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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

INVESTIGATION OF CHEMICAL CONTENT AND ANTIMICROBIAL ACTIVITIES OF DIFFERENT PLANT SOURCES OF ANATOLIAN PROPOLIS SAMPLES

Farklı Bitki Kaynaklı Anadolu Propolis Örneklerinin Kimyasal İçeriği ve Antimikrobial Aktivitelerinin Araştırılması

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ABSTRACT

The ethnopharmacological approach combined with chemical and biological methods can be a useful model in the field of pharmacology. One of these approaches, apitherapy, is the use of bee and hive products for therapeutic purposes. Propolis is among the best known of these bee products. The chemical composition of propolis varies according to the local or endemic flora, bee species, geographical origin and season. This study is to determine the antimicrobial activity differences between chestnut and polyfloral origin propolis against various pathogenic bacterial species. First of all, the Liquid Chromatography-Mass Spectrometry (LC-MS/MS) method was used for the determination of bioactive components known to be responsible for antimicrobial activity. Folin-Ciocalteu method and colorimetric aluminum chloride assay were used to determine the total phenolic (TP) and flavonoid (TF) amounts. 19 different pathogenic microorganisms were selected to test the antimicrobial activity levels of propolis samples with agar well diffusion and minimum inhibitory concentration (MIC) methods. TP and TF values of chestnut propolis (71.06 mg GAE/mL-11.75 mg QE/mL) were significantly higher than polyfloral sample (36.84 mg GAE/mL-7.04 mg QE/mL). Chrysin, a flavone derivative, was the most abundant compound in both samples. The MIC values of chestnut propolis ranged from 19.5 to 2500 µg/mL, while the MIC value of polyfloral origin propolis was between 39.06 and 5000 µg/mL. The most susceptible strain was *Mycobacterium smegmatis* for both samples with different concentration. Notably, it was observed that the botanical origins affect the chemical composition of propolis, and this situation can also be effect antibacterial and antifungal activity in respective propolis because of the different amount and diversity of bioactive compounds. Consequently, chestnut propolis is a promising candidate for drug discovery that can be used to treat some infectious diseases, including diseases related with resistant bacteria.

Keywords: Chestnut propolis, total phenolic, flavonoid, phenolic composition, antimicrobial and antifungal activity

ÖZ

Kimyasal ve biyolojik yöntemlerin entegre çalışılması ile oluşturulan etnofarmakolojik yaklaşım, farmakoloji alanında faydalı bir model olabilir. Bu yaklaşımlardan biri olan apiterapi, arı ve kovan ürünlerinin tedavi amaçlı kullanılmasıdır. Bu arıcılık ürünleri içinde propolis, en iyi bilinenler arasındadır. Propolisin kimyasal bileşiminin yerel veya endemik floraya, arı ırkına, coğrafi kökene ve mevsime göre değiştiği bilinmektedir. Bu bilgiler doğrultusunda çalışma, kestane ve polifloral orijinli propolis örneklerinin farklı patojenik mikroorganizma suşlarına karşı antimikrobiyal aktivite

farklılıklarını belirlemek amacıyla yapılmıştır. Antimikrobiyal aktiviteden sorumlu olduğu bilinen biyoaktif bileşenlerin tayini için öncelikle Sıvı Kromatografi-Kütle Spektrometresi (LC-MS/MS) yöntemi kullanıldı. Toplam fenolik (TP) ve flavonoid (TF) miktarlarını belirlemek için Folin-Ciocalteau yöntemi ve kolorimetrik alüminyum klorür testleri kullanıldı. Propolis örneklerinin antimikrobiyal aktivite düzeyleri seçilen 19 farklı patojenik mikroorganizmaya karşı agar kuyu difüzyonu ve minimum inhibitör konsantrasyon (MIC) yöntemleri ile belirlendi. Kestane propolisinin TP ve TF değerleri (71.06 mg GAE/mL-11.75 mg QE/mL), polifloral örnekle (36.84 mg GAE/mL-7.04 mg QE/mL) kıyaslandığında anlamlı olarak yüksek bulunmuştur. Bir flavon türevi olan Chrysin, her iki örnekte de en yüksek oranda bulunan bileşik olarak tespit edildi. Kestane propolisinin MİK değerleri 19,5 ile 2500 µg/mL arasında değişirken, polifloral orijinli propolisin MİK değeri 39,06 ile 5000 µg/mL arasında belirlendi. Her iki örneğe karşı farklı konsantrasyonlarda en duyarlı suş *Mycobacterium smegmatis*'di. Bu çalışma ile botanik orijinlerin propolisin kimyasal bileşimini etkilediği ve bu durumun biyoaktif bileşiklerin farklı miktar ve çeşitliliğinden dolayı ilgili propoliste antibakteriyel ve antifungal aktiviteyi de etkileyebileceği doğrulandı. Sonuç olarak, kestane propolisi, dirençli bakteriler de dahil olmak üzere bazı bulaşıcı hastalıkları tedavi etmek amacıyla kullanılabilecek ilaç geliştirme çalışmaları için umut vaad edici bir aday olarak kullanılabileceği önerilmektedir.

Anahtar Kelimeler: Kestane propolisi, toplam fenolik madde, flavonoid, fenolik kompozisyon, antimikrobiyal ve antifungal aktivite

GENİŞLETİLMİŞ ÖZET

Amaç: Bu çalışmanın amacı farklı orjinli propolis örneklerinin biyoaktif çeşitliliğini ve miktarını belirleyerek, seçilen farklı patojen mikroorganizmalara karşı antimikrobiyal aktivite düzeylerini karşılaştırmaktır.

Giriş: Dünya genelinde artan antibiyotik direnci sebebiyle insanlar sentetik ürünler yerine doğal ürünlere yönelmektedir. Doğalürünler, tarih boyunca geleneksel tıpta kullanılmış ve potansiyel bir yeni ilaç kaynağı olmuştur. Propolis, eski Mısırlılar ve Yunanlılar zamanından beri bilinen ve bazı hastalıkların tedavisinde kullanılanantimikrobiyal ajan örneğidir. Propolisin antimikrobiyal aktivitesi, farklı araştırmacılar tarafından kapsamlı bir şekilde incelenmiş; Gram pozitif veya Gram negatif bakterilerin yanı sıra mayalar ve küfler gibi çok çeşitli mikroorganizmaların büyümesini inhibe veya kontrol edebildiği bildirilmiştir. Propolis, polifenol (flavonoidler, fenolik asitler ve esterler), fenolik aldehitler ve ketonlar gibi 300'den fazla farklı bileşenden oluşur. Polifenoller ve terpenoidler de en aktif grup olarak kabul edilir. Bu biyoaktif bileşiklerin sayısı ve konsantrasyonu bal arısının yaşadığı coğrafyaya, mevsime, arı ırkına ve kovanının belirli bitki kaynaklarına yakınlığına bağlı olarak değişkenlik gösterir.

Gereç ve Yöntem: Bu çalışmada etkinliği araştırılan propolis örnekleri Düzce Üniversitesi Arıcılık Uygulama ve Araştırma Merkezi'nden (DAGEM) temin edildi. Örnekler Haziran ve Temmuz aylarında propolis tuzakları kullanılarak kovanlardan toplandı. Labaratuvara getirilen ham propolis örnekleri (Kestane ve polifloral orjinli) öğütüldükten sonra etanolik ekstraksiyon metoduna tabi tutuldu. Kullanıma hazır hale gelen örneklerin toplam fenolik (TP) miktarları Folin-Ciocalteau yöntemi ile toplam flavonoid (TF) miktarları ise kolorimetrik alüminyum klorür testi ile tespit edildi. Propolis örneklerinin biyoaktif bileşenlerinin tespiti için Sıvı Kromatografi-Kütle Spektrometresi (LC-MS/MS) yöntemi kullanıldı. Seçilen 19 farklı patojene karşı örneklerin antimikrobiyal aktivite düzeylerini belirlemek için ilk basamakta agar kuyucuk, ardından minimal inhibisyon konsantrasyonu (MİK) deneyleri yapıldı.

Bulgular: Araştırmalar sonucunda kestane propolisinin polifloral örneğe göre daha yüksek oranda antimikrobiyal aktivite sergilediği tespit edilmiştir. Her iki örneğe karşı da farklı konsantrasyonlarda en duyarlı suş *Mycobacterium smegmatis* olarak belirlenmiştir. Bu yüksek etkinliğin de içeriğindeki biyoaktif bileşenlerin farklılığından kaynaklandığı düşünülmektedir. Kestane propolisinin toplam fenolik ve flavonoid miktarı polifloral örneğe göre anlamlı düzeyde farklılık göstermiştir. Her iki propolis örneğinde de en yüksek oranda tespit edilen bileşik bir flavon türevi olan Chrysin'dir. Kestane propolisinde hesperidin ve protokatekuik asit saptanmazken, polifloral orijinli propoliste bu bileşenler tespit edilmiştir. (±)-Kateşin, siringik asit, (-)-epikateşin ve rutin polifloral kökenli propolis bileşenlerinde tespit edilemezken, bu biyoaktif maddelerin konsantrasyonları kestane

propolisinde kayda değer düzeyde tespit edilmiştir. Sadece bir flavonoid türevi olan daidzein her iki numunede de bulunamamıştır.

Sonuç: Bu çalışmanın sonuçları propolis içeriğinin orjinlendiği bitki kaynaklarına göre değiştiği bilgisini doğrulamaktadır. Kestane propolisinin seçilen patojenlere karşı çok düşük dozlarda etkili olması, bulaşıcı hastalıkların önlenmesinde ve tedavisinde kullanım potansiyeline sahip olduğunu gösteren önemli bir sonuç olarak değerlendirilmektedir.

INTRODUCTION

Propolis, which has great potential as a medicine and has many biological properties, is more effective than medicinal plant extracts, because its composition is extraordinarily variable. The bioactive components of the propolis samples may vary according to the different geographic origin, race, climate, flora and bud exudates (Bankova et al. 2000, Kartal et al. 2003). Propolis consists mainly of polyphenols (phenolic aldehydes, phenolic acids and their esters, flavonoid aglycones, alcohols and ketones), but it also contains terpenoids, amino acids, steroids and inorganic substances (Moreno et al. 2000). It is known that bees collect secretion from buds of poplar (*Populus* spp.), alder (*Alnus* spp.) in Poland and Central Europe (Przybyłek and Karpiński 2019). In other European countries such as Albania, Bulgaria, Hungary, different types of poplar are known as sources of propolis (Zabaiou et al. 2017). In some regions of Turkiye, chestnut (*Castanea sativa*) trees are common and honey bees often use these trees to produce propolis (Kekecoglu et al. 2021). Many previous studies have shown that different types of propolis exhibits great potential as an antioxidant, antimicrobial and antiviral agent because of the its rich content (Fatima et al. 2014, Al-Juhaimi et al. 2022, Kekecoglu et al. 2021, Yıldız 2020, Uçar 2021). It is thought that the main source of antimicrobial activity originates from pinocembrin, galangin and caffeic acid phenethyl esters, and this effect is caused by the inhibition of bacterial RNA-polymerase by phenolic compounds (Takaisi-Kikuni and Schilcher 1994). In this process, where the incidence of antimicrobial resistance is constantly increasing, the demand for natural products is increasing rapidly. Propolis is effective on many microorganisms such as viruses, fungi, including resistant bacteria (Bankova et al. 1996, Koru et al. 2007). For example, Veiga et al. (2017) showed that poplar propolis had antimicrobial activity against both Gram-positive and Gram-negative bacteria, including Methicillin-resistant *Staphylococcus aureus* (MRSA). In addition, it is known that ethanolic extracts of propolis have antifungal effect against different strain of yeast (Bankova et al. 2014). In addition to the therapeutic properties of propolis, it has reported that it has no side effects in animals or humans as a result of toxicity tests (Demir et al. 2016).

All countries have honeybee races of local ecotypes that adapt to its own ecological conditions. Although there are different bee races in our country, ecotypes of these races have spread in different areas (Ruttner, 2013, Kekecoglu, 2018). Honey bees have some characteristics that are different from each other in every race. Accordingly, propolis collection behavior also varies according to different honey bee races and ecotypes (Eroglu et al. 2021). *Apis mellifera anatoliaca*, which is found in Yıgılca district of Düzce province, is a special ecotype belonging to this region.

The aim of this study is to investigate and compare the bioactive components and antimicrobial activities against pathogenic microorganisms including resistant bacteria of propolis samples obtained from different botanical origins. Secondly, to test whether the Yığılca ecotype, a special bee subspecies, affects this biological activity.

MATERIAL AND METHODS

Sample collection, Extraction and Preparation

Propolis samples were collected from Duzce University Beekeeping Research and Development Center (DAGEM) located in the north-east area of Duzce. Propolis samples were collected with propolis traps placed in hives in June and July. The samples were kept in a dry place and stored at 4℃ until its complete process. For extraction the samples were disintegrated with a grinder and 30 g of the propolis mixed in 90 mL of 96% ethanol and shaken at 30 ℃ for two weeks. Then, centrifuged at 26,000× g for 30 min and the supernatant was filtered twice with Whatman No. 4. The remaining ethanol was allowed to evaporate to obtain a completely dry sample from this final solution. The sample was kept at 4 ℃ in the dark until use.

Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

LC-MS/MS method was used in the content analysis of the samples, as it is a reliable and successful technique for the characterization of active compounds in biological products such as propolis. For component analysis of the samples Thermo-Scientific LC coupled with a TSQ Quantum Access Max triple-stage quadruple-mass spectrometer (San Jose, CA, USA) was used. LC separations were performed in a C18 analytical column (15 cm x 3 mm x 5 µm; Torrance, California, USA). The run time was 5.5 minutes, the temperature of the column was 40°C, and the injection volume was 10µL. The mass-spectrometer was working with an electrospray ion source (ESI) in negative mode under the selected ion monitoring (SRM) condition (Nichitoi et al. 2020).

Determination of Total Phenolic (TP) and Flavonoid (TF) Content

The total phenolic content of both propolis samples was determined using the Folin–Ciocalteu colorimetric method mentioned in Singleton and Rossi (1965) with minor modifications. First, 20 µL of propolis extract was mixed with 680 µL of distilled water. 400 µL of 0.2 N Folin-Ciocalteu was added to this mixture and vortexed, this mixture was incubated for 2 minutes. After incubation, 400 µL of Na_2CO_3 (10%) was added, the mixture was shaken at regular intervals and incubated for 2 hours at room temperature. The absorbance of the mixture was measured at 760 nm and the total amount of phenolic substance was calculated as mg gallic acid equivalent per gram sample.

The total flavonoid amount of propolis samples was determined by making minor changes in the aluminum chloride colorimetric method described by Fukumoto and Mazza (2000). Quercetin was used as a standard to generate the calibration curve. The results were expressed as mg of quercetin equivalents (QE) per g pollen sample.

Test Microorganisms

For determination of the antimicrobial activityof propolis samples, seven Gram-negative, nine Gram positive and three yeast-like fungi were used. Gram-negative bacteria consisted of *Aeromonas sobria* ATCC 43979, *Aeromonas hydrophila* ATCC 7966, *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 18883, *Escherichia coli* ATCC 25922, *Vibrio* sp. Clinic strain, *Yersinia pseudotuberculosis* ATCC 911, *Pseudomonas aeruginosa* ATCC 27853, while Gram positive bacteria consisted of *Bacillus* sp. Clinic strain, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* 702 Roma, *Staphylococcus aureus* MRSA Clinic strain, *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* Clinic strain, *Listeria monocytogenes* ATCC 11994, *Mycobacterium smegmatis* ATCC 607 and *Enterococcus faecalis* ATCC 29212. Yeast-like fungi group contained *Candida tropicalis* ATCC 13803, *Candida albicans* ATCC 60193, *Saccharomyces cerevisiae* RSKK 251

Culture media and preparation of inoculum

All bacteria were transferred from stock cultures to Tryptic Soy Agar (TSA) (Merck), Blood base agar (for *S. pyogenes*) and Brain Heart Infusion (BHI) Agar (for *M. smegmatis*) and incubated overnight at 37 °C. Potato Dextrose Agar (PDA) (Merck) was preferred for the growth of yeast-like fungi. Single colonies from plates were transferred into tubes containing 2 ml of Mueller Hinton Broth (MHB), except *M. smegmatis*. Yeast-like fungi were inoculated in tubes which include 2 ml Malt extract broth. All tubes were incubated at 37 ℃ and 120 rpm for 1-3 hours. The turbidity of the suspensions were adjusted spectrophotometrically to the McFarland 0.5 turbidity standart (1.5 x 10^8 colony forming unit per ml (cfu/ml) for bacteria, 6 x 10^8cfu/ml for yeast fungi).

Test for antimicrobial activity

Agar well diffusion method

Test plates were prepared with suitable medium and wells of 6 mm in diameter were punched in the agar plates by using sterile glass tube. Overnight cultures (100 µL) spread on the petri surface with a sterile swap. 50 µL of propolis extracts were transferred to each well. Negative control was %96 ethanol and standard controls were Ampicillin (10 µg) for bacteria, streptomycin (10 µg) for *M. smegmatis* and fluconazole (5 µg) for the yeasts. Propolis extracts were tested at 4 different concentrations (1/2, 1/4, 1/8, 1/16) in the agar well method. Zones of inhibition formed by the extracts were determined using caliper after incubation and those that formed larger than 6 mm were used in the MIC experiment (Kuppulakshmi et al., 2008).

Evaluation of Minimum Inhibitory Concentrations (MIC)

For determination of MIC values, inoculum suspensions were prepared from 24 h overnight cultures. 100 µL of propolis extracts were diluted

with the liquid medium to reach a final bacterial and yeast-like fungi count in ELISA plates (96-Well ELISA Microplates) by microdilution technique. The final concentration of propolis samples ranged from 5000 to 39 µg/mL. The MIC values were determined as the lowest concentration of propolis extracts that inhibit microbial population growth.

Statistical Analysis

The analyses results of bioactive compounds in propolis samples were expressed in mean + standard deviation by using Microsoft Office Excel 2019 (Microsoft Corporation, Redmond, WA, USA). Significant differences between means were determined by T-test (SPSS version 25 for Windows 11; post hoc-one way ANOVA).

RESULTS

The bioactive components of ethanol extracts of propolis samples used in the study are given in Table 1. LC-MS/MS analysis showed that different amounts of bioactive components were detected in both propolis samples. Chrysin which is the flavone derivative, was the most abundant of all these components. While hesperidin and protocatechuic acid were not detectable in chestnut propolis, they were present in polyfloral origin propolis. (±)-Catechin, syringic acid, (-)-epicatechin and rutin were absent in polyfloral origin propolis components. Only daidzein, which is a flavonoid derivative, was not found in both samples.

Table 1. Analysis of phenolic composition in propolis samples (µg/ml)

Tablo 1. Propolis örneklerinin fenolik kompozisyonu (µg/ml)

Compounds	**Chestnut Propolis (µg/ml) (MEAN±SD)**	**Polyfloral Propolis (µg/ml) (MEAN±SD)**
Gallic acid	0,422±0,002	*nd
Protocatheuic acid	nd	1,46±0,04
Benzoic acid	95,7±0,011	2,31±0,005
(±)-Catechin	31,33±0,06	nd
Caffeic acid phenethyl ester	725,3±0,02	94,82±0,007
Syringic acid	18,12±0,03	nd
(−)-Epicatechin	1,77±0,024	nd
p- Coumaric acid	375,73±0,03	65,85±0,01
Ferulic acid	633,26±,07	69,17±10,3
Rutin	4734,5±0,47	nd
Myricetin	2596,37±0,025	2,07±0,056
Resveratrol	737,27±0,025	176,28±0,017
Daidzein	nd	nd
Luteolin	90,57±0,03	14,65±0,004
trans-Cinnamic acid	219,43±0,06	103,63±0,003
Hesperidin	nd	12,87±0,004
Chrysin	7214,42±0,07	2301,65±0,005
Pinocembrin	2272,72±0,04	910,4±0,006
CAPE	3593,27±0,06	1209,99±0,008

*nd: not detected

The TPC of the samples, measured by the Folin–Ciocalteu method, the TFC of measured by the aluminum chloride colorimetric method. After the necessary dilution of the ethanolic propolis extracts, the total amount of phenolic and flavonoid substances were determined according to the gallic acid and quercetin standard respevtively. When the data is evaluated the TPC value of chestnut propolis was nearly two times polyfloral origin propolis sample (Table 2). The obtained TPC value was 71,06±1,4 mgGAE/mL for chestnut propolis, 36,84±1,4 mgGAE/mL for polyfloral origin sample. The total flavonoid amounts of the samples were different from each other. The amount of TFC of chestnut propolis was higher than the polyfloral origin propolis sample. (t-test should be added to explain differences between two samples)

Table 2. Total phenolic and flavonoid content of propolis extracts

Tablo 2. Propolis ekstraktlarının toplam fenolik ve flavonoid madde içeriği

	Total Phenolic (mg GAE/mL)	Total flavonoids (mgQE/mL)
Chestnut propolis	71,06±1,40	11,75±0,15
Polyfloral propolis	36,84±1,40	7,04±0,30

Propolis samples obtained from two different sources were effective against all selected test microorganisms. Agar well diffusion and MIC values of propolis samples are summarized in Table 5. As a result of one-way variance analysis (ANOVA), it was seen that there was statistical differences in terms of inhibition zones ($F_{chestnut\ propolis}$=4,300, p<0,05; $F_{polyfloral\ propolis}$=7,420, p<0,05). As a result of the multiple comparison analysis, it was seen that there were significant differences in the effectiveness of chestnut propolis between Gr (-) and Gr (+) bacteria according to the results of the agar well method (x= 4,50; p<,030). Similarly, it was observed that there were significant differences in the effect of the polyfloral propolis sample against Gram (+) and Gram (-) bacteria (x= 4,010; p<,017) (Table 3). According the agar well diffusion method with four different concentration (1/2, 1/4, 1/8, 1/16) among the samples obtained by ethanolic extraction, we obtained the highest antimicrobial activity from chestnut propolis. The microorganism in which both propolis samples were most effective was *M. smegmatis* and their effect zones sizes were differed. The highest susceptible zone was obtained from *M. smegmatis* with the value of 26 and 22 mm for chestnut and polyfloral origin popolis respectively. Gram positive bacteria were more sensitive than Gram-negative one for both propolis samples.

Table 3. Statistical analysis of chestnut and polyfloral samples' inhibition zone between microorganism groups

Tablo 3. Kestane ve polifloral propolis örneklerine ait inhibisyon zonlarının mikroorganizma grupları arasındaki istatistiksel analiz sonuçları

Inhibition Zone	Factors	s	Mean Differences	F	P
Chestnut Propolis	Gr (-)/Gr (+)	1,470	4,500	4,300	,030*
	Gr (+)/Yeast fungi	1,570	2,220		,376
	Gr (-)/Yeast fungi	1,480	2,280		,511
Polyfloral propolis	Gr (-)/Gr (+)	1,270	4,010	7,420	,017*
	Gr (+)/Yeast fungi	1,680	1,330		,306
	Gr (-)/Yeast fungi	1,740	2,670		,715

* Statistical significance was defined as *P*<0.05

Table 4. Statistical analysis of chestnut and polyfloral samples' MIC value between microorganism groups

Tablo 4. Kestane ve polifloral propolis örneklerinin MIC değerlerinin mikroorganizma grupları arasındaki istatistiksel analiz sonuçları

MIC (µg/ml)	Factors	s	Mean Differences	F	P
Chestnut Propolis	Gr (-)/Gr (+)	300,500	1347,660	10,450	,001*
	Gr (+)/Yeast fungi	397,300	253,910		,801
	Gr (-)/Yeast fungi	411,500	1093,740		,047*
Polyfloral propolis	Gr (-)/Gr (+)	610,410	2460, 930	8,680	,003*
	Gr (+)/Yeast fungi	807,500	273,430		,939
	Gr (-)/Yeast fungi	835,840	2187,500		,041*

* Statistical significance was defined as *P*<0.05

Table 5. Agar well diffusion and MIC values of the Propolis extracts against the tested microorganisms

Tablo 5. Propolis ekstraktlarının test edilen mikroorganizmalara karşı agar kuyucuk difüzyonu ve MİK değerleri.

	Microorganisms	Chestnut propolis		Polyfloral propolis		Antibiotics*	
		Inhibition zone (mm)	MIC (µg/ml)	Inhibition zone (mm)	MIC (µg/ml)	Inhibition Zone (mm)	MIC (µg/ml)
Gr (-)	*Escherichia coli*	10	2500	8	5000	10	10
	Klebsiella pneumoniae subsp. *pneumoniae*	10	2500	9	5000	10	32
	Yersinia pseudotuberculosis	12	1250	10	2500	10	32
	Vibrio sp.	14	312.5	13	625	NT	NT
	Aeromonas hydrophila	10	2500	8	5000	NT	NT
	Aeromonas sobria	12	1250	10	2500	NT	NT
	Pseudomonas aeruginosa	14	625	12	1250	18	>128
Gr (+)	*Enterococcus faecalis*	14	312.5	12	1250	10	2
	Listeria monosytogenes	16	156.25	14	312,5	NT	NT
	Streptococcus pyogenes	15	156.25	12	1250	NT	NT
	Staphylococcus aureus	18	39.06	14	312.5	35	2
	S. aureus MRSA+	15	156.25	13	625	NT	NT
	Bacillus subtilis	15	156.25	13	625	NT	NT
	Bacillus sp.	14	312.5	14	312.5	NT	NT
	Bacillus cereus	13	625	12	1250	NT	NT
	Mycobacterium smegmatis	26	19.5	22	39.06	35	<1
Yeast fungi	*Candida albicans*	13	625	12	1250	25	<8
	Candida tropicalis	13	625	12	1250	25	<8
	Saccharomyces cerevisiae	16	156.25	14	312.5	25	<8

*The test control antibiotics used: Ampicillin for Gram (-) and Gram (+) bacteria (10 µg/ml), Streptomycin for ARB+ bacteria (10 µg/ml), and Fluconazole for the yeast fungi (5 µg/ml). (-): No activity, NT, Not tested.

*Kullanılan kontrol antibiyotikleri: Gram (-) ve Gram (+) bakteriler için ampisilin (10 µg/ml), ARB+ bakterileri için Streptomisin (10 µg/ml) ve maya mantarları için Flukonazol (5 µg/ml). (-): Etkinlik yok, NT, Test edilmedi.

According to the MIC results obtained from the propolis samples, significant differences were obtained among the microorganisms ($F_{chestnut\ propolis}$=10,450, p<0,05; $F_{polyfloral\ propolis}$=8,680, p<0,05). When the MIC results of chestnut propolis were evaluated, significant differences were observed between the activity values between Gram (+) and Gram (-) bacteria (x = 1347,660; p<,001). Similarly, significant differences were observed between the efficacy values of the MIC results of polyfloral propolis (x = 2460,930; p<,003). The differences between other groups (Gram (+) /Yeast fungi, Gram (-) /Yeast fungi) are summarized in table 4. The efficacy dose of chestnut propolis was between 19,5 and 2500 µg/mL while the polyfloral origin propolis sample was between 39,06 and 5000 µg/mL. Chestnut propolis showed remarkable bactericidal effect against *M. smegmatis* with the dose of 19,5 µg/mL. The most resistant strains were *E. coli*, K. *pneumoniae* subsp. *pneumoniae* and *A. hydrophila* with inhibition dose of 5000 µg/mL. Two propolis samples exhibited moderate antifungal activity against selected yeast like fungi. Most resistant yeast were *C. albicans* and *C. tropicalis* with the dose of 2500 for polyfloral origin propolis and 1250 for chestnut propolis, most sensitive was *S. cerevisiae* (Table 5).

DISCUSSION

It is known that the chemical content of propolis depends on the origin of the plant, geographical location and the harvest season (Al-Ani et al. 2018). In this study, content differences due to the plant origin of propolis samples were observed. While some bioactive components were found in chestnut propolis, some of them were not detected in the polyfloral origin propolis sample. In addition, the amounts of the analyzed components were different from each other. Previous studies have shown that European, African and Asian propolis mostly contains phenolics and flavonoids such as pinocembrin, *p*-coumaric acid, cinnamic acid, chrysin, naringenin, galangin, quercetin, apigenin, pinobanksin, kaempferol, caffeine (Huang et al. 2014; De Groot et al. 2013). Among these components polyphenols and terpenoids are the most active group (Pimenta et al. 2015). The flavonoid group consists of chrysin, pinostrobin, galangin, pinocembrin, quercetin, apigenin, kaempferol and other components (Przybyłek, and Karpiński 2019). Our chestnut and polyfloral origin propolis samples contained the highest rate of chrysin, which is a flavonoid derivative. Another critical group constituting the content of propolis is aromatic acids, among which cinnamic, ferulic, caffeic, *p*-coumaric and benzoic acids are the most common (Kędzia and Hołderna-Kędzia 2017; Bankova 2000). Almost all of these aromatic esters were detected at high rates in chestnut propolis.

The present study aimed to investigate the antimicrobial properties of chestnut and polyfloral origin propolis samples. The influence of ethanol extraction in different concentrations on the growth of bacteria and fungi was determined. Incubation of propolis samples with higher concentrations resulted in higher inhibition of growth zones. Some researchers reported that propolis samples were only effective against Gram-positive bacteria and fungi, while others reported that the activity was not high against Gram-negative bacteria (Nieva et al.1999; Kujumgiev et al. 1999; Sforcin et al. 2000) . In this study, it was confirmed that Gram-positive bacteria were sensitive to low concentrations for both samples and that Gram-negative bacteria growth was inhibited to a lesser extent than Gram-positive bacteria. Chestnut propolis was the most effective against test microorganisms, followed by polyfloral origin sample. In previous studies, the best anti-staphylococcal effect levels of propolis ethanolic extract were reported for extracts derived from Turkey (8 µg/mL), Oman (42 µg/mL) and Ireland (80 µg/mL) (Uzel et al. 2005; Popova et al. 2013; AL-Ani et al. 2018). The antimicrobial activity of chestnut propolis against this bacterial species that causes pneumonia, osteomyelitis, septic arthritis, bacteremia, endocarditis and various skin infections is quite low as compared to previous studies (39.06 µg/mL). It is known that the presence of phenolic compounds in the chemical structure of chrysin is responsible for the antibacterial effects of propolis, as well as other flavonoids (Warfvinge et al.1985; Sforcin and Bankova, 2011; Sharifi et al. 2020). The slightlyhigh detection of chrysin in our chestnut propolis sample may be explained bylow MIC concentration against *S. aureus*.

We obtained strong antimicrobial activity from both propolis samples against *M. smegmatis* which is a saprophytic acid-resistant bacterium that also causes skin diseases. It has been reported in previous studies that pinocembrin and its 3-OH analog galangin, flavonoids such as quercetin, myricetin and rutin are the components responsible for the most potent microbicidal compounds via

increasing bacterial membrane permeability (Vică et al. 2022; Das et al. 2015; Stepanovic et al. 2003; Kosalec et al. 2003). Otherbioactive compounds which are identified and studied inpropolis are caffeic acid phenethyl ester (CAPE), which exhibit good antimicrobial properties by inhibiting bacterial RNA polymerase (Šuran et al. 2021; Speciale et al. 2006). Considering that the most abundant bioactive components as a result of LC-MS/MS analyzes of the chestnut propolis was chrysin, rutin, CAPE, myricetin and pinocembrin. It is possible to obtain MIC values at such low concentrations against *M. smegmatis.* Because one of these components, rutin, cannot be detected in the polyfloral origin propolis sample, while myricetin is present in trace amounts. These results demonstrated that chestnut propolis is a promising candidate for using as an antimicrobial product.

Ristivojević et al. (2016) tested the efficacy of 53 propolis samples on *L. monocytogenes* and reported the lowest efficacy dose as 100 µg/mL and the highest as 10.600 m/mL. The MIC values of chestnut and polyfloral origin propolis, whose antimicrobial activity levels were investigated in this study, against this bacterium causing meningitis, septicemia and monocytosis were 156.25 µg/mL and 312.5 µg/mL respectively. The fact that the propolis samples have such a low MIC values can be explained by the synergistic effect of the phenolic compounds with high level in the samples or special bee subspecies of Yığılca ecotype that collect propolis. Al-Ani et al. (2018) investigated the effect of different propolis samples against *S. pyogenes*, which causes dermal diseases such as impetigo and necrotizing fasciitis and they obtained different MIC values ranging from 80 to 600 µg/mL. The effect concentration of chestnut propolis against this bacterium is still quite low (156.25), which is below the average dose compared to previous studies.

Previous studies reported that different propolis samples have significant antifungal activity against a wide range of pathogen like Candida species which were isolated from patients and show antibiotic resistance (De Castro 2001; Cornara et al. 2017; Vica et al. 2021; Lan et al. 2016). AL-Ani et al. (2018) evaluated the effect of propolis samples from Germany, Ireland and Czech against different Candida species and reported the effective values against *C. albicans* as 5000, 600 and 1200 µg/mL, respectively. MIC values of the same samples against *C. tropicalis* were reported as 5000, 200 and 600 µg /mL, respectively. Chestnut propolis, which antifungal effect was tested in this study, showed a very low activity value on the same yeast-like fungus, and MIC values were determined as 625 µg /mL against both Candida species. It has been previously reported that the amount of CAPE in propolis significantly affects the antifungal activity (Cornara et al. 2017). Considering the CAPE amount of chestnut propolis, it is not surprising that such a low MIC value was obtained.

Conclusion

The antimicrobial activities of two different floral origin propolis from Anatolia against various pathogenic bacterial strains were determined by a MIC method. It was confirmed that chestnut propolis sample has higherphenolic and flavonoid contents and also it was found to be more effective against both Gram positive and Gram-negative bacteria. In the study, it was determined that the most abundant bioactive component in chestnut propolis samples, wwere chrysin followed by rutin, CAPE, myricetin and pinocembrin. The results suggest that the high content of bioactive components inhibit the growth and proliferation of bacteria by acting alone or synergistically. It was concluded that MIC values were obtained at lower concentrations from chestnut propolis than other sample according to this reason. Among the Gram-positive strains, *M. smegmatis* was the most susceptible strain for chestnut propolis, while the most resistant strains were *E. coli*, *Klebsiella pneumoniae* subsp. *pneumoniae* and *A. hydrophila*. The knowledge gained through this study may be a comparative analysis of the content to attribute the antimicrobial activity of propolis to specific chemical compounds and to confirm that these components are related to the floral origin.

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