. 2016, Pomastowski et al. 2019). Polymyxins, produced by *P. polymyxa*, are particularly effective against Gram-negative bacteria, including multidrug-resistant strains, targeting the outer membrane to cause cell lysis (Poirel et al. 2017). *P. polymyxa* TH13 isolates from honey produce polymyxin E, showing broad antibacterial activity, including against *P. larvae* (Lee et al. 2009). *P. alvei* MP1 from buckwheat honey demonstrates activity against *L. monocytogenes*, *S. aureus*, and *E. coli* O157 (Pajor et al. 2020).

Microbial contaminants in honey, while a safety concern, can also produce beneficial antimicrobial compounds. These include *Lactobacillus*, *Bifidobacterium*, *Bacillus*, and yeast (*Saccharomyces cerevisiae*), which prevent food spoilage (Xiong et al. 2022; Brudzynski 2021). Studies show that raw honey can inhibit various food spoilage microorganisms and human pathogens, including *Aspergillus* niger, *Penicillium expansum*, *Lactobacillus acidophilus*, *Pseudomonas fluorescens*, *Bacillus cereus*, *E. coli* O157, *Listeria monocytogenes*, *Salmonella enterica* Ser. *Typhimurium*, and *S. aureus* (Xiong et al., 2022; Carter et al. 2016, Brudzynski 2021). Over 90% of

bacterial strains found in honey show in vitro antimicrobial activity, highlighting the significant role of microbial strains in honey's antibacterial properties (Chanclud & Lacombe 2017, Xiong et al. 2022).

The honey microbiota is influenced by the microbial colonization of nectar, honey, and honey bees, shaping the metabolites produced through competition (Brudzynski 2021). This microbiota includes dominant bacterial orders Lactobacillales and Bacillales (genera *Bacillus* and *Paenibacillus*), along with fungi and yeasts, forming the core microbiota of honey and contributing to its antimicrobial activity (Brudzynski 2021).

Microbial contamination in honey originates from various environmental sources such as air, water, and pollination environments, with bees acting as primary vectors through their foraging activities (Alvarez-Pérez et al. 2012). During the transformation of nectar into honey, the diversity and composition of the microbiota decrease due to ripening processes (Wen et al. 2017). Metagenomic analyses reveal an overlap between the core microbiota of honey and those of nectar, pollen, bee stomachs, and crops, with Actinobacteria, Firmicutes, and Proteobacteria being dominant, and *Bacillus* and *Lactobacillus* the most abundant families (Anderson et al. 2013, Corby-Harris et al. 2014, Bovo et al. 2018).

While the composition of the honey microbiota can vary based on botanical and geographical origins, *Bacillus* and *Lactobacillus* consistently play significant roles and contribute to honey's antimicrobial properties (Alvarez-Pérez et al. 2012, Lee et al. 2008). The Lactobacillaceae and Bacillaceae families are key producers of antibacterial chemicals such as bacteriocins, surfactants, and siderophores (Caulier et al.,2019, De Vuyst & Leroy 2007, Zacharof & Lovitt 2012). These families significantly influence honey's antibacterial efficacy (Khan et al. 2016). The dynamic interactions within the honey microbiota are crucial for maintaining honey's quality and antimicrobial activity (Figure 3) (Brudzynski 2021).

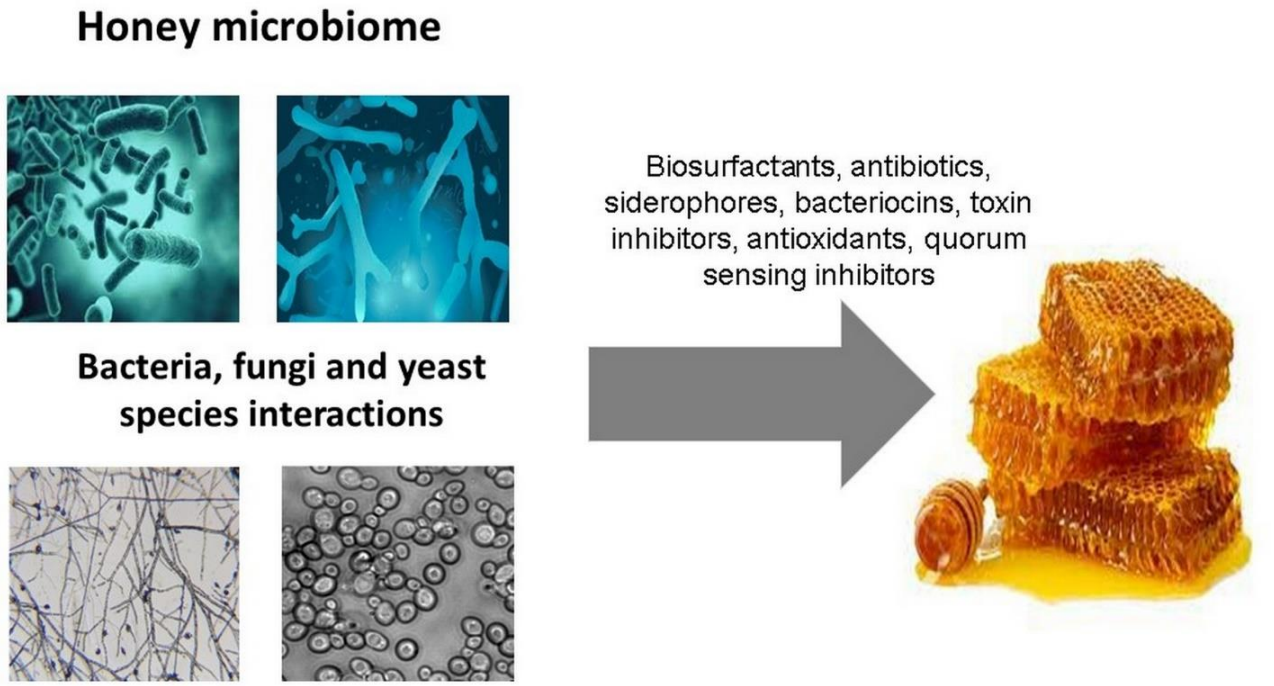

Figure 3. Contribution of the honey microbiota in the formation of prebiotic properties of honey.

Compared to other fermented foods, honey contains fewer microorganisms. Its microbiota mainly originates from pollen, flowers, soil, air, dust, and the digestive tract of honey bees (Snowdon & Cliver, 1996; Kafantaris et al., 2020). This microbiota includes bacteria, yeasts, and molds, with

environmental factors influencing its composition (Kafantaris et al. 2020, Wen et al. 2017). Human processing can introduce secondary contaminants (Snowdon & Cliver 1996, Olaitan et al. 2007). Plant-associated microorganisms are transferred to hives during pollination, with the honey bee gut being a significant source of microbial transmission into honey. Lactobacilli and Bifidobacteria, conserved components of the honey bee gut microbiota, are also found in honey and bee nectar (Anderson et al. 2013, Raymann et al. 2018, Olofsson & Vásquez 2008).

Although most gut bacteria thrive only in the gut, *Lactobacillus kunkeei* and Acetobacteraceae (*Asaia spp.*) are exceptions, found in extreme conditions such as honey and royal jelly (Anderson et al. 2013, Xiong et al. 2022). Honey's physicochemical properties—high sugar content, low water activity, and low pH—mean most bacteria found in honey are osmotolerant, xerotolerant, and acidotolerant. Consequently, many bacteria in honey remain metabolically dormant (Brudzynski 2021, Xiong et al. 2022). The honey microbiota is influenced by factors like titratable acidity, water activity, and color (Balzan et al. 2020, Kňazovická et al. 2019, Xiong et al. 2022).

The honey microbiota serves as a bioindicator, reflecting agricultural and urban landscapes, microbial environments to which honey bees are exposed, and chemical contaminants along foraging routes. It also indicates hive immune status and overall hive health (Xiong et al. 2022). Next-generation sequencing (NGS) metagenomics provides taxonomic classification of the honey microbiota and serves as an indicator of honey origin and hive health (Bovo et al. 2020, Xiong et al. 2022).

Prebiotic potential of honey due to Lactic Acid Bacteria and Bifidobacteria derived compounds

Honey bees deploy both cellular and humoral immune defenses in response to infections caused by various pathogens, including bacteria, fungi, viruses (Evans et al. 2006). Honey bees have own individual and social immunity, but their humoral immune responses are relatively limited, primarily involving the production of a few antimicrobial peptides. These include proline-rich apidaecins, abaecins, cysteine-rich defensins 1 and 2, and glycine-rich hymenoptaecin (Casteels-Josson et al. 1994). In honey, antimicrobial peptides such as defensin-1, hymenoptaecin, and jelleins, derived from honey bees, contribute to its antimicrobial properties (Brudzynski & Sjaarda, 2015, Di Girolamo et al. 2012, Klaudiny et al. 2005, Kwakman et al. 2010;). Defensin-1 and royalisin (defensin 2), found in honey and royal jelly respectively, exhibit distinct antimicrobial activities against Gram-positive bacteria and fungi (Fujiwara et al. 1990, Brudzynski 2021). Royalisin additionally shows activity against *Paenibacillus larvae larvae* (Bachanova et al. 2002). Hymenoptaecins, inducible antimicrobial peptides synthesized in response to bacterial infections, require proteolytic processing for activation and are introduced into honey through bee hypopharyngeal gland secretions (Xu et al. 2009). Jelleins, located within the major royal jelly protein 1 (MRJP 1), exert antimicrobial effects primarily against Gram-positive bacteria, following proteolytic release from MRJP 1 (Brudzynski & Sjaarda 2015).

Honey bees possess additional defenses against pathogens through their microbiota, particularly lactic acid bacteria. Lactic acid bacteria play a crucial role in the honey bee gut microbiota, contributing to the bees' overall health and immune function (Ilyasov et al. 2024). These bacteria produce lactic acid as a metabolic product, creating an acidic environment in the bee gut that inhibits the growth of harmful pathogens (Vásquez & Olofsson 2009).

Several species of lactic acid bacteria have been identified in the honey bee gut, including *Lactobacillus*, *Bifidobacterium*, and others (Anderson et al. 2013). Lactic acid bacteria and fructophilic lactic acid bacteria are particularly prevalent in carbohydrate-rich environments, such as honey and other bee products (Forsgren et al., 2009; Endo & Salminen, 2013). The honey bee's foregut serves as a food storage area and the starting point for lactic acid bacteria to break down carbohydrates, deriving from both the pollination environment and the bee's digestive system (Anderson et al. 2013, Corby-Harris et al. 2014, Olofsson et al. 2014).

The lactic acid bacteria not only compete for nutrients and space with potential pathogens but also produce antimicrobial compounds such as bacteriocins and organic acids that directly inhibit pathogen growth (Olofsson & Vásquez 2008). Moreover, lactic acid bacteria have been shown to stimulate the honey bee immune system, enhancing the expression of antimicrobial peptides and other immune-related genes (Forsgren et al. 2009, Raymann et al. 2017). This immune stimulation further strengthens the bees' ability to fend off

infections caused by various pathogens, including bacteria, fungi, and viruses.

The antibacterial activity of bee-derived products is significantly influenced by lactic acid bacteria. The antibacterial activity of bee-derived products is significantly influenced by lactic acid bacteria (Ilyasov et al. 2024). Various lactic acid bacteria isolated from pollen, honey, bee bread, and crops demonstrate antimicrobial activity against foodborne and multidrug-resistant pathogens, as well as bee pathogens (Ramos et al. 2020). For instance, *Lactobacillus johnsonii*, *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Lactobacillus apis* inhibit *Melissococcus plutonius* and *Paenibacillus larvae*, causative agents of European and American foulbrood infections (Forsgren et al. 2009). Lactic acid bacteria strains from Malaysian honey, like *Lactobacillus acidophilus*, show inhibition of antibiotic-resistant *S. aureus, Staphylococcus epidermis*, and *Bacillus subtilis*, and *L. kunkeei* prevents *P. aeruginosa* biofilm development (Berríos et al. 2018).

Lactic acid bacteria also produce potent antifungal compounds against various fungi and yeasts. Lactobacilli isolated from beebread, such as *Fructobacillus fructosus*, *Fructobacillus tropaeoli*, and *L. kunkeei*, show strong antagonism against *Zygosaccharomyces rouxii*, a common spoilage yeast (Ramos et al. 2020). When honey's moisture content exceeds 18%, spoilage yeasts like *Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii* can develop and ferment honey, but lactic acid bacteria's antifungal activity can prevent this (Chaven, 2014; Muhialdin et al., 2018). Lactic acid bacteria also deactivate mycotoxins produced by fungi such as *Penicillium*, *Fusarium*, and *Aspergillus*, either through binding and adhering to cell walls or by metabolizing mycotoxins into non-toxic derivatives (Brudzynski 2021, Muhialdin et al., 2018, Sadiq et al., 2019).

Honey can contain up to 10^8 colony-forming units per gram of viable lactic acid bacteria (Vásquez et al. 2012). Honey bees transfer bacteria from their gut microbiota into nectar, influencing honey's bacterial composition, which includes species from Enterobacteriaceae and Firmicutes such as *Lactobacillus*, *Bacillus*, and *Weissella* (Kafantaris et al. 2020, Naseer et al. 2015; Olofsson et al. 2016). Commonly identified bacteria in honey also include *Achromobacter*, *Citrobacter*, *Enterobacter*, *Flavobacterium*, *Proteus*, and *Pseudomonas* species (Kafantaris et al., 2020).

Studies on stingless bee honey reveal *Lactobacillus malefermentans* as the most abundant species (Li et al. 2020, Xiong et al. 2022). However, lactobacilli may be absent in honey with less than 18% moisture during ripening (Wen et al. 2017, Xiong et al. 2022). The presence of *L. kunkeei* varies depending on bloom source and season (Vásquez et al. 2012, Wen et al. 2017, Xiong et al. 2022). *Enterobacter*iaceae in honey likely originate from the pollination environment and are often isolated from foraging honey bees (Corby-Harris et al. 2014; Xiong et al. 2022).

Honey's bacterial composition remains relatively conserved during ripening, with *Bacillus* and *Lactococcus* being the most common phylotypes, while fungal communities show more diversity (Xiong et al. 2022). Flowers such as Chasteberry harbor *Metschnikowia*, *Cladosporium*, and *Alternaria*, while Chasteberry honey contains *Metschnikowia*, *Phoma*, and *Candida*, highlighting a divergence in fungal communities (Kafantaris et al. 2020, Wen et al. 2017).

Beneficial plant microorganisms, such as actinobacteria, also contribute to bee health by producing secondary chemicals that inhibit fungal growth and spoilage (Anderson et al. 2013, Kurek-Gorecka et al. 2020). Secondary metabolites produced by lactic acid bacteria, like *L. kunkei*, support hive health by combating spoilage microbes and pathogens (Arredondo et al. 2018, Xiong et al. 2022).

A recent study using Illumina MiSeq NGS analyzed bacterial diversity in honeys from various countries, identifying 52 bacterial genera. *L. kunkei* and *L. apinorum* were most abundant in some samples, while pathogens like *Melissococcus plutonius* and *Enterococcus faecalis* were also present, likely due to sample contamination (Kafantaris et al. 2020, Kňazovická et al. 2019).

Lactobacillus kunkei, commonly found in flowers, fruits, soil, hive environments, and fermented bee products (Anderson et al. 2013), has been identified in honey bee bread and influences honey composition under various conditions (Xiong et al. 2022). Another prevalent bacterium in honey, *Lactococcus lactis*, universally present in all sequenced honeys (Xiong et al. 2022), participates in the fermentation of honey carbohydrates, producing lactic acid (Sinacori et al. 2014, Xiong et

al. 2022).

Lactic acid bacteria, including *Lactococcus*, are introduced into bee species through horizontal transfer between hives and their environment. These bacteria, during their logarithmic growth phase, synthesize bacteriocins, small cationic antimicrobial peptides (Rutherford & Bassler 2012). Reviews have extensively covered the structure, classification, and mechanism of action of these bacteriocins (Alvarez-Sieiro et al., 2016, De Vuyst & Leroy 2007, Zacharof & Lovitt 2012). Despite the abundance of lactic acid bacteria in honeycomb products, only one bacteriocin, kunkecin A, has been identified in honey bee *L. kunkeei* to date (Zendo et al. 2020). However, other lactobacilli like *L. johnsonii*, *L. plantarum*, *L. brevis*, and *L. apis* show potential for producing bacteriocins with activity against honey bee pathogens (Endo & Salminen 2013, Forsgren et al. 2009).

Nisin A, also known as kunkecin A, derived from *Lactococcus lactis subsp. lactis*, is a class I lantibiotic bacteriocin that targets lipid II in Gram-positive bacterial cell walls (Zendo et al. 2020). It exhibits broad-spectrum antibacterial activity against bacteria such as staphylococci, streptococci, bacilli, clostridia, and mycobacteria. In contrast, kunkecin A specifically targets *Melissococcus plutonius*, the causative agent of European foulbrood (Zendo et al. 2020). Lantibiotics like nisin can disrupt Gram-negative bacteria when their outer membrane integrity is compromised by chelating agents such as EDTA (Parada et al. 2007).

Apart from bacteriocins, lactic acid bacteria produce biosurfactants, which alter cell envelope properties and inhibit biofilm formation by interacting with membrane components. These biosurfactants influence biofilm development by interfering with quorum sensing systems, crucial for bacterial survival and pathogenicity regulation (Rutherford & Bassler 2012). *Lactobacillus* species-derived biosurfactants demonstrate potent anti-biofilm activity against clinical isolates of Gram-positive and Gram-negative bacteria, as well as against fungi (Table 2) (Sharma & Saharan 2016).

Table 2. Prebiotic potential of honey due to Lactic Acid Bacteria and Bifidobacteria.

Species	Antimicrobial compounds	Target species	References
Lactobacillus acidophilus	acidocin, lactacins	*Lactobacillus sp., Listeria monocytogenes, Enterococcus faecalis, Limosilactobacillus fermentum, Enterococcus faecalis, Lactobacillus delbrueckii, Lactobacillus helveticus, Lactococcus lactis.*	Oscáriz and Pisabarro, 2001; Parada, et al., 2007; Brudzynski, 2021
Lactobacillus helveticus	helveticin J, lactocin 27	*Lactobacillus sp., Lactobacillus bulgaricus, Lactococcus lactis*	Upreti and Hinsdill, 1975; McAuliffe et al., 2001
Lactobacillus johnsonii	lactacin F	*Lactobacillus sp., Enterococcus faecalis, Lactobacillus delbrueckii, Lactobacillus helveticus*	Abee et al., 1994; Allison et al., 1995; McAuliffe et al., 2001
Lactobacillus kunkeei	kunkicin	*Listeria monocytogenes, Staphylococcus aureus*	McAuliffe et al., 2001; Brudzynski, 2021
Lactobacillus plantarum	plantaricin A	*Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium, Escherichia coli, Bacillus cereus, Bacillus pumilus, Bacillus megaterium*, *Pediococcus*, *Carnobacteria*, *Clostiridia*, *Propionobacteria*	Gong et al., 2010; Meng et al., 2022
Lactobacillus lactis	nisin, lacticin 3147	*Staphylococcus aureus, Listeria innocua, Lactobacillus sakei, Lactiplantibacillus plantarum, Enterococcus faecalis, Propionibacterium acne, Streptococcus mutans, Bacillus sp., Micrococcus sp., Clostridium sp.*	Parada, et al., 2007; Alegría et al., 2010; Brudzynski, 2021
Pedicoccus pentosaceus	pediocin	*Listeria monocytogenes, Lactobacillus sp., Lactococcus sp., Leuconostoc spp, Pediococcus sp., Staphylococcus sp., Enterococcus sp., Clostridium sp.*	Jack et al., 1995; Parada, et al., 2007; Brudzynski, 2021

Prebiotic potential of honey due to Bacillales derived compounds

Bacillales, a dominant order within Firmicutes, thrives in nectar and honey environments, constituting a substantial portion of honey microbiota, ranging from 60% to 90% of all bacteria present. The genera *Bacillus* and *Paenibacillus* are particularly prominent within this order (Pajor et al. 2018; Pomastowski et al. 2019). Through advanced molecular techniques like 16S rRNA gene sequencing and MALDI-TOF analysis, *Bacillus* isolates have been categorized into three main phylogenetic clusters: the *B. subtilis* group (including *B. subtilis*, *B. methylotrophicus*, *B. atrophaeus*, *B. licheniformis*, and *B. amyloliquefaciens*), the *B. cereus* group (comprising *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis*), and the *B. pumilis* group (represented by *B. pumilis*, *B. safensis*, and *B. altitudinis*) (Brudzynski, 2021, Chaven 2014, Zacharof & Lovitt 2012).

The family Bacillaceae dominates honey microbiota due to its robust production of antimicrobial compounds aimed at outcompeting other microorganisms (Caulier et al. 2019). *Bacillus* and *Paenibacillus* genera are prolific producers of antimicrobial substances such as bacteriocins, lipopeptides (surfactants), and siderophores, which confer a competitive advantage by inhibiting the growth of neighboring species (Jack et al. 1995).

Specific *Bacillus* groups present in honey, including *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. thuringiensis*, and *B. cereus*, produce strain-specific bacteriocins like subtilin, subtilosin, lichenicidin, thuricins, and cereins (Caulier et al. 2019). These bacteriocins exert their antimicrobial effects through mechanisms such as pore formation and membrane permeabilization, leading to cell death (Brudzynski 2021).

One notable example is subtilin, structurally similar to nisin from *Lactococcus lactis*, which disrupts bacterial cell walls by binding to lipid II, thereby inhibiting peptidoglycan synthesis and inducing cell lysis (Caulier et al. 2019). Despite their production by *Bacillus* strains in honey, many of these bacteriocins have not been directly detected in the honey itself (Brudzynski 2021).

Bacillus species also produce lantibiotic bacteriocins like thuricins, which exhibit potent activity against various pathogens including *Clostridioides difficile* and other Gram-positive bacteria (Lee et al. 2009, Rea et al. 2010). These bacteriocins induce cell death through mechanisms involving membrane disruption and interference with peptidoglycan synthesis, similar to the action of β-lactam antibiotics (Cho et al. 2014).

In addition to bacteriocins, *Bacillus* strains are capable of synthesizing diverse non-ribosomal peptides and polyketides, which contribute to their competitive survival in microbial communities (Straight & Fischbach 2016, Zhao & Kuipers 2016). These compounds target various cellular components in competing species, providing *Bacillus* with a substantial survival advantage in diverse environments (Caulier et al. 2019).

Moreover, *Bacillus* and *Paenibacillus* genera are known for producing lipopeptides such as surfactin, fengycin, and iturin, which exhibit broad-spectrum antibacterial and antifungal activities by disrupting membrane integrity (Vlamakis et al. 2013, Janek et al. 2020). These lipopeptides function as biosurfactants, reducing membrane surface tension and inhibiting biofilm formation, thereby further enhancing *Bacillus*' competitive edge (Table 3) (Vlamakis et al. 2013; Deleu et al. 2013; Janek et al. 2020).

Furthermore, siderophores produced by *Bacillus* and *Paenibacillus* species sequester iron from the environment, depriving competing microorganisms of this essential nutrient and inhibiting their growth (Miethke et al., 2006). Bacillibactin, a catecholate siderophore produced by various *Bacillus* species, including *B. subtilis* and *B. cereus*, enhances iron uptake under low-iron conditions, contributing to their competitive success (Khan et al. 2016).

Table 3. Prebiotic potential of honey due to Bacillales.

Species	Antimicrobial compounds	Target species	References
Bacillus subtilis	subtilin, subtilosin A, sublancin, surfactin, fengycin, bacillomycin, bacillibactin, bacitracin, bacilysin, bacillaene	Gram+, *Listeria monocytogenes*, *Gardnerella vaginalis*, *Streptococcus agalactiae*, *Bacillus cereus*, *Streptococcus pyogenes*, *Staphylococcus aureus*	Abriouel et al., 2011; Zhao & Kuipers, 2016; Caulier et al., 2019
Bacillus licheniformis	lichenin, bacitracin, lichenicidin, lychenisin	Gram+, *Listeria monocytogenes*. *Streptococcus bovis*, *Staphylococcus aureus*	Abriouel et al., 2011; Zhao & Kuipers, 2016; Brudzynski, 2021
Bacillus amyloliquefaciens	amylolysin, iturin, bacillaene, bacilysin, subtiliosin, fengycin, surfactin	Gram+, *Listeria monocytogenes*, *Staphylococcus aureus*	Zhao & Kuipers, 2016; Brudzynski, 2021
Bacillus cereus	cereins, bacillibactin, thuricin	Gram+, *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Bacillus pumilus*	Abriouel et al., 2011; Zhao & Kuipers, 2016; Brudzynski, 2021
Bacillus thuringiensis	thuricin 17, thurincin H, thuricin CD	Gram+, *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus megaterium*, *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Staphylococcus aureus*, *Carnobacterim psicola*, *Geobacillus stearothermophillus*, *Clostridium difficile*, *Escherichia coli* MM294	Rea et al., 2010; Abriouel et al., 2011; Zhao & Kuipers, 2016
Bacillus pumilis	pumilicin, surfactin, bacilysin, pumilacidin, bacitracin	Gram+, *Staphylococcus aureus*, *Enterococcus faecalis*	Abriouel et al., 2011; Sumi et al., 2015; Brudzynski, 2021
Bacillus megaterium	megacin, surfactin, fengycin, bacillomycins	Gram+, *Staphylococcus aureus*	Sumi et al., 2015
Paenibacillus larvae	paenibacterin, iturin, tridecaptin, fusaricidin	Gram+, Gram-, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*	Sood et al., 2014; Keller et al., 2018
Paenibacillus polymyxa	paenibacillin, bacillibactin, bacillaene, polymyxin, paenimacrolidin	Gram+, Gram-, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*	Zhao & Kuipers, 2016; Keller et al., 2018; Brudzynski, 2021
Brevibacillus brevis	gramicidin	Gram+, Gram-, *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*	Sumi et al., 2015; Brudzynski, 2021

Prebiotic potential of honey due to fungi derived compounds

Yeasts and fungi are prevalent contaminants in nectar and honey, thriving under conditions such as low pH, high sugar concentration, and antagonistic interactions among microbes (Alvarez-Pérez et al. 2012, Snowdon & Cliver 1996). These resilient microorganisms, categorized as osmotolerant, xerotolerant, and acidotolerant, include predominant genera like *Candida*, *Eremascus*, *Metschnikowia*, *Bettsia*, *Monascus*, *Oidiodendron*, *Pichia*, *Saccharomyces*, *Skoua*, *Torulopsis*, and *Zygosaccharomyces* (Sinacori et al. 2014).

Ascomycetes, including filamentous fungi such as *Aspergillus*, *Penicillium*, *Cladosporium*, and *Fusarium*, also flourish, with less frequent occurrences of *Arthrinium*, *Chaetonium*, *Daldinia*, and *Emericella* (Sinacori et al. 2014). Notably, xerotolerant genera like *Bettsia*, *Ascosphaera*, *Metschnikowia*, and *Eremascus* survive at very low water activity levels (up to 0.82), while acidophilic or acidotolerant genera like *Pichia*, *Saccharomyces*, and *Zygosaccharomyces* thrive even in acidic environments below pH 2 (Brudzynski, 2021, Snowdon & Cliver 1996, Rodríguez-Andrade et al. 2019).

Serratia symbiotica constitutes about 4.8% of bacteria in polyfloral honey from Italy (Bovo et al. 2020), commonly found in Hymenoptera species (Xiong et al., 2022). Originating as a secondary endosymbiont of aphids, some *Serratia* species have evolved through aphid honeydew consumption by bees, influencing honey production (Bovo et al. 2020, Xiong et al. 2022). Certain *Serratia* strains can act as opportunistic pathogens for honey bees, impacting hive health (Raymann et al. 2018, Xiong et al. 2022).

Honey often contains yeast and mold (Kačániová et al. 2009, Kafantaris et al. 2020), with common fungal taxa including *Bettsia*, *Yarrowia*, *Skoua*, *Zygosaccharomyces*, and *Metschnikowia*. Chasteberry honey displays a diverse fungal profile, featuring prevalent genera like *Waitea*, *Phoma*, *Metschnikowia*, and *Cryptococcus*, reflecting their prevalence in chasteberry flowers (Xiong et al. 2022). Genera such as *Waitea* and *Cryptococcus* may be absent from certain honeys due to their floral specificity, commonly found in Vitex flowers (Wen et al. 2017, Xiong et al. 2022). Culture-dependent methods highlight *Zygosaccharomyces* and Debaryomyces as the most abundant yeast taxa in honey (Sinacori et al. 2014, Xiong et al. 2022), consistently identified via culture-independent ITS2 metabarcoding (Balzan et al. 2020, Xiong et al. 2022).

Fungal and yeast genera found in honey encompass *Aspergillus*, *Penicillium*, *Monascus*, *Bettsia*, *Skoua*, *Oidiodendron*, *Eremascus*, *Ascosphaera*, Schizo*saccharomyces*, *Zygosaccharomyces*, *Candida*, *Saccharomyces*, Cyberlindnera, *Starmerella*, *Cladosporium*, *Alternaria*, *Stemphylium*, *Fusarium*, and *Mucor* (Brudzynski, 2021, Sinacori et al. 2014, Rodríguez-Andrade et al. 2019). Notably, *Cladosporium*, a filamentous fungus widely distributed in the environment, is commonly found in honey and may coexist with bees, possibly transmitted from plants or through bee interactions, and persists in bee products (Martinson et al. 2012). Similarly, filamentous fungi like Botrytis, *Penicillium*, and *Mucor*, prevalent in plant pollen, are transferred to honey bees and are frequently found in beebread (Disayathanoowat et al. 2020). Common yeast genera isolated from pollen and beebread include *Candida*, *Cryptococcus*, Kloeckera, *Metschnikowia*, and Rhodotorula (Brudzynski 2021).

Certain fungi, such as *Aspergillus* and *Penicillium* species, are considered environmental contaminants in honey (Kačániová et al. 2009), with *Aspergillus flavus* identified as a significant fungus in polyfloral Italian honey, known for causing stonebrood disease in honey bees (Xiong et al. 2022). *Ascosphaera apis*, responsible for chalkbrood disease, predominates in various wildflower honeys (Xiong et al. 2022). Despite their presence, the mere detection of these potentially harmful fungi does not necessarily indicate hive infection, as evidenced by their asymptomatic presence in colonies over extended periods (Xiong et al. 2022).

Fungi and yeasts employ various survival strategies within the honey microbial ecosystem, including spore formation to endure adverse conditions and the production of secondary metabolites such as mycotoxins, antibiotics, siderophores, and surfactants (Deshmukh et al. 2015). Mycotoxins, known as killer toxins, are produced by yeasts like *Metschnikowia*, *Zygosaccharomyces*, *Saccharomyces*, and *Candida*, as well as by filamentous fungi including *Aspergillus*, *Fusarium*, and *Penicillium* species (Liu et al. 2015). These toxins exert lethal effects by disrupting essential cellular functions like DNA and cell wall synthesis

(Liu et al. 2015). For example, zygocin from *Zygosaccharomyces bailii* selectively targets *Candida* species, facilitating its own growth. Lactic acid bacteria and *Bacillus spp.* in honey possess enzymatic systems capable of degrading and transforming mycotoxins into non-toxic derivatives (Kačániová et al. 2009).

Some yeast genera, like *Aspergillus* and *Penicillium*, are known producers of β-lactam antibiotics such as penicillin and cephalosporin, which inhibit cell wall synthesis by targeting penicillin-binding proteins (Venkatesh & Keller 2019). *Candida* species produce sophorolipids with anti-biofilm properties, inhibiting adhesion and biofilm formation of various species including *Candida* and *Pichia*, as well as Gram-positive bacteria (Paraszkiewicz et al. 2019). Yeasts also secrete siderophores during iron starvation to chelate essential metals like iron Fe (III), manganese, and zinc from the environment, thereby restricting the growth of competing microorganisms (Brudzynski 2021).

Prebiotic potential of honey due to plant plant derived compounds

Plant metabolites in honey play a crucial role in shaping the prebiotic properties of honey, influencing its nutritional and therapeutic benefits. Honey, a complex mixture produced by honey bees from floral nectar and enriched with plant-derived compounds, exhibits diverse prebiotic effects due to these metabolites.

Plant-derived essential oils and terpenoids in honey provide potent antimicrobial effects, inhibiting pathogens and enhancing its shelf-life and therapeutic uses (Brudzynski & Sjaarda 2015). Plants use R genes to produce pathogenesis-related (PR) proteins like defensins (PR-12), thionins (PR-13), thaumatin-like proteins (PR-5), and lipid transfer proteins (PR-14), known for broad-spectrum antibacterial and antifungal properties (Sudisha et al., 2011). Enzymes such as chitinases (PR-2, PR-4, PR-8, PR-11) and glucanases degrade fungal cell wall components, while PR-8 chitinases also include lysozymes targeting bacterial peptidoglycan. PR-10 proteins with ribonuclease activity may combat RNA viruses, and the nectar redox cycle produces hydrogen peroxide, bolstering plant antimicrobial defenses (Carter & Thornburg 2004). These mechanisms collectively enhance the antimicrobial properties of honey infused with plant-derived compounds.

Additionally, defense compounds like polyphenols, phenolic acids, flavonoids, terpenes, and alkaloids further bolster plant defenses. Proteomic analyses of honey have identified proteolytic enzymes, including serine-proteases like trypsin and chymotrypsin, which likely contribute to honey's anti-fungal activity through plant-derived defense molecules (Brudzynski 2021).

Plant polyphenols, such as flavonoids and phenolic acids, are abundant in honey due to their presence in floral nectars. These compounds contribute significantly to honey's antioxidant capacity, protecting against oxidative stress and reducing the risk of chronic diseases (Samaranayaka & Li-Chan 2011). Polyphenols scavenge free radicals and enhance the stability of honey's bioactive components during storage (Aumeeruddy et al. 2018).

Oligosaccharides derived from plant nectars act as prebiotics in honey, promoting the growth and activity of beneficial gut bacteria such as Bifidobacteria and Lactobacilli (Tiihonen et al. 2010). These compounds ferment in the colon, producing short-chain fatty acids that support gut health and immune function (Hiel et al. 2019).

Plant-derived compounds in honey, including organic acids and enzymes, contribute to its digestive benefits. These components aid in nutrient absorption, alleviate digestive discomfort, and promote overall gastrointestinal well-being. Flavonoids and phenolic acids in honey exhibit anti-inflammatory properties by inhibiting pro-inflammatory cytokines and enzymes. This modulation of inflammatory pathways contributes to honey's therapeutic potential in treating inflammatory conditions such as gastritis and arthritis (Erban et al. 2019, Khalil et al., 2011).

Conclusions: This review has sought to unravel the intricate biological processes that contribute to honey’s renowned therapeutic properties. Although honey’s medicinal benefits are widely recognized, there is a lack of understanding about the specific mechanisms through which these effects are achieved. Our review reveals that honey’s bioactivity is a result of the complex interplay between its chemical composition and its microbiota, including contributions from both the honeybee’s gut and the nectar’s microbiota. The role of key compounds such as hydrogen peroxide, methylglyoxal, and plant-derived polyphenols is well-established, with these agents providing honey with its potent antibacterial,

antioxidant, and anti-inflammatory properties. However, beyond these chemical constituents, the honey microbiota plays a critical role in enhancing honey's bioactivity. The bacteria, yeasts, and fungi present in honey contribute antimicrobial peptides, enzymes, and organic acids that further potentiate its therapeutic effects. Moreover, the honey microbiota's prebiotic effects are an emerging area of interest, with honey's oligosaccharides promoting the growth of beneficial gut bacteria and supporting overall gastrointestinal health. This review article underscores the importance of understanding the dynamic interactions between plants, bees, and microorganisms in shaping honey's medicinal properties. The nectar-honeybee-honey microbiota axis is a critical determinant of honey's composition, and the environmental factors that influence these interactions must be taken into account when evaluating honey's therapeutic potential. As climate change and other ecological factors continue to impact global honey production, it is vital that we deepen our understanding of these relationships to ensure the continued availability of high-quality, bioactive honey.

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DERLEME /REVIEW

DOĞANIN ŞİFASI: ARI ZEHRİNİN SAĞLIK ÜZERİNDEKİ ETKİLERİ VE UYGULAMALARI

Healing Power of Nature: Effects and Applications of Bee Venom on Health

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ÖZ

Apiterapi, başta bal arısı zehri olmak üzere bal arısı ürünlerinin kullanımıyla yüzyıllardır birçok insanın şifa edindiği ve sıklıkla başvurulan bir integratif tedavi yöntemidir. Bal arısı zehri, insan vücuduna manuel enjeksiyonla topikal olarak veya doğrudan arı sokmasıyla uygulanabilmektedir. Bal arısı zehri içerdiği peptit ve enzimler sayesinde sahip olduğu yüksek biyoterapötik potansiyeli ile başta enflamatuvar hastalıklar olmak üzere nörodejeneratif hastalıklar ve romatoid artrit gibi kas-iskelet sistemi hastalıklarının tedavisinde kullanılmaktadır. Literatürdeki birçok çalışma, bal arısı zehri bileşenlerinin biyolojik aktivitelerini tanımlamış ve bu bileşenlerin yeni nesil ilaçlar olarak potansiyel kullanımını geliştirmek etrafında şekillenmiş durumdadır. Bu derlemenin amacı, bal arısı zehrinin toplanmasını, ana bileşenlerini, temel biyolojik özelliklerini ve terapötik uygulamalarını özetlemektir.

Anahtar Kelimeler: Apiterapi, Bal arısı zehri, Apitoksin, Enflamasyon

ABSTRACT

Apitherapy is an integrative treatment method that has been relied upon for centuries, using honeybee products, primarily bee venom, for healing many human ailments. Bee venom can be administered to the human body through manual injection, topically, or directly via bee stings. Bee venom contains various bioactive molecules such as peptides and enzymes, which possess significant biotherapeutic potential in treating inflammatory diseases, neurodegenerative disorders, and musculoskeletal conditions like rheumatoid arthritis. Numerous studies in the literature have identified the biological activities of bee venom components and have focused on developing the potential use of apitoxin and its constituents as next-generation drugs. The aim of this review is to summarize the collection of bee venom, its main components, fundamental biological properties, and therapeutic applications.

Keywords: Apitherapy, Bee venom, Apitoxin, Inflammation

EXTENDED ABSTRACT

Purpose: Bee venom is a defensive mechanism product produced by bees. They metabolically produce bee venom within their venom sacs located in their bodies and apply it through a stinging apparatus called a stinger when threatened. Biochemically, bee venom contains various peptides, enzymes, and other bioactive components such as phenolic compounds. The collection, composition, and biotherapeutic effects of bee venom are frequently studied topics in the literature, particularly in Apitherapy, analytical chemistry, and medicine. The interest in bee venom has also increased due to its use for healing purposes since ancient times. In this review, the components and potential effects of bee venom are compiled and presented.

Discussion: Major components of bee venom include melittin, apamin, adolapin, and phospholipase A2. Melittin, the primary component, constitutes about 50-60% of BV's dry weight and is known for its strong antimicrobial, anti-inflammatory, and anticancer properties. Melittin can disrupt cell membranes, leading to cell lysis and death, making it a potent antimicrobial and anticancer agent. It also inhibits the activation of nuclear factor kappa B and Tumor necrosis factor-alpha, key regulators of inflammatory responses, thus exhibiting anti-inflammatory properties. Apamin blocks calcium-activated potassium channels, which can help modulate neurological functions and potentially treat neurodegenerative disorders like Parkinson's and Alzheimer's. The enzyme phospholipase A2 degrades phospholipids in cell membranes, releasing arachidonic acid and subsequent production of pro-inflammatory eicosanoids, thus modulating the immune response. Additionally, bee venom has great biotherapeutic potential synergistically. Its anti-inflammatory properties have been explored for the treatment of arthritis, multiple sclerosis, and other inflammatory diseases. Clinical studies have demonstrated that bee venom therapy can reduce pain and inflammation in patients with rheumatoid arthritis. The antimicrobial properties of bee venom, especially melittin, have been investigated for the treatment of bacterial and viral infections, showing efficacy against pathogens such as *Staphylococcus aureus* and Human immunodeficiency virus. In oncology, bee venom has shown potential in inhibiting the growth of various cancer cell lines, including breast, prostate, and lung cancers.

Conclusion: Bee venom holds considerable promise as a biotherapeutic agent due to its diverse biological activities and potential applications in various fields, including immunology, oncology, and neurology. However, further clinical research is necessary to overcome current challenges and fully realize its therapeutic potential as a toxin. Standardization, safety, and advanced delivery methods will play crucial roles in the successful integration of bee venom into mainstream medical practice.

GİRİŞ

Bal arısı (*Apis mellifera*) ile insanların yakın ilişkileri ve arıların çeşitli sebeplerle kullanımı insanlığın varoluşundan bu yana süregelen bir durum olarak karşımıza çıkmaktadır. Arı ve insan temaslarının temellerinin atıldığı erken dönemden itibaren de "apiterapi" kavramının doğduğu düşünülmektedir (Kritsky 2017). İlk arı figürlerinin tasvirleri Neolitik Çağ dönemine denk geliyor olup yine aynı dönemde farklı mağara çizimlerinde de arı ürünlerine yer verilmiştir (Ransome 2004). O zamanlardan günümüze dek arı ürünlerinin tedavi amaçlı kullanılması, farklı iyileştirici özelliklerinin keşfedilmesi süreçleri devam etmektedir (Pucca vd. 2019). Arı ürünlerinden bal arısı zehri, kendisini oluşturan biyoaktif bileşenlerin potansiyel biyoterapötik kullanımları açısından 19. yüzyılın sonlarından bu yana bir araştırma konusu haline gelmiştir (Carpena vd. 2020). Arı zehrinin şifasının kanıta dayalı keşiflerinin arıcılar kaynaklı olduğu, arılar tarafından sıklıkla sokulan bu kişilerde öncelikle artrit ve diğer kas-eklem problemlerinin ve sonrasında farklı bağışıklık sistemi aracılı "gözlemlenebilir" hastalıklara karşı direncin olağan bir sonucu olduğu düşünülmektedir (Kim 2013). Tunç Çağından bu yana insanoğlu, kadim tıp uygulamaları aracılığıyla bal arısı zehrini, enflamatuvar hastalıklar başta olmak üzere farklı hastaların şifasına ulaşabilmek kaynaklı kullanmaktadır. Bal arısı zehri, kadim zamanlardan beri geleneksel Doğu tıbbında çeşitli sağlık sorunlarının tedavisinde kullanılmıştır. Ayurveda uygulamalarında bal arısı zehrinin Rasayana (gençleştirici) ve bağışıklık sistemini güçlendirme özellikleri üzerinde durulurken Geleneksel Çin Tıbbında ise "ısı" ve "yang" enerjilerinin dengelenmesine ve bununla ilişkilendirilen hastalıkların tedavisinde kullanılmaktaydı (Gokulakrishnaa vd. 2020). Avrupa'da ise apiterapi

terimini ilk kullanan ve bal arısı zehrini kelliğe bir tedavi olarak öneren ilk kişi Hipokrat olmuştur. Yine Osmanlı İmparatorluğu dönemi halk hekimliğinde de bal arısı zehri tedavisinin kullanıldığı bilinmektedir. Tüm bu bilgilerin ışığında bal arısı zehrinin tarihsel bağlamda antienflamatuvar, farklı cilt rahatsızlıklarının giderilmesi ve eklem ağrılarında biyoterapötik etkileri sebebiyle geleneksel uygulamalar ile ilişkilendirilmiştir ancak 19.yüzyıl sonrasında bal arısı zehrinin nöroprotektif, antikolesterol ve antifungal gibi çok geniş biyoterapötik etkileri olduğu keşfedilmiştir ve günümüzde de aktif olarak araştırılmaktadır (Ullah vd. 2023).

BAL ARISI ZEHRİNİN ELDE EDİLİŞİ VE ÜRETİM YÖNTEMLERİ

Elektrik Stimülasyonu

Bal arısı zehrinin toplanmasının ana yöntemleri ilk olarak Markovic ve Mollnar tarafından 1954 ile Palmer tarafından 1961 senelerinde yapılan yayınlar ile literatüre kazandırılmıştır (Markovic vd. 1954, Palmer 1961). Sonrasında 90'lara kadar literatürde yer alan toplama metotları geliştirilerek sürece devam edilmiştir. Günümüz modern bal arısı zehri toplama tekniklerinin temeli bu çalışmalara dayanmaktadır (de Graaf vd. 2021). İlgili elektrik stimülasyonu yönteminin temel avantajı bal arılarının zehir toplanması aşamasında iğnelerini kaybetmemeleri ve hayatta kalabilmelerinden ileri gelmektedir. Bu nedenle bu yöntem, en güvenli bal arısı zehri toplama yöntemi olarak kabul edilmekte ve uygulanmaktadır. Bal arısı zehri toplanmasında maksimum verim arıların yoğun sezonu olan yaz ve sonbahar mevsimlerinde ve akşam saatlerinde elde edilmektedir. Elektrik stimülasyonu için günümüzde 12-15 Volt arasında değişen ve güç şebekesine bağlanabilen cihazlar kullanılmaktadır. Bu cihazlar 50 ila 1000 Hz frekansta, 2–3 saniye süreli ve 3–6 saniye duraklamalı elektriksel darbe üretimi sağlayabilmektedirler (Bogdanov 2016). Cihazların alt kısmında bulunan cam slayt uyarılmış bal arısı zehirlerinin dipte toplanmasına ve yine bu yüzeyden elde edilebilmesine olanak tanımaktadır. Tavsiye edilen bal arısı zehir toplama yöntemi, tüm kovan ve kolonin strese girmesini engelleyen ayrı bir toplama düzeneği ve belirli sayıda bal arısının kullanıldığı yöntemdir. Elektro-stimülasyon için en uygun süre 30-60 dakika, en uygun mola süresi ise 45-90 dakika olarak belirlenmiştir.

Rezervuar Diseksiyonu

İlgili protokol laboratuvar ölçeğinde zehir toplama çalışmaları için tasarlanmış olup, elektrik stimülasyonu sırasında meydana gelen birikme, oksitlenme ve bozunum dezavantajlarının önlenebilmesi amacıyla tasarlanmıştır (Wieser 1973). Bu yöntem ile zehir elde edilmesi için öncelikle bal arıları -20 °C'de uyuşturulmakta ve buz üzerinde tutulan bal arılarının gövdeleri dışarı çekilerek zehir rezervuarlarıyla birlikte sokma aparatı (iğnesi) çıkartılmaktadır. Çıkarılan zehir keseleri distile suda yıkanarak olası kontaminasyonlar önlenmekte ve elde edilen numune kurutularak kullanılmaktadır (Carreck 2011).

Manuel Sağım

Bu zehir toplama yönteminin çıkış noktası kütle spektroskopisi olmuştur. Kütle spektrometresinin bal arısı zehrinin bileşimini belirlemede oldukça hassas olması nedeniyle araştırmacılar, rezervuarın dış tarafında yer alan hemolenf proteinlerinin toplama aşamasında örneği kirletmesinin önüne geçmek istemişlerdir (Van-Vaerenbergh vd. 2014). Bu yöntem ile bal arısı zehri eldesinde yine rezervuar diseksiyonuna benzer şekilde bal arıları uyuşturularak rezervuarları çıkartılmaktadır. Sonrasında rezervuara iki taraftan uygulanan kuvvet ile bal arısı zehri numunelerinin tampona geçmesi sağlanmakta ve örnek toplanmaktadır (Peiren vd. 2005).

Bal Arısı Zehri İçeriği

Biyokimyasal perspektifte insanları sokan böceklerin büyük çoğunluğu peptitler, proteinler, enzimler ve diğer küçük moleküllerden oluşmaktadır ve bal arısı zehri de genel olarak bu biyoaktif bileşenlerden oluşmaktadır. Bal arısı zehri bileşimi aminoasitler, peptitler, proteinler, enzimler, şekerler, biyojenik aminler, uçucu bileşikler, fosfolipitler ve feromonlara dayanmaktadır ve bileşimin %80'i sudan oluşmaktadır.

Bal arısı zehrinin biyokimyasal profilinin karakterizasyonu, biyoaktif bileşenlerin tanımlanması ve miktarının belirlenmesi amacıyla çeşitli analitik yöntemler kullanılarak gerçekleştirilmektedir. Bu süreçte, öncelikli olarak kromatografi teknikleri önemli bir rol oynamaktadır. Yüksek performanslı sıvı kromatografisi (HPLC), melittin ve apamin gibi peptitlerin ayrılması ve miktar tayininde yaygın olarak kullanılırken (Samancı ve Kekeçoğlu, 2019), gaz kromatografisi-kütle

spektrometrisi (GC-MS) ise bal arısı zehrinde yer alan uçucu organik bileşiklerin ve küçük moleküllerin tespiti için tercih edilmektedir (Isidorov vd. 2023) . Matriks destekli lazer desorpsiyon/iyonizasyon zaman uçuşlu kütle spektrometrisi (MALDI-TOF/MS), peptitlerin ve proteinlerin moleküler ağırlıklarının belirlenmesi ve tanımlanması için sıklıkla kullanılan bir tekniktir (Baracchi ve Turillazzi, 2010).

Sıvı kromatografisi ile birleştirilen elektrosprey iyonizasyonu (ESI-MS) ise peptit ve proteinlerin yapısal ve kantitatif bilgilerinin edinilmesini sağlamaktadır (Sobral vd. 2016). Elektroforez yöntemleri, özellikle sodyum dodesil sülfat poliakrilamid jel elektroforezi (SDS-PAGE), bal arısı zehri proteinlerin moleküler ağırlıklarına göre ayrılmasında kullanılmaktadır ve protein saflığı ile bileşimini değerlendirmek amacıyla tercih edilmektedir (Kolaylı 2017).

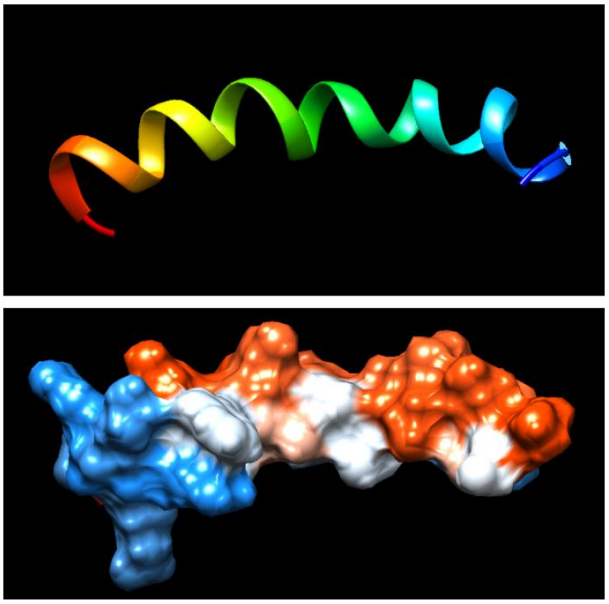
PEPTİTLER

Melittin

Alerjen bir yapı göstermesi sebebiyle Api-m4 olarak da adlandırılan Melittin, H-Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH_2 dizisinde 26 aminoasitten oluşmaktadır. Katyonik özellik göstermekte olup alfa-sarmal bir polipeptittir ve molekül ağırlığı 2846,5 g/mol'dur (Şekil 1). Fizikokimyasal olarak suyu seven bir yapısı olup, su ve diğer polar çözücülerle rahat çözünebilmektedir. Dönebilen 99 tane bağ sayısına sahip olduğundan esnek bir moleküldür ve konformasyonel değişiklikler yapabilme potansiyeli taşımaktadır. Ayrıca Topolojik Polar Yüzey Alanı geniş olduğundan (1150 $Å^2$) diğer biyomoleküllerle ve hücre zarlarıyla yüksek etkileşim potansiyeli göstermektedir. Melittin arı zehrinin %50-60'ını oluşturulan ve en çok çalışılan bileşiği olduğundan farklı biyoterapötik özellikleri de araştırmacılar tarafından ortaya konulmuştur. Melitinin en bilinen ve çalışılmış biyoaktif özelliği antienflamatuvar aktivitesidir. Temel olarak bu etki, Toll benzeri reseptörler (TLR) 2 ve 4'ün, doğal immün sistemin bir bileşeni olan CD14'ün, nükleer faktör kappa-B (NF-kB) ve trombosit türevi büyüme faktörü reseptör betasını inhibe etme mekanizmasına dayandırılmaktadır (Lee vd. 2016, Ahmedy vd. 2020). Melittinin bir diğer önemli özelliği de biyolojik membranlardaki gözenekleri şekillendirme kapasitesinden kaynaklanan sitolitik aktivitesidir (Klocek vd. 2009). Melittin yapısında 2 Lizin ve 2 Arjinin barındırmaktadır ve bu aminoasitlerin yan zincirleri fizyolojik pH'de pozitif yüklüdür. Lizin yan zincirinde bir amino grubu ($-NH^+_3$), arjinin ise guanidino grubu ($=NH^+$ - NH_2) taşımaktadır. Bu gruplar melittine toplamda dört pozitif yük kazandırmakta ve katyonik bir davranış sergilemesine aracı olmaktadır. Bu katyonik davranış biçimi de anyon lipit membranları ile oluşacak bir çekime öncülük etmekte ve melittinin hidrofobik doğası da bu etkileşimler aracılığıyla lipit membranlarına yerleşmesini sağlamaktadır. Bu yerleşim membran üzerinde şiddetli dalgalanmalara neden olmaktadır ve yük dengesizliği kaynaklı fosfolipitler arasında deformasyonlar oluşturmaktadır (Liu vd. 2018). Bu deformasyonlar membran yüzey basınçlarında dengesizlikler yaratarak gözenekler oluşmasına neden olmaktadır. Bu hücre zarı deformasyonları, melittinin antimikrobiyal, antikanser ve hemolitik aktivitesinin biyokimyasal yolağının temelini oluşturmaktadır (Lee vd. 2013). Melittinin bir diğer önemli biyoterapötik özelliği de antiviral etkisidir. Melittinin farklı *in vitro*, *in vivo* ve *in silico* çalışmalar aracılığıyla büyük bir antiviral potansiyel barındırdığı ortaya konmuştur (Memariani vd. 2020). Nanopartiküller ile fonksiyonelleştirilmiş melittinin HIV-1NLHX ve HIV-1 NLYU2 viral suşlarının enfektivitesini inhibe etme ve genom taşıyan viral kapsidi devre dışı bırakma özelliği keşfedilmiştir (Hood vd. 2013). Melittin sadece viral yapı ile etkileşime girip onu inhibe etmekle kalmayıp aynı zamanda viral replikasyonu interferon tip I'in (I-IFN) uyarılması aracılığıyla inhibe edebilmektedir (Uddin vd. 2016).

Melittinin antimikrobiyal ve antiviral etki gösterdiği biyokimyasal mekanizmasının antikanser etkisi de bulunmakta ve tümör bölgesi ile etkileşime girebilmektedir. Farklı kanser tipleri üzerinde gerçekleştirilen çalışmalar melittinin antikanser etkisinin çok geniş bir yelpazesi olduğunu *in vitro* ve *in vivo* olarak kanıtlamış durumdadır. Örneğin melittin, ölüm reseptörlerinin (ölüm reseptörleri-DR3, DR4 ve DR6) ekspresyonunu ve transkripsiyon-3 (STAT3) yolunun sinyal transdüserleri ve aktivatörlerinin inhibisyonunu artırarak insan yumurtalık kanseri hücrelerinin hücre büyümesini engellemiştir (Jo vd. 2012). Başka bir çalışmada, mitokondri yolakları aracılığıyla mide kanseri hücrelerinde apoptozu indükleyebildiği ve buna eşlik eden serbest radikal oluşumunu arttırabildiği ortaya konulmuştur (Kong vd. 2016). Melittin ayrıca

enflamasyon temel proteinlerden olan fosfoinositid 3-kinaz (PI3K), protein kinaz-B (AKT) ve 5' adenosin monofosfatla aktifleştirilen protein kinazın (MAPK) aşağı regülasyonunu ve kısmi inhibisyonunu sağlayarak cilt kanseri kaynaklı apoptozu da düzenleyebilmektedir (Lim vd. 2019). Antikanser etkisinin yanından melittinin kanser önleyici (protektif) etkisinin de olabileceği ile ilgili Yu ve arkadaşları (2019) tarafından karaciğerin immünolojik toleransı yoluyla karaciğer sinüzoidal endotel hücrelerinde (LSEC'ler) metastazı önlemek için nanopartiküller aracılı *in vivo* aktivite incelenmiştir (Yu vd. 2019). Sentezlenen melittin içerikli nanopartiküllerin LSEC'leri hedeflediğini ve negatif modüle ettiğini ortaya koymuşlardır ki bu durum karaciğer metastazını inhibe eden ve karaciğer kanserinin ortaya çıkma olasılığını azaltan immünolojik bir yanıtla sonuçlanmaktadır.



Şekil 1. Melittin 3D yapısı (üstte zincir ve altta hidrofobik katı yüzeyi olmak üzere) (Pettersen vd. 2004)

Figure 1. 3D structure of melittin (chain structure on top and hydrophobic solid surface on bottom)

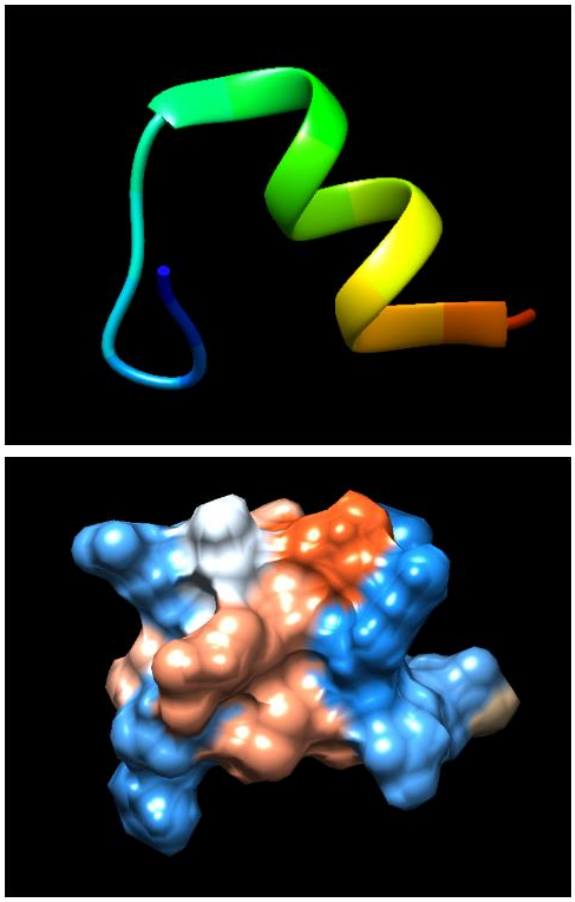
Apamin

Apamin, iki disülfit bağıyla çapraz bağlanmış 18 aminoasitten oluşmaktadır ve dizisi H-Cys(1)-Asn-Cys(2)-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys(1)-Ala-Arg-Arg-Cys(2)-Gln-Gln-His-NH_2 şeklindedir. Molekül ağırlığı 2027,4 g/mol olan bileşen nörotoksik bir peptittir. Yapısal olarak iki disülfit bağı 1-11 ve 3-15 pozisyonlarını birbirine bağlamaktadır ve üç boyutlu olarak halka şeklini alabilmektedir (Şekil 2). Ek olarak apamin, sarmal bölgenin merkezinde yer alan arginin-13 ve arginin-14'ün bitişiğinde yer aldığı bir a-sarmal çekirdeğe sahiptir ve bu iki aminoasit bölgesi nörotoksik aktivitenin sürdürülmesi açısından önem taşımaktadır. Apamin hidrofilik bir özellik sergilemekte ve suda çözünebilmektedir (Kuzmenkov vd. 2022). Melittine benzer şekilde yüksek dönebilir bağları sebebiyle oldukça esnektir ve büyük makromoleküllerle etkileşimlere girebilme potansiyeli taşımaktadır. Apamin nötr bir yükü olduğundan biyolojik yapılarda iyonize olmamaktadır. Farmakolojik vizyonda büyük ve kararlı yapıların kan beyin bariyeri (BBB) geçirgenliği ve merkezi sinir sistemine erişimi ya yavaş olmaktadır ya da olmamaktadır. Ancak apaminin bir ilginç özelliği de BBB geçirgenliğini sağlayabilmesidir (Palma 2013). Apamin, allosterik bir inhibitör olduğundan, Ca2+ ile aktifleşen K+ kanallarını bloke etme yeteneğine sahiptir ve bu durum özellikle sinir sisteminde sitotoksik aktivite kazandırmaktadır. Bunun yanında yine nörotoksik olmasından kaynaklı olarak muskarinik M2 reseptörlerini aktive edebilmekte ve kas-sinir iletkenliğini azaltabilmektedir (de Matos Silva vd. 2010). Bu özellikleri, farklı sinir sistemi hastalıklarından büyük bir potansiyel barındırdığını da ortaya koyar niteliktedir (Mohammadi-Rad vd. 2019). Melittinin antienflamatuvar aktivitesine benzer şekilde apamin de siklooksijenaz-2 (COX-2) yapısını inhibe ederek TNF-a, IL-1 ve IL-6 regülasyonlarını düşürerek yüksek antienflamatuvar etki de göstermektedir (Kim vd. 2020). Bu bağlamda yapılan bir çalışmada apaminin insan keratinosit hücre dizisinde Th2 ile ilişkili kemokinleri ve diğer proinflamatuar sitokinleri baskılayabildiğini, aynı zamanda NF-κB ve STAT yolaklarını inhibe edebildiği belirlenmiştir (Kim vd. 2017).

Mast Hücresi Degranüle Edici Peptit (MCD)

MCD, 22 aminoasitten oluşmaktadır ve yapısı H-ξIle-Lys-Cys(1)-Asn-Cys(2)-Lys-Arg-Ala-Val-ξIle-Lys-Pro-Ala-ξIle-Cys(2)-Arg-Lys-ξIle-Cys(1)-Gly-Lys-Asn-NH_2 şeklindedir. Molekül ağırlığı 2587,2 g/mol olan peptitin aminoasitlerden 3-15 ve 5-19 bölgeleri arasında iki disülfit köprüsü bulunmaktadır. Fizyolojik pH değerinde net yükü +8'dir ve bu duruma yapıda bolca bulunan lizin, arjinin ve histidin pozitif yüklü aminoasitleri neden olmaktadır. Arı zehrinin kendisi sadece az miktarda histamin içerdiğinden, MCD peptiti, granül membranlarının mast hücre membranı ile füzyonu ve granül içeriklerinin mast hücrelerinin parçalanması olmadan ekzositozu yoluyla mast hücre

granüllerinden salınarak ilave histamine katkıda bulmaktadır ki bu durum da arı sokması ve zehir maruziyeti sonrası kızarıklık, iltihaplanma ve ağrıya neden olmaktadır (Gaudenzio vd. 2016). Bu nedenle, MCD peptidi, alerjik reaksiyon için sayısız kimyasalın büyük miktarda salınmasını tetiklemek üzere hücreye bağlı spesifik IgE ile reaksiyona giren temel bir arı zehri alerjeni olarak da tanımlanabilmektedir (Elieh vd. 2018). Ek olarak MCD, apamine benzer şekilde, nöral uyarılabilirlikte bir artış meydana getirerek Ca2+ ile aktifleşen K+ kanallarını bloke etme kapasitesi nedeniyle bir nörotoksin görevi görebilmektedir (Cornara vd. 2017).



Şekil 2. Apamin 3D yapısı (üstte zincir ve altta hidrofobik katı yüzeyi olmak üzere) (Pettersen vd. 2004)

Figure 2. 3D structure of apamin (chain structure on top and hydrophobic solid surface on bottom)

Adolapin

Adolapin, Shkenderov ve Koburova (1982) tarafından bal arısı zehrinden izole edilmiş ve isimlendirilmiş bir polipeptittir. 103 aminoasitten oluşmaktadır ve bazik bir yapıdadır (Shkenderov ve Koburova 1982). Bal arısı zehrinin kuru ağırlığının ortalama %1'ini oluşturmaktadır. Literatür araştırmaları, adolapinin prostaglandin sentezini bloke ederek ve siklooksijenaz aktivitesini inhibe ederek antienflamatuvar, antinosiseptif ve antipiretik etkilere sahip olduğunu ortaya koymaktadır (Koburova vd. 1985).

Tertiapin

Tertiapin 21 aminoasit içeren küçük bir peptit olup bal arısı zehrinin içerisinde yaklaşık %0,1 kadar bulunur. Çözelti içerisindeki tertiapinin üç boyutlu NMR spektroskopi çalışması, tertiapinin yan zincir etkileşimlerinden kaynaklanan oldukça kompakt bir molekül olduğunu göstermektedir (Drici vd. 2000). Tip-4 ters dönüşten ve uzatılmış bir β tabakası tarafından oluşturulan bir döngü ile bağlanan bir α-sarmalından oluşmaktadır ve polipeptit zincirindeki dört sistein, iki disülfit bağı oluşturmaktadır (Jin ve Lu 1999). Tertiapin'in K+ kanallarını bloke ettiği farklı literatür çalışmaları ile kanıtlanmıştır (Bidaud vd. 2020, Kanjhan vd. 2005). Bu çalışmalar, tertiapinin fonksiyonel kanalların saflaştırılmasında ve aynı zamanda bu kanallara karşı farmasötik ajanların taranmasında güçlü bir ligand olarak potansiyeli olduğunu ortaya koymaktadır. Bu durum tertiapinin, kardiyoprotektif ve nöroprotektif etkisinin olabileceğini göstermektedir.

Secapin

Secapin, büyük oranda prolin ve bir disülfit köprüsü içeren 24 aminositten oluşan bir peptittir. (Meng vd. 2012). Temel olarak toksik değildir ancak farelerde yüksek dozda belirgin hipotermi ve sedasyon belirtileri oluşturduğu belirlenmiştir (Gauldie vd. 1976). Secapinin güçlü bir nörotoksin olarak hareket ettiği birçok çalışma ile kanıtlanmış olmasına rağmen, diğer biyoterapötik potansiyelleri henüz tam olarak aydınlatılmış değildir (Ye vd. 2016). Son yıllarda gerçekleştirilen çalışmalar secapinin, hiperaljezik, ödematojenik (Mourelle vd. 2014) ve antimikrobiyal özellikler (Kim vd. 2017) gösterdiğini ortaya koymuştur. Bunlara ek olarak secapin sadece bal arıları tarafından değil, diğer böcek ve hayvan türlerinde de bağışıklık sistemi ve savunma mekanizmasının olağan bir durumu olarak sentezlenmektedir (Touchard vd. 2018).

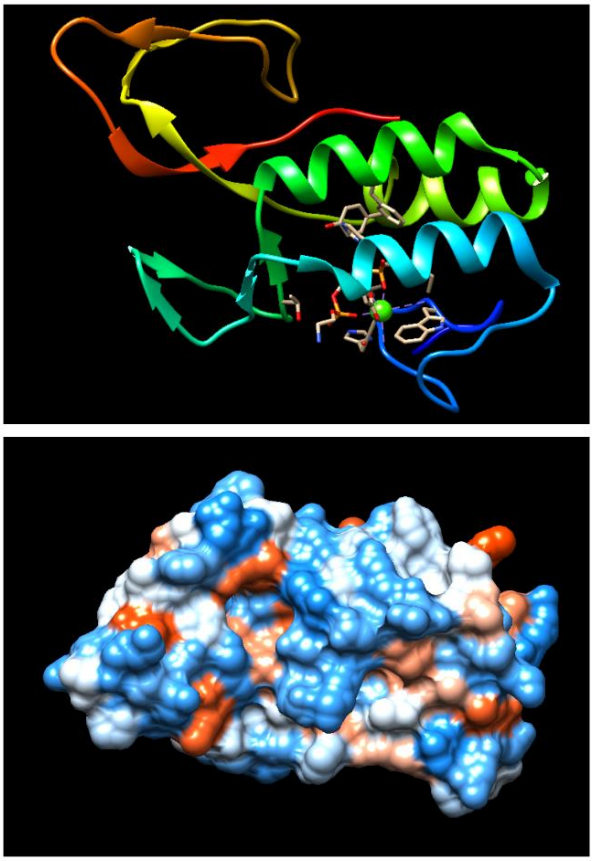
ENZİMLER

Fosfolipaz A_2

Fosfolipaz A2 (PLA2) veya Api-m1, 15.8 kDa ağırlığa sahip ve 134 aminoasitten oluşan bir polipeptittir. Konformasyonel olarak sabit bir davranış sergileyebilmek adına 9-31, 30-70, 37-63, 61-95 ve 105-113 aminoasit bölgeleri arasında toplamda 5 disülfit bağı bulunmaktadır (Şekil 3). Enzim grubu doğada çok fazla canlıda bulunması ve her birinde farklı bir yapı sergilemesi sebebiyle 16 farklı gruba ayrılmış olup *Apis mellifera*'dan elde edilen PLA2 grup 3'e aittir (Lee ve Bae 2016). PLA2 enzim grubu katalitik aktivite göstermektedir ve enzimatik aktivitesi kalsiyuma bağlıdır. PLA2'nin katalitik aktivitesi, membran gliserol-3-fosfolipidlerin sn-2 yağ açil ester bağının hidrolizi olup bu da yağ asitlerinin ve lizofosfolipitlerin serbest bırakılmasıyla sonuçlanmaktadır (Putz vd. 2007). Katalitik aktivite sonucunda hücre zarı bileşenleri hidrolize edilir ve sindirilir ki bu da zar boyunca deformasyon ve bozulmalar meydana getirir. PLA2'nin, bu membran bozulması yoluyla kanser hücrelerine karşı yüksek sitotoksik aktivite gösterdiği belirlenmiştir (Putz vd. 2007). Ek olarak bu mekanizmanın, PLA2'nin antimikrobiyal etkisine de katkısı olduğu düşünülmektedir (Nevalainen vd. 2008). Arı zehrinde bulunan PLA2'nin M (kas hücreleri) ve N (beyin zarı hücreleri) tipi reseptörlere bağlanarak bir ligand görevi de üstlenebildiği belirlenmiştir (Lee ve Bae 2016). N tipi reseptörlere bağlanması da nörotoksik aktivitesi ile ilişkilendirilmiştir (Ong vd. 2010). Ek olarak PLA2 enflamasyon ile ilişkili Foxp3'ün yukarı regülasyonunu sağlayabilir ve T hücrelerinin farklılaşması ile antienflamatuvar etki gösterebileceği için nöroenflamatuvar ve nörodejeneratif hastalıklar için de büyük bir biyoterapötik potansiyeli barındırmaktadır (Premrajan vd. 2023).

Hiyalüronidaz

Hiyalüronidaz veya Api-m2, 40.9 kDa ağırlığında ve 350 aminoasitten oluşan 1 disülfit köprüsüne sahip bir proteindir. Hiyalüronidaz, bağ dokusunun ana glikozaminoglikanı olan hiyalüronanın bölünmesini indüklemekte ve zehir bileşenlerinin dokulara ve kan dolaşımına difüzyonunu kolaylaştırarak sistemik zehirlenmeye neden olmaktadır (dos Santos-Pinto vd. 2018). Hiyalüronidaz, bağışıklık sistemi tarafından antijenik olarak algılanabilmesi sebebiyle bazı bireylerde arı sokmasına karşı şiddetli alerjik tepkiler (anafilaksi) oluşmasına sebep olabilmektedir. Hiyalüronidaz biyoterapötik açıdan, lokal anesteziklerin yayılımını artırmak için kullanılır. Enjeksiyon bölgelerinde doku geçirgenliğini artırarak ilacın daha geniş bir alana dağılmasını sağlayabilir ve travma veya cerrahi sonrası oluşan ödemlerin tedavisinde dokulardaki sıvı birikiminin çözülmesine yardımcı olabilir (Buhren vd. 2016). Son yıllarda kozmetik uygulamalarda hiyalüronidaz, yanlış yapılan veya aşırı hiyalüronik asit verilen dolgu bölgelerinde çözündürme amaçlı kullanılabilmektedir (Cohen vd. 2015).



Şekil 3. PLA2 3D yapısı (üstte zincir ve altta hidrofobik katı yüzeyi olmak üzere) (Pettersen vd. 2004)

Figure 3. 3D structure of PLA2 (chain structure on top and hydrophobic solid surface on bottom)

Fosfataz

Bal arısı zehrindeki başlıca alerjenlerden bir diğeri, asidik bir fosfataz olan Api-m3'tür (ortofosforik monoester fosfohidrolaz). Asidik pH değerlerinde fosfomonoesterleri hidrolize eden bir enzim grubuna dahil olup hidroliz yoluyla organik bileşiklerden bir fosfat grubunu uzaklaştırmaktadır. Enzimin moleküler ağırlığı 43,9 kDa'dır, 373 aminoasitten

oluşmaktadır ve izoelektrik noktası 5,64'tür (Georgieva vd. 2009). Fosfataz ile ilgili çalışmalarda enzimin vasküler endotelyal büyüme faktörü gibi belirli bir büyüme faktörünün fosforilasyonunu inhibe ederek tümör anjiyogenezini ve metastazı engelleyebilme potansiyeli ortaya konulmuştur (Huh vd. 2010).

DİĞER BİLEŞİKLER (MİNÖR BİLEŞİKLER)

Tüm bu içeriğe ek olarak bal arısı zehri %1'in altında kalan ve kuru ağırlıkta minör miktarlarda bulunan birtakım peptitler ve enzimler içermektedir. Ham bal arısı zehrinin büyük ölçekli fraksiyonlanması, melittinin bir türevi olan 19 amino asitlik melittin F'nin izolasyonuyla sonuçlanmıştır (Moreno ve Giralt 2015). Benzer şekilde kromatografik kolon üzerinde izole edildiğinde tüm arı zehrinin yaklaşık %0,7'sini oluşturan cardiopep, beta-adrenerjik benzeri bir yapıdır ve anti-aritmik etkisi bulunmaktadır (Shipman ve Cole 1969).

ARI ZEHRİ BİYOLOJİK AKTİVİTESİ

Bal arısı zehrinin hastaların yaşam kalitesini iyileştirmedeki tedavi edici değeri 100 yılı aşkın süredir iyi bilinmektedir. Modern zehir yaklaşımları, farmakolojik öneme sahip olduğu kanıtlanmış zehir bileşenlerinin keşfedilmesine olanak tanımış ve melittin ve apamin gibi aktif bileşenlerin kullanımı yoluyla terapötik stratejilerin optimizasyonunun yolunu açmıştır. Daha sonra, bal arısı zehrinin uygulama kapsamı, geleneksel antinosiseptif etkiden, sinir sisteminin dejeneratif hastalıklarına kadar genişlemiştir. Bunun nedeninin, zehir enzimlerinin ve peptitlerin, enjekte edilebilir çözünmüş maddeler olarak doğal stabiliteleri ve hedef dokulara ulaşmadaki etkinlikleri nedeniyle olduğu anlaşılmaktadır.

Antioksidan Aktivite

Bal arısı zehri antioksidan aktivitesi çoğunlukla melittin, PLA2 ve apaminin konsantrasyonuna bağlıdır. Antioksidan etkiler, bu bileşiklerin lipit peroksidasyon sürecini engellemesi ve süperoksidaz dismutaz aktivitesini arttırma kapasitesinden kaynaklı görülebilmektedir (Sobral vd. 2016). Sobral ve ark., çalışmalarında Kuzeydoğu Portekiz'den toplanılan bal arısı zehrinin 2,2-difenil-1-pikrilhidrazil (DPPH) inhibitör aktivitesini, β-karoten ağartma inhibisyonunu ve tiyobarbitürik asit reaktif maddelerin (TBARS) inhibisyonunu ayrı ayrı değerlendirmişlerdir (Sobral vd. 2016). Elde edilen sonuçlar, farklı *in vitro* metotlar için yüksek antioksidan aktiviteyi ve buna ek olarak meme kanseri ve rahim ağzı kanseri üzerinde de yüksek antikanser aktivite ortaya koymuştur. Somwongin ve ark., farklı *Apis* türlerinden elde edilen zehirlerin antioksidan etkilerini karşılaştırmış ve tüm zehir ekstraktlarının DPPH inhibisyonu gösterdiğini, buna karşılık en yüksek aktivitenin *Apis dorsata* ve ardından *Apis mellifera* türlerinden elde edilen zehir numuneleri tarafından sergilendiğini bulmuşlardır (Somwongin vd. 2018).

İn vivo çalışmalarla ilgili olarak El-Hanoun ve ark., tavşanlara 20 hafta boyunca haftada iki kez deri altı uygulama yoluyla tavşan başına 0.1, 0.2 ve 0.3 mg bal arısı zehri enjekte etmişlerdir (El-Hanoun vd. 2020). Sonuçlar, tedavi edilen tavşanlarda glutatyon-S-transferaz (GST) aktivitesinde ve glutatyon (GSH) içeriğinde bir artış ve malondialdehit (MDA) ve TBARS seviyelerinde bir azalma ortaya koymuştur; bu da bal arısı zehrinin antioksidan etkisini *in vivo* olarak doğrular niteliktedir. Ayrıca Mohamed ve ark., arı zehri ve asetilsalisilik asit ile tedavi edilen gastrik ülseri olan sıçanların lipid peroksidasyonunu zayıflattığını bulmuşlardır (Mohamed vd. 2019). Ahmed ve ark., Freund adjuvanı ile indüklenen artritik sıçanlarda antioksidan savunma sistemindeki bozulmanın, arı zehri uygulamasının bir sonucu olarak önemli ölçüde düzeldiğini bildirmişlerdir (Ahmed vd. 2018). Antioksidan sistemindeki bu iyileşme, karaciğer lipid peroksidasyonunda azalma ve glutatyon içeriğinde ve glutatyon peroksidaz ve GST dahil antioksidan enzimlerin aktivitelerinde artışla anlaşılmış ve raporlanmıştır.

Antimikrobiyal Aktivite

Bal arısı zehri antimikrobiyal etkisinin büyük oranda melittin aracılı olduğu düşünülmektedir. Melittinin antimikrobiyal etkisinin temel mekanizması, bahsedildiği üzere biyolojik membranları parçalama kapasitesinden ileri gelmektedir. Bal arısı zehri bileşenleri Gram + ve Gram - bakterilere karşı antibakteriyel aktiviteye sahiptir ve ayrıca *Candida* cinsine ait bazı türlere karşı antifungal etkiler de bildirilmiştir. Ayrıca bal arısı zehrinin yapısal savunma molekülü olan Secapin aracılı da bir antifungal etkisi bulunmaktadır.

Bal arısı zehrinin hücre zarlarını bozma ve hücrelerin yüzeysel molekülleri ile etkileşime girme kapasiteleri aynı zamanda antiviral tedavide kullanılma potansiyelini de göstermektedir. Hayvan ve bitki

virüsleri üzerinde yapılan çalışmalar, bal arısı zehrinin potansiyel antiviral aktivitesini kanıtlamış durumdadır (Picoli vd. 2018, Shi vd. 2016). Ayrıca melittinin, influenza A virüsüne (PR8), veziküler stomatit virüsüne (VSV), solunum sinsityal virüsüne (RSV) ve herpes simpleks virüsüne (HSV) karşı antiviral etkileri de *in vitro* ve *in vivo* çalışmalar ile raporlanmıştır (Uddin vd. 2016). Ek olarak arı zehrinin, *Trypanosoma brucei brucei* ve *Plasmodium falciparum* gibi bazı mikroorganizmalara karşı tedavide antiparaziter bir ajan olarak kullanılması da önerilmiştir (Boutrin vd. 2008, Dacheux vd. 2019)

Antikanser Aktivite

Literatürde yer alan birçok çalışma, bal arısı zehri ve bileşenlerinin, apoptoz indüksiyonu ve nekrozu ile farklı tümör hücrelerinin büyümesinin inhibisyonu gibi antikanser özellikler gösterdiğini bildirmiştir (Kwon vd. 2022, Małek vd. 2023). Kansere karşı apoptotik aktivite, tümör hücresi büyümesini azaltmak için en etkili yöntemlerden bir tanesidir. Melittin, bal arısı zehrinin tümör hücrelerine karşı en yüksek sitotoksik aktiviteye sahip bileşenidir. Park ve ark., tarafından gerçekleştirilen prostat kanseri üzerindeki çalışmalar, arı zehrinin Bcl-2, XIAP, iNOS ve COX-2 gibi antiapoptotik genlerin aşağı regülasyonu aracılı antikanser aktivite gösterdiğini belirlemiştir (Park vd. 2011). Bal arısı zehri aracılı apoptozun yumurtalık kanser hücresi üzerindeki etkisinin incelendiği başka bir çalışmada ise JAK2/STAT3 yolunun inhibisyonu ile antikanser etki belirlenmiştir (Jo vd. 2012). Yumurtalık kanseri hücrelerinin büyümesinin azaltılmasına yönelik etki, STAT3'ün etkisizleştirilmesi ve ölüm reseptörleri DR3, DR4 ve DR6'nın aşırı ekspresyonu sonucunda gözlemlenmiştir.

Bal arısı zehri ana bileşeni melittinin apoptozdan farklı antikanser aktivitesi Lewis akciğer karsinomu bulunan farelerde incelenmiştir (Lee vd. 2017). Melittin tedavisi, tümörle ilişkili makrofajların (TAM), özellikle tümör stromasındaki CD206+ M2 benzeri TAM'lerin sayısını azaltmıştır. CD206+ M2 benzeri TAM'lerdeki azalma nedeniyle tümör dokularındaki VEGF+ ve CD31+ hücrelerinin sayısı azalmıştır. Bu durum melittinin anti-anjiyogenik etkisini ortaya koymaktadır. Melittinin kolorektal kansere karşı *in vitro* etkilerinin incelendiği bir çalışmada ise 20 µg/mL melittin uygulamasıyla kanser hücrelerinin *in vitro* reaksiyonunun oldukça hızlı gerçekleştiği belirlenmiştir (Soliman vd. 2019). Melittin uygulamasının başlamasından sadece 15 dakika sonra hücre hattında tam ölüm meydana geldiği bildirilmiştir.

Ek olarak bal arısı zehrinin epigenetik bir ajan olarak anti-tümör etkisi, son yıllarda kanser tedavisinde potansiyel bir araştırma konusu haline gelmiştir. Bal arısı zehrinin içerdiği melittin ve apamin gen ekspresyonunu ve hücresel mekanizmaları etkileyerek kanser hücrelerinde apoptosisi indükleyebilir, hücre çoğalmasını baskılayabilir ve metastazı engelleyebilir. Uzuner ve ark. çalışmalarında bal arısı zehrinin kanser tedavisinde seçici bir DNA (de)metilasyon ajanı olarak kullanılabileceğini ortaya koymuşlardır (Uzuner vd. 2021).

Nöroprotektif Aktivite

Nörodejeneratif hastalıklar, glia hücrelerinin ve mikrogliaların kronik aktivasyonunun nöroinflamasyonu ile ilişkilidir. En önemli nöronal hastalıklardan bazıları Parkinson, Alzheimer, multipl skleroz ve amyotrofik lateral sklerozdur (ALS). PLA2 ve apamin gibi bazı arı zehri bileşenleri, birtakım ilaçların nörodejeneratif bozukluklara karşı etkinliğini artırmak için anti-nöroenflamasyon ajanları olarak incelenmiştir (Mohammadi-Rad vd. 2019). Akut Parkinson hastalığının 1-metil-4-fenil-1,2,3,6-tetrahidropiridin (MPTP) ile indüklenen fare modelinde gerçekleştirilen bir çalışma, 2 hafta boyunca her 3 günde bir bal arısı zehri akupunkturunun MPTP'nin neden olduğu enerji kaybını önlediğini ortaya koymuştur (Doo vd. 2010). Ayrıca, bal arısı zehri akupunkturu substantia nigrada MPTP'nin neden olduğu fosfo-Jun immünoreaktivitesini zayıflatmıştır; bu durum, dopaminerjik nöronların MPTP toksisitesine karşı korunmasına atfedilmiştir. Deneysel Parkinson fare modelinde yapılan daha sonraki bir çalışma, bal arısı zehrinin MPTP enjeksiyonundan sonra şiddetli dopaminerjik hücre kaybını baskıladığını göstermiştir (Kim vd. 2011). Ek olarak bal arısı zehri, nöroenflamasyon mekanizması olarak substantia nigrada MAC-1 ve iNOS ekspresyonunun artmasıyla ilişkili olan MPTP'nin indüklediği mikroglial aktivasyonu azaltmıştır. Bu bulgulara dayanarak, bal arısı zehri akupunkturunun, proenflamatuvar faktörlerin baskılanması yoluyla farelerde MPTP ile ilişkili Parkinson modeline karşı dopaminerjik nöroprotektif etki sağlayabileceği öne sürülmüştür. ALS, beyin sapı ve omurilikteki motor nöronların seçici kaybıyla karakterize, yetişkin başlangıçlı, yıkıcı bir nörodejeneratif hastalıktır. En yaygın klinik tablo yüz kaslarında veya uzuvlarda zayıflık,

fasikülasyon ve aşırı refleksivitedir (Hardiman vd. 2017)

ALS hayvan modeli olarak kullanılan hSOD1G93A transgenik farelerinde, melittin uygulaması akciğerlerde Iba-1 ve CD14, dalakta ise enflamasyonla ilişkili CD14 ve COX2 ekspresyonunu azaltmıştır (Lee vd. 2014). Akupunktur noktası yoluyla yapılan bal arısı zehri tedavisinin, enjeksiyon tedavisine göre daha etkili olduğu belirlenmiş ve buna ek olarak bal arısı zehri akupunkturu, CNS'deki endojen bağışıklık modülatör sisteminin devreye girmesiyle omurilikteki motor fonksiyonunu arttırmış ve motor nöron ölümünü azaltmıştır (Cai vd. 2015). Semptomatik hSOD1G93A transgenik farelerinde gözlemlenen beyin sapı ve omurilikte a-sinüklein fosforilasyonu ve nitrasyonun artan ekspresyonu, akupunktur noktası uyarımı yoluyla melittin veya bal arısı zehri uygulamasıyla azaltılmıştır (Yang ve Choi 2013).

Antienflamatuvar Aktivite

Bal arısı zehrinin antienflamatuvar etkinliği melittin ile ilişkilendirilmektedir. Melittin ile yapılan tedaviler, TLR yollarının aktivasyonunu modüle ederek enflamatuvar sitokinlerin oluşumunu inhibe etmektedir. Melittin in vitro olarak nükleer NF-kB p65'in aktivasyonunu baskılayabilmekte ve p38 MAPK sinyalini engelleyebilmektedir. (Lee vd. 2014). *İn vivo* olarak melittinin ayrıca, NF-kB ve AP-1 transkripsiyon faktörlerinin modülasyonu yoluyla da antienflamatuvar özellikler gösterdiği belirlenmiştir (Lee vd. 2014). Son çalışmalarda arı zehrinin atopik dermatite karşı topikal uygulama yoluyla antienflamatuvar aktiviteye sahip olduğu belirlenmiştir (An vd. 2018). Bu etkinin IgE seviyesinin, sitokin salınımının ve NF-kB ve MAP kinaz aktivitelerinin azalmasından kaynaklandığı belirlenmiştir. Romatoid artrit prevalansı ülkelere bağlı olarak %0,2 ila %0,9 arasında değişen en yaygın enflamatuvar patolojilerden birisidir (Radu ve Bungau 2021). Artritin indüklendiği sıçanlarda bal arısı zehrinin etkilerini bir çalışmada tedaviye en iyi yanıtı veren grup, 15 gün boyunca deri altından uygulanan 2 mg/kg bal arısı zehri ile tedavi edilen grup olmuştur (Kocyigit vd. 2019). Ayrıca bu grupta, pozitif kontrole göre IL-1β, IL-6, TNF-a ve TGF-β1 gibi enflamatuvar sitokinlerin daha düşük seviyelerde olduğu belirlenmiştir. Bir diğer önemli enflamatuvar hastalık olan gut artritinde ise bal arısı zehri ve apaminin (0,5 ve 1 mg/kg) intraperitoneal ve oral uygulanması, sadece enflamatuvar sitokinlerde bir azalma göstermekle kalmamış, aynı zamanda gut farelerinde pençe ödemi ve ağrıyı da ortadan kaldırmıştır (Lee vd. 2020).

ARI ZEHRİ UYGULAMALARI

Klinik Uygulamalar

Geleneksel olarak bal arısı zehri, antienflamatuvar, anti-apoptoz, anti-fibroz ve anti-artroskleroz etkileriyle bilinmekte ve yüzyıllardır çeşitli şekillerde uygulanmaktadır. Bal arısı zehri farklı teknikler aracılığıyla uygulanabilmektedir: doğrudan arı sokması, bal arısı zehri enjeksiyonu veya bal arısı zehri akupunkturu (aynı zamanda apiterapinin çıkış noktası olarak da bilinmektedir). Doğrudan sokturma, bal arısının cildi sokması sağlanarak zehrin vücuda enjekte ettirilmesi yöntemidir. Bu uygulamada bal arısı dikkatlice tutulur ve belirli noktalara sokması sağlanır ve bu durum bal arısının ölümü ile sonuçlanmaktadır. Geleneksel olarak her ne kadar doğrudan sokturma tekniği kullanılsa da günümüzde çoğu çalışma ve uygulama, bal arısı zehrinin fonksiyonelleştirilmiş uygulaması ve akupunkturun şifası ile birleşmesi sebebiyle bal arısı zehri akupunkturunu kullanmaktadır. Bal arısı zehrinin terapötik potansiyelini değerlendirmek amacıyla, insanlarda gerçekleştirilen bir çalışma, yetişkinlerin 10 akupunktur noktası üzerinde 8 gün boyunca haftada iki kez uyarılması durumunda, bal arısı zehri akupunkturunun Parkinson tedavisinde adjuvan olarak etkinlik gösterdiğini ortaya koymuştur (Cho vd. 2012).

Arı zehri akupunkturunda kullanılan bazı temel akupunktur noktaları şunlardır:

- LI4 (Hegu): Başparmak ve işaret parmağı arasındaki bu bölge, baş ağrısı ve stresin hafifletilmesinde kullanılmaktadır (Hwang 2021).
- ST36 (Zusanli): Diz kapağının altındaki kaval kemiğinin dış tarafında yer alan bu nokta, sindirim sorunları ve enerji eksikliği için oldukça etkilidir (Kwon vd. 2001).
- GB20 (Fengchi): Boynun arka kısmında, kafatasının tabanında yer alan bu nokta nörodejeneratif hastalıklarda etkilidir (Kim vd. 2017).
- LV3 (Taichong): Ayak başparmağı ile ikinci parmak arasındaki kemiklerin birleştiği noktada bulunan bu bölge, stres ve adet kramplarının hafifletilmesi için kullanılmaktadır (Lin ve Hsieh 2020).

Bu noktalar, arı sokması veya zehir enjeksiyonu yoluyla tedavi edilerek çeşitli sağlık sorunlarının yönetiminde etkili bir şekilde kullanılmaktadır. Bal arısı zehri akupunkturu, geleneksel akupunkturun enerji düzenleyici etkilerini bal arısı zehrinin antienflamatuvar ve ağrı kesici özellikleriyle birleştirmektedir.

Ürün Aracılı Uygulamalar

Bal arısı sokmaları ve bal arısı zehri enjeksiyonlarının birtakım dezavantajları olduğundan, uzun vadeli ve sürekli bir terapötik etki sağlayan uygun bir sürekli salım sisteminin tasarımı gerekli hale gelmiştir ve son yıllardaki araştırmalar artmıştır. Son zamanlarda nanopartiküller poli-D, L-laktik-ko-glikolik asit (PLGA), aljinat ve kitosan gibi biyolojik olarak parçalanabilen polimerlerden hazırlanmaktadır. Bu polimerler ve bunların nanopartikülleri biyoaktif bileşikler için mükemmel ve etkili taşıyıcılardır ve böylece bal arısı zehri, dağıtımını ve salınımını iyileştirmek için bu polimerlere ve bunların nanopartiküllerine yüklenebilmektedir. Bu durum, sık enjeksiyon ihtiyacını ortadan kaldırarak hasta uyumunun iyileştirilmesine neden olmakta ve bal arısı zehrine biyouyumlu ve biyobozunur özellikler kazandırmaktadır. Bu bağlamda, Qiao ve ark., bal arısı zehri ile kopolimer poli(dl-laktit-ko-glikolid-b-etilenglikol-b-dl-laktit-ko-glikolid) (PLGA-PEG-PLGA) arasındaki etkileşimleri araştırmışlardır (Qiao vd. 2007). Çalışma sonucunda bal arısı zehri salınımının azaldığını ve hidrojelin zayıf bir şekilde parçalandığını, buna karşın uygulanan terapötik aktivitelerin devam ettiğini fark etmişlerdir. Başka bir ilaç dağıtım yönteminde Xing ve ark., kolona özgü ilaç dağıtımı için model ilaç olarak kaplanmış kalsiyum aljinat jel lipozomlarını ve bal arısı zehri peptitlerini kullanarak ağızdan uygulamaya yönelik yeni bir formülasyon tasarlamışlardır (Xing vd. 2003). Bal arısı zehri kolon iletiminin 4-5 saat kadar sürdüğünü ve biyoterapötik aktivitenin azalmadığını ortaya koymuşlardır.

2018 yılında Lee ve ark., kitosan/aljinat nanopartiküllerinin bal arısı zehrini kapsülleme konusundaki etkinliğini ve bunların domuz üreme ve solunum sendromu virüsüne karşı potansiyellerini değerlendirmişlerdir (Lee vd. 2018). Çalışma sonucunda nanopartiküllerin, Th1 bağışıklık tepkisini indükleyebildiğini ve küme farklılaşması (CD4+), T lenfositleri, hafıza T hücresi popülasyonları, sitokinlerin üretimini artırabildiğini belirlemişlerdir. Başka bir çalışmada, Alalawy ve ark., bal arısı zehrinin nano-mantar kitosan üzerine yüklenmesinin serviks karsinomu (HeLa) hücrelerine karşı anti-kanser biyoaktivitesini arttırdığını ve HeLa hücrelerinde zamana ve doza bağlı olarak ciddi apoptoz belirtilerini tetikleyebileceğini ortaya koymuşlardır (Alalawy vd. 2020). Partiküller dışındaki bir çalışmada ise, bal arısı zehri ve propolis yüklü nanofiberlerin geniş spektrumlu antibakteriyel aktiviteler gösterdiği ve deri enflamasyonlarının tedavisinde yüksek potansiyel barındırdığı bulunmuştur (Aburayan vd. 2022).

SONUÇ VE ÖNERİLER

Bal arısı zehri, geleneksel tıptaki uzun tarihçesine ek olarak modern tıpta da ilgi çekici bir araştırma konusu haline gelmiştir. Gelecekte bal arısı zehrinin tıbbi uygulamaları ve terapötik potansiyeli üzerine daha fazla çalışma yapılması, bu doğal ürünün sağlık ve kozmetik sektöründe daha geniş çapta uygulamalarda kullanılmasının önünü açabilir. Örneğin, bal arısı zehrinin bileşenlerinin genetik mühendislik teknikleriyle üretimi, bu değerli bileşenlerin laboratuvar ortamında sentezlenmesini mümkün kılabilir ve rekombinant DNA teknolojisi kullanılarak bal arısı zehrindeki spesifik proteinlerin ve peptitlerin büyük ölçekli üretimi, farmasötik endüstride yeni tedavi yöntemlerinin geliştirilmesine olanak tanıyabilir (Moridi vd. 2020). Bunun yanında bal arısı zehri, diğer doğal ürünler ve farmasötik ajanlarla kombine edilerek daha etkili tedavi protokolleri oluşturulabilir. Örneğin, bal arısı zehri ile diğer apiterapi ürünlerinin kombinasyonu, sinerjistik etkilerle daha güçlü terapötik sonuçlar elde edilmesini sağlayabilir. Ayrıca, bal arısı zehrinin mevcut ilaç tedavileri ile kullanımı, tedavi sürecinin etkinliğini artırabilir ve hastaların yaşam kalitesini iyileştirebilir. Bal arısı zehri, geleneksel tıpta uzun süredir kullanılan ve biyoterapötik özellikleri nedeniyle kapsamlı araştırmalara konu olan karmaşık bir biyolojik yapı ürünüdür. Temel bileşenleri arasında proteinler ve peptitler bulunmakta olup melittin en bol bulunan ve üzerinde en çok çalışılan bileşendir. Melittin, bal arısı zehrinin antienflamatuvar ve immünomodülatör etkilerinde önemli bir rol oynarken, PLA2 gibi bileşenler de önemli diğer biyoterapötik etkilere sahiptir. Araştırmalar, bal arısı zehrinin çeşitli biyoterapötik etkilerine yoğunlaşmış olup, bu etkilerin mekanizmalarının daha iyi anlaşılması için hem *in vitro* hem de *in vivo* deneylerin artırılması büyük bir önem arz etmektedir. Bal arısı zehri bileşenlerinin

etkilerinin ve yollarının tam olarak anlaşılması, terapötik uygulamalarının değerlendirilmesi açısından kritik bir öneme sahiptir. Bu bağlamda, gelecekte yapılacak kapsamlı araştırmalar ve yenilikçi yaklaşımlar, arı zehrinin sağlık sektöründe daha geniş ve güvenli bir şekilde kullanılmasına olanak tanıyacaktır.

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DERLEME /REVIEW

A LITTLE KNOWN BEE PRODUCT WITH THE POTENTIAL TO BECOME A FUNCTIONAL FOOD AND NUTRITIONAL SUPPLEMENT: APILARNIL

Fonksiyonel Bir Gıda ve Besin Takviyesi Olma Potansiyeline Sahip Az Bilinen Bir Arı Ürünü: Apilarnil

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ABSTRACT

Beekeeping plays a crucial role in supporting agricultural sustainability and the economy through pollination and the production of honey and other bee products. Among these products is apilarnil, a less known substance derived from drone larvae that provides health benefits. Apilarnil is rich in essential nutrients and has been reported to have favorable effects on the reproductive system, autonomic nervous system and cardiovascular health. In addition to its natural medicinal properties, its nutritional and pharmaceutical potential is increasingly recognized, leading to the commercial production of apilarnil. This bee product is very important for health as it contains amino acids, fatty acids, vitamins, minerals, hormones and antioxidants. Apilarnil is recognized as a complete food and is included in various food products. Research emphasizes the androgenic, estrogenic, antioxidant and immune system boosting effects of apilarnil. Animal studies indicate its potential to improve reproductive health, reduce stress and promote growth and development. It also shows promise in protecting against oxidative stress and improving general health. In this review, information on apilarnil and its uses is compiled.

Keywords: Honeybee Products, Drone Brood Homogenate, Apitherapy, Edible Insects, Nutritional Supplement

ÖZ

Arıcılık, tozlaşmanın yanında bal ve diğer arı ürünlerinin üretimi yoluyla tarımsal sürdürülebilirliğin ve ekonominin desteklenmesinde çok önemli bir rol oynamaktadır. Bu ürünler arasında, erkek arı larvalarından elde edilen ve sağlık açısından fayda sağlayan, az bilinen bir madde olan apilarnil de yer almaktadır. Apilarnil temel besinler açısından oldukça zengindir ve üreme sistemi, otonom sinir sistemi ve kardiyovasküler sağlık üzerinde olumlu etkileri olduğu bildirilmiştir. Doğal tıbbi özellikleri yanında, besleyici ve farmasötik potansiyeli giderek daha fazla kabul görmekte ve apilarnilin ticari üretimine yol açmaktadır. Bu arı ürünü amino asitler, yağ asitleri, vitaminler, mineraller, hormonlar ve antioksidanlar içerdiğinden sağlık için oldukça önemlidir. Apilarnil tam bir gıda olarak kabul edilmekte ve çeşitli gıda ürünlerine dahil edilmektedir. Araştırmalar apilarnilin androjenik, östrojenik, antioksidan ve bağışıklık sistemini güçlendirici etkilerini vurgulamaktadır. Hayvanlar üzerinde yapılan çalışmalar, üreme sağlığını iyileştirme, stresi azaltma ve büyüme ve gelişmeyi destekleme potansiyeline işaret etmektedir. Ayrıca oksidatif strese karşı koruma ve genel sağlığı iyileştirme konusunda da umut vaat etmektedir. Bu derlemede apilarnil ve kullanım alanlarına ilişkin bilgiler derlenmiştir.

Anahtar Kelimeler: Balarısı Ürünleri, Erkek Arı Larva Homojenatı, Apiterapi, Yenilebilir Böcekler, Besin Takviyesi

GENİŞLETİLMİŞ ÖZET

Amaç: Bitkilerin tozlaşmasına katkı sağlayan arılar, tarımın verimliliğinin artmasına destek olmaktadır. Bu nedenle arıcılık, tarımsal sürdürülebilirlik için son derece önemli bir sektördür ve küresel ölçekte kırsal ekonomiye katkı sağlamaktadır. Arıcılık faaliyetleri sırasında arıcılar bal ve balmumunun yanı sıra polen, arı ekmeği, arı zehri, propolis ve apilarnil gibi farklı ürünler de üretmektedir. Bu ara ürünler, arıcılık sektörüne ürün portföyünü çeşitlendirme ve gelirini artırma fırsatı sunmanın yanı sıra sürdürülebilirlik konusunda da etkin bir katkı sağlamaktadır. Bal ve diğer tüm arı ürünleri, günümüzde apiterapi olarak isimlendirilen geleneksel ve tamamlayıcı tıpta yaygın olarak kullanılmaktadır. Doğal gıda takviyelerine ve tamamlayıcı tedavilere artan ilgiyle birlikte bu ürünlere olan talep de giderek artmaktadır. Bu ürünler arasında erkek arı larvalarından elde edilen apilarnil, zengin amino asit, vitamin, yağ asidi, antioksidan ve mineral içeriği ile öne çıkan değerli bir üründür. Bu derlemede, apilarnilin üretimi ve kullanım alanlarına ilişkin bilgiler sunulmaktadır.

Tartışma: Tam bir gıda olarak kabul edilen apilarnilin bağışıklık sistemini desteklediği, antioksidan etkilere sahip olduğu, üreme sistemi, otonom sinir sistemi ve kardiyovasküler sağlık üzerinde olumlu etkiler gösterdiği çeşitli çalışmalarda rapor edilmiştir. Hayvanlar üzerinde yapılan çalışmalarda da üreme sağlığını iyileştirme, büyüme ve gelişmeyi destekleme ve stresi azaltma potansiyeline sahip olduğu gösterilmiştir. Çalışmalar, apilarnilin oksidatif strese karşı koruma sağladığını ve genel sağlığın iyileştirilmesine katkıda bulunabileceğini göstermektedir. Apilarnil içeren gıda takviyeleri ve tamamlayıcı tedavi amaçlı üretilen ürünler çeşitli ülkelerde farklı isim ve markalar altında uzun zamandır üretilmektedir. Bu tür ürünlere artan ilgi sebebiyle Avrupa Birliği tüketici güvenliğini sağlamak amacıyla ara arı ürünlerine üretim standartlarını da içeren sıkı yasal düzenlemeler getirmiştir.

Sonuç: Arı ürünlerinin alerjik reaksiyon oluşturma riski daima göz önünde bulundurulması gereken önemli bir husustur. Bu nedenle arı ürünleri üzerindeki etiketler mutlaka tüketiciyi yeterince bilgilendirecek gerekli tüm uyarıları içermelidir. Yasal düzenlemelerde bu etiketleme uygulamasını zorunlu kılmaktadır. Ayrıca apilarnil yüksek su içeriği ve hassas besinsel öğeleri sebebi ile son derece kolay bozulabilen bir ürün olduğu için üretiminde hijyen ve güvenlik standartlarına uyulması büyük önem taşımaktadır. Son olarak, apilarnil ve diğer ara arı ürünlerinin hak ettikleri değeri bulmaları için alternatif gıda veya besin takviyesi olarak tamamlayıcı tıpta kullanımları üzerine daha fazla bilimsel çalışma yapılması gerekmektedir.

INTRODUCTION

Beekeeping is an important agricultural production sector that not only ensures the vegetative continuity of nature and the sustainability of agricultural production, but also makes a significant economic contribution to local economies. Many agricultural products are pollinated by bees and increase their productivity. On a global scale, 35% of agricultural products are pollinated with the help of bees and other pollinators, and the economic value of this service is estimated at approximately 235-577 billion dollars per year (IPBES 2016). Annual honey production worldwide is around 1.8 million tons, and the commercial value of this production is quite high (FAO 2024). For this reason, important beekeeping activities are carried out in different regions of the world under various climatic conditions. In addition to honey, beekeepers produce a wide variety of bee products such as beeswax, pollen, bee bread, royal jelly, bee venom, propolis and apilarnil (Bogdanov 2012; 2017; Silici 2023). All these honeybee products were used by many ancient civilizations in traditional medicine to treat diseases and injuries. Today, these bee products are continuing to be used in complementary medicine called apitherapy in different doses and compositions and provide important health benefits (Olas 2022). With the increasing interest in natural remedies and food supplements in recent years, the demand for bee products has also begun to increase (Akcicek and Yucel 2015). This situation has lead beekeepers who want to increase production efficiency and their income to start producing variety of bee products and to increase production diversity in recent years (Akcicek and Yucel 2015).

One of the lesser known and invaluable honeybee products is drone bee larvae or drone brood homogenate called apilarnil (Bogdanov 2012; 2017). This bee product produced from 7 days old drone larvae was first patented in Romania and named by apitherapist Nicolae V. Iliesiu, using the Latin abbreviations "Api" for bees, "lar" for larvae, and "nil", the first letters of his own name (Ilesiu 1991). Apilarnil is known for its positive effects on the male and female reproductive glands, the autonomic

nervous system and the cardiovascular system of the elderly and is used in many countries as a natural nutritional supplement containing drone milk or a mixture of drone milk and propolis. In addition, with the increasing trend towards natural products and traditional treatment methods such as apitherapy, apilarnil also began to be used in the pharmaceutical and cosmetic industry. As a result of these developments, the number of studies on its large scale production and biological effects has increased in recent years. So, this review was prepared to evaluate apilarnil production and the experimental animal studies on its biological effects in order to draw a projection for its increasing use.

Apilarnil Production

Male bees, which constitute approximately 5-10% of the populations, are produced from spring to autumn, mostly between April and June, for fertilization of the queen bee. The surplus males that do not have pollen collecting combs, stingers or wax glands and die after mating are usually destroyed by beekeepers as soon as possible in order to prevent worker bees from spending unnecessary energy to feed them and to protect hives from the *Varroa* parasite (Jensen et al. 2019, Sawczuk et al. 2019, Ulmer et al. 2020). This practice turns this bee product, which has high nutritional value and various pharmaceutical effects, into waste. However, in recent years, with the increasing interest in natural products with pharmaceutical properties, the number of research on the commercial production of apilarnil has begun to increase. In a study conducted in Denmark (Lecocq et al. 2018), it was shown that drone combs, which are used effectively in the biological control of *Varroa* mite, can be used to produce high amounts of apilarnil. As a result of the study, it was determined that the average total biomass of drone larvae extracted from each colony during a production season can reach over 1,000 kg per colony. The researchers suggested that drone larvae, which has a national production potential of 80 tons/year, could be used as a raw food material (Lecocq et al. 2018) and thus promote sustainable beekeeping (Ulmer et al. 2020). In another study, it was shown that production can be stimulated by removing combs carrying drone larvae from the hive every 7-11 days (Jensen et al. 2019).

Physical Properties and Chemical Composition of Apilarnil

Apilarnil is produced from 3–11 day old male bee larvae before pupation. It can be yellow, cream or gray in color, has a thick viscous liquid structure, a characteristic egg odor, slightly acidic taste and low solubility in water and alcohol (Barnutiu et al. 2013, Sidor and Dzugan 2020). It has a high water content, which makes it susceptible to rapid deterioration (Jensen et al. 2019). To preserve it without losing its quality and biological activity, different storage methods such as mixing with honey, freezing or absorbing lactose are recommended (Bogdanov 2012, 2017). If the cold chain rules are strictly followed, the product has been shown to be stored at -15°C for 1 year without much deterioration in its quality (Topal et al. 2018). For longer-term storage, it needs to be subjected to processes such as grinding, homogenization, filtration, and lyophilization (Topal et al. 2018). Lyophilization has been proven the most effective technique in preserving the active ingredients of apilarnil. In this technique, larvae dried by sublimation can be stored for a long time without changing the active ingredient content (Sidor et al. 2021a).

The nutritional components of fresh apilarnil, which may contain some honey, propolis and royal jelly, may vary depending on the region, season or larval age, as in all other bee products. Together with this, studies show that it contains all essential amino acids necessary for human and animal health, is a rich source of palmitic, stearic, and oleic acids as well as polyunsaturated fatty acids, and is a good source of B complex vitamins ve vitamin C and E, choline, and coenzyme Q10 as well as minerals such as phosphorus, potassium and magnesium (Table 1). In addition, it contains hormones such as testosterone, estradiol, progesterone and prolactin, phenolic (such as ferulic and ellagic acid) and flavonoid compounds that have antioxidant effects (Table 1). Studies have shown that drone larvae can be considered as a potential health enhancing agent due to their levels of amino acids, fatty acids, vitamins, minerals, antioxidant substances and hormones (Silici 2023).

Table 1. Physical properties and chemical composition of fresh and lyophilized apilarnil

	Fresh	Lyophilized	References
Sensory characteristics	Yellow colored thick viscous liquid	Yellow or beige colored amorphous powder	
Solubility	Low in water and alcohol		Balkanska et al. 2014, Sidor and Dzugan 2020, Kosum et al. 2022, Moraru et al. 2024
pH	5.5-7.5	4.5-6.8	
Acidity (ml 0.1 N NaOH/g)	0.7-2.6	-	
Conductivity (µS/cm)	144-178	-	
Proximate composition			Lazaryan 2002, Finke 2005, Lipinski et al. 2008, Barnitiu et al. 2013, Balkanska et al. 2014, Isidorov et al. 2016, Margaoan et al. 2017, Shoinbayeva et al. 2017, Silici 2019, Sidor et al. 2021a, Borkovcova et al. 2022, Kosum et al. 2022
Total energy (kcal/100 g)	111.9-120.3	472-501.4	
% Moisture	68.5-78.5	3.5-6.0	
% Ash	0.7-3.0	2.7-4.1	
% Protein	7.2-15.4	32.0-52.4	
% Carbohydrate	6.9-12.2	17.8-38.9	
% Lipid	3.1-8.4	20-24.2	
Amino acids (%)			Lazaryan 2002, Finke 2005, Isodorov et al. 2016, Margaoan et al. 2017, Silici 2019, Gosh et al. 2020, 2021, Sidor et al. 2021a
Alanine	0.17-2.4	1.83-2.36	
Arginine	0.11-2.1	2.18-3.00	
Asparagine	0.77	2.4	
Aspartic acid	0.08-3.6	3.23-3.57	
Phenylalanine	0.63-1.8	1.84-2.08	
Glutamic acid	2.13-6.9	5.63-7.94	
Glycine	1.15-1.8	1.5-2.29	
Glycine-Proline	0.012	-	
Histidine	0.41-1.1	0.99-1.21	
Methionine	0.38-0.8	0.50-1.15	
İsoleucine	0.5 -1.9	2.02-2.43	
Lysine	1.21-3.2	3.52-7.20	
Leucine	1.07-4.0	3.26-3.96	
Proline	2.78-3.6	1.58-3.92	
Serine	0.09-0.87	1.4-2.03	
Cysteine	0.016-1.8	0.25-1.61	
Threonine	1.23-1.7	1.30-1.86	
Tryptophan	0.32-0.5	-	
Tyrosine	0.77-2.4	2.02-2.55	
Valine	0.81-2.6	2.27-2.87	
Phosphoserine	0.11-0.12	-	
% Essential amino acids	0.071	-	
% Free amino acids	0.022	-	
Fatty acids (%)			Finke 2005, Yucel et al. 2019, Gosh et al. 2020, Erdem and Inci 2022
10-Heptadecenoic acid	1.41	-	
Lauric acid	0.2	0.03	
Myristic acid	1.2	0.36-2.61	
Palmitic acid	14.7	4.81-42.6	
Palmitoleic acid	0.2	0.06-0.59	
Stearic acid	4.3	1.11-10.42	
Oleic acid	18.2	4.72-42.69	
Alpha-Linoleic acid	0.4	1.06	
Gamma-Linoleic	0.3	2.54	
Linolenic acid	0.4	ND	
Arachidic acid	0.2	ND	
Eicosenoic acid	0.1	1.11	
Behenic acid	0.1	0.97	
% Saturated fatty acids	51.75	63.06	
% Mono saturated fatty acids	46.25	47.77	
% Poly saturated fatty acids	2.0	0.11	

Minerals (µg/g)			
Calcium	138	194.2-1336	Finke 2005, Gosh et al. 2020, Sidor et al. 2021a, Borkovcova et al. 2022
Phosphorus	1790	3021.7-6868.8	
Magnesium	211	382.6-680.6	
Sodium	128	80.4-300.8	
Potassium	2690	2887.7-8910.8	
Chlorine	870	-	
Iron	12.9	11.8-60.87	
Zinc	16.0	14.4-257	
Manganese	0.6	2.4-8.7	
Copper	4.0	1.1-54.8	
Selenium	0.06	-	
Sulphure	-	941.8	
Carbohydrates (%)			
Fructose	0.3-8.4	0.38	Lipinski et al. 2008, Barnitiu et al. 2013, Balkanska et al. 2014, Isidorov et al. 2016, Margaoan et al. 2017, Sidor and Dzugan 2020
Glucose	3.61-72.7	3.55	
Sucrose	0.05-1.5	-	
Turanose	0.05-2.4	-	
Maltose	0.33-5.3	0.9	
Trehalose	0.44-6.6	0.25	
Isomaltose	0.11-4.2	-	
% Total sugar	6.2-8.2	-	
% Glycogen	9.2-11.7	-	
Vitamins			
Vitamin A (IU/g)	<1	0.31-14.7	Finke 2005, Sawczuk et al. 2022
Beta-carotene (µg/g)	<0.2	-	
Vitamin B1 (µg/g)	4.1	-	
Vitamin B2 (µg/g)	9.1	-	
Vitamin B3 (µg/g)	36.7	-	
Vitamin B5 (µg/g)	11.9	-	
Vitamin B6 (µg/g)	1.2	-	
Vitamin B7 (µg/g)	0.23	-	
Vitamin B12 (µg/g)	<0.0012	-	
Choline (µg/g)	1684	-	
Vitamin C (µg/g)	38.0	650-3360	
Vitamin D (IU/g)	<0.25	-	
Vitamin E (IU/g	<0.005	-	
Antioxidants			
Fumaric acid (µg/g)	5.03	-	Hryniewicka et al. 2016, Silici 2019, Gosh et al. 2020, Sidor et al. 2021a, 2021b, Kosum et al. 2022, Sawczuk et al. 2022
trans-Aconitic acid (µg/g)	11.20	-	
p-Benzoquinone (µg/g)	0.95	-	
Catechin hydrate (µg/g)	1.85	-	
α-tocopherol (µg/g)	7.2-8.4	0.53-24.1	
Coenzyme Q10 (µg/g)	19.4-21	0.03-114	
Total phenolic (mg GAE/g)	1.8-3.2 180.05-	0.61-9.4	
Total flavonoid (mg QE/g)	320.43	0.63-5.8	
Total antioxidant activity(mg	0.04-0.1	90.91	
AAE/g)	-	-	
(µmol TE/g)	0.008-0.016	1.01-81.6	
Antiradical activity (%DPPH)	6.91-24.76	2.2-3	
(FRAP, µmol TE/100 g)	0.79-1.63		
Hormones		45.6-51.32	
Testosterone (pmol/g)	0.47-9.1	10-151.25	Bogdanov 2017, Shoinbayeva et al. 2017, Yucel et al. 2019, Sidor et al. 2021a
Estradiol (nmol/g)	2.18-6.8	0.81-457.87	
Progesterone (nmol/g)	0.51	8.0	
Prolactin (nmol/g)	4.11		

At this point, we should also emphasize that as any natural product, chemical components of apilarnil may vary depending on the age of larvae collected, season, vegetation of the collection location, handling methods or the methodological differences used in the detection (Gosh et al. 2020, Moraru et al. 2024). For example, a recent study (Abd El-Whaed et al. 2024) showed that the flavonoid content (13.16 ± 0.94%) and the antioxidant activity (DPPH IC_{50} = 179.93 ± 2.46 µg/ml) of lyophilized apilarnil may be much higher than those previously reported (in Table 1). Additionally, researchers reported different number of volatile metabolites with two different spectrometric detection approaches (Abd El-Whaed et al. 2024). Another study focusing on the phytochemical content with anticholinergic, antiglaucoma, antiepilepsy and antioxidant properties and the antioxidant activity of apilarnil reported that it showed generally lower antioxidant activity in comparison to the standard molecules (namely butylated hydroxyanisole, butylated hydroxytoluene, trolox, α-tocopherol and vitamin C) with the methods used (Inci et al. 2023). However, except vitamin C, it had higher radical scavenging activity than the standard antioxidants. Furthermore, it showed higher anticholinergic, antiglaucoma, antiepilepsy enzyme inhibitory activities than the standard antioxidants, indicating that it can be used in treatment of these diseases. Upon these outcomes, the researcher concluded that apilarnil has a more effective antioxidant profile compared to standard antioxidants (Inci et al. 2023).

Use of Apilarnil as Food and in Health Area

Apilarnil is recommended to be considered as a complete food in human and animal nutrition because it contains all essential amino acids and is a rich source of vitamins and minerals (Topal et al. 2018). In fact, products containing bee larvae have begun to be developed in the field of gastronomy. Examples of these developments are products such as snacks called "Bienengrammeln" or "bee crackle", or chocolate mousse and ice cream containing bee larvae (Ramos-Elorduy et al. 2007, Mishyna et al. 2019). A product that can be used as a meat substitute in foods such as hamburgers has also been developed by combining drone pupae with soya bean concentrate (Ulmer et al. 2020).

A recent study (Ghosh et al. 2020) suggested that late pupae and adult drones, which have high amino acid and mineral content and low fatty acid content, are beneficial for human health and therefore should be used as human food, while larvae and early pupa stages can be used in animal feeds. However, it is recommended that all drone products, regardless of developmental stage, be subjected to processes such as drying, blanching, etc. before consumption, thus increasing safety in consumption (Ghosh et al. 2020). Additionally, they can only be used as human food or in animal feeds if they are produced in accordance with standard production protocols that ensure hygiene and food safety (Ghosh et al. 2021). At this point, it is worth emphasizing that EU legislation requires regular microbiological tests and examinations for risky compounds, and therefore insects must be subjected to risk-reducing processes before consumption. In this respect, the International Platform of Insects for Food and Feed (IPIFF, 2022) has published guidelines on the cultivation of insects for human and animal consumption and instructions for food production. Drone brood intended for consumption is notified under the definition of "food" in Regulation (EC) No 178/2002. It is classified as a new food under Regulation (EU) 2015/2283 and can only be placed on the market after safety assessment and approval by the European Food Safety Authority (EFSA). In addition, other regulations specify legal provisions generally applicable to food safety, production and processing and to products on the market (e.g. Regulation (EC) No. 178/2002; in Germany, for the requirements of the Food Hygiene Regulation (Lebensmittelhygiene-Verordnung - LMHV); for labelling requirements, Regulation (EU) No. 1169/2011; for hygiene regulations, Regulation (EU) No. 1169/2011; for hygiene regulations, Regulation (EC) No. 852/2004 and Regulation (EC) No. 853/2004; and for the assessment of microbiological criteria, Regulation (EC) No. 2073/2005) (Schiel et al. 2022). In addition, both EU and non-EU countries are required to legislate on the use of apilarnil.

Another important point is that apilarnil often contains honey components such as glands, wax containing propolis, nectar and pollen. Although bee allergy is rare but possible, consuming bee products can lead to the development of food and respiratory allergies that begin with the development of sensitivity. For this reason, it is of great importance to investigate the allergenicity of bee products that will be offered for consumption through comprehensive tests and to determine the scope and frequency of the risks posed by their consumption. In addition, necessary legal regulations should be established to ensure that

products offered for consumption carry labels with appropriate warnings for consumers who are allergic to bee products. It should also always be recognized that, unlike other edible insect species, honey bees reared in an open environment cannot be strictly managed.

In apitherapy, drone larvae are commonly used to treat psychotic and neurodegenerative diseases in the elderly, reproductive problems and to enhance libido (Bolatovna et al. 2015, Shoinbayeva et al. 2017). It has also been used to lower cholesterol and triglyceride levels (Vasilenko et al. 2002), maintain liver health and support the immune system (Vasilenko et al. 2005), treatment of mental and nervous disorders (Meda et al. 2004, Bogdanov 2017), thyroid irregularities (Osnicewa et al. 2009), and promote youth and vigor (Bieljajew and Safonowskaja, 2009).

It is well known that pro-oxidants, which are a natural product of aerobic metabolism and whose levels increase under stress, negatively affect reproductive success, especially in males (Schreck 2010, Ubilla and Valdebenito 2011, Baskaran et al. 2021). Apilarnil contains essential amino acids, minerals and vitamins, as well as flavonoids and polyphenols and has a high degree of antioxidant activity (Silici 2019). It is also rich in reproductive and sex hormones. Indeed, drone brood has been used for many years as a natural medicine in some Eastern European, Far East, African and South American countries to support fertility and to eliminate male sexual problems such as sperm deficiency and immobility, and erectile dysfunction (Sidor and Dzugan 2020, Kekecoglu et al. 2021). Recently, it has also been evaluated as an alternative to testosterone replacement therapy (Erdem and Ozkok 2017). In addition, studies to standardize its production and application as a fertility booster were conducted. For example, a company called Vitaliter has carried out studies to purify the lipid part of apilarnil to produce gel capsules (ApiREX) that can be used in testosterone treatment (https://silo.tips/download/apilarnil-lipid-zt-ile-testosteron-artrc-doal-gda-takviyesi-gelitirilmesi). Two other companies submitted patent applications for their already developed products (Erdem et al. 2017, Vakina et al. 2017).

Nowadays, the tendency to meet the needs of the body from natural products, which has increased with the concept of healthy living, has led to a rapid process of developing new commodities with added value from different bee products (Cosmia et al. 2016, Marangoz and Dolu 2019). Under the influence of this trend, many food supplements under various names containing apilarnil in powder, viscous liquid or tablet form have begun to be produced in some European, Far Eastern and South American countries (Hroshovyi et al. 2021, Kekeçoğlu et al. 2021). In a recent study on the development of dietary supplements containing apilarnil (Dzugan et al. 2023), a product containing frozen drone brood (DB) enriched with calcium ions from calcium carbonate (CC) or eggshell (ES) was designed. It was shown that the bioavailability of DB components was better in DB + ES than in DB + CC and DB capsules. It was reported that the two-component food supplement proposed in this study, which demonstrated the synergistic effect between DB and ES calcium, may be an effective therapeutic alternative for balancing osteoporosis-related hormone and calcium deficiency (Dzugan et al. 2023).

Experimental Studies on the Biological Activities of Apilarnil

Many studies have been conducted on various animal groups to investigate the effects of apilarnil from different aspects. In an earlier study, Kogalniceanu et al. (2010) reported that apilarnil is an agent that increases glycogen consumption in muscle tissue and has a catabolic effect on the carbohydrate metabolism of white Winstar rats. Yucel et al. (2011) reported that male broilers given 4 g apilarnil per day had better body weight gain. Researchers also observed that daily application of apilarnil enhanced the secondary sexual characteristics such as crown length and beard width. Thereupon, they stated that apilarnil may have an androgenic effect rather than an anabolic effect (Yucel et al. 2011). In their later study, they investigated the possibility of stimulation of sexual development at an earlier age by administering apilarnil to 28 and 55-day-old male and female broilers in the prepubertal period (Altan et al. 2013). In that study, low (2.5 g/animal) and high (7.5 g/animal) dose daily oral apilarnil administration showed no significant effect on the growth performance of male and female broilers. However, increases in testicular weight, plasma testosterone level and crest size were observed in apilarnil-treated males and apilarnil reported to decrease the age at sexual maturity and reduce stress and fear behaviors. In addition, significant decreases in total cholesterol and glucose levels and increases in good

cholesterol levels were detected (Altan et al. 2013). Based on these results, the researchers stated that apilarnil have a higher biological activity in males. Similarly, feeding fresh drone larvae for a month before attempting to spawn has been reported to be more effective in improving reproductive performance in virgin yellow princess (*Labidochromis caeruleus*) males than in females, and spawning began approximately 10 days earlier in aquariums with males and females fed fresh apilarnil (Sahin 2020).

Seres et al. (2014) investigated the androgenic effect of apilarnil by giving drone milk, which is used in larvae and adult nutrition of male bees, to castrated male rats. They also observed that the relative weight of androgen-related organs (glans penis and seminal vesicle) and plasma testosterone levels increased in the rats fed male royal jelly. In addition, they determined that the milk exerts this effect by increasing the expression of Spot14-like androgen-inducible protein in the prostate and that its active components with androgenic effects may be palmitate and oleate methyl esters. In another study, Bolatovna et al. (2015) reported that the weight of the seminal glands increased by 20.1–21.9%, the weight of the epididymis increased by 21.8–25.8%, and sexual dysfunction decreased by 83.3% when piglets administered apilarnil extract via parenteral injection. In addition to increasing the weight of the seminal glands and epididymis, apilarnil injection was reported to improve reproductive functions in terms of ejaculate volume, spermatozoa density, sperm motility, fertility rate, offspring survival rate, and the rate of acrosome-damaged spermatozoa. Moreover, researchers observed that apilarnil calmed injected animals, suggesting that it may have a stimulatory effect on the central nervous system (Bolatovna et al. 2015). Apilarnil extract given to wild boars was also found to improve semen productivity quantitatively and qualitatively (Bolatovna et al. 2015). Similarly, Yemets et al. (2020) showed daily feeding male piglets with 0.5 g of drone homogenate stimulates reproductive functions and improves fertility during puberty. Kosum et al. (2018) gave 2 ml apilarnil twice a day to 75-day-old Sanen goats found larger testicles and higher testosterone hormone levels in the apilarnil group compared to the control group. In follow-up studies, they also observed that the effect size was directly proportional to the dose of apilarnil administered, but no doses of apilarnil had an effect on growth (Kosum et al. 2022).

In addition to having an androgenic effect on sexually mature or immature animals, apilarnil has been found to stimulate the immune system and improve general health in pigs by increasing the antibody production and the response of T-lymphocytes (Mitrofanov and Budnikova 2021). A study conducted on dogs (Efanova et al. 2019) showed that the number of red blood cells and leukocytes, as well as the levels of thyroxine, testosterone, hemoglobin, total protein and globulin in the blood, increased in animals fed apilarnil at a rate of 15 mg/kg per day for two months. The preparation called Apistimul, which consists of apilarnil and sodium chloride, was also increased the amount of hemoglobin and erythrocytes in ram's blood, improved the qualitative and quantitative characteristics of ejaculate, and had a general stimulating effect on all their reproductive functions (Shoinbayeva et al. 2017).

Besides the androgenic effect, Seres et al. (2013) investigated the estrogenic effect of drone milk. They reported that male royal jelly increased uterine weight in juvenile female rats by inducing the expression of estrogen-linked peptide complex component C3 and the active component that may cause this estrogenic effect is E-dec-2-enedioic acid (Seres et al. 2013). An increase in body weight and ovarian length was also observed on day 145 in white sows fed DB homogenate (Kistanova et al. 2020). Animals fed DB homogenate appeared to have a larger pool of primordial follicles with dense growth, as well as ovaries with larger diameter primary and tertiary follicles. Atresia symptoms were also observed in the Graafian follicles of the animals. Based on these findings, the researchers reported that DB homogenate supplementation stimulated the early stages of folliculogenesis in the ovaries, but triggered atresia in the final stage of follicular development (Kistanova et al. 2020).

Many researchers focused on the antioxidant and cell protective effects of apilarnil. Kuzmenko et al. (2018) reported that apilarnil supplementation helps to maintain antioxidant-prooxidant balance in the uterus of pregnant pigs and supports normal embryonic development, especially by increasing non-enzymatic antioxidant levels. At the end of the study investigating the effect of apilarnil on prooxidant-antioxidant homeostasis in pigs during puberty, it was found that the administration of apilarnil to pigs has significantly positive effect on the formation of reproductive function during puberty and had antioxidant effects that significantly slowed

the course of peroxidation processes (Shostya et al. 2019). Therefore, it was inferred that apilarnil can be used as a gonad protector by the breeders. This property of apilarnil has also been demonstrated in a recent toxicology study (Elashal et al. 2024). Researchers experimentally showed that apilarnil supplementation may ameliorate a widespread environmental pollutant (Bisphenol A) induced reproductive impairment symptoms in adult male rats. A 0.6 g/kg body weight daily apilarnil administration was effective to restore the serum glutathione, testosterone and gonadotropin levels, improve the counts, motility and morphology of sperm. Additionally, it increased the proliferating cell nuclear antigen gene expression while decreasing the malondialdehyde levels in testis (Elashal et al. 2024).

In the hepatitis model induced by exposure to carbon tetrachloride in rats, apilarnil was determined to improve liver functions as well as stimulating the immune system (Vasilenko et al. 2002, 2005). Doganyigit et al. (2019a) investigated the effects of different doses (0.2-0.8 g/kg) of apilarnil in the diabetes model induced by the intra-abdominal lipopolysaccharide (LPS) injection. They reported that simultaneous apilarnil injection reduces the DNA damage in kidney tissue 6 h after LPS injection and shows the highest protection at a dose of 0.8 g/kg. Additionally, they observed increased amounts of sperm in the testicular lumen sections of adult male rats given LPS, depending on the dose of apilarnil (Doganyigit et al. 2019b). Consistent with this, subsequent studies supported that apilarnil prevents apoptosis by dose-dependently reducing the expression of precursor genes related to cytokine production in liver and brain tissues, reduces DNA damage, and protects cells against oxidative stress by increasing enzymatic antioxidant levels (Doganyigit et al. 2020a; Hamamcı et al. 2020). Apilarnil also inhibited apoptosis and DNA damage in lung cells (Doganyigit et al. 2020b) and induced autophagy pathway in liver cells (Doganyigit et al. 2020c). Under an LPS-induced sepsis model, apilarnil was reported to reduce inflammation in the kidneys and exhibit anti-apoptotic effects, again by suppressing the expression of inflammatory cytokines in the TLR4/NF-κB pathway (Inandiklioglu et al. 2021).

Okan et al. (2022) investigated the protective effect of apilarnil in endotoxic shock, which is one of the important causes of mortality in intensive care units, through histopathological changes and immune system-related gene (tumor necrosis factor-alpha (TNF-α) and natriuretic peptide (BNP)) expression changes in the heart tissue of male rats with LPS induced sepsis. In this study, where differences in TNF-α expression in heart tissue and BNP expression in brain tissue were evaluated immunohistochemically, edema, bleeding and infiltration were observed in the LPS-administered control group, while these damages were significantly reduced in the LPS-administered apilarnil (0.8 g/kg) group. Additionally, while the expression levels of TNF-α and BNP genes increased significantly in the LPS-administered control group, the expression of these genes was reported to be suppressed in the LPS-administered apilarnil group. Based on these results of the study, the researchers concluded that apilarnil had a therapeutic effect against heart damage caused by LPS due to its anti-inflammatory and antioxidant contents.

Finally, a new study examining the effects of dietary bee product supplementation on reproductive success and oxidative stress levels showed that adding apilarnil and royal jelly to the diet or calorie restriction could be implemented to delay the age-related decline in semen production. It has also been shown that it slows down the aging process and extends reproductive life in male breeder broilers (Seremet-Tugalay and Altan 2020).

Conclusion: As implicated by the animal studies summarized above, apilarnil is a bee product with antioxidant, anti-inflammatory, antitumor, anti-apoptotic, androgenic and anabolic effects due to its rich content in amino acids, fatty acids, vitamins, minerals, hormones and antioxidants. It supports general health by strengthening the immune system and antioxidant defense mechanism. Therefore, this bee product has a high potential to make serious contributions to the fields of health and nutrition. However, it should not be overlooked the fact that more detailed studies still need to be carried out on its usage areas, production and usage standards.

It is also important to note that, despite apilarnil or apilarnil-containing various products are being marketed on a global scale via the internet, neither the FAO nor local organizations maintain records of bee products other than honey and beeswax. The lack of records pertaining to the production of alternative bee products hinders the full realization of the potential yield of beekeeping and the accurate quantification of the production of such bee

products. Furthermore, it impedes the broader utilization of these products or raw materials, as it engenders uncertainty regarding their continuity, sources and supply. It is hoped that in the near future, the production quantities of alternative bee products, such as apilarnil, will also be recorded.

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DERLEME /REVIEW

REVEALING THE MULTIFACETED LANDSCAPE OF PROPOLIS RESEARCH (1945-2023): A COMPREHENSIVE ANALYSIS OF DYNAMICITY, SPATIOTEMPORAL TRENDS, AND EMERGING PARADIGMS IN SCHOLARLY DISCOURSE

Propolis Araştırmalarının Çok Yüzlü Görünümünü Açığa Çıkarma (1945-2023): Dinamiklik, Zamansal-Mekansal Eğilimlerin veYeni Paradigmaların Kapsamlı Analizi

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ABSTRACT

Propolis, a natural resinous substance produced by bees, has long been known for its potential health benefits. This study aims to present a comprehensive bibliometric investigation, exploring the dynamicity, spatiotemporal trends, and emerging patterns in the scholarly discourse surrounding propolis research. The study tailed PRISMA guidelines and used MeSH databases and Scopus to retrieve relevant bibliographic data spanning 75 years. R-based Bibliometrix and VOSviewer applications were employed for data analysis. A noticeable increase in scholarly production was observed in the last two decades. Active participation in propolis research was identified from Brazil, China, and Türkiye. The multidimensional nature of propolis research was evident through the diversity of topics covered in highly impactful research and intellectual maps of information sources. Thematic evolution highlighted the dynamic nature of propolis research, with emerging areas of investigation and an enhanced understanding of its therapeutic applications. Five prominent themes emerged: "propolis," "oxidative stress," "honey," "beeswax," and "allergic contact dermatitis." Additionally, emerging themes included chronic kidney disease, COVID-19, and metabolomics. Mapping international cooperation and co-citation of authors demonstrated multiple research activities. The findings of this study hold implications for researchers, healthcare professionals, and policymakers, providing insights into the current landscape of propolis research.

Keywords: Propolis, Bioactive Compounds, Therapeutic Potential, Antimicrobial, Bibliometrics

ÖZ

Arılar tarafından üretilen doğal reçinemsi bir madde olan propolis, sağlık açısından potansiyel faydaları ile uzun zamandır bilinmektedir. Bu çalışma, propolis araştırmalarını çevreleyen bilimsel söylemdeki dinamikliği, mekansal-zamansal eğilimleri ve ortaya çıkan kalıpları keşfederek kapsamlı bir bibliyometrik araştırma sunmayı amaçlamaktadır. Çalışmada PRISMA yönergeleri izlenmiş ve 75 yılı

kapsayan ilgili bibliyografik verileri elde etmek için MeSH veri tabanları ve Scopus kullanılmıştır. Veri analizi için R tabanlı Bibliometrix ve VOSviewer uygulamaları kullanılmıştır. Son yirmi yılda bilimsel üretimde gözle görülür bir artış gözlemlenmiştir. Brezilya, Çin ve Türkiye'den propolis araştırmalarına aktif katılım tespit edilmiştir. Propolis araştırmalarının çok boyutlu doğası, son derece etkili araştırmalarda ele alınan konuların çeşitliliği ve bilgi kaynaklarının entelektüel haritaları ile ortaya çıkmıştır. Tematik evrim, propolis araştırmalarının dinamik doğasını, ortaya çıkan araştırma alanlarını ve terapötik uygulamalarının daha iyi anlaşılmasını vurgulamıştır. Öne çıkan beş tema olarak: "propolis", 'oksidatif stres', 'bal', 'balmumu' ve 'alerjik kontakt dermatit'tir. Ek olarak, ortaya çıkan temalar arasında kronik böbrek hastalığı, COVID-19 ve metabolomikler yer almaktadır. Uluslararası işbirliğinin ve yazarların ortak atıflarının haritalanması, çoklu araştırma faaliyetlerini göstermiştir. Bu çalışmanın bulguları, araştırmacılar, sağlık uzmanları ve politika yapıcılar için çıkarımlar içermekte ve mevcut durum hakkında içgörü sağlamaktadır.

Anahtar Kelimeler: Propolis, Biyoaktif Bileşikler, Terapötik Potansiyel, Antimikrobiyal, Bibliyometri

GENİŞLETİLMİŞ ÖZET

Amaç: Arılar bitkilerden reçine toplayıp kendi salgılarıyla karıştırarak propolis adı verilen harika bir madde üretir ve kovanlarını dışarıdan gelebilecek tehlikelere karşı korumak için kullanırlar. Birçok uygarlık boyunca, geleneksel ilaçlar bu doğal bileşenden geniş ölçüde yararlanmıştır. Propolis son zamanlarda bilimsel açıdan oldukça ilgi çekmiş ve tıbbi olanaklarını ortaya çıkarmaya yönelik çalışmalarda bir patlama yaşanmıştır. Bu araştırma, arılar tarafından üretilen ve sağlık açısından potansiyel faydaları olan doğal bir madde olan propolis dünyasını incelemeyi amaçlamaktadır. Kapsamlı bir bibliyometrik analiz yaparak, propolis araştırmalarını çevreleyen bilimsel tartışmalarda değişen eğilimleri, coğrafi kalıpları ve yeni içgörüleri ortaya çıkarmak amaçlanmıştır.

Gereç ve Yöntemler: PRISMA tarafından özetlenen yönergeleri izleyerek, MeSH veritabanlarını ve Scopus'u titizlikle tarayarak 75 yıllık etkileyici bir süreyi kapsayan zengin bibliyografik veriler toplanmıştır. Verileri analiz etmek için R tabanlı Bibliometrix ve VOSviewer araçları kullanılmıştır.

Bulgular: Araştırmamız, son yirmi yılda bilimsel çıktılarda çarpıcı bir artış olduğunu ortaya koyarak propolis araştırmalarına olan ilginin arttığını göstermektedir. Brezilya, Çin ve Türkiye gibi ülkeler propolisin potansiyelini keşfetme konusundaki aktif katılımlarıyla öne çımaktadır. Propolis araştırmalarının çok yönlü doğası, ele alınan çeşitli konular aracılığıyla canlı bir şekilde tasvir edilmiş, etkili araştırma bulgularına ve aydınlatıcı bilgilere yol açmıştır. Uluslararası işbirliği ve yazar ortak atıflarının incelenmesi, propolis araştırmalarını ileriye götüren küresel erişimi ve işbirlikçi ruhu ortaya koyan, birbirine bağlı araştırma faaliyetleri ağını ortaya çıkarmıştır.

Sonuç: Sonuç olarak, çalışmanın bulguları propolis araştırmaları için akademi, sağlık uygulamaları ve politika oluşturma alanlarında yankı uyandıran geniş kapsamlı çıkarımların altını çizmektedir. Propolis araştırmalarının dinamikliği ve gelişen manzarası, keşif ve yenilik için olgunlaşmış canlı bir alana işaret etmektedir. Sağlam klinik deney kanıtlarından yararlanarak propolis, kanıta dayalı profesyonel onaylarla desteklenen ana akım sağlık hizmetlerinde önemli bir yer edinmeye hazırdır. Propolisin tüm terapötik potansiyelini ortaya çıkarmak için, etki mekanizmalarını aydınlatmaya, disiplinler arası ortaklıkları teşvik etmeye ve titiz klinik deneyler yürütmeye yönelik ortak bir çaba zorunludur. Bu uyumlu yaklaşım sadece propolis anlayışımızı geliştirmekle kalmayıp, aynı zamanda propolisin terapötik müdahalelerin geleceğini şekillendirmede oynayabileceği önemli rolü vurgulayarak daha iyi sağlık sonuçlarının yolunu açmaktadır.

Gelecekte yapılacak çalışmalar, propolisin etki mekanizmalarının kapsamlı bir şekilde araştırılması için stratejiler geliştirmeli, özellikle ikincil metabolitlerinin ve sağlık yanlısı etkileri için konjugatlarının özelliklerini belirlemelidir. Farmakoloji, immünoloji ve doğal ürünlerin kimyası gibi şu anda çok ayrı olan alanların entegrasyonu, sinerjik etkisi de dahil olmak üzere propolis hakkındaki bilgileri ilerletecek ve tıbbi faaliyetlerinin en iyi şekilde kullanılmasıyla yeni formülasyon ve tedavilerle sonuçlanacaktır.

Bununla birlikte, çeşitli popülasyonlar için propolisin güvenliğini, etkinliğini ve etkili dozlarını kanıtlamak için uygun klinik çalışmalar da gereklidir. Böylece bu doğa ürünü, uygulayıcılar için kanıta dayalı önerilerle

geleneksel tıbbın bir parçası olacaktır. Etki mekanizmasını daha derinlemesine inceleyerek, disiplinler arası işbirliklerini teşvik ederek ve iyi klinik çalışmalar yaparak, propolisin gerçek terapötik Değerini takdir edebiliriz ve sağlık hizmetleri gelişecektir.

INTRODUCTION

Propolis (bee glue) is a remarkable material that bees create by collecting resins from plants, combining them with their own secretions, and utilizing them to protect their hives from external threats (Irigoiti *et al.* 2021; Park, Alencar and Aguiar 2002). This natural substance has been widely used in traditional remedies across different cultures. In recent times, propolis has gained significant scientific interest, leading to a surge in research aimed at unravelling its therapeutic potential (Peršurić and Pavelić 2021; Roquetto *et al.* 2015).

The chemical composition of propolis underlies its potential health benefits. Propolis is a multipart mixture of chemicals derived from botanical sources. It contains various compounds, including flavonoids, phenolics, and terpenoids. The occurrence of these bioactive ingredients gives propolis a wide spectrum of properties, including antioxidant, antimicrobial, and anti-inflammatory effects (Ding *et al.* 2015; Jansen-Alves *et al.* 2023; Suleiman *et al.* 2021). Flavonoids contribute to propolis' antioxidant and anti-inflammatory properties, protecting cells from oxidative stress and reducing inflammation. Phenolic compounds in propolis exhibit antimicrobial and antifungal effects, inhibiting the growth of microorganisms (Fonseca *et al.* 2011; Ożarowski and Karpiński 2023; Salami *et al.* 2024). Terpenoids found in propolis contribute to its antimicrobial and antiviral activities. The specific composition of propolis varies based on factors like location and season, influencing its bioactivity. The synergistic effects of its compounds further add to its therapeutic properties (Suleiman 2021; Valverde *et al.* 2023; Zullkiflee, Taha and Usman 2022).

Bibliometric studies play a vital role in research by providing a quantitative assessment of the scientific literature. They offer valuable insights into publication trends, citation patterns, collaboration networks, and emerging research areas. By analyzing the scientific landscape, bibliometric studies help researchers identify knowledge gaps, influential authors, and top contributing institutions and countries (Fardi, Kodonas and Gogos 2023; Fox *et al.* 2023; Guo *et al.* 2023). These studies aid in understanding the current state of any research, guiding researchers in selecting research directions, fostering collaborations, and promoting advancements. Bibliometric studies are essential for evidence-based decision-making, shaping research strategies, and facilitating the dissemination of knowledge in the field of research (Fardi, Kodonas and Gogos 2023; Guo 2023). Therefore, the present study was intended to conduct a holistic bibliometric analysis of propolis research, mapping the scientific landscape in terms of publication outputs, collaboration networks, citation patterns, and key research topics. The study aims to provide understandings into the current state of propolis research, identify influential authors, institutions, and countries, and highlight emerging research areas. The findings will contribute to a deeper understanding of the field, guide future research directions, foster collaborations, and facilitate evidence-based decision-making in propolis research.

MATERIALS AND METHODS

Data Source, Search Strategy, and Data Collection

The Scopus database was utilized as the main source for this bibliometric study. Scopus provides a comprehensive collection of scholarly publications from various disciplines (Falagas *et al.* 2008). The search strategy involved the use of relevant keywords and MeSH terms (Dhammi and Kumar 2014) to retrieve publications related to propolis research. The keywords used were "Bee Bread," "Bee Glue," and "Propolis." These terms were selected based on their association with propolis in the MeSH Browser (https://meshb-prev.nlm.nih.gov/record/ui?ui=D011429). The search was conducted within Scopus using the identified keywords to retrieve data-driven research. Therefore, the search results were filtered to include only articles, excluding other types of documents such as conference papers, reviews, and editorials. Articles written in languages other than English or those that were still in the press were also not included. The PRISMA guidelines (Şalvarlı and Griffiths 2021) were followed to ensure a systematic and apparent approach to data collection and analysis (Figure 1). PRISMA, or Preferred Reporting Items for Systematic Reviews and Meta-Analyses, is

a framework that was conceived with the intention of using heretofore untapped ideas in a systematic review increasing the quality of reporting and accountability therein. Some of it incorporated a checklist that we followed which contained, inter alia, the title, abstract, background, aims, methodology (eligibility criteria as well as search strategy), outcome (in this setting: selection and characteristics of studies) and conclusion (in this context, interpretation of the evidence and its weaknesses). Also, we oriented the reader with step-wise representation in the form of flow diagram in which the process of selecting the studies was demonstrated along with how many studies were identified, how many more were screened and how many were finally excluded and why were they excluded. Embedded in each of these activities, we incorporated PRISMA with respect to different domains of research enhancing the reproducibility of our work. These guidelines ensured that readers soothe their critics by understanding our clinical methods as well as the evidence we found which will ultimately enhance the knowledge base of clinicians and nurture evidence-based care. We also implemented some changes of PRISMA to keep up with the changing needs of reporting, employing special mechanisms like PRISMA-P for protocol to the extensions for various types of research.

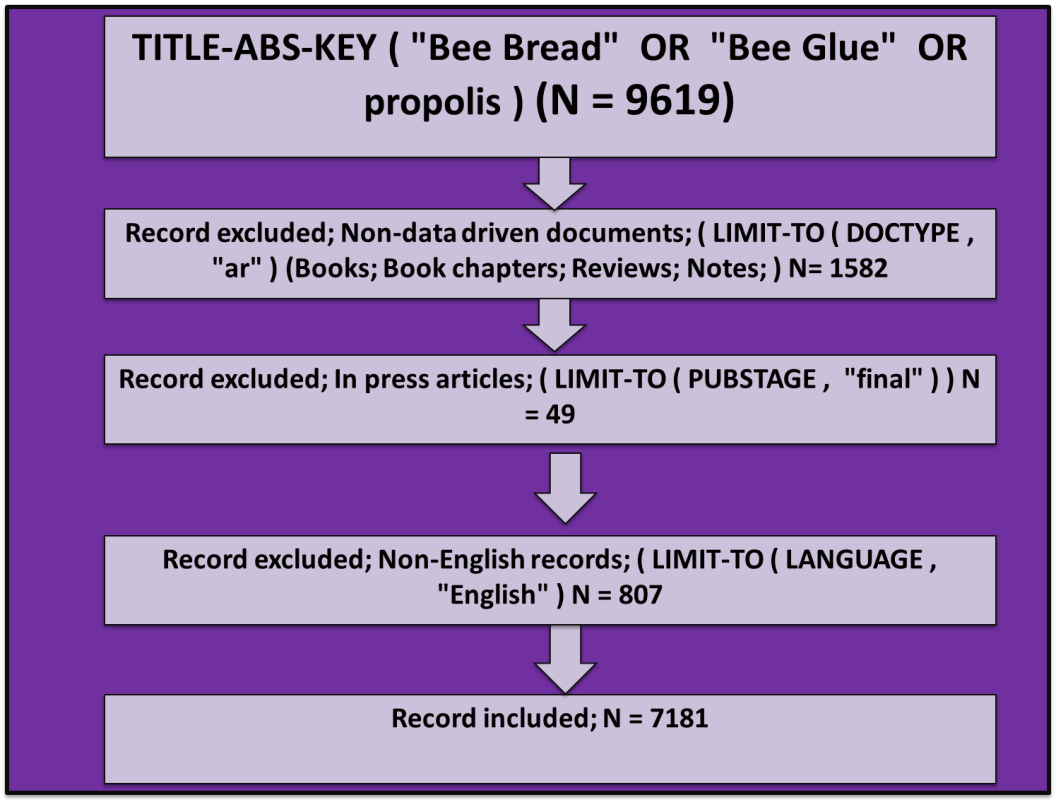

Figure 1. The PRISMA guidelines were diligently followed to ensure a systematic and transparent approach to data collection and analysis. The search and selection criteria were meticulously employed in accordance with the PRISMA guidelines. "ar" denotes the article type, and "DOCTYPE" refers to the type of documents included in the study. The data was extracted on October 2, 2028.

Data Analysis

The collected dataset was analyzed using bibliometric techniques to extract relevant information using VOSviewr (Van Eck and Waltman 2011) and Bibliometrix (Aria and Cuccurullo 2017) applications. The analysis encompassed various aspects, including publication outputs, citation patterns, collaboration networks, and key research topics. The number of publications related to propolis research was recorded and analyzed to identify trends in publication output over time using

regression. This included the total number of publication and annual publication counts. The citation patterns of the identified countries and publications were examined to determine the influence of propolis research. This involved analyzing the number of citations received by each publication, identifying highly cited articles, and exploring the citation networks among the identified publications. Co-authorship analysis was performed to assess the collaboration networks within propolis research. This involved identifying the most prolific countries contributing to propolis research and mapping their collaborative relationships. Keyword co-occurrence analysis was conducted to identify the key research topics within propolis research. This analysis involved identifying the most frequent keywords in the publications and exploring their relationships to uncover emerging research areas and popular themes. The findings from the data analysis were interpreted to provide an understanding of the present state of propolis research. The results were used to identify knowledge gaps, prominent authors and institutions, collaboration opportunities, and emergent research areas.

Ethical Considerations

Ethical considerations regarding data privacy and confidentiality were taken into account during the data collection and analysis process. No ethical approval is required as no human subjects are involved.

RESULTS

Hotspots

The hotspots reveal key contributors in terms of authors, affiliations, and countries. In propolis research, there are a total of 25,004 authors involved, indicating the collaborative nature of the field. Among these authors, 204 have contributed as single authors, implying that they have authored research documents individually without any co-authors. Among the top authors, Bankova, V. (Bulgaria) leads with 106 publications, followed by Bastos, J.K. (Brazil) with 80 publications, and Sforcin, J.M. (Brazil) with 75 publications. The most contributed topics by Bankova, V. include pinobanksin and stingless bees, eutectics and choline, electroplating, geraniin, antioxidants, and tannins. Notable affiliations include the University of São Paulo, with 340 publications; São Paulo State University, with 190 publications; and Public Research University in Campinas (Brazil), with 148 publications. Brazil emerges as the leading country in propolis research with 1,197 publications (Figure 2), followed by Türkiye (627), the United States (532), China (462), and Japan (404). The cumulative production of the top ten countries accounts for 53.70% of the global production in propolis research. These hotspots highlight the noteworthy contributions made by authors, affiliations, and countries in progressing propolis research.

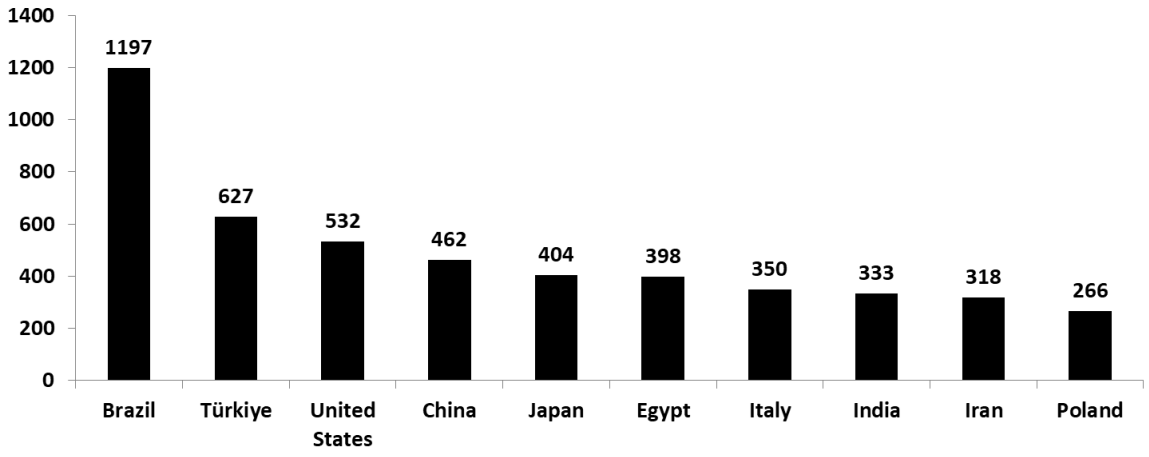

Figure 2. Top-Publishing Countries in Propolis Research. In this figure, the number of articles is represented on the x-axis, while the countries contributing to propolis research are listed on the y-axis. The chart highlights the distribution of research output across different countries in the field of propolis studies.

Sources

With 2110 sources (journals) in propolis research, it demonstrates the extensive and robust body of literature dedicated to studying and exploring the various aspects of this natural substance. Scholars from different disciplines contribute to these journals, further advancing our understanding of propolis and its prospective applications in various fields. The abundance of sources reflects the ongoing interest and significance of propolis as a subject of scientific inquiry and its potential for various therapeutic and agricultural applications. In propolis research, the top sources contributing to the field include "Molecules" with 145 publications, "Evidence-Based Complementary and Alternative Medicine" with 143 publications, "Journal of Apicultural Research" with 102 publications, "Contact Dermatitis" with 97 publications, "Journal of Agricultural and Food Chemistry" with 88 publications, "Food Chemistry" with 86 publications, and "Journal of Ethnopharmacology" with 85 publications. These sources have played a crucial role in disseminating knowledge and advancements in propolis research. Regarding the classification of subjects, the field of propolis research spans various disciplines. The primary subject areas include Agricultural and Biological Sciences with 2064 publications, Medicine with 1988 publications, Biochemistry, Genetics and Molecular Biology with 1914 publications, Pharmacology, Toxicology and Pharmaceutics with 1808 publications, and Chemistry with 1269 publications. Additionally, other subject areas contributing to propolis research include Immunology and Microbiology, Chemical Engineering, Environmental Science, Dentistry, Materials Science, Veterinary, Engineering, Multidisciplinary, Nursing, Physics and Astronomy, Computer Science, Health Professions, Neuroscience, Social Sciences, Energy, Economics, Econometrics and Finance, Earth and Planetary Sciences, Mathematics, Business, Management and Accounting, Psychology, Arts and Humanities, and Decision Sciences. This multidisciplinary nature of propolis research reflects its significance and wide-ranging implications across various scientific domains.

Expansion

From 1945 to 2023, propolis research has demonstrated consistent growth in annual production, as depicted in Figure 3. The average annual growth rate of 1.4% reflects a gradual escalation in the number of research documents over the years. Based on the data, the mean age of the papers in the dataset is 9.46 years. While there have been occasional fluctuations and plateaus in annual production, the overall trajectory showcases an expanding body of research in propolis. The annual growth was analyzed using polynomial regression, and the R-squared value obtained was 0.887. Recent years have witnessed a significant surge in propolis research output, with 434 documents published in 2023, 590 in 2022, and 641 in 2021. This upward trend underscores the growing interest and importance of propolis as a subject of scientific investigation. The early years of propolis research saw relatively few publications, but as the field has advanced, there has been a substantial upsurge in research output. Collectively, these findings reflect the rising attention and research activity in the field of propolis, emphasizing its recognized potential benefits and applications across diverse disciplines.

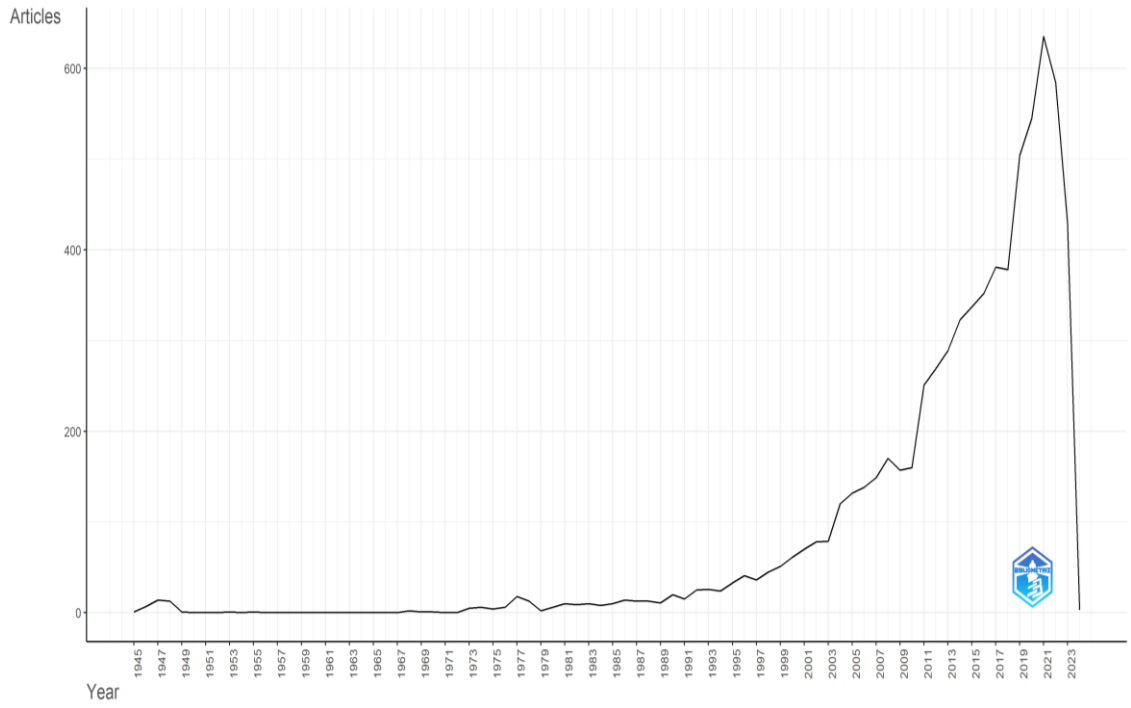

Figure 3. Annual Growth of Propolis Research (1945-2023). The y-axis represents the number of articles published, while the x-axis denotes the years since the first article was published on the topic discussed in this paper. This chart illustrates the progression of propolis research over time, showcasing the increasing trend in scholarly output related to propolis from 1945 to 2023.

Three-field plot

The Sankey diagram, also known as a three-field plot, visually represents the flow of relationships between authors, countries, and sources in a normalized manner. The larger rectangular within each category allow for easy assessment of the relationships among the elements. The color intensity and size of the rectangles indicate strong connections between authors such as "Bastos J.K.," "Berretta, A.A.," and "Rosalen, J.M.," and countries like "Brazil," "China," and "Poland." Similarly, the diagram highlights significant associations with sources such as "Evidence-based Complementary and Alternative Medicine," "Molecules," and "Journal of Ethnopharmacology" (refer to Figure 4). This visualization offers a widespread overview of the interconnectedness and prominence of specific authors, countries, and sources within the analyzed dataset. The examination reveals that Bastos J.K. did not participate in any joint study with countries such as Iran and Indonesia. Moreover, the most productive nations effectively communicated their propolis-related research across all the outlets illustrated in Figure 4. Brazil is the most networked country, followed by China and Poland. Popova, M. and Bankova, V. are the least prolific authors in the domain of propolis research papers, as seen in Figure 4.

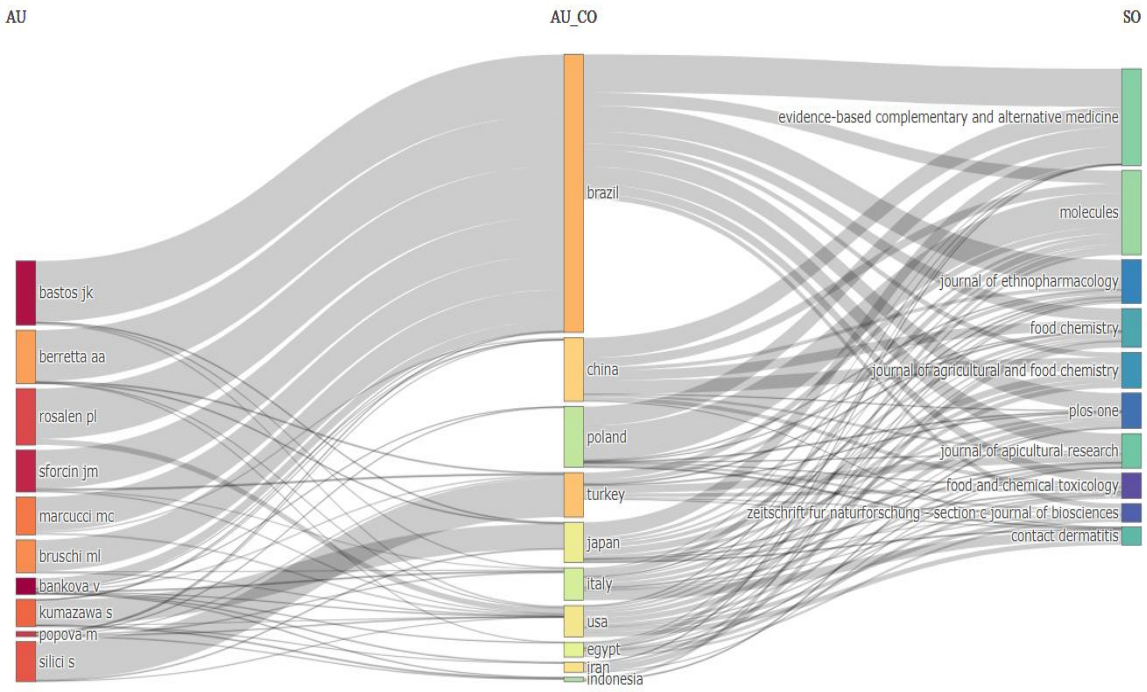

Figure 4. Three-Field Sankey Diagram. This Sankey diagram visualizes the interconnections between authors (AU), authors' countries (AU_CO), and sources (SO). The thickness of the lines represents the number of papers co-authored by authors from different countries and the number of papers published in each source by each country. Each rectangle in the diagram represents a source, a nation, or an author, with the size of the rectangle indicating its significance within the network. The data for this visualization was derived from BibTex files and processed using the Bibliometrix application, providing a comprehensive overview of the relationships within the scholarly network.

Social structure

Impactful components

The top-cited papers in propolis research along with information on their titles, citation counts, publication years, sources, and citation averages are shown in Table 1. "Estimation of total flavonoid content in propolis by two complementary colorimetric methods," which was published in the Journal of Food and Drug Analysis in 2002, is the most frequently referenced paper (ranked #1). This work has received 3,719 citations in total, with an average citation count of 169.05. The item rated second, "Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-κB," was published in the Proceedings of the National Academy of Sciences of the United States of America in 1996. It has received 1,052 citations, averaging 37.57 citations. Titled "Analysis of propolis: Some parameters and procedures for chemical quality control," the third-place paper was released in 1998 and appeared in the Journal of Apicultural Research. With 914 citations overall, its average citation count is 35.15. The fourth-ranked paper, (Title: Antibacterial, antifungal and antiviral activity of propolis of different geographic origin) which was released in 1999 in the Journal of Ethnopharmacology, examines the antiviral, antibacterial, and antifungal properties of propolis sourced from various regions. It has received 858 citations with a citation average of 34.32. Table 1 highlights the top-cited documents in propolis research, covering a broad range of topics. These include flavonoid content estimation, inhibitory effects of caffeic acid phenethyl ester on NF-κB, quality control procedures, antibacterial and antiviral activities, antioxidant properties, cytotoxicity against tumors, botanical origin of Brazilian propolis, and gut bacteria in honeybee development. These topics showcase the multidimensional nature of propolis

research, encompassing chemistry, biological activities, and interactions with honeybees. The average number of citations per document in the entire dataset of 7,181 documents is 27.23.

Table 1. Top-cited articles in propolis research

Rank	DOI number of the publication	Published journal	Publication year	Citations	Citation average
1st	doi.org/10.38212/2224-6614.2748 (Chang *et al.* 2002)	Journal of Food and Drug Analysis	2002	3719	169.05
2nd	10.1073/pnas.93.17.9090 (Natarajan *et al.* 1996)	Proceedings of the National Academy of Sciences of the United States of America	1996	1052	37.57
3rd	10.1080/00218839.1998.11100961 (Woisky and Salatino 1998)	Journal of Apicultural Research	1998	914	35.15
4th	10.1016/S0378-8741(98)00131-7 (Kujumgiev *et al.* 1999)	Journal of Ethnopharmacology	1999	858	34.32
5th	10.1016/S0378-8741(99)00189-0 (Moreno *et al.* 2000)	Journal of Ethnopharmacology	2000	681	28.38
6th	10.1016/S0308-8146(03)00216-4 (Kumazawa, Hamasaka and Nakayama 2004)	Food Chemistry	2004	678	33.90
7th	10.1016/0014-5793(93)80184-V (Sud'ina *et al.* 1993)	FEBS Letters	1993	486	15.68
8th	10.1007/BF01941717 (Grunberger *et al.* 1988)	Experientia	1988	474	13.17
9th	10.1021/jf011432b (Park, Alencar and Aguiar 2002)	Journal of Agricultural and Food Chemistry	2002	471	21.41
10th	10.1128/AEM.07810-11 (Martinson, Moy and Moran 2012)	Applied and Environmental Microbiology	2012	371	30.92

Impactful scholars

Table 2 presents data on the influence of many scholars' publications on various metrics, including total citations (TC), number of publications (NP), H-Index, G-Index, and M-Index, as well as the year their publishing record began (PY_Start). These indicators provide insights into the researchers' productivity, impact, and citation performance in their respective fields. The H-Index represents the number of papers with a corresponding number of citations, while the G-Index and M-Index offer alternative measures considering the distribution of citations. The TC indicate the overall impact of the researchers' work, and NP showcases their productivity. The PY_Start provides information on the year when their publication record began. Bankova, V. has an H-Index of 43, a G-Index of 78, and an M-Index of 1.162. With a total of 6,319 citations and 104 publications since 1987, their research impact is notable. Rosalen, P.L. has an H-Index of 30, a G-Index of 55, and an M-Index of 1.154. Sforcin, J.M. has an H-Index of 30, a G-Index of 51, and an M-Index of 1.111. They have accumulated 2,846 citations from 75 publications since 1997.

The strength of the H-index is that it assesses the number of citations left at a given pass mark along with the number of articles written. This is the primary authorship index. Based on the number of citations a specific paper has, the G-index first sets itself apart. This finding highlights the importance of publishing in high-impact journals. The M-index was calculated using unique authors per article in relation to the total number of published articles on particular influential factors that foster teamwork. TC: Total citations; NP: number of papers; PY_start: the year of first published article.

Table 2. Author's impact. The influence of many scholars' publications on various metrics, including total citations (TC), number of publications (NP), H-Index, G-Index, and M-Index, as well as the year their publishing record began.

Author	H-Index	G-Index	M-Index	TC	NP	PY_Start
Bankova, V.	43	78	1.162	6319	104	1987
Rosalen, P.L.	30	55	1.154	3501	55	1998
Sforcin, J.M.	30	51	1.111	2846	75	1997
Bastos, J.K.	28	46	1.4	2227	80	2004
Marcucci, M.C.	27	48	0.931	2766	48	1995
Ikegaki, M.	25	38	0.962	2526	38	1998
Popova, M.	25	46	1.087	2197	55	2001
Silici, S.	25	44	1.25	2018	52	2004
Kumazawa, S.	23	48	1.045	2729	48	2002
Park, Y.K.	23	31	0.852	3172	31	1997

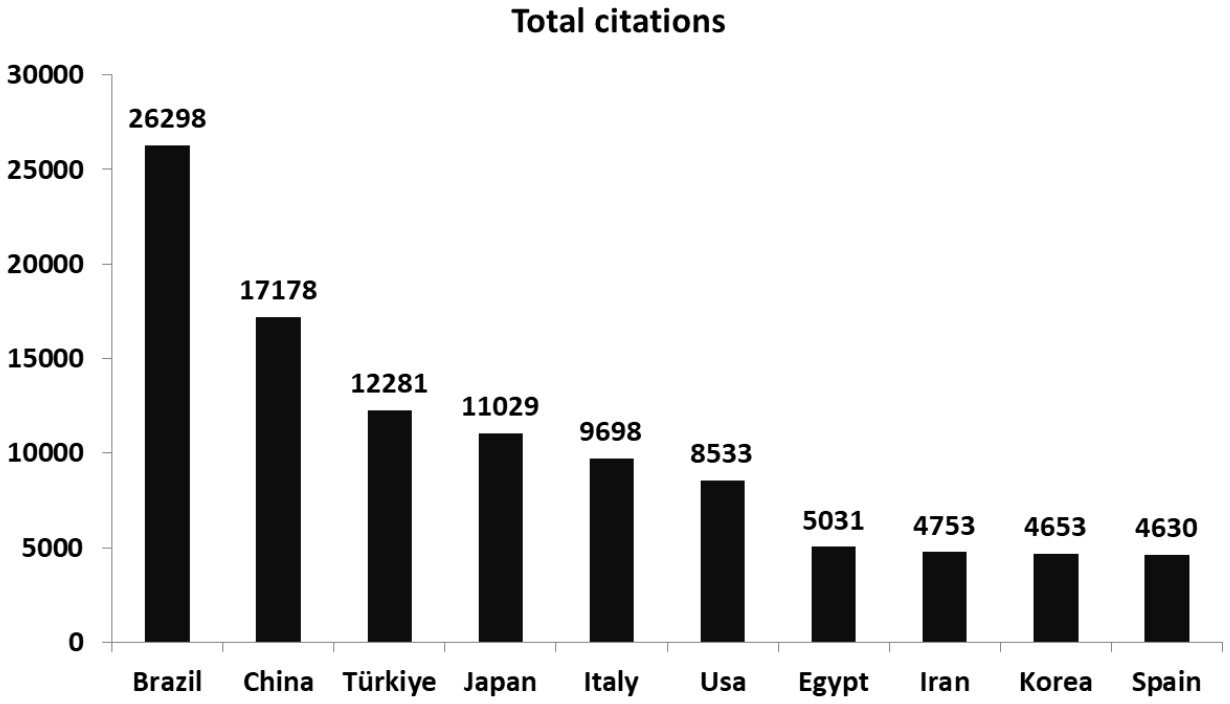

Figure 5. Most-cited countries. This figure showcases the countries that have been most frequently cited in the context of the research presented. The citation frequency serves as a metric for the impact and influence of these countries within the scholarly discourse examined in the study.

Keywords co-occurrence

The conceptual structure and intellectual dynamics of propolis research are characterized by the frequency and co-occurrence of specific keywords. The data contains 28,056 keywords plus (ID) and 12,642 author's keywords. These two keyword categories contain all the terms and phrases used to classify and describe propolis research publications. Bibliographic indexing systems may add keywords to keywords plus (ID) to improve searchability and discoverability. However, the author's keywords (DE) category includes keywords selected by the authors to highlight their research's primary themes, emphasis areas, and findings. These two keyword groups provide a deep overview of propolis study subjects, allowing scholars to examine many pertinent concepts and themes. The dataset

includes a total of 28,056 keywords plus (ID) and 12,642 author's keywords (DE). Among the top author's keywords, "propolis" stands out with a frequency of 2,860 occurrences, indicating its central role in propolis research. Other notable keywords include "flavonoids" (278 occurrences), "antioxidant activity" (234 occurrences), "antioxidant" (215 occurrences), and "antimicrobial activity" (193 occurrences). Additionally, keywords such as "caffeic acid phenethyl ester" (185 occurrences), "honey" (178 occurrences), "beeswax" (173 occurrences), and "Apis mellifera" (170 occurrences) are also frequently mentioned. These numbers provide an insight into the prominence of specific keywords and highlight the areas of focus within propolis research. Propolis research covers its composition, biological activities (such as antioxidant and antimicrobial properties), potential therapeutic applications (such as wound healing and anti-inflammatory effects), and specific compounds (phenolic compounds and polyphenols). The co-occurrence of these keywords reveals propolis' main study fields. The section is regarded as straightforward, and further analyses will be seen in the succeeding portions of this work, which will delve deeper into many aspects such as the conceptual map, thematic development, and emerging trends in propolis research.

Conceptual dynamics

Thematic evolution in propolis research since 1945 was analyzed using Bibliometrix software, providing insights into the progression of research themes over time (Figure 6). From 1945 to 2016, the focus was primarily on topics such as allergic contact dermatitis, antioxidants, artepillin C, bee pollen, beeswax, caffeic acid phenethyl ester, Candida albicans, natural products, and propolis itself. However, a shift in research themes occurred from 2017 to 2024, emphasizing antioxidant activity, oxidative stress, propolis, honey, and the continued exploration of propolis' potential benefits. This analysis highlights the evolving interests and priorities within the propolis research field, reflecting emerging areas of investigation and the changing landscape of scientific inquiry. The year 2016 marks a pivotal point in the conceptual development of the propolis research field. After 2016, there was a noticeable shift in the research landscape, with an expanded focus on antioxidant activity, oxidative stress, propolis, honey, and related bee products. This shift suggests a broader recognition of the importance of propolis as a natural antioxidant and its potential role in addressing oxidative stress-related conditions. This thematic evolution and conceptual development demonstrate the dynamic nature of the propolis research field as new areas of investigation emerge, and the scientific community deepens its understanding of propolis and its therapeutic applications.

Thematic map

The thematic map reveals five distinct themes in propolis research (Figure 7). The first theme, emerging, focuses on allergic contact dermatitis. It has a high Callon density, indicating its development. This theme encompasses research on allergic contact dermatitis and its relationship to propolis. The second theme, classified as motor, represents a significant research focus on propolis itself. It has high Callon centrality and density, indicating its importance. This theme includes topics such as propolis, flavonoids, antioxidant activity, antimicrobial properties, and the phytochemical ingredients of propolis. The third theme, classified as emerging or declining, is centered around beeswax. It has a low Callon density, suggesting a lesser degree of research compared to other themes. This theme focuses explicitly on beeswax and its properties. The fourth theme, classified as motor, revolves around oxidative stress. The values of Callon centrality and density indicate its significance in propolis research. This theme includes studies on oxidative stress, caffeic acid phenethyl ester, apoptosis, inflammation, and their relationships to propolis. The fifth theme, classified as central, is centered around honey. It has moderate Callon centrality and density, indicating its status in the propolis research field. This theme encompasses research on honey, including topics such as *Apis mellifera*, bee pollen, bee products, and the analysis of honey using GC-MS. These five themes represent the key research focus areas within the propolis field. Each theme has its level of relevance, development, and centrality, showcasing the diverse aspects of propolis research that scientists are exploring.

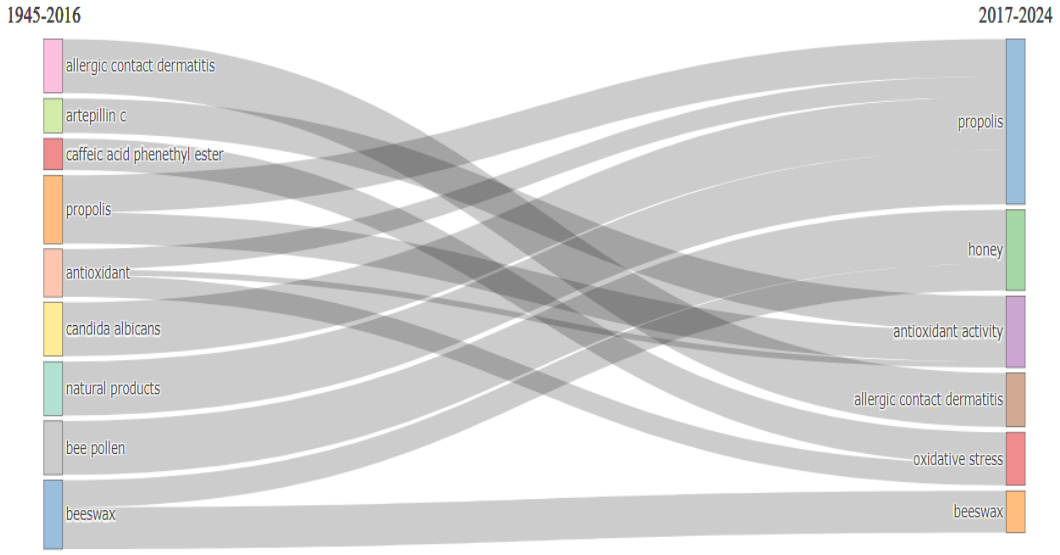

Figure 6. Conceptual Dynamics and Transformation. This figure visualizes the evolution of main topics and the pivotal year of transformation in 2016. The conceptual dynamics within the research field are illustrated, highlighting shifts and trends in the subject matter over time. Generated using the Bibliometrix application and the BibTex data file, this visualization provides insights into the changing landscape of scholarly topics and their development within the specified timeframe.

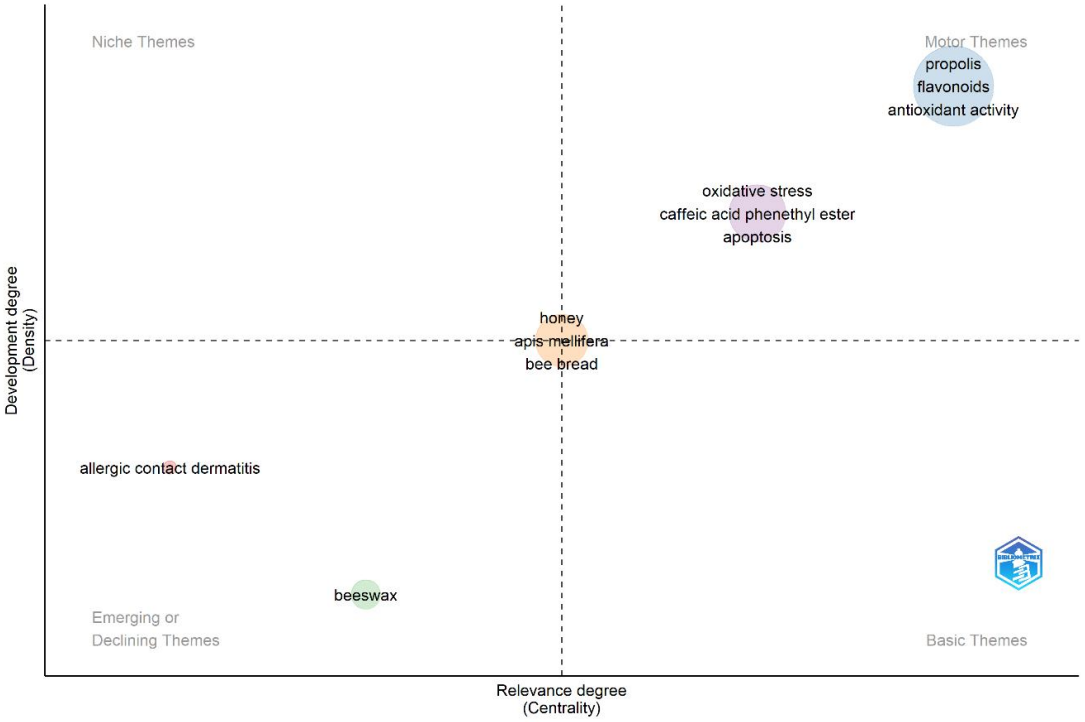

Figure 7. Thematic map. Based on centrality and density, the four quadrants of thematic maps indicate the significance and evolution of the research themes. The BibTex data file and the Bibliometrix application were used to create this figure.

Table 3. Thematic map terms

Cluster	Classification of the themes	Callon-Centrality	Callon-Density	Rank-Centrality	Rank-Density	Cluster Frequency	Keyworker of the cluster
Propolis	Motor	0.067	2.067	5	5	5897	Propolis, flavonoids, antioxidant activity, antioxidant, antimicrobial activity, antibacterial activity, phenolic compounds, cytotoxicity, antimicrobial, polyphenols, propolis extract, antioxidants, artepillin c, antibacterial, HPLC, flavonoid, natural products, wound healing, chemical composition, antifungal activity, phenolics, anti-inflammatory, *Candida albicans*, red propolis, *Staphylococcus aureus*, brazilian green propolis, green propolis, brazilian propolis, caffeic acid, *Streptococcus mutans*, *Baccharis dracunculifolia*, calcium hydroxide, galangin, biofilm, phenolic acids, chlorhexidine, nitric oxide, stingless bees
Oxidative stress	Motor	0.036	1.993	4	4	1185	Oxidative stress, caffeic acid phenethyl ester, apoptosis, inflammation, chrysin, cape, rat, pinocembrin, lipid peroxidation, rats, caffeic acid phenethyl ester (cape), cytokines, COVID-19, liver, breast cancer
Honey	Central	0.028	1.936	3	3	852	Honey, *Apis mellifera*, bee bread, pollen, bee pollen, chitosan, bee products, GC-MS, royal jelly, honey bee, stingless bee
Beeswax	Emerging or declining	0.004	0.578	2	1	173	Beeswax
Allergic contact dermatitis	Emerging or declining	0.000	1.724	1	2	58	Allergic contact dermatitis

Trending topics

Figure 8 provides an overview of the trending topics in propolis research, highlighting their frequency and the years they gained prominence. Chronic kidney disease emerged as a trending topic in propolis research, with five occurrences between 2022 and 2023 indicating a growing interest in exploring propolis' potential benefits for this condition. Antimicrobial research has been highly prevalent, with 113 instances from 2016 to 2022 demonstrating sustained interest in investigating the antimicrobial properties of propolis. “Propolis extract” has also garnered significant attention, appearing 90 times between 2015 and 2022, suggesting a continued focus on exploring its potential benefits and applications. Bee bread, a bee-made product, has been a trending topic with 100 occurrences from 2018 through 2022, indicating ongoing research into its properties and potential advantages. The broader category of bee products, including propolis, bee bread, and honey, has been a topic of interest, with 51 instances from 2019 to 2022, reflecting exploration into the diverse range of bee products and their potential applications. The global COVID-19 pandemic has influenced propolis research, with 37 occurrences as a trending topic from 2021 to 2022, highlighting efforts to investigate propolis' potential antiviral properties in the context of the epidemic. Metabolomics, a comprehensive examination of metabolites, has gained attention with 17 occurrences from 2020 to 2022, indicating the use of metabolomic approaches to understand propolis' composition and effects. Lastly, antiviral research has been notable, with 15 occurrences from 2016 to 2022, indicating ongoing investigations into propolis' potential antiviral properties. Overall, these trending topics demonstrate the diverse areas of interest and ongoing research within the field of propolis, including its potential applications in various diseases, antimicrobial and antiviral activities, and the exploration of specific propolis components and metabolomic analysis.

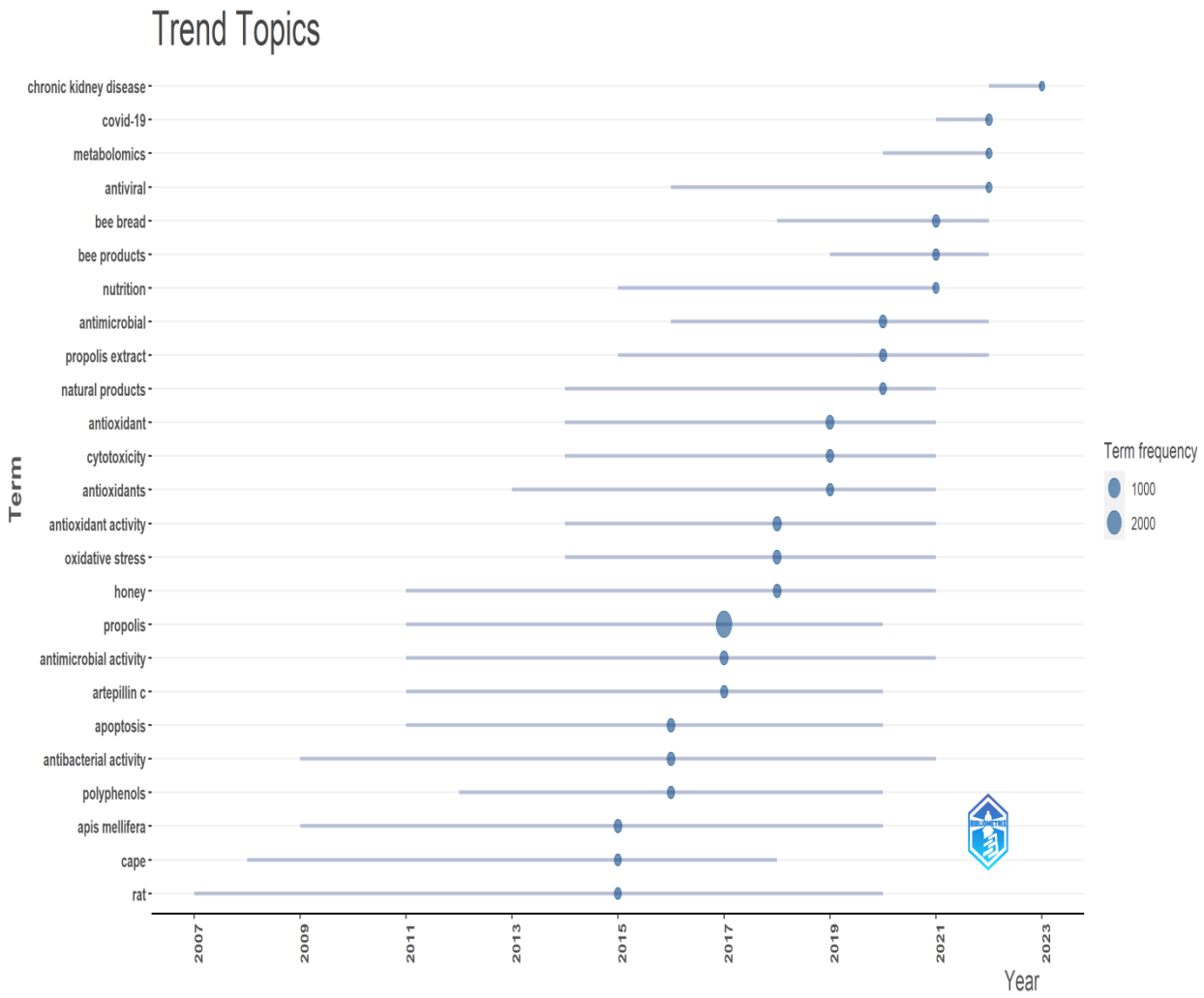

Figure 8. Trending topics. The research topic's temporal span is shown by the graph, where blue circles denote the term's frequency and horizontal lines show the duration. Data files from BibTex and Bibliometrix were used to create this figure.

Social exploration

The data reveals that out of 7181 documents, 20.6% of co-authorships were international collaborations. On average, each document had 5.57 co-authors, indicating a high level of collaboration in the research. These results highlight the significance of teamwork and the exchange of knowledge across borders in the field. Figure 9 presents key findings on research collaboration and publication trends for several countries. Brazil leads in article publications, displaying a significant level of international collaboration and a relatively high MCP (multiple country publications) ratio. Türkiye follows showing moderate collaboration and MCP ratio. China ranks third with 491 articles, indicating a moderate level of collaboration but a higher MCP ratio (Figure 9a). Japan and Egypt demonstrate relatively lower collaboration frequencies but have notable MCP ratios. Other countries, including Iran, India, Italy, Indonesia, and the USA, also show varying levels of collaboration and MCP ratios. These findings highlight the research landscape and international cooperation patterns, shedding light on the countries' scientific productivity and their involvement in multinational publications. Figure 9a gives the number of multi-country documents but does not assess the strength of a country's research cooperation. The single country publications ratio

(SCP) shows the number of published articles with national collaboration. As shown in Figure 9a, the USA has the highest SCP ratio.

Figure 9b represents the strength and progression of global collaboration in propolis research. The United States emerges as the most cooperative nation, closely followed by Brazil. The analysis of collaboration in propolis research goes beyond the leading countries and reveals interesting insights. Figure 9B visually represents the strength and development of collaboration, providing a deeper understanding of the research landscape. Violet-colored rectangles represent leading countries with established networks and partnerships in propolis research. Yellow-colored rectangles indicate countries engaged in recent research collaborations, including Indonesia, Saudi Arabia, Iraq, Iran, and Portugal, suggesting their active efforts to expand research networks. Notably, Egypt and Saudi Arabia have the most significant collaboration, with over 80 joint research initiatives.

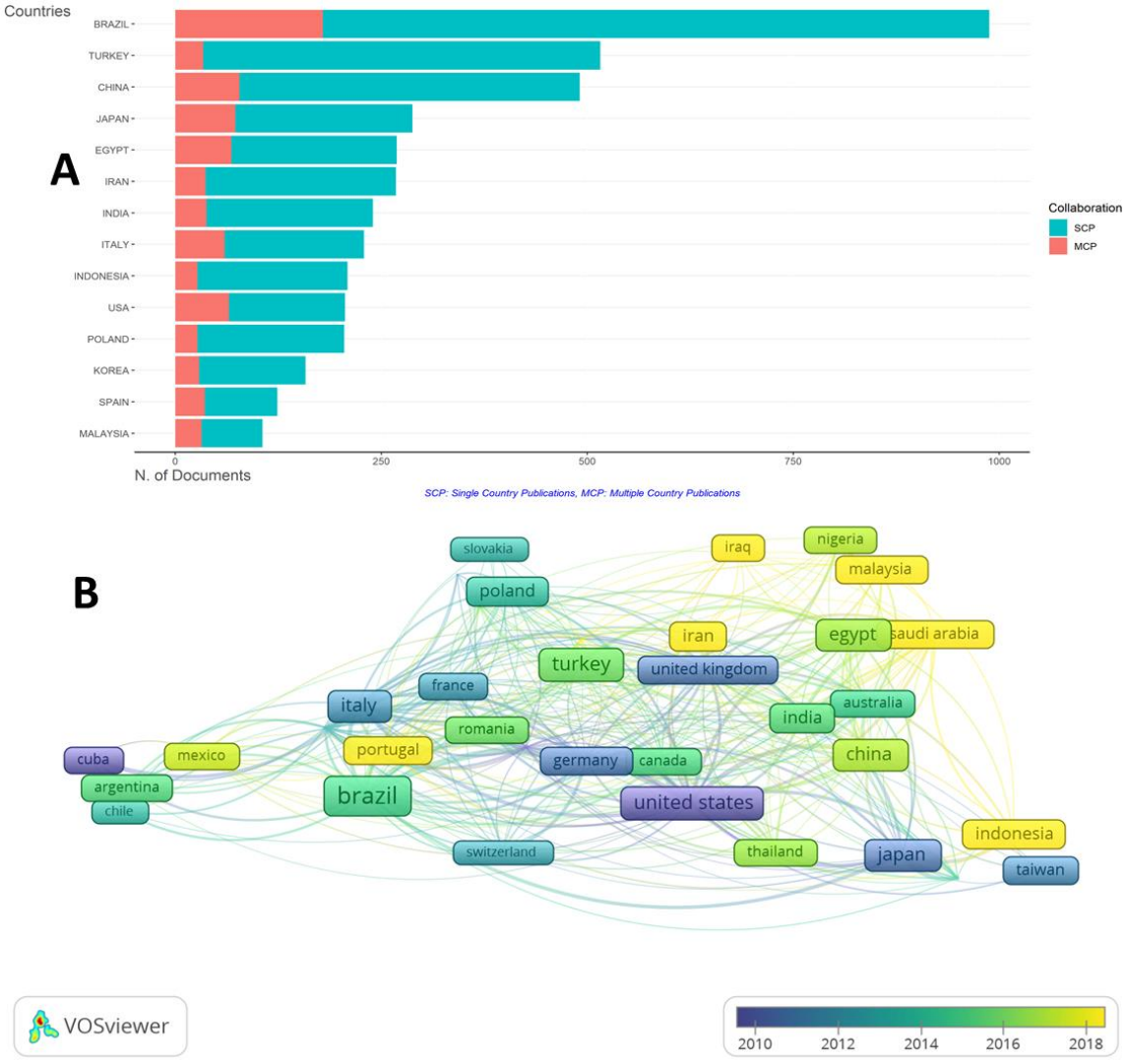

Figure 9. Mapping of international collaboration. A: SCP and MCP analysis of countries' publications. B. the temporal analysis of the global collaboration.

Author's co-citation

Author co-citation is a bibliometric method that examines the co-citation patterns of authors in scholarly publications. It can provide a visual representation of the intellectual map of a field, highlighting the key contributors. Using VOSviewer, this study mapped the author's co-citation with a threshold of 700 as a minimum number of citations of an author (Figure 10). Of the 321838 co-cited authors, 36 meet the threshold. Four clusters were generated (green, red, blue, and yellow) with a total link strength of 421812 and 630 links. Bankova, V. is the leading co-cited author and pioneered the green cluster. Marcucci, M.C. anchored the red cluster. Sforcin J.M. and Park, Y.K. lead the blue and yellow clusters, respectively.

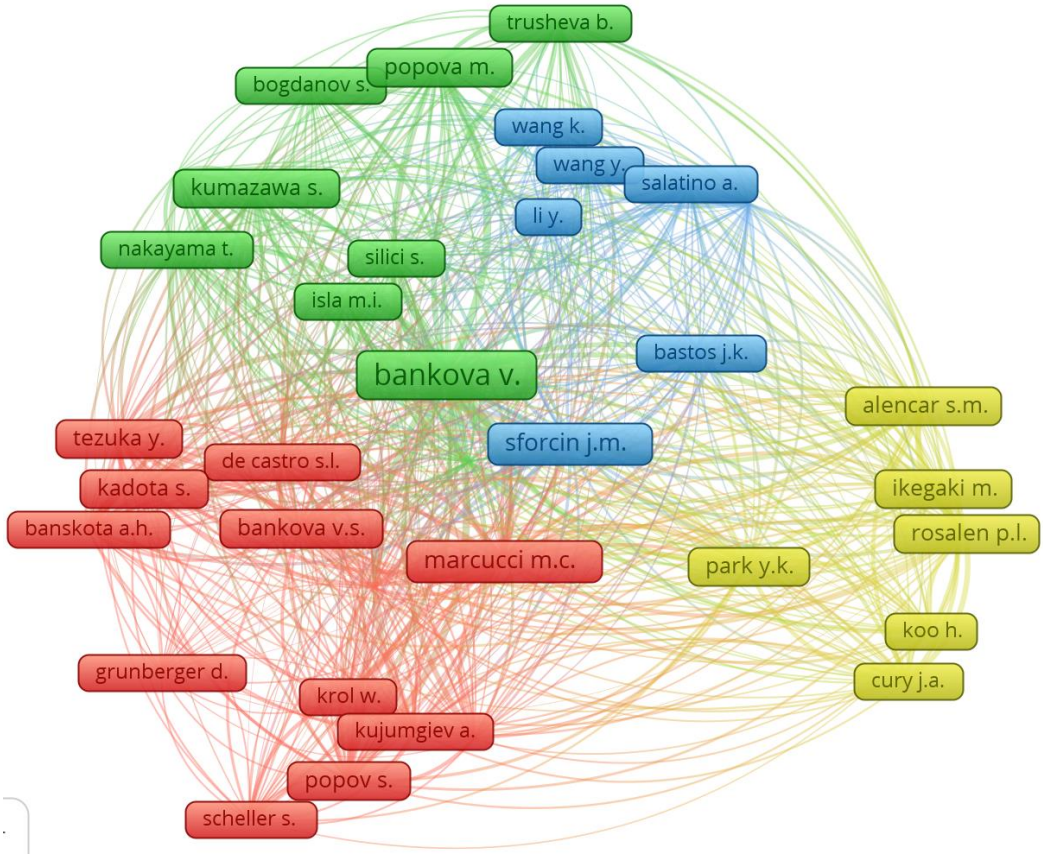

Figure 10. Author's co-citation. Two clusters were detected. A total of 421812 of authors were co-cited. Thirteen of them have been co-cited 630 times.

DISCUSSION

In the area of propolis, recent research uptakes can be explained by some key components. The development of innovative technologies for propolis extraction has increased the efficiency and the cleanliness of this natural product thus making it suitable for research and application purposes (Gupta, Naraniwal and Kothari 2012; Valverde 2023). Besides, notable research in the discipline has caused more interest. Also, the increasing trend of population towards the use of natural products and alternative medicine has created a market increase for researching on propolis and other natural products as there is more demand in health and wellness products that are natural (Glänzel, Leta and Thijs 2006; Salatino, Teixeira and Negri 2005; Zullkiflee, Taha and Usman 2022). All these factors work strongly towards the advancing field of propolis

research and therefore its increased use in scientific research and practical aspects.

Due to its versatility and prospective uses, propolis research spans several fields. Propolis contains many beneficial chemicals, such as polyphenols, terpenoids, and flavonoids. These chemicals have antioxidant, anti-inflammatory, antibacterial, and anticancer effects (Corrêa *et al.* 2017; Gleiznys *et al.* 2019; Hayama *et al.* 2015; Suleiman 2021; Valverde 2023; Zullkiflee, Taha and Usman 2022). Propolis research in biology and ecology examines bee propolis production, hive defense and immunity, and bee health and behavior. These studies advances bee biology and affect pollination and conservation (Shanahan 2023; Simone-Finstrom *et al.* 2017). Pharmacology and medicine study propolis for medicinal uses. Researchers from these fields study its antibacterial, anti-inflammatory, and antioxidant characteristics to produce natural antibiotics, cure inflammatory illnesses, and counteract oxidative stress (Kantrong *et al.* 2023; Omar *et al.* 2023; Valverde 2023; Vilhelmova-Ilieva *et al.* 2023). Propolis may also fight cancer and produce new drugs (Ding 2015; Frión-Herrera *et al.* 2019; Zou *et al.* 2016; Zullkiflee, Taha and Usman 2022). The antibacterial and anti-inflammatory properties were studied in dentistry to prevent and treat oral infections, gum disorders, and tooth caries (Alizadeh Tabari *et al.* 2023; Karaoğlu *et al.* 2023; Kujumgiev 1999; Sales-Peres *et al.* 2023; Shamma *et al.* 2023; Valverde 2023). Natural alternatives to conventional oral care products include propolis-based mouthwashes, toothpaste, and dental materials (Alizadeh Tabari 2023; Karaoğlu 2023; Sales-Peres 2023). Additionally, propolis research connects with food science and agriculture. Propolis's antimicrobial and antioxidant properties are studied for food preservation and functional food additives (Irigoiti 2021). Propolis is studied as a natural pesticide and plant growth enhancer in agriculture (Fuat Gulhan *et al.* 2012). Bioactive substances in propolis and their potential applications make research interdisciplinary. By bridging disciplines, researchers can discover new insights and uses and add to existing knowledge. Recent evaluations concur with this work about the many bioactivities of this natural chemical (Chavda *et al.* 2024; El-Sakhawy, Salama and Tohamy 2023; Salami 2024; Tek, Şentüre Ş and Ersoy 2024).

Brazil's prominence as the most cited country in propolis research can be attributed to various factors. Firstly, Brazil's abundant propolis resources stemming from its diverse ecosystems and geographic regions attract researchers worldwide (Salatino, Teixeira and Negri 2005), leading to more studies conducted on it. Secondly, Brazil has a strong research tradition in natural products and traditional medicine, with experts actively investigating propolis's chemical composition, biological activities, and therapeutic potential (Alves and Rosa 2007). Collaborative efforts and networking with international scientists further enhance visibility and impact. Additionally, the prolific publication output of Brazilian researchers in reputable scientific journals and their significant contributions to propolis research influence the field and garner citations (Glänzel, Leta and Thijs 2006). However, it's essential to acknowledge that other countries and researchers have also made valuable contributions to propolis research. This study concurs with prior research indicating that Brazilian propolis has been extensively examined globally owing to its distinctive chemical makeup and biological attributes, including antioxidant, antibacterial, and anti-inflammatory activities (de Sousa Silveira *et al.* 2024; Franchin *et al.* 2024; Scorza *et al.* 2024).

A positive inclination in the annual production of propolis research during the analyzed period discloses. The number of research documents has steadily increased, indicating a growing interest in propolis and its potential applications. Recent years have seen a distinguished surge in the number of documents, with 2021 recording the uppermost number, followed by 2022 and 2023. Fluctuations and plateaus in annual production reflect variations in research output, potentially influenced by factors such as shifting priorities, funding accessibility, and emergent trends. The data also highlights a significant expansion in research output in recent decades, underscoring the growing recognition of propolis' benefits and applications. Overall, the findings demonstrate the increasing attention and research activity dedicated to propolis, indicating a growing understanding of its potential across various disciplines. The present analysis concurs with prior research indicating a substantial increase in studies about honey and associated products, as documented by earlier bibliometric analyses (Andreo-Martínez *et al.* 2020; Stefanis *et al.* 2023).

The analysis of keywords revealed that wound dressing is a study subject associated with this natural chemical. Propolis has demonstrated significant benefits in wound dressings due to its

superior antiseptic efficiency and antibacterial characteristics. Multiple reports showed the skin wound healing process, related evaluation criteria, and emphasized the improved propolis-based material dressings, including antibacterial properties, adhesion, hemostasis, anti-inflammatory effects, and substance distribution. Moreover, prior reports have documented the use of propolis wound dressing for the treatment of many wound types, including healing wounds, burns, and ulcers (Canales-Alvarez *et al.* 2024; Doodmani *et al.* 2024; El-Sakhawy, Salama and Tohamy 2023; Manginstar *et al.* 2024; Necip *et al.* 2024; Zayed *et al.* 2024). Future directions for propolis-based wound dressings in wound healing are offered. The observed findings align with previous studies (El-Sakhawy, Salama and Tohamy 2023; Manginstar 2024) indicating that propolis-based materials may serve as a potential novel dressing for wound occlusion and tissue restoration.

The evolution in propolis research themes after 2016 can be linked to developments in scientific knowledge, the appreciation of propolis' potential benefits, the broader interest in natural remedies, and the changing priorities of the scientific community (Sandberg and Corrigan 2001). Improved analytical methods and technology (Gupta, Naraniwal and Kothari 2012) allowed researchers to explore new aspects of propolis, particularly its antioxidant activity and potential for addressing oxidative stress (Fonseca 2011). The growing understanding of propolis's bioactive compounds and its status as a valuable natural product also contributed to increased research. Additionally, the expanded focus on honey and related bee products reflected an interest in alternative remedies (Peršurić and Pavelić 2021). Overall, evolving interests and advancements in scientific understanding drove the shift in research themes, shaping the progression of propolis research since 2016.

The trending topic of COVID-19 in propolis research can be attributed to several factors. Propolis is being explored for its potential antiviral activity against coronaviruses, including COVID-19. Its antimicrobial properties and immunomodulatory effects have sparked interest in understanding how propolis may support the immune system and mitigate the impact of the virus. The COVID-19 epidemic has also heightened attention in natural products and traditional medicine, leading researchers to investigate propolis as a potential treatment or supportive therapy (Silveira *et al.* 2021). Additionally, the public's curiosity and concern regarding COVID-19 have contributed to increased attention on propolis research. As a natural substance with potential health benefits, propolis has garnered interest as a possible solution for combatting the virus (Karaoğlu 2023; Omar 2023; Ożarowski and Karpiński 2023; Sales-Peres 2023; Taysi *et al.* 2023; Vilhelmova-Ilieva 2023). The combination of propolis' bioactive compounds and the urgent need for effective COVID-19 treatments has driven research in this area. The trending topic of COVID-19 in propolis research reflects the ongoing efforts to find solutions and explore the potential of natural compounds in addressing the global pandemic.

Author co-citation is a bibliometric analysis that examines the co-citation patterns of authors in scholarly publications (Zhang *et al.* 2023). It involves identifying and analyzing the occurrence with which two or more authors are cited together in the reference lists of articles. Author co-citation analysis helps identify influential authors and research themes within a field and the relationships and networks among researchers. It can provide a visual representation of the thematic structure of a field, highlighting the key contributors and the extent of their interactions. It can also assist researchers in determining areas that require more study and gaps in the body of knowledge. All things considered, author co-citation analysis is a useful method for comprehending the academic environment and recognizing important individuals and their contributions (Carollo *et al.* 2023; Tan *et al.* 2023; Zhang 2023). The most contributed topics by Bankova, V. include pinobanksin and stingless bees, eutectics and choline, electroplating, geraniin, antioxidants, and tannins. Marcucci, M.C., the top-cited author in the red cluster, is a researcher with a diverse range of interests spanning multiple fields. Researchers in this cluster are interested in various topics, including dental materials and bonding agents in restorative dentistry, female reproductive health, nonvital tooth treatments, honey and stingless bees, and regenerative dentistry focusing on tooth pulp and stem cells. This demonstrates their multidisciplinary approach and contributions to different areas of study (Adiningrat *et al.* 2023; Alizadeh Tabari 2023; Jauhar *et al.* 2023; Kantrong 2023; Mandil *et al.* 2023; Rasool *et al.* 2023; Saleh *et al.* 2023; Shamma 2023). Researchers in the blue clusters are interested in apoptosis, 8-bromo-7-methoxychrysin, and flavones (Ding 2015; Frión-

Herrera 2019; Zou 2016). Dietary carbohydrates, allicin, garlic, diallyl trisulfide, biogenic amines, antimicrobial, histamines, and tyramine are the primary research for the scholars in the yellow cluster (de Figueiredo *et al.* 2017; Lee *et al.* 2019; Park *et al.* 2020; Park, Lee and Mah 2019; Roquetto 2015; Takeshita *et al.* 2013).

Many clinical applications of propolis research have been reported. But at the same time, certain critical limitations need to be addressed. There are issues such as geographical composition variations where one region may produce propolis that may be very different from that produced in another, thus impacting its safety and effectiveness (Zullkiflee, Taha and Usman 2022). Moreover, unformulated variations and indefinite formulations of the compound also make it impractical for clinic or hospital use, which raises concerns about uniformity in doses and results (Valverde 2023). In addition, propolis cannot be applied or used in a larger scope due to regulatory requirements since there is always limited data to support the claims (Ding 2015; Valverde 2023). Understanding these challenges is important in order to broaden the clinical uses of propolis and protect its safety when used in medicine.

This bibliometric research has certain major limitations. Firstly, all these studies have reported in published scientific literature only, making it impossible for unpublished or non-indexed research, therefore biasing the outcomes either way. The selection criteria used to look for such papers in this case has been criticized for inducing selection bias therefore making it less generalizable. At any case, the better the study, the more the data there will be, and in most cases, the data may be insufficient or inaccurate, thereby influencing the study's findings. Owing to its time cutoff knowledge, this July 2023 study has not been able to identify new developments or research directions. Different bibliometricians employ bibliometric data analysis to make classification as well as analysis that tends to deviate from one researcher to another researcher. Also, citation analysis can outline patterns and relationships, but not determinants and processes. Finally, the use of numbers perhaps may hide useful information affecting the way one would assess the value of all the studies such as the clinical relevance and even outcomes measured in humans, which are in real life more logically observable than in laboratory settings as the progression of many diseases does not fit the clinical trials. Overcoming these drawbacks is important in increasing the quality and relevance of bibliometric approaches to the analysis of scientific activity.

Conclusion: At the end of the study, a comprehensive bibliometric review of propolis research was carried out, showing its growth, new trends, and the spatiotemporal distribution of studies conducted in various spheres. The interesting fact that emerged from analysis of the research literature includes the strong growing tendencies of interest towards the use of propolis in medicine which makes this topic of scientific research quite pressing and important in the world today. The research output development throughout the countries and regions demonstrates the wider growing awareness of the health benefits and healthcare relevance of propolis. Recognizing patterns and trends in propolis research has significant implications, such as the ability to identify hotspots and research gaps for future work. These temporal and geographical patterns must be clearly understood in order to foster connectivity and cross-pollination among researchers in order to advance the issues at hand. In the future studies should develop strategies for thorough exploration of the action mechanisms of propolis, especially determining the properties of its secondary metabolites and their conjugates for pro-health effects. Integration of currently very separate fields including pharmacology, immunology, and chemistry of natural products will advance the knowledge of propolis including its synergistic effect resulting in new formulation and treatments with the best use of its medicinal activities. Along with this, proper clinical studies are required also to prove the safety, efficacy, as well as effective doses of propolis for various populations so that this product of nature would be part of conventional medicine with evidence-based recommendation for practitioners. By delving deeper into its mechanism of action, promoting cross-disciplinary collaborations, and performing good clinical studies, we can appreciate the true therapeutic Value of propolis and health care will improve.

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DERLEME /REVIEW

APITHERAPY AND APPLICATIONS IN VETERINARY MEDICINE

Veteriner Hekimlikte Apiterapi ve Uygulamaları

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ABSTRACT

The use of bees and bee products for therapeutic purposes in human and veterinary medicine is called apitherapy. Natural products have been used throughout human history to protect against and treat diseases. In recent years, the emergence of drug resistance and the occurrence of adverse effects associated with the indiscriminate and irregular use of pharmacological agents have prompted humanity to pursue alternative solutions. One of the most significant solutions is the administration of bee products. The use of bee products in apitherapy applications includes honey, bee venom, pollen, propolis, beeswax, royal jelly, perga and apilarnil (bee larvae). In general, apitherapy products with a wide range of indications are widely used in various system diseases and some dermatologic problems and various effects of these products such as antibacterial, antifungal, antiviral, antioxidant, anticarcinogenic, anti-inflammatory, antidiabetic and immunomodulatory effects *in vitro* and *in vivo* have been investigated in many different studies. Apitherapy is less common in veterinary practice than in human medicine. Additionally, bee products are employed in the treatment of other animal diseases, including gastrointestinal disorders, otitis, sinusitis, ophthalmic conditions, dermatological disorders, and skin care. Additionally, bee products are employed as food supplements for animals. The most prevalent additive in animal food is bee pollen. It has been demonstrated to promote growth, reduce mortality and prevent morbidity. Apitherapy has gained importance in the field of veterinary medicine in recent years in order to prevent both human health and economic losses, especially in the treatment of animals consumed as food.

Keywords: Apitherapy, Bee Products, Veterinary Medicine

ÖZ

Arı ve arı ürünlerinin insan ve veteriner hekimliğinde tedavi amaçlı kullanımına apiterapi denir. Doğal ürünler, insanlık tarihi boyunca hastalıklara karşı korunmak ve tedavi etmek için kullanılmıştır. Son yıllarda ilaç direncinin ortaya çıkması ve farmakolojik ajanların gelişigüzel ve düzensiz kullanımıyla ilişkili olumsuz etkilerin ortaya çıkması, insanlığı alternatif çözümler aramaya yöneltmiştir. En önemli çözümlerden biri de arı ürünlerinin uygulanmasıdır. Arı ürünlerinin apiterapi uygulamalarında kullanımı bal, arı zehri, polen, propolis, balmumu, arı sütü, perga ve apilarnil (arı larvaları) içerir. Genel olarak, çok çeşitli endikasyonlara sahip apiterapi ürünleri çeşitli sistem hastalıklarında ve bazı dermatolojik problemlerde yaygın olarak kullanılmaktadır ve bu ürünlerin antibakteriyel, antifungal, antiviral, antioksidan, antikarsinojenik, antiinflamatuar, antidiyabetik ve in vitro ve in vivo immünomodülatör etkileri gibi çeşitli etkileri birçok farklı çalışmada araştırılmıştır. Apiterapi, veteriner hekimliğinde insan hekimliğine göre daha az yaygındır. Ayrıca, arı ürünleri gastrointestinal bozukluklar, otitis, sinüzitis, oftalmik durumlar, dermatolojik bozukluklar ve cilt bakımı dahil olmak üzere diğer hayvan hastalıklarının tedavisinde kullanılır. Ayrıca, arı ürünleri hayvanlar için gıda takviyesi olarak kullanılır. Hayvansal gıdalardaki en yaygın katkı maddesi arı polenidir. Büyümeyi teşvik ettiği, ölüm oranını

azalttığı ve morbiditeyi önlediği gösterilmiştir. Apiterapi, özellikle gıda olarak tüketilen hayvanların tedavisinde hem insan sağlığını hem de ekonomik kayıpları önlemek amacıyla son yıllarda veterinerlik alanında önem kazanmıştır.

Anahtar Kelimeler: Apiterapi, Arı Ürünü, Veteriner Hekimlik

GENİŞLETİLMİŞ ÖZET

Amaç: Son yıllarda gelişen ilaç direnci ve ilaçların yan etkiler nedeni ile hem insan hem de hayvan sağlığında tıbbı alternatif çözümler aranmaktadır. Arı ürünleri de bu alternatif tedavi seçeneklerinin en önemlileri arasında yer almaktadır. Hayvanlarda apiterapi uygulamaları insanlarda olduğu kadar yaygın değildir. Antik uygarlıklar arı ürünlerini hayvanlar için de kullanmıştır, ancak günümüzde arı ürünlerinin pahalı, üretimin sınırlı olması, üretimin güvenilirliği ve canlılarda yan etkisi olup olmadığı konusundaki kuşkular sonucu uygulamalar sınırlı kalabilmektedir. Arılar, yüzyıllardır farklı medeniyetler tarafından çeşitli hastalıkların tedavisinde kullanılan bal, propolis, arı sütü, arı poleni, balmumu ve arı zehri gibi biyoaktif bileşenler içeren çok sayıda ürün üretir. Doğal ürünlerin araştırılması, çeşitli hastalıkları önlemek veya tedavi etmek için son zamanlarda önem kazanmıştır. Arı ürünlerine ve apiterapiye olan ilgi de artmıştır. Apiterapi, hastalıkları önlemek veya ilerlemelerini kontrol etmek için terapötik/profilaktik ajanlar olarak arılar veya ürünleriyle yapılan tedavidir. Günümüzde, apiterapi birçok ülkede tamamlayıcı ve bütünleştirici tıbbın bir parçasıdır. Ayrıca, içerdikleri besin maddeleri nedeniyle, arı ürünlerinin nutrasötik ve diyet takviyesi olarak tüketimi artmıştır. Arı ürünlerinin farmakolojik aktivitesi üzerine yapılan araştırmalar son yıllarda artmış ve çok sayıda biyolojik özellik ortaya çıkmıştır. *In vitro* ve *in vivo* çalışmaların yanı sıra klinik deneyler, arı ürünlerinin çeşitli hastalıkların tedavisinde ve sağlık dengesi ve homeostaz için endike olabileceğini göstermiştir. Buna ek olarak, arı ürünleri gıda, kozmetik ve ilaç endüstrisi tarafından yeni ilaç arayışlarında sürekli olarak kullanılmaktadır. Apiterapi veteriner hekimlikte farklı hayvan türlerine uygulanabilmektedir; Evcil hayvanlar, egzotik hayvanlar, çiftlik hayvanları, vahşi hayvanlar, kuşlar, sürüngenler. Apiterapi ile tedavi edilen hastalıklar çok çeşitlidir ve arı ürünlerinin yaygın kullanımı, anti-mikrobiyal, anti-enflamatuar, anti-radyoaktivite, anti-kanser ve yara iyileştirici özellikleri nedeniyle dikkat çekicidir. Balın floral kaynaklarına ve içeriğine bağlı olarak, antimikrobiyal, antiparaziter, antitümoral, immunmodülatör, antienflamatuar, antioksidan, gastroprotektif, kardiyoprotektif, hepatoprotektif, boğaz ağrısı, öksürük, astım, alerji, semtoplarını azaltıcı, analjezik, antianemik, antiosteoporotik, prebiotik, performans arttırıcı, fertilite arttırıcı ve yara iyileştirici birçok özelliği bulunmaktadır. Polenin antibakteriyel, antifungal, antioksidan ve antikanser özelliklere sahiptir. Polenlerin antimikrobiyal özelliği quersetin, mirsetin, kampferol gibi yapısında bulunan fenolik bileşiklerden kaynaklanmaktadır. Genellikle doğal antibakteriyel özellikleri nedeniyle, binlerce yıldır hem insanların hem de hayvanların sağlığını desteklemeye yardımcı olmak için kullanılmıştır. Evcil hayvanlardan çiftlik hayvanlarına kadar birçok hayvan arı propolisinden faydalanabilir. Genellikle solunum ve bağışıklık sistemlerinin sağlığını desteklemek ve antibakteriyel özelliklerinden dolayı daha spesifik hastalıklar için kullanılır. Arı propolisinin, hayvanlarda ve evcil hayvanlarda kanseri, inflamation ve tümörleri önlemede iyi bir doğal takviye olduğu gösterilmiştir. Birçok hastalıkta infilamasyona sebep olan T hücrelerinin üretimini engeller. Arı sütü, işçi arılar tarafından üretilen bir salgıdır ve yalnızca kraliçe arıya verildiği için etkili bir şekilde bir "süper besin"dir. Ayrıca çeşitli vitaminler ve amino asitler içerdiği anlaşılmıştır ve yüzlerce yıldır sağlığı desteklemek için kullanılmıştır. Arı sütünün koyun gebelik oranları ve kuzulama oranları üzerindeki etkilerini, incelenmiş ve etkili bulunmuştur. Hücre yenilenmesi, üretimi ve metabolizma üzerinde etkilidir. Bütün vücut dokularında canlılık, sağlık, enerji ve bağışıklık sağlamaktadır. Evcil hayvanlar için bal ve arı zehri karışımı uygulanan köpekler, kediler ve safkan yarış atları dahil olmak üzere evcil hayvanlardaki artritis ağrısını azaltmıştır. Eklem, kas hastalıkları ve kasların fonksiyonel bozuklukları bulunan evcil hayvanlarda etkili bulunmuştur. Arı zehiri çok güçlü bir ağrı kesici ve anti-inflamatuar etki sağlar. Düzenli olarak alındığında, zamanla eklem rahatsızlığını en aza indirmeye yardımcı olur, eklem hareketliliğini korur ve geri kazandırır. Apilarnil yüksek oranda protein, vitamin ve hormon içerir. Yapısındaki bileşikler sayesinde androjenik, anabolik, anti-lösemik, anti-anemik, antiaterosklerotik, renoprotektif, hepatoprotektif, nöroprotektif, hipolipidemik, üretrotropik, biyolojik

uyarıcı, immünmodülatör, enerji verici ve hücre yenilenmesini uyarıcı etkilerinden yararlanarak hayvan besleme, hayvan sağlığını koruma ve tedavide kullanılabilir.

Sonuç: Arı ürünleri hayvanlarda farklı etkiler gösterebilir. Arı ürünleri uygulandıktan sonra hayvanların ürüne olumlu tepki gösterme süresi değişiklik gösterebilir. Uygulanacak arı ürünlerini seçerken, mümkün olduğunca saf ve doğal bir ürün seçilmelidir. Eğer ürün uygulanacak hayvana bir tedavi uygulanıyorsa, arı ürünü kullanılmadan önce veteriner hekime danışılmalıdır. Arı ürünleri genellikle güvenli olsa da henüz tam olarak uzun hatta kısa vadede etkileri bireysel ya da tür olarak farklılık gösterebilir. Bazı insanlar ve hayvanlar, özellikle arı sokmalarına alerjisi olanlarda, arı ürünlerine alerjik olabilir. Uygulanacak arı ürününün hayvanların diyetine küçük miktarlardan başlayarak kademeli olarak arttırmak güvenli olur. Ayrıca doğal arı ürünlerinin renginde farklılıklar görülebilir. Bu durum normaldir, çünkü doğal ürünler oldukları için, renklerinde farklılıklar olabilir ve doğa tarafından standart şekilde üretilmemiş olmaları oldukça normaldir.

Bu ürünler geçmişten günümüze insanlar tarafından gıda maddesi olarak kullanılmaktadır. Bununla beraber sağlık üzerine olumlu etkileri olan bu ürünlerin tedavi amaçlı kullanımı da gün geçtikçe artmaktadır. Bu derlemenin amacı apiterapi ürünlerinin hayvanlar üzerinde, sağlıklı yaşamı korumak, desteklemek ve çeşitli hayvan hastalıkları üzerine olan etkilerini kullanım alanlarıyla birlikte vurgulamaktır. Bir arı ürünü bir hayvanda etkili olmasına rağmen bir sonraki hayvanda etkili olamayabilir. Her hayvanın ilaca ve diğer takviyelere tepkisi farklılık göstermektedir. Hayvanların apiterapi ürünlerine farklı sürede tepki verirler. Genel olarak, arı ürününü seçerken, doğal olması amaçlanan ürünlerden maksimum potansiyeli elde etmeye yardımcı olmak için mümkün olduğunca saf ve doğal bir ürün seçmeye çalışılmalıdır. Tüm tamamlayıcı tedavilerde olduğu gibi, bir arı ürününü kullanmadan önce, özellikle de hayvanların mevcut ilaçlarını kullanıyorsa, öncelikle veteriner hekim tavsiyesine başvurulmalıdır

INTRODUCTION

Recently, there has been an increasing focus on alternative medical solutions, driven by the need to address the challenges posed by drug resistance and adverse effects. These developments have been observed across both human and animal health. Of these alternative treatment options, bee products represent one of the most significant and promising areas of research. The use of apitherapy in animals is less prevalent than in humans. Ancient civilisations also used bee products for animals, but today applications may be limited due to the high cost of bee products, limited production, doubts about the reliability of production, and side effects that are not fully understood (Boukraâ 2023).

Apitherapy is an alternative treatment that uses honey, pollen, propolis, royal jelly and bee venom for health benefits. It has been used for thousands of years. The diseases treated with apitherapy are very diverse. Bee products are used for their antimicrobial, anti-inflammatory, anti-radiation, anti-cancer and wound healing properties. The medical treatments applied to humans and animals are similar. In this context, the concepts of human and animal health are not different. From a broad perspective, veterinary medicine aims to protect both human and animal health. It is reported that complementary and alternative therapies are not very common in basic veterinary education and practice worldwide.

Honey

Honey has a highly variable structure, comprising carbohydrates (glucose, fructose, maltose, sucrose, maltulose, isomaltose, and turanose), a water fraction, and a range of other components, including flavonoids, amino acids, phenolic acids, antioxidants, enzymes, vitamins and minerals (Alvarez-Suarez et al. 2014, Cengiz et al. 2018). One of the most important properties of honey is its antimicrobial effect. This activity is based on two principal sources. The first of these is the effect of H_2O_2 produced by glucose oxidase in honey in the presence of light and heat; the other is the non-peroxidal activity that inhibits microbial growth independently of light and heat. Honeys have two different characteristics as nectar and secretion honeys. Nectar-sourced honeys exhibit a higher concentration of glucose and fructose than their secretion-sourced counterparts. Secretory honeys are characterised by a higher mineral content. Furthermore, the pH of nectar-derived honeys is more acidic than that of secretion-derived honeys (Öner and Usta 2022).

The antimicrobial, antiparasitic, anti-inflammatory, antitumour, immunomodulatory, antioxidant,

gastroprotective, hepatoprotective, cardioprotective, respiratory system, cough, allergy, asthma, sore throat, painkiller, antianemic, performance enhancer, prebiotic, antiosteoporotic, fertility enhancer and wound healing properties of honey depend on the source and content of the honey in question (Sorucu 2019).

The most well known and demonstrated effectiveness of honey in animals, especially in treating burns and wounds resistant to conventional therapies (Chatzimisios et al., 2023, Lukanc et al. 2020, Vogt et al., 2021).

In clinical trials, the effects of honey on a wide variety of animals, including cattle, horses, dogs, cats, and swine have been extensively studied (Vogt at al, 2021).

Wound Treatment

When incorporated into an animal's diet, honey has been shown to accelerate wound healing and provide health benefits. It is hypothesised that the health-promoting properties of honey assist the immune system, including in animals with seasonal allergies. The health-promoting effects of honey are beneficial to a wide range of animals, including horses, dogs, other domestic animals and farm animals. A combination of honey and silver sulphadiazine has been employed in the treatment of burns in 15 adult dogs aged 4-5 years. Honey was applied to burn wounds in the dorsolateral region of the body. The reepithelialisation process was more pronounced in the dogs treated with honey, with the affected collagen fibres forming more regularly (Jalali et al., 2007). In cats, dogs, horses, and other animals, the administration of honey in conjunction with other therapeutic modalities has demonstrated efficacy in the management of laminitis, digestive problems, diarrhea, irritability, liver pathology (hepatopathy), arthritis, chronic cough, sinusitis, dermatological conditions, respiratory disorders, cardiac issues, hoof quality (hoof health), and renal dysfunction (Boukraâ 2023).

In one study, honey dressings were applied to canines with dermal wounds. The researchers reported that the healing of the wounds was rapid, that bacterial infections were successfully controlled, that necrotic wounds required less surgical intervention, and that honey was well tolerated by patients. In this study, the standard wound treatment applied to a 12-year-old Mechlin Shepherd dog with diffuse myiasis was unsuccessful in curing the disease. Subsequently, a honey compress treatment was initiated, and upon the animal's return for a follow-up examination five days later, it was observed that the superficial back wounds had almost completely healed, the deep wounds had diminished in size, and healthy granulation tissue had formed (Boukraâ 2023, Rooster and Declercq 2008).

The second case was a 9-year-old shar-pei referred for poor general condition, ulcerations on the tongue and a swelling on the medial side of the left heel marked by skin discolouration. The only treatment that was applied was honeydressing. By the seventh day, a clear granulation layer had formed at the wound site, and by the time that the sixth week had elapsed, the defect was almost completely healed with less scarring. (Boukraâ 2023, Rooster and Declercq 2008).

A nine-month-old boxer was burned on all four paws while playing with a plastic bottle containing a sodium hydroxide-based chemical in powder form. The dog developed exudative, malodorous necrosis, digital and interdigital skin loss, tendon exposure and large foot pad defects in all four paws. The wounds were treated with the application of gauze compresses impregnated with honey, which were then covered with a bandage. The bandages were changed on a daily basis. Initially, the animal showed pain for a short time after honey application, but after 7 days necrotic tissue and odour disappeared, and a clear granulation layer was formed at the wound sites on the paws (Boukraâ 2023, Rooster and Declercq 2008).

A five-year-old male Bernese mountain dog developed a parapreputial fistula following a celiotomy and prostate operation. Despite intravenous broad-spectrum antibiotic treatment, deep sampling and culture of the fistula was performed 5 days after the last surgical intervention and antibiotic-resistant Enterobacter cloacae was isolated. Topical treatment was initiated, given that the general condition was not impaired. Honey was applied to the fistula as deeply as possible with a 1mL syringe. During the first week, drainage from the fistula was significantly reduced. When the fistula was sampled and cultured again, E. cloacae did not grow. It took approximately 1 month for the fistula to disappear completely (Rooster and Declercq 2008).

Additionally, honey has been employed in the treatment of wounds in horses. The application of honey to leg wounds in horses has been

demonstrated to result in a reduction in wound size and an acceleration of the healing process. The healing time was found to be approximately 27% shorter (Bischofberger et al., 2011).

In one study, medicinal honey was used to treat an 8-year-old cat with complete skin loss over 100% of the limb circumference from elbow to claw and a contemporary ulnar fracture. In 49 days, 80% regression of the wound was observed. The wound fully healed with hair regrowth and minimal scarring. Furthermore, full functionality was restored to the affected limb (Figür 1) (Lukanc et al. 2020).

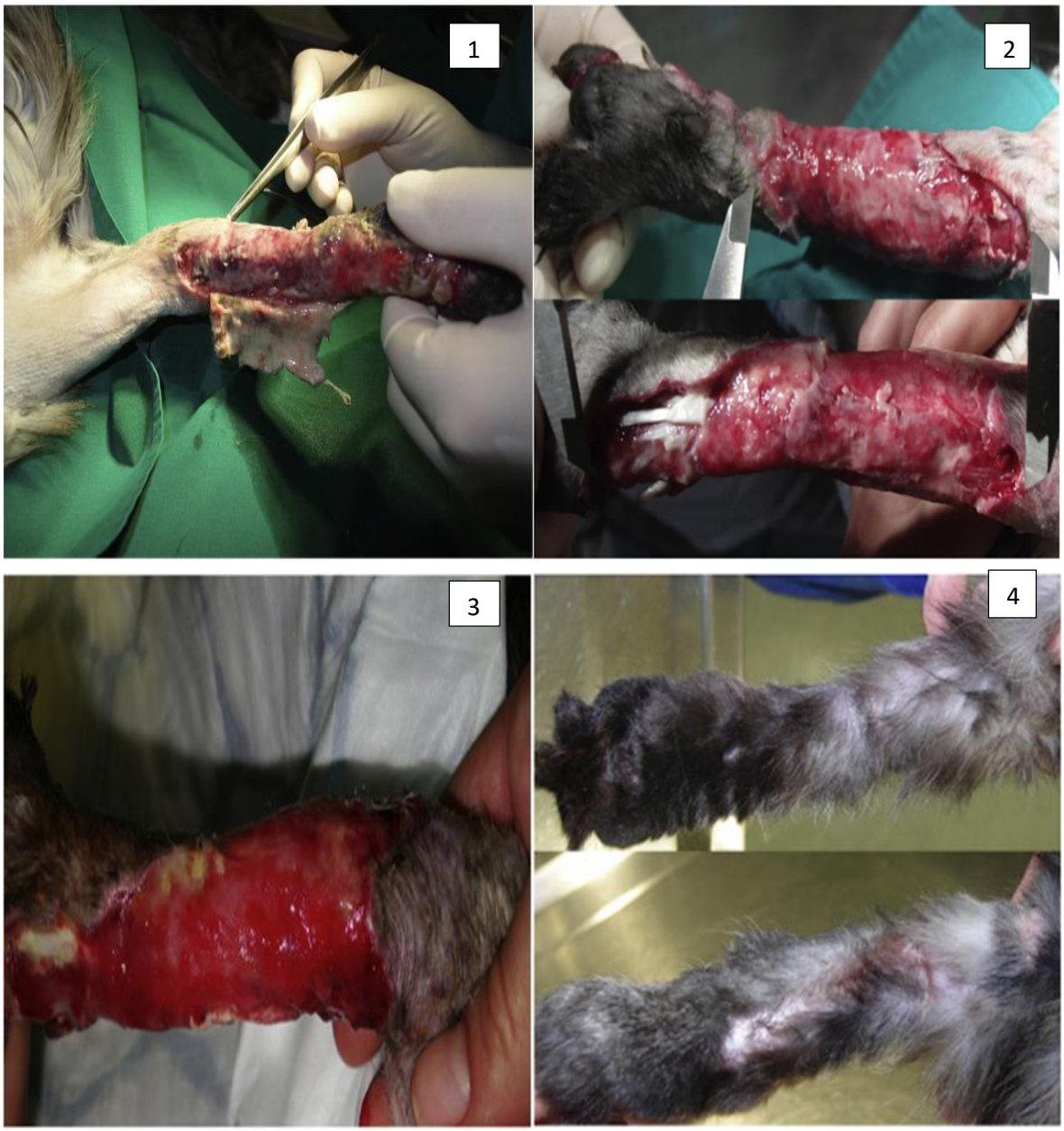

Figure 1. Wound healing after honey treatment application: **1.** Following the necrotomy, 2. On the seventh day following the application of honey, 3. On the fourteenth day following the application, 4. On the one hundred and fifth day (Original photo, V.Erjavec).

Honey has been used as a topical treatment for wounds in humans and animals for a considerable period of time due to its beneficial healing properties. In addition to its healing properties, honey has also demonstrated deodorising effects in malodorous wounds (Boukraâ 2023). The results of these studies demonstrate that honey is an effective and beneficial substance for promoting healing in wounds. Furthermore, it has been established that the treatment of such wounds with honey is more efficacious and cost-effective than other conventional ointments and wound dressings (Boukraâ 2023, Lukanc et al. 2020, Rooster and Declercq 2008).

Mastitis

Mastitis is defined as the inflammation of the mammary gland, which typically occurs in response to an intramammary bacterial infection. Mastitis represents a significant challenge globally, particularly within the context of dairy cow breeding. A study was conducted to assess the efficacy of honey in treating various bacterial strains associated with mastitis. The findings indicated that honey demonstrated sensitivity to these bacteria. Mastitis can be treated by administering honey via the udder duct to the infected udder. Honey does not damage the udder tissues and does not leave any unwanted residues in the milk. The administration of honey to animals with mastitis, even when it exhibits comparable efficacy to antibiotics, represents a viable and innocuous alternative to antibiotic treatment (Molan 2002).

The in vitro activity of different honeys against some mastitis-causing bacterial strains was investigated by Minimum Inhibitory Concentration (MIC) method and found to be effective. (Table 1).

Table 1. Efficacy on some strains of bacteria causing mastitis of natural and artificial honey

Bacteria species	Manuka Honey (%)	Rewarewa Honey (%)	Artificial Honey (%)
Actinomyces pyogenes	1-5	1-5	5-10
Klebsiella pneumoniae	5–10	5–10	>10
Nocardia asteroides	1–5	5–10	>10
Staphylococcus aureus	1–5	1–5	>10
Streptococcus agalactiae	1–5	5–10	>10
Streptococcus dysgalactiae	1–5	5–10	>10
Streptococcus uberis	1–5	5–10	>10

Source: Molan, P.C. 2002

Honey can be used for many purposes in veterinary medicine. The objective of the study was to assess the combined and individual effects of grayanotoxin-rich Turkish mad honey and 5-fluorouracil (5-FU) on colon cancer modelling in rats using N-methyl-N-nitrosourea (MNU) as a carcinogen. The findings indicated that mad honey and 5-FU reduced anaplastic cell growth and oxidative stress by inhibiting antiapoptotic activity. Additionally, the histopathological examination of the liver and kidney revealed no evidence of toxicity associated with the metabolism of mad honey and 5-FU. Consequently, it can be postulated that the concurrent administration of these two agents may represent a promising approach for the management of colon cancer (Kurtdede, et al. 2023).

Pollen

Pollen is a substance collected by bees from flowers with nectar, and it is a bioactive structure formed by flowering plants for the purpose of reproduction. It is notable for its high protein content. It has been demonstrated to possess anti-inflammatory, antioxidant and immunostimulatory properties. While it has the potential to be an effective treatment for allergic diseases, there is also a risk of an allergic reaction due to its protein density (Mărgăoan et al., 2019).

Pollen has antibacterial, antifungal, antioxidant and anticancer properties. This antimicrobial property of pollen is due to phenolic compounds such as quersetin, myrcetin, campferol (Onbaşlı 2019).

Bee pollen is frequently administered to animals for the purposes of bolstering immunity, enhancing digestive health, providing energy, and as an antibiotic. Furthermore, it is frequently employed as a means of mitigating the effects of pollen allergies.

Bee pollen is typically available in tablet or granule form. Pollen has been demonstrated to stimulate ovarian function. It has been determined that laying increased in hens whose ration was supplemented with 2% pollen and 5% animal proteins. Concurrent with the observed increase in egg-laying behaviour, there was also a notable enhancement in the resilience of eggs to the rigours of incubation. (Boukraâ 2023).

The sperm quality, fertility and blood profiles of male rabbits that were administered bee pollen demonstrated a notable enhancement. Furthermore, the offspring of rabbits that were administered bee pollen exhibited increased body weights and superior survival rates. The administration of bee pollen has been demonstrated to enhance reproductive performance in animals, as evidenced by increased rates of both birth and live offspring (Boukraâ 2023).

Bee pollen fed chickens had better development of duodenum, ileum and jejunum villi. The results of this study indicate that bee pollen may play a role in the early development of the digestive system (Boukraâ 2023).

There is some evidence to suggest that the ingestion of pollen may offer protection against the adverse effects of X-ray radiation treatments for both animals and humans (Schmidt and Buchmann 1992). The addition of pollen to the diet has been demonstrated to enhance the health, growth and utilisation of nutrients in domestic animals (Crane 1990). The addition of 2.5% pollen to the diets of chickens and piglets was observed to enhance their capacity to utilise feed (Ettle et al., 2006, Rodrigues et al., 2018). Furthermore, beekeepers provide their colonies with pure pollen, pollen supplements, or pollen substitutes when the availability of natural pollen sources is limited (Boukraâ 2023).

In a study examining the impact of pollen on immunity, it was reported that the addition of 1.5% pollen to the diet of broiler chickens over a 21-day period resulted in an increase in IgM levels (De Oliveira et al., 2013). It has been documented that the addition of pollen to poultry diets has been observed to have a stress-reducing effect and to positively influence animal performance (Seven et al., 2010).

Propolis

Trees secrete a resinous substance, which is collected by bees. Following a series of processing steps, the substance is transformed into propolis, a substance known to have beneficial properties. Bees utilise propolis to protect the colony against microorganisms by neutralising and coating the hive or creatures entering the hive from outside. The substance exhibits antibacterial, antifungal and anti-inflammatory properties (Prabhakar et al., 2016). It has been demonstrated that propolis is effective in the eradication of *Penibacillus larvae*, which are the causative agents of American foulbrood. (Sonmez et al., 2023, Toutiaee et al., 2023). The use of this substance has been documented for thousands of years, with a focus on its beneficial effects on human and animal health. Its natural antibacterial properties have been identified as a key factor in this regard. The benefits of bee propolis are not limited to humans; many animals, including pets and farm animals, can also derive advantages from it. The substance is frequently employed in the treatment of immune system and respiratory disorders, as well as more particular diseases, due to its antibacterial properties. The utilisation of bee propolis in the form of a natural supplement has been demonstrated in animal and veterinary studies to constitute an effective methodology for the prevention of cancer, inflammation and tumour formation in a variety of animal species. It has been demonstrated that this substance inhibits T cell production, which is responsible for the inflammatory processes observed in numerous pathological conditions. Propolis is a natural medicine that is safe for use in animals. No adverse effects apart from allergic reactions have been documented. Bee propolis is available in tablet form and can also be incorporated into toothpastes and skin care lotions (Abu-Seida 2023, Almuhayawi 2020, Kasote et al., 2022). Additionally, propolis has been demonstrated to enhance the immune system of animals. The administration of propolis to the diet of laying hens resulted in an increase in the production of immunoglobulin G (IgG) and immunoglobulin M (IgM). This consequently led to an enhancement of the immune system (Cetin et al., 2010).

The effects of propolis treatment on weight gain, growth rate, reproduction and meat quality were investigated (Bogdanov 2011.).

The utilisation of water-extracted propolis (WPP) as a treatment for Cushing's syndrome in canines represents a promising avenue of investigation, particularly given that there is currently no known cure for this syndrome. Moreover, the cost of treatment is considerable, and the efficacy of

available therapies is often limited. In certain instances, it has been associated with significant adverse effects, including mortality. The administration of propolis was observed to result in the remission of Cushing's syndrome after a period of three months (Glenn 2006).

It has been demonstrated that the incorporation of 500 ppm propolis into the diet of broiler chickens can lead to a notable increase in body weight, with a potential gain of up to 20% (Ghisalberti 1979).

The incorporation of 30 ppm propolis into the dietary regimen of laying hens led to a 5-6% enhancement in egg production, feed conversion efficiency, and chicken weight (Bonomi et al.,1976).

Staphylococcus aureus, which is an important pathogen for human and animal health, is an etiological factor causing mastitis in dairy cattle farms. It is very difficult to destroy *S. aureus* with antibiotics. In a study investigating the effect of propolis on mastitis, it was reported that propolis extract may be effective against the *S. aureus* species causing mastitis. However, milk components may reduce the effects of propolis. (Santana et al., 2012).

In order to investigate the effects of propolis on newborn calf diarrhea, a solution of propolis prepared with 2 cc of 96% ethyl alcohol was administered to the calves. Neonatal diarrhoea was not observed in calves at the end of one month (Tolon et al., 2002).

In a study, the antiparasitic effect of propolis in sheep was investigated. In this study, sheep naturally infected with *Trichuris* spp., *Trichostrongylus* spp. and *Ascaris* spp. were treated with a solution of 33% propolis extract. The total number of eggs in faeces was found to have decreased by 59.7% in the group of animals that had been treated with propolis, in comparison to the control group, which exhibited an increase of 63.6% in the same parameter (Morsy vd., 2013).

A study conducted by Aguiar et al. (Aguiar vd., 2014) to determine the effects of propolis on milk yield and quality showed that cows fed with propolis supplementation had higher protein content in milk and also had higher milk yield. Propolis is a rich source of flavonoids. In a study, an ethanol extract of propolis was administered at a dosage of 150 mg/kg in the form of a diet supplement. The results demonstrated that this treatment led to an increase in sexual activity, sperm concentration, sperm quality, and testosterone concentration (Hashem et al., 2013).

Dogs afflicted with a superficial dermal infection, attributable to pathogenic dermatophytes of the genera *Epidermophyton*, *Trichophyto*n and *Microsporum*, were subjected to a therapeutic regimen comprising weekly baths with a commercial soap formulated with propolis, along with a daily topical ointment for a period of three to eight weeks. Following a two-week course of treatment, all microbial cultures yielded negative results, and all three dogs were found to be free of lesions at the conclusion of the treatment period (Sánchez et al., 2014).

It has been used in dogs and cats with blepharitis, keratoconjunctivitis sicca, infectious conjunctivitis, corneal ulcers, corneal edema, tear duct obstruction, and glaucoma. A study was conducted to investigate the efficacy of propolis in the treatment of eye diseases in 25 dogs and 5 cats. A comparison of the recovery time for animals treated with allopathic eye washes and those treated with propolis revealed a notable difference. Those treated with propolis demonstrated a recovery time of five to seven days for acute cases and 10 to 15 days for chronic cases. It can be reasonably proposed, therefore, that propolis may prove an efficacious therapeutic agent for ophthalmic disorders without the attendant risks and at a relatively modest cost (Giral et al., 2007).

Propolis application was performed in periodontal diseases which are frequently seen in dogs, cause recurrent gingivitis and periodontitis and can cause tooth loss if left untreated. t was established that the utilisation of ethanol extracts of propolis in the treatment of dental caries in canines has the capacity to diminish inflammatory processes, facilitate the reorganization of tissue at the surface level and reduce bacterial activity. It is hypothesised that propolis may be utilised as an oral antiseptic without the potential for adverse effects (Ahuja and Ahuja 2011, Ilewicz vd., 1979).

Information on different clinical events and effects of propolis in dogs are shown in Table 2.

Notably, propolis, in its aqueous or ethanolic extract form, has been demonstrated to be an effective adjuvant in veterinary vaccines. Its efficacy in this role has been shown to be superior to that of conventional vaccine adjuvants (Ma et al., 2011). Additionally, propolis has been incorporated into canine parvovirus and canine coronavirus vaccines

(Das Neves Ferreira et al., 2012), as well as a vaccine against porcine parvovirus (Ma et al., 2011) and a vaccine against the bacterium *Aeromonas salmonicida*, the causative agent of furunculosis in fish. (Liu et al., 2020) resulted in a notable increase in antibody production.

Table 2. Areas and modes of action of propolis in dogs*

Therapeutic effect	Disease or microorganism
Antimicotic	**Canine otitis** *Malassezia pachydermatis* *Candida spp.* **Dermatophytosis** *Microsporum canis* *Microsporum gypseum*
Antineoplasic	Transmissible venereal tumor Osteosarcoma Cushing's syndrome
Bactericide	*Staphylococcus aureus* *Pseudomonas* ssp. *Proteus* ssp. *Escherichia coli*
Immunostimulant	Canine distemper Canine parvovirus
Oftalmic Blefaritis	Conjuntivitis Keratoconjuntivitis
Peridontal Clinical	Gingivitis Peridontitis
Metabolic	Liver diseases
Antiparasitic	Giardiais Trypanosomiasis

*(Betancourt et al. (2015)

Different forms of propolis have been used in various problems in animals and found to be effective. The efficacy of propolis has been demonstrated in the treatment of mastitis, gynaecological disorders, gastrointestinal and respiratory system problems, diarrhea in malnourished calves, wound management, local anaesthetics in surgical procedures, paratyphoid in ducks, foot-and-mouth disease and enzootic pneumonia in pigs. Additionally, propolis has been shown to stimulate growth in animals, with favourable outcomes (Bogdanov 2011).

Royal Jelly

A secretion of worker bees, royal jelly is a 'superfood' as it is only given to the queen bee. It has been used for hundreds of years to promote health and is known to contain a wide range of vitamins and amino acids. The impact of royal jelly on ewe pregnancy and lambing rates has been the subject of scientific investigation and has yielded positive results (Kridli et al., 2006). It provides vitality and therefore health, energy and immunity in all tissues of the body by influencing cell regeneration, production and metabolism (Aydın and Tekeoğlu 2018). It has been found that royal jelly can reduce cholesterol levels in the blood plasma, triglyceride levels and cholesterol deposits in the arteries of rabbits (Nakajin et al., 1982). Royal jelly has been reported to inhibit tumour growth in mice following prophylactic and therapeutic oral administration (Tamura et al., 1987). Furthermore, research has shown that royal jelly can reduce the healing time of skin lesions, exert an anti-inflammatory effect and accelerate wound healing. In rats, these effects have been evidenced by the acceleration of wound healing and the reduction of inflammatory responses (Fujii et al., 1990).

A study investigating the effect of royal jelly on the yield characteristics of laying hens revealed that the administration of lyophilised royal jelly at doses of 10 and 15 ppm led to a significant increase in egg production (10.5%–11%), feed consumption (21%–22%), egg weight (5%–4.8%), live weight gain (7%–6.5%), and yolk pigmentation (9.5%–9.7%) compared to the control group (Bonomi et al., 2000).

The combination of royal jelly and honey demonstrated a synergistic effect against pathogenic bacteria, indicating a potential use as an antimicrobial agent (Boukraa 2008).

In an experimental study, royal jelly was administered to mice with perforated eardrums for therapeutic purposes. Following the administration of royal jelly, it was observed that the eardrum tissue fibres and connective tissues of the mice exhibited a greater degree of fusion than in the control group. It was thus concluded that royal jelly application resulted in the successful healing of eardrum rupture (Calli et al., 2008).

A study examining the effects of royal jelly on reproduction reported that it increased spermatozoa density and spermatozoa motility, decreased the rate of abnormal spermatozoa, and positively affected semen quality in mice (Temamoğulları et al., 2006). It has been determined that royal jelly has positive effects on oestrus and fertility in sheep (Atabay 2012).

Royal jelly can help animals live a longer and healthier life. It has been demonstrated that royal jelly can mitigate the effects of cellular stress, which is a contributing factor in the development of age-related illnesses and diseases. Especially congenital joint, arthritis, osteoarthritis, disorders obesity or old age problems in pet animals can be reduced by giving royal jelly. Royal jelly early free radical

protection can slow down the development and reduce the severity of the disease. It should be noted that royal jelly may cause allergic and adverse reactions in some individuals. It is therefore advised that royal jelly should not be administered to pregnant animals or nursing mothers. The prevalence of allergic reactions in animals that consume royal jelly remains inconclusive. Nevertheless, it has been hypothesised that the likelihood of developing an allergy to royal jelly is increased in animals with a history of allergic disease (Boukraâ 2023).

It should be noted, however, that royal jelly has a limited shelf life. The process of refrigeration and freezing causes chemical changes that delay and reduce the favourable effects of the substance in question. It is recommended that royal jelly be cooled to a temperature between 0°C and 5°C. However, the optimal storage temperature is below -17°C. The average recommended storage period for products produced after this date is 18 months in the refrigerator. The recommended storage period for products stored at -17°C is 24 months, as this is the temperature that offers optimal stability. Freeze-dried royal jelly and royal jelly-based products can typically be stored at room temperature, with the potential for prolonged storage periods, sometimes exceeding several years (Boukraâ 2023).

Bee Venom

Bee venom is a secretion produced by the abdominal glands of honey bees. It contains a variety of bioactive peptides, including melitin, apamin and adolapin, as well as other components such as histamine, noradrenalin and dopamine. Additionally, it comprises enzymes (Aydın and Tekeoğlu 2018).

A range of biochemically and pharmacologically active substances have been identified in bee venom. These include polypeptides, including melittin, apamin, and mast cell degranulating peptides; amines such as histamine, serotonin, dopamine, and norepinephrine; and enzymes such as phospholipase, hyaluronidase, and histidine decarboxylase (Khalil et al. 2021).

Bee venom has been demonstrated to possess a number of beneficial biological activities. Of particular note is its efficacy in modulating the immune system. For a considerable period, this substance has been utilised in alternative medicinal practices with the aim of alleviating a range of pathological conditions, including those associated with discomfort and inflammatory responses (Jung et al., 2013).

A combination of honey and bee venom has demonstrated efficacy in alleviating arthritis pain in a range of animal species, including dogs, cats and thoroughbred racehorses. The treatment has been demonstrated to be efficacious in the management of joint and muscle diseases, as well as functional muscle disorders. Bee venom has been demonstrated to possess potent analgesic and anti-inflammatory properties. When administered on a regular basis, it has been demonstrated to reduce joint discomfort over time, while also maintaining and restoring joint mobility (Boukraâ 2023).

A study investigating the efficacy of bee venom in alleviating hip dysplasia in canines with arthritis revealed that cage activity in the treated group exhibited a marked increase, reaching up to 70% compared to the control group. Nevertheless, it should be noted that the use of this treatment is contraindicated in animals with a known allergy to bees (Vick, and Brooks 1972).

It has been reported that the application of cream containing 0.06% bee venom in corneal injury in dogs has high biostimulative, antiseptic and anti-inflammatory effects and provides faster and better quality healing compared to those in the control group (Krylov and Bardahchieva 1997).

The effect of bee venom supplementation on the performance, antioxidant activity and liver functions of broiler chicks was investigated in a scientific study. The findings indicated a notable increase in live weight gain by the 28th day. A notable increase in feed consumption was observed (Han et al., 2010).

The objective of the study was to assess the efficacy of bee venom in the treatment of mastitis. A total of 15 cows with confirmed mastitis were selected for participation in the study and were randomly divided into four groups. Each group received a different dose of bee venom administered subcutaneously: group 1 received 3 mg, group 2 received 6 mg, group 3 received 12 mg, and group 4 received 24 mg of the venom. The results of the analysis of milk samples collected three and six days after administration demonstrated a reduction in the levels of pathogens across all administered doses (Rahimjanova et al., 2022).

Perga (Bee Bread)

The main ingredient of perga, known as bee bread, is pollen. The worker bees mix the pollen with

enzymes they secrete and use it in the nutrition of the young and the queen. The distinction between pollen and perga is that the latter is fermented with bee enzymes. Perga is three times more nutritionally dense than pollen (Mărgăoan et al. 2019). It contains a higher concentration of free amino acids and readily assimilated sugars. Consequently, it has a high digestibility. It has been demonstrated to exert a beneficial influence on reproductive hormones. Thus, it is used to increase reproductive ability and to increase muscle strength and volume. It is employed in the management of blood pressure and chronic constipation as a result of the acetylcholine it contains (Parlakpınar and Polat 2021).

The bactericidal and bacteriostatic properties of bee bread are noteworthy characteristics. This study was conducted with the aim of evaluating the antimicrobial activity of bee bread extracts at varying concentrations against a range of bacterial strains. The bacterial strains tested were *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Salmonella enterica*. The results of the study indicate that the initial two dilutions of bee bread extract (33% and 16.66%, respectively) demonstrated heightened antimicrobial activity, while the remaining dilutions exhibited diminished yet discernible activity contingent on the pathogen under examination. The highest antimicrobial efficacy was observed in the case of the *S. aureus* strain, with all dilutions demonstrating an inhibitory effect at both time points, 8 and 12 hours. (Urcan et al., 2018).

Apilarnil

It is a bee product called drone brood homogenate. The product is obtained by collecting and homogenising larvae of the drone larvae, which are approximately seven to eight days old and have not yet completed their development (Parlakpınar and Polat 2021). Apilarnil can be used in animal nutrition, animal health protection and treatment by taking advantage of its androgenic, anabolic, hepatoprotective, renoprotective, neuroprotective, hypolipidemic, anti-anemic, anti-leukaemic, antiatherosclerotic, urethrotropic, biological stimulant, immunomodulatory, energising and cell regeneration stimulating effects thanks to its high protein, vitamin and hormone levels (Sawczuk et al., 2022, Sidor and D˙zugan 2020).

In a study, it was determined that apilarnil had no effect on growth performance in broiler chickens. However, it was observed to stabilise blood glucose and cholesterol levels and to induce premature sexual maturity in male broiler chickens (Altan et al., 2013). Apilarnil is a highly androgenic compound that exerts a dual influence on the body, both androgenic and anabolic (Altan et al., 2013, Erdem and Özkök 2018).

Apilarnil has been demonstrated to increase blood testosterone levels, promote the development of secondary sexual characteristics, enhance the weight of sexual organs, improve sperm quality and quantity, and alleviate reproductive difficulties when administered to a range of animals, including rats, pigs, rams, and poultry (Bolatovna et al., 2015, Shoinbayeva 2017, Yucel et al., 2011).

It can have a direct effect on the quantity and quality of the sperm, allowing the male breeding animals to be used for a longer period. Apilarnil has been demonstrated to stimulate spermatogenesis in males due to its androgenic properties (Altan et al., 2013, Bolatovna et al., 2015). It has been found to increase the amount of sperm, improve sperm quality and quantity and relieve reproductive difficulties. As a result, Apilarnil has both an androgenic and an anabolic effect and can be used as a natural alternative to drugs and chemicals for the stimulation of growth and sexual development. Additionally, apilarnil can be employed as a natural substance due to its anabolic effects, which have been demonstrated to positively influence growth rate, feed conversion, and meat quality in animals (Yucel et al., 2011). The administration of Apilarnil to young pigs resulted in a 20.1-21.9% increase in seminal gland weight and a 21.8-25.8% increase in epididymis weight. Additionally, sexual dysfunction parameters in pigs exhibited an 83.3% improvement. Additionally, there was a 54.3% increase in ejaculate volume, a 27.1% increase in germ cell density, a 51.2% increase in survival rate, and a 14.2% increase in mobility. The percentage of damaged spermatozoa acrosomes decreased by 2.1%, while fertility increased by 76.4% (Bolatovna et al.,2015).

The study on correcting endocrine and metabolic conditions in dogs using apilarnil showed that administering apilarnil to dogs at a daily dose of 15 mg/kg for two months resulted in a significant increase in blood levels of thyroxine, testosterone, erythrocytes, haemoglobin, total protein, globulins and leucocytes (Efanova et al., 2019).

The beneficial effects of bee products on animals have been established through clinical studies, as evidenced in Table 3.

Table 3. A list of bee products whose positive effects have been demonstrated in animal studies*

Bee Product	Animals studied in vivo	Effects
Honey	Horse	Wound healing
	Cat	Wound healing
	Dog	Wound healing
	Mouse	Wound healing
	Rat	Gastroprotective effect Gastric ulcer treatment Hypoglycaemic and antioxidant effects Pancreas protection Cardioprotective effect Antidiabetic effect Anti-atherogenic effect
Propolis	Mouse	Antifungal activity in vulvovaginal candidiasis Antiparasitic (anti-malarial activity) against *Plasmodium chabaudi* Antiparasitic (anti-malarial) action against *Plasmodium falciparum* and *P. berghei* Antiparasitic effect against *Schistosoma mansoni* Anti-carcinogenic potential Reduction of oxidative stress to reduce hepatotoxicity and nephrotoxicity
	Honey bee	Antiparasitic effect against *Nosema ceranae*
	Rat	Antiparasitic effect against *Trypanosoma brucei* and *T. congolense* Wound healing Anti-carcinogenic potential Gastro-protective properties due to antioxidant and anti-*Helicobacter pylori* activity Protective role in the early stages of tongue cancer development Antimicrobial activity against mutans streptococci Prevention of tooth decay Effects of anti-caries Effects on chemoprevention and gastroprotection Healing of corneal wounds
	Dog	Wound healing Storage medium (in the case of tooth replacement) Antimicrobial effects for otitis externa
	Pig	Healing of burn wounds
	Chicken	Protective effect on kidneys and liver
Bee Venom	Dog	Anti-inflammatory and analgesic effects Healing effect on otitis externa caused by *Malassezia* Healing effect for facial paralysis
	Mouse	A neuroprotective effect in Parkinson's disease Neuroprotection in multiple sclerosis Antiviral activity against a wide range of viruses Neuro-protective effects
	Rabbit	Positive effects on the reproductive performance and on the immune response of the male animal Improving reproductive characteristics (sexual stimulant), immune response and health
	Chicken	Promoters of growth Effects as an immunoprophylactic
	Pig	Stimulation of antibody production and viral clearance in cases of infection with the PRRS virus Positive effects on growth, survival and the immune system

	Rat	Gastro-protective effect Anti-diabetic activity Analgesic activity Anti-bacterial activity against *Staphylococcus aureus*
Pollen	Rat	Anti-inflammatory and protective effects for treatment of prostatitis Healing effects in cases of prostatic hyperplasia and inflammation Protective role in diabetes-related problems with blood glucose control and sexual dysfunction in men
	Chicken	Positive effect on colonisation of intestinal microflora Promoters of growth Improvement of the growth performance and the immune system Improvement in weight gain and the rate at which food is digested Positive effect on daily gain, feed conversion and gut microbial composition
Royal Jelly	Rat	Liver and kidney protection during chemotherapy Nephro-protective effect Anti-fibrotic effect against pulmonary fibrosis Stimulation of the folliculogenesis and the secretion of the steroid hormones Improvement of bone strength after ovariectomy Prevention of osteoporosis after ovariectomy Improving male fertility and reproductive success
	Mouse	Immunomodulatory, antioxidant and anticancer effects Antioxidant, anti-inflammatory, apoptotic and anticancer effects Anti-aging effect Improving male fertility and reproductive success
	Rabbit	Relieving neurological problems after ovariectomy Improving male fertility and reproductive success
Apilarnil	Sheep	Reproductive stimulation in rams
	Rat	Androgenic effect on castrated male animals Hepatoprotective effects Neuroprotective effect
	Broiler	Androgenic effects, Reduced levels of blood glucose and cholesterol
	Pig	İncrease in the production parameters of the females Anabolic effect on females Improves fertility; stimulates reproductive function (by reducing time to first estrous cycle for insemination)

*Source: Stevanović J. et al. (2024)

The effects of a bee product on animals may exhibit variability. Following the administration of bee products, the time required for animals to demonstrate a favourable response to the product may vary. In selecting bee products for application, it is advisable to opt for those that are pure and natural to the greatest extent possible. In the event that a treatment is to be applied to the animal, the advice of a qualified veterinarian should be sought prior to the use of the bee product. While bee products are typically regarded as safe, the precise long- or short-term effects may vary considerably between individuals or species. It is possible that some individuals and animals may exhibit an allergic response to bee products, particularly those who have previously experienced an allergic reaction to bee stings. It is safe to introduce bee products into the diet of animals gradually, commencing with minimal quantities.

It should be emphasised that in veterinary practice apitherapy can only be administered after examination by a licensed veterinarian. Apitherapy has great potential for use in veterinary medicine. Officially, however, it can only be used as a complementary treatment. A growing body of evidence highlights the value of apitherapy in promoting animal health and vitality. As a result, there is increasing interest among pet owners and veterinarians in the use of bee products. However, they need to be aware of the potential risks

associated with apitherapy, including potentially fatal anaphylactic reactions in certain cases, However, further preclinical and clinical studies are needed to fully understand the basic mechanisms of action of bee products and to determine optimal doses and methods of application in animals.

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