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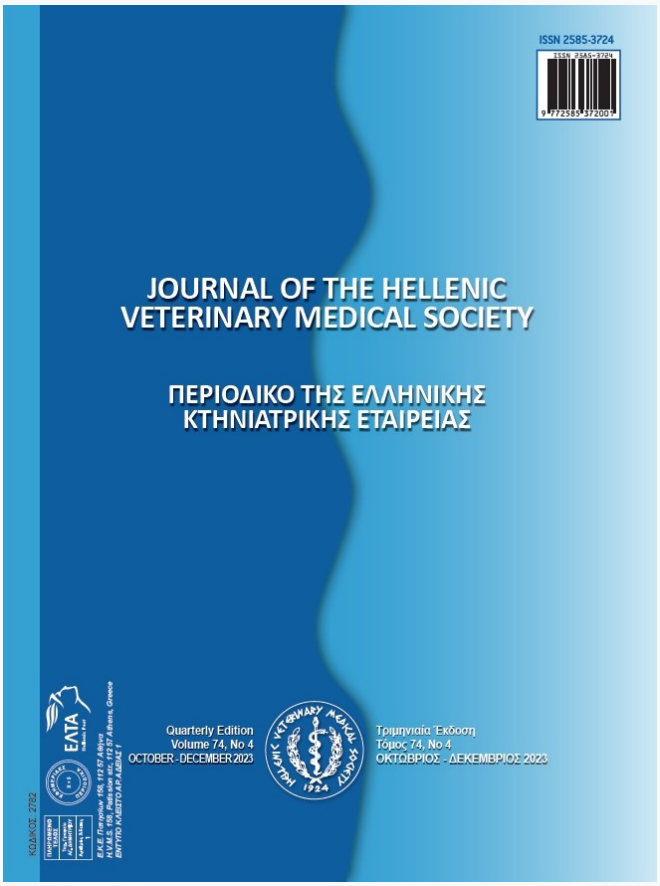

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Comparison of some biological activities of propolis and bee bread samples obtained from *Apis mellifera Anatoliaca* and *its* Muğla and Efe ecotypes

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ABSTRACT: Honey and other bee products have been extensively researched in recent years due to the demand for natural products with nutritional and therapeutic qualities, as they contain high levels of biologically active compounds and valuable nutrients. In this study, antioxidant, anti-inflammatory and antimicrobial activities of bee bread and propolis samples produced after colony development by bees of different races and ecotypes accepted to core colonies in the same location were evaluated. Total phenolic, flavonoid and antioxidant content values were the the highest in propolis samples, especially in propolis from *Apis mellifera anatoliaca*. The most effective results were also recorded in almost all of the other tests performed for the same sample, such as DPPH free radical scavenging, lipid peroxidation inhibition, anti-inflammatory and antimicrobial activity. In addition, according to the results of phenolic component analysis, the amount of phenolic substance detected in the sample belonging to this species was higher. It was surprising that propolis samples obtained from Efe and Muğla ecotypes showed higher values in the test for chelating activity with Fe. The values calculated for the bee breads in all examined parameters were lower than the propolis samples.The highest values were calculated for the bee bread harvested from the efe ecotype.None of the tested bee breads showed antimicrobial activity on selected pathogenic bacteria.In this study, it was evaluated how the differences arising from both race and ecotype and the type of bee product reflected on biological activities.

Keywords: Antioxidant; anti-inflammatory; antimicrobial; ecotype; honeybee; race

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INTRODUCTION

Bee products have been widely used since ancient times due to their high nutritional value and many therapeutic benefits (Martinello and Mutinelli, 2021). Propolis, one of these bee products, is a bio-product produced by honey bees (*Apis mellifera* L.) to be used as a hive adhesive and antimicrobial agent. The properties of propolis, a complex and resinous natural substance containing functional compounds collected from plant leaves, flower buds, branches, bark and plants, vary according to geographical regions (Peixoto et al., 2021; Marcucci et al., 2001; Shehata et al., 2020; Kekeçoğlu et al., 2021). Propolis is a mixture of resin and balm, beeswax, pollen, organic compounds and honey bee saliva (Bulman et al., 2011). Most of these organic compounds are polyphenolics and flavonoids, which contribute to the renowned antioxidant properties of propolis (Kurek-Górecka et al., 2014). It has been reported that propolis exhibits a higher level of antioxidant activity than honey, which is proportional to its antimicrobial activity (Nakajima et al., 2009; Ahmad et al., 2019; El Sohaimy and Masry, 2014; Bouarab-Chibane et al., 2019). In addition, it is present in lesser amounts of active ingredients such as polyphenols, terpenoids, steroids, sugars, amino acids in propolis (Benzie and Strain, 1999; Sun et al., 2015).Bee bread, on the other hand, is another valuable bee product containing antioxidant and phenolic compounds, consisting of bee pollen pellets packed by bees and transported to the hive on their hind legs in the pollen bag. Bee bread is mainly composed of, 24-35% carbohydrates, 20% protein, 3% minerals and vitamins (B1, B2, C, E, K, biotin, folic acid and nicotinic acid), 3% lipids, pantothenic acid, carotenoids, phenolic acids and flavonoids, sterols and several enzymes such as amylase, saccharase and phosphatases (Suleiman et al., 2021).Furthermore, bee bread is a good source of polyunsaturated fatty acids (PUFA), which are important for human nutrition and cannot be synthesized in the body. However, the number of scientific studies on bee bread is less than other bee products (Silici, 2014). Bee bread, which is known to exhibit antimicrobial, antioxidant, antiradical, anticancer and anti-inflammatory activities, has been used in the treatment of many diseases in recent years (Khalifa et al., 2020).

There are several studies documenting that external factors such as geographical conditions, climate change, season and botanical source show a change in propolis properties (Do Nascimento et al., 2019; Yuan et al., 2020). In addition, it has been revealed that seasonal and geographical factors cause the differentiation of biological activities such as antioxidant and antimicrobial activities (Filipič et al., 2019; López-Romero et al., 2018; Kumazawa et al., 2004). The race and subspecies of honey bee that produces propolis is also an important factor in these characteristics (Kekeçoğlu et al., 2021).

In the literature, there are reports on various biological and pharmacological activities of propolis related to antibacterial, antifungal, antiviral, antioxidant, cytotoxic, anti-inflammatory, immunomodulatory, hepatoprotective and anticancer activities. These known activities lead propolis to have a wide range of applications. Despite such widely known applications, propolis does not get the value it deserves around the world. Propolis which used for its biological activities should be collected using propolis traps. Thus, it can create a source of income for beekeepers in addition to honey (Peixoto et al., 2021). Similar biological activities have been reported for bee bread (Zerdani et al., 2011; Imran et al., 2019; Orsolic, 2013), and it is emphasized that it is one of the natural products with the highest anti-inflammatory activity (Gómez-Caravaca et al., 2006).

The homeland of honey bees is Asia, Europe and Africa and they have been living on earth for millions of years. *Apis mellifera* is the most common and has the highest economic value in the genus Apis, and within this species, regional races and ecotypes (subspecies) adapted to different regions have been formed with adaptation to various regions. Five of the 27 bee races defined throughout the world (*Apis melifera caucasica, A.m. syriaca, A.m. anatoliaca, A. m. meda* and *A.m. carnica*), which differ due to their special geographical location and climate range on the migration routes, are distributed in Turkey (Ruttner, 1988). The *A. Mellifera* races have adapted to the climatic conditions and floral structure of the region where they spread. While these adaptations are effective on the morphological and physiological structure of bees, they may lead to the formation of different races and ecotypes. In addition, the adaptations of different races can affect on anatomical and morphological characteristics, such as gland sizes and secretions. This change also affects the composition and activity of honey bee products (Kekeçoğlu et al., 2021). Morphological differences in honey bee races also cause differences in propolis collecting behavior (Winston, 1991; Kekeçoğlu et al., 2020). The amount and quality of propolis collected by honey bees varies

depending on botanical sources, season, year, propolis collection techniques and genetic origin of honey bee races (Kekeçoğlu et al., 2020; Mobus, 1972; Ghisalberti, 1979; Crane, 1990). In addition, the extraction method and the chosen solvent greatly affect the measurable biological activity of propolis (Kekeçoğlu et al., 2021).

Today, bee products are at the forefront of the most valuable natural products, and the physicochemical and biological properties of them are affected by many factors. For this reason, biological activities of products from different geographical regions are frequently examined in research. In the current study, by following a different method, bees belonging to different races and ecotypes were accepted to core colonies in the same location and bee bread and propolis were harvested following the colony development. Harvested bee products were investigated in terms of their antioxidant, anti inflamatory and antimicrobial activities. Furthermore total phenolic and flavonoid contents were determined and phenolic compound analysis was done. Thus, propolis and bee bread samples taken from the same hive were compared only according to the difference caused by the bee race.

MATERIALS AND METHODS

Collection and extraction of samples

In total nine queen bees, three of each belonging to different races and ecotypes were obtained from different institutions as the source of propolis and bee bread used in the study. *Apis mellifera anatoliaca*, Muğla ecotype and Efe ecotype were obtained from Macahel Queen Bee Enterprise, Muğla Beekeepers Association and Aegean Agricultural Research Institute, respectively (Figure 1) . The queen bees, which were obtained from the specified enterprises and institutes in April and May, were accepted to the core colonies in the Yumrutaş village of Persembe district of Ordu province, and the colony development was followed. The propolis traps placed under the hive cover of the aforementioned colonies were taken after a sufficient time and left to freeze in the refrigerator, and then the propolis samples were stretched out from the traps and placed in jars. On the other hand, the bee breads were collected from the 8 and 9 frames in which the bee bread was stored, with the help of a perga (bee bread) spoon, leaving empty frames in the colonies whose colony development was followed, and put into jars. Both bee bread and propolis samples were collected in September and kept at -20°C until analysis. Since propolis and bee bread production changes seasonally, only one sample was taken from each colony.

In order to prepare the bee bread ethanol extract, firstly, a certain amount of powdered bee bread sample was treated with approximately 10 times the

Figure 1: The location of the study area on the map of Turkey and the sampling points,

amount of ethanol at room temperature in the dark for 72 hours. After this period, wet-softened mixture formed was mixed in a magnetic stirrer at 500 rpm for 10 minutes and then centrifuged at 4000 g for 10 minutes. The supernatant was taken and filtered through Whatman No 1. The solvent was removed by rotary evaporator (Othman et al., 2019). On the other hand, in order to prepare propolis extract, propolis samples that were frozen at-20 °C were crushed into powder in a mortar. Extraction was carried out in an ultrasonic bath (40 °C for 45 min and 756 W) by adding 10 times the amount of ethanol on a certain amount of powdered propolis sample. After that, the mixture was centrifuged at 4000 g for 10 minutes. The supernatant was taken and filtered through Whatman No 1. The solvent was removed by rotary evaporator (Okińczyc et al., 2021).At the end of the evaporation, the weighed residues were dissolved in an appropriate amount of ethanol, and bee bread and propolis extracts of known concentrations were prepared and the yield of the exraction were calculated.

The short names of the samples were made as follows: Bee bread (ABB) and propolis (AP) harvested from *Apis mellifera anatoliaca*; Bee bread (MBB) and propolis (MP) harvested from Muğla ecotype; Bee bread (EBB) and propolis (EP) harvested from Efe ecotype.

Total phenolic and flavonoid content of the samples

Content of the total phenolics in the ethanol extracts of the bee bread and propolis samples was measured by Folin-Ciocalteu reagent (Singleton and Rossi, 1965) and the total amount of the phenolic compounds was determined as mg of gallic acid equivalent, using a calibration curve(0.001-0.01 mg/mL gallic acid concentration range). Aluminium chloride method was used to reveal the flavonoid content and the results were calculated as catechin equivalents by using the catechin calibration curve (0.002-0.01 mg/mL catechin concentration range) drawn for this purpose (Kim et al., 2003).

Antioxidant activity assays

The total antioxidant capacity (TAC) of the ethanol extracts was evaluated by the phosphomolybdenum method according to method of Prieto et al (1999). The TAC values of the samples were expressed as ascorbic acid equivalents (mg AAE/g extract) by using the calibration curve drawn for different concentrations (0.004-0.02 mg/mL) of ascorbic acid.

DPPH radical scavenging activity (%) of the bee bread and propolis extracts was calculated for each concentration by using the following formula according to the values obtained from the spectrophotometric measurement made at 517 nm after incubation of varying concentrations of the extracts with methanolic DPPH solution for 30 minutes in the dark (Sànchez-Moreno et al., 1998).

Scavenging activity (%)= (Absorbance$_{control}$) - (Absorbance$_{sample}$)/ (Absorbance$_{control}$) x 100

By plotting the percent scavenging values found for each concentration, the extract concentration that scavenged half of the DPPH radicals in the reaction medium was calculated (SC_{50}; mg/mL).

Another indication of their antioxidant activity was observed through the ferrous ion-ferrozine complex method. Herewith, metal chelating effect was calculated using the equation used for DPPH scavenging activity after measuring the absorbance values at 562 nm (Dinis et al., 1994).

In order to examine the potential of the samples to inhibit *ABAP induced lipid peroxidation*, reaction mixtures were prepared in methanol (final volume 1.2 mL) containing 26.5 µM linoleic acid, 0.4 mM 2,2-azobis(2-amidinopropane)-dihydrochloride (ABAP) and 0.1 mg/mL extract. The well-mixed mixtures were allowed to stand at room temperature for 20 minutes, after which time, changes in absorbance were measured against methanol at 234 nm. The mixture without the extract was used as a blank (Pryor et al., 1993). The potential to prevent lipid peroxidation was calculated with the help of the above equation. The same procedures were performed for the known concentration of ascorbic acid and the comparison was made.

Anti-inflamatory activity assay

The anti-inflammatory activities of the bee products were investigated according to the method defined by Williams et al (2008) by evaluating their inhibition potential on bovine serum albumin (BSA) denaturation. The reaction mixture was prepared in phosphate buffered saline (PBS) buffer, which was formed to contain 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 and prepared by adjusting the pH to 6.3 with HCl. Reaction mixtures formed by adding the sample to be tested at varying concentrations or ibuprofen used as a standard to the reaction medium containing 0.8% BSA, firstly kept at

37 °C for 20 minutes and then at 71 °C for 15 minutes, and the turbidity that occurs in each tube was read in the spectrophotometer at 660 nm against the blank tube prepared by adding only the solvent instead of the sample. The percentage inhibition of protein denaturation was calculated using the following equation:

$$\% \text{ Inhibition} = [(Abs_{blank} - Abs_{sample}) / Abs_{blank}] \times 100$$

Antimicrobial activity assays

Antimicrobial detection of bee bread and propolis samples were performed on *Aeromonas hydrophila, Aeromonas sobria, Yersinia ruckeri, Vibrio anguillarum*and *Lactococcus garvieae* strains isolated from diseased rainbow trout in Fethiye/Turkey (Ürkü and Önalan, 2017). All bacteria were cultured for 18 h at 21-22 °C in Mueller HintonBroth and used as inoculums.To meausere antimicrobial activity,disc diffusion method was used and obtained values for samples were compared with a commercial antibiotic disc (oxytetracycline- 30 µg/disc) (Oxoid, England)known antibiotic (oxytetracyline). Petri dishes with 10 mLof Mueller-Hiton agar were prepared, previously inoculated with 0.1 mL of a 24 h broth culture of test bacteria. Filter-paper-discs (6mm in diameter) were impregnated with 20µL of sample and another with ethanol (negative control). The inoculated plates were incubated at 21 - 22°C for 24 h. Diameters of the inhibition zones were measured in millimeters according to Gonsales et al. (2006). This test was performed in triplicate for all samples.

Micro-broth dilution method was applied in order to calculate the minimum inhibition concentration (MIC) values for the samples with significant results according to the disk diffusion method (Qaiyami, 2007).Briefly, serial two-fold dilutions (10 times) of all propolis were prepared in 96-well microtiter plate containing cation-adjusted Mueller-Hinton broth. Control microtiter plates containing medium and 80% ethanol at the same dilutions were also made. Bacterial suspensions were adjusted to the 0.5 McFarland standards (approximately 1 to 2 × 10^8 CFU/mL). A constant amount of bacteria was added to all wells and the plate was incubated at 21 - 22°C for 18-24 hour.

RP-HPLC analysis and sample preparation

To preparation of the ethanolic extracts for HPLC analysis, solutions were firstly evaporated and the residue dissolved in 10 mL of purified water (pH 2).The aqueous solution was extracted 3 times with both, respectively, under the same conditions (15 min, 200 rpm, 25 °C) with diethyl ether and ethyl acetate.The resulting organic phase, which was collected in a flask after each extraction, was evaporated. Just before being fed to the HPLC device, the residue was filtered in 2 mL of methanol and then filtered by 0.45 µm filters. The phenolic content analysis of the samples were done in triplicate (Kara et al., 2022).

Each sample extract was injected into the HPLC system with a reverse phase C18 column (250 mm × 4.6 mm, 5 µm; GL Sciences) at 250, 280, 320 and 360 nm. The mobile phase consisted of (A) acetonitrile: water (70:30) and (B) 2% acetic acid in water. The samples to be tested were injected as 20 µL. The column temperature was set at 30°C, and the flow rate at 1.0 mL/min. The phenolic content of the samples

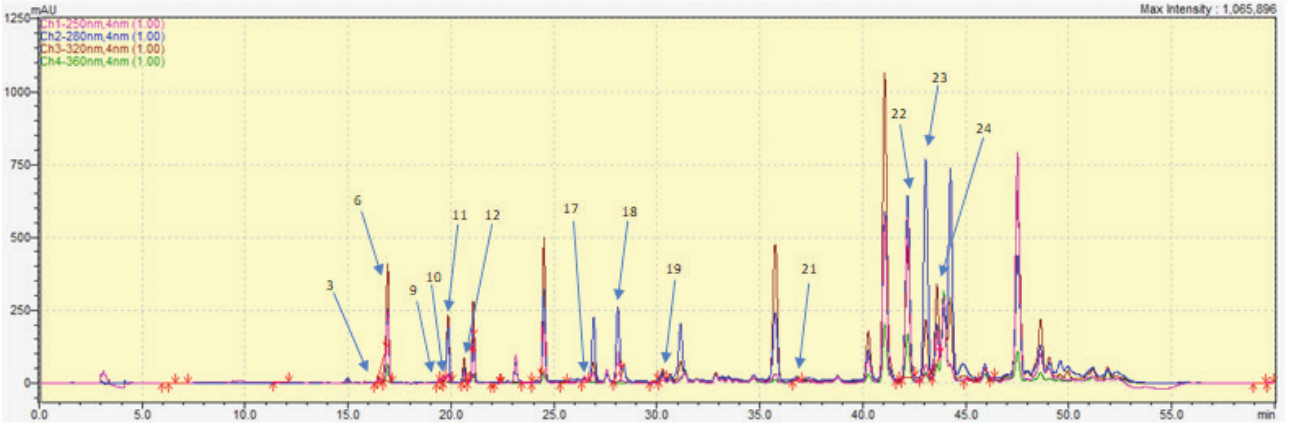

Figure 2: HPLC-UV chromatograms of phenolic standards (40 ppm) for MP;Gallic acid (1), protocatechuic acid (2), Chlorogenic acid (3), p-OH benzoic acid (4), epicatechin (5), caffeic acid (6), syringic acid (7), m-OH benzoic acid (8), Rutin (9), Ellagic acid (10), p-Kumaric Acid (11), ferulic acid (12), myricetin (13), resveratrol (14), daidzein (15), luteolin (16), quercetin (17), t-cinnamic acid (18), Apigenin (19), Hesperidin (20), Ramnetin (21), chrysin (22), Pinocembrin (23), CAPE (24) and curcumin (25)

was determined by the gradient program in the method specified by Kara et al. (2022).This procedure was performed in three repetitions. A standard chromatogram for the 25 phenolic standards (gallic acid, protocatechuic acid, chlorogenic acid, *p*-OH benzoic acid, epicatechin, caffeic acid, syringic acid, *m*-OH benzoic acid, rutin, ellagic acid, *p*-kumaric acid, ferulic acid, myricetin, resveratrol, daidzein, luteolin, quercetin, *t*-cinnamic acid, apigenin, hesperidin, rhamnetin, chrysin, pinocembrin, caffeic acid phenethyl ester (CAPE) and curcumin) at 40 ppm concentration applied at HPLC-PDA is shown in Figure 2 (Kara et al., 2022).The LOD and LOQ values of the analyzed components are available in the study by Kara et al. (2022).

Statistical analysis

The statistical significance of the results was determined by using a one-way ANOVA analysis then the ranking of significance was determined using the Tukey post-hoc test. The results were given as means ± SD. Significance levels were defined as $p < 0.05$. Data were analyzed by the program MINITAB 18 (Minitab, State Collage, PA, USA).

RESULTS

Total phenolic and flavonoid contents and antioxidant activities

As can be easily seen from the Figure 3, the highest values in terms of total phenolic and flavonoid content and total antioxidant capacity belong to the sample harvested from the colonies formed from the Efe ecotype (EBB) in the case of bee bread samples, while the sample harvested from the *Apis mellifera anatoliaca* (AP) race in the case of propolis extracts. The total phenolic content (TPC) values of bee bread (12.18-29.53 mgGAE/g extract) are lower than the values calculated for propolis samples (104.83-144.26 mgGAE/g extract). The same applies in the case of total flavonoid content values. While the average value of the flavonoid content for bee bread sample was 31.04 mgCTE/g extract, this value is 308.80 mgCTE/g extract for propolis samples.

Total antioxidant activity values calculated in accordance to these first two findings were higher for propolis samples. While the variation of the total antioxidant activity is between48.26-115.62 mg AAE/g extract for bee breads, it is between 158.76-346.09mg AAE/g extract in the presence of propolis samples.

The DPPH radical scavenging values of the samples were expressed as SC_{50}, which means the extract concentration that can scavenge half of the DPPH radicals in the environment. Therefore, the smaller value means that the sample to which it belongs more effectively scavenges DPPH radicals. According to this, it can be said that propolis samples are almost equal among themselves, but have a much superior scavenging feature of DPPH radicals compared to bee bread samples. The minimum SC_{50} value as 0.0011 mg/mL was obtained for propolis sample from *Apis mellifera* anatoliaca race. In the case of bee bread samples the lowest SC_{50} value (0.1053 mg/mL) was calculated forthe sample belong to Efe ecotype. Similarly, it was concluded that the chelation percentage of 0.25 mg/mL portions of the samples with Fe^{+2} ions in the medium was higher in the case of propolis samples.

Consistent with that obtained in the first two antioxidant test methods, only 0.001 mg/mL fractions of the tested propolis samples inhibited ABAP-induced lipid peroxidation well. However, only 0.05 mg/mL concentrations of bee bread samples showed equivalent inhibition.

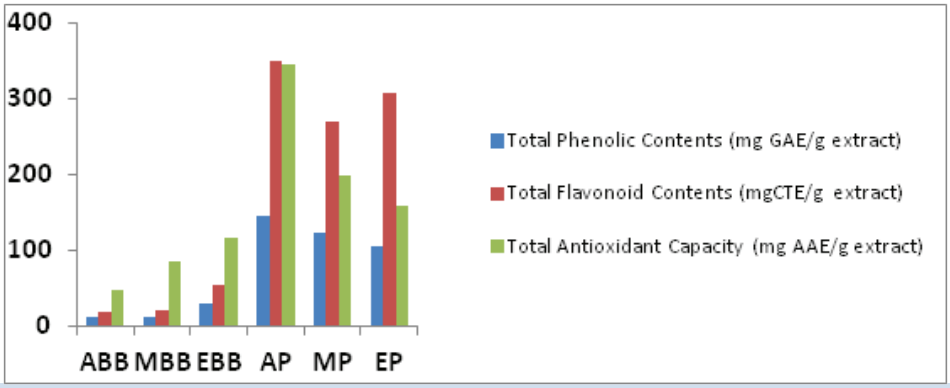

Figure 3: Total phenolic, flavonoid and antioxidant activities of samples

Table 1. Antioxidant and antiinflamatory activities

Samples	DPPH (SC_{50}µg/mL)	% Chelating Activity* (0.25 mg/mL)	Antiinflamatory Activity (IC_{50}; mg/mL)	Lipid Peroxidation Inhibition (%)**
ABB	290.9±1.2[b]	1.126±0.113[e]	0.33±0.02[c]	14.63±1.56[c]
MBB	868.5±5.8[a]	0.866±0.078[f]	1.34±0.65[b]	13.97±1.04[c]
EBB	105.3±0.5[c]	3.636±0.256[d]	1.68±0.13[a]	16.74±0.98b[c]
AP	1.1±0.3[d]	4.935±0.874[c]	0.019±0.004[e]	21.54±2.25[a]
MP	1.8±1.0[d]	7.446±0.668[b]	0.047±0.014[e]	16.01±1.00b[c]
EP	1.4±0.9[d]	8.052±1.003[a]	0.242±0.017[d]	18.63±0.65[ab]

*Chelating Activity values were calculated for 0.25 mg/mL concentrations of all samples.
** Lipid peroxidation inhibition values were obtained for 0.05 mg/mL concentrations of bee bread samples and 0.001 mg/mL for propolis samples.

Table 2. Zone diameter (mm) measurements and minimum inhibition concentration (MIC) of propolis samples

Pathogen bacteria	Zone diameter (mm) MIC Value (µg/ml)		
	AP	**EP**	**MP**
Aeromonas hydrophila	-	-	-
Aeromonas sobria	-	-	-
Yersinia ruckeri	-	-	-
Vibrio anguillarum	12	13	15
	6.2 ±1.7	8.2±1.2	29.1±5.5
Lactococcus garvieae	11	12	14
	3.1 ±0.8	4.1±0.9	7.3±2.0

Anti-inflamatory activity assay

In particular, the beneficial effect of propolis samples on inflammation is clearly seen from the results (Table 1). The IC_{50} value for ibuprofen, a non-steroidal anti-inflammatory drug active ingredient, was calculated as 0.0709 mg/mL. When this value is compared with the values calculated for the tested samples listed in Table 1, it was concluded that the propolis samples harvested from *Apis mellifera anatoliaca* (AP) and Muğla ecotype (MP) were much more effective than the reference drug with the IC_{50} values 0.019 and 0.047 mg/mL, respectively.

Antimicrobial activities

Contrary to its antioxidant and antimicrobial activity, the antimicrobial activities of thesamples examined on the tested microorganisms are limited. *Aeromonas hydrophila, Aeromonas sobria, Yersinia ruckeri, Vibrio anguillarum* and *Lactococcus garvieae* pathogens isolated from diseased rainbow trout were used as bacterial strain. It was concluded that none of the bee bread samples showed antimicrobial activity at a level to prevent the growth of these species, while propolis samples were effective only on *V. anguillarum* and *L. garvieae* (Table 2).

Phenolic profiles

Of the 25 phenolic acids whose presence was investigated, 10 (Gallic acid, protocatechuic acid, epicatechin, syringic acid, m-OH benzoic acid, myricetin, resveratrol, daidzein, Hesperidin, curcumin (they are not placed in the table because they do not contain numerical data)) could not be detected in any of the tested bee bread and propolis species.On the other hand, while chlorogenic acid was detected only in propolis of Muğla ecotype, p-OH benzoic acid was detected only in propolis from *Apis mellifera anatoliaca*. Only *p*-coumaric acid was detected at varying rates in both propolis and bee bread samples.Caffeic acid, on the other hand, was detected only in propolis samples, especially in *Apis mellifera anatoliaca* propolis with a concentration of 18.684 mg/g among the samples tested.

Among the phenolics of hesperidin, pinocembrin, chrysin, coumaric acid, caffeic acid and CAPE, which are known to be effective agents of propolis (Malkoç et al., 2019), hesperidin was not found in any of the tested propolis samples. However, the others mentioned were significantly detected in all propolis samples (Table 3).Especially pinocembrin and chrysin are quite high in all propolis samples.

Table 3. Quantitative amounts (mg/g extract) of phenolic compounds obtained by HPLC analysis

Standarts (ug/g sample)	ABB	EBB	MBB	AP	EP	MP
Chlorogenic acid	n.d.*	n.d.	n.d.	n.d.	n.d.	514.344±15.235
p-OH Benzoic Acid	n.d.	n.d.	n.d.	98.659±7.425	n.d.	n.d.
Caffeic Acid	n.d.	n.d.	n.d.	8146.450±100.589	4888.560±43.836	5120.023±56.088
Rutin	n.d.	143.132±13.98	n.d.	n.d.	n.d.	98.887±9.5
Ellagic Acid	n.d.	n.d.	n.d.	n.d.	n.d.	1051.174±23.822
p-Kumaric Acid	70.024±5.383	36.595±4.500	156.083±12.500	2903.882±18.600	3365.997±41.100	2589.523±14.666
Ferulic Acid	n.d.	n.d.	n.d.	875.812±11.400	1313.045±20.874	1210.443±14.100
Luteolin	n.d.	n.d.	n.d.	92.665±10.102	9.974±1.216	n.d.
Quercetin	n.d.	n.d.	22.444±2.177	735.602±23.164	369.426±42.132	366.599±2.588
t-Sinnamic Acid	n.d.	n.d.	n.d.	1006.886±8.900	1822.325±153.800	1847.049±25.667
Apigenin	n.d.	n.d.	n.d.	1134.384±13.500	459.091±33.865	565.873±7.900
Ramnetin	n.d.	n.d.	n.d.	1276.977±13.924	n.d.	412.085±3.147
Chrysin	26.918±1.856	53.532±4.789	n.d.	14529.339±160.871	17320.796±158.988	14427.877±156.995
Pinocembrin	29.082±2.666	36.156±4.265	n.d.	15641.482±106.963	16249.461±153.455	14226.449±140.007
CAPE	n.d.	n.d.	n.d.	5505.103±88.988	9779.230±100.025	6647.129±80.125

*n.d: not determined

DISCUSSION

In this study, the differences in the biochemical properties of propolis and bee bread samples, which are bee products of different races and ecotypes, were investigated, regardless of their growing conditions. First of all, in order to reveal how the known antioxidant and antimicrobial properties of bee products vary according to race and ecotype differences, antioxidant activity values were investigated by different methods in determining and following up the total phenolic and flavonoid content. In addition, by HPLC analysis, the amount of a series of standard phenolics and flavonoids in the samples in question was calculated.

Since it is known that the extraction solvent has an effect on the biological activity, we preferred to work with ethanol solvent so that there could not be another variability parameter due to the fact that ethanolic propolis extract has higher activity among different solvents such as ethanol, water, methanol and ethyl acetate. Also,ethanol is not toxic and can be easily removed after extraction if propolis extracts are to be used as a food ingredient (Ma et al., 2016; Usman et al., 2016).

Total antioxidant activity findings calculated for both propolis and bee bread samples show a high correlation with total phenolic (R^2=0.8035) and flavonoid (R^2=0.7364) content amounts. Guzelmeric et al. (2021) also calculated high correlations between the phenolic content values of propolis samples and the antioxidant activity findings, although they made the following comment: Although a high TPC value means antioxidant potential, for the estimation of the sample with the highest antioxidant activity, it is preferred to perform more than one antioxidant test, especially in complex matrices such as propolis, because it has a different mechanism of action. It can be said that the results obtained are very high compared to some data reported in the literature. Shehata et al., (2020) investigated propolis samples from different geographic regions and while the values they obtained vary as 210-313 mg GAE for phenolic content for 100 g sample, it is in the range of 96.30-162.03 mg CAT/100 g for flavonoid content. Whereas, it is clear that both phenolic and flavonoid content values of propolis samples obtained from the Western, Central and Eastern Black Sea Regions are more compatible with our findings (Guzelmeric et al., 2021). Our findings are also consistent with the bee bread sample obtained from Morocco (Bakour et al., 2019).

It is estimated that the antioxidant effect of phenolic and flavonoids, which are detected at varying rates in bee bread and propolis samples, can eliminate the negative effects of free radicals in various ways, namely by different mechanisms. They can exert their effects in various ways, such as removing radicals that ignite peroxidation, preventing the initiation of reactive species by binding metal ions, and interrupting the auto-oxidation chain reaction.

The antioxidant activities of the samples, whose total antioxidant activities were calculated in this way, were also tested with different methods. Based on these findings, the antioxidant activities of the samples were also evaluated by two different methods such as DPPH free radical scavenging and Fe^{2+}

chelating tests. Thus, it can be said that the results obtained from these two methods are correlated in a way that supports each other.

Apart from these, the lipid peroxidation inhibition activities of our study samples were examined and very small concentrations of tested propolis samples inhibited ABAP-induced lipid peroxidation in the medium, which was in agreement with that obtained in the first two antioxidant test methods. The inhibitory effect of propolis is roughly 50 times more effective than bee bread.

It is known that oxidative stress, especially lipid peroxidation, is important in carcinogenesis. Increased levels of lipid peroxidation products are involved in the early stages of tumor growth (Padmavathi et al., 2006). Determination of lipid peroxidation inhibition property of chemicals represents a reliable measure of antioxidant properties as it mimics the efficacy of an antioxidant compound in preventing oxidative damage to lipoproteins or cell membrane more than other methods (Gregoris and Stevanato, 2010). There is evidence that propolis attenuates aluminum-induced lipid peroxidation in male rats (Newairy et al., 2009). Gregoris and Stevanato (2010), who studied Venetian propolises, attributed the lipid peroxidation inhibition potential they observed in the propolis they tested to the presence of caffeic acid and its derivatives. Indeed, the amounts of α-caffeic acid and CAPE in the propolis samples examined in the present study vary in proportion to their lipid peroxidation inhibition potentials.

It is also known that propolis prevents inflammation by inhibiting the release of various mediators, platelet aggregation and the synthesis of exazonoids (prostaglandin and leukotrienes).Galangin and quercetin components are the most active flavonoids in preventing inflammation. In addition, there are reports that the caffeic acid phenyl ester component, which is abundant in propolis samples collected from temperate regions such as Turkey, reduces inflammation by inhibiting lymphokine production and T cell proliferation, as well as cytokine and chemokine production(Valenzuela-Barra et al., 2015). It can be said that the types of inflammation in which these components responsible for the anti-inflammatory activity are effective are different. Studies have shown that CAPE and galangin are effective in preventing foot edema, pleurisy and joint inflammation (arthritis), while quercetin is effective in preventing rheumatoid arthritis (Borrelli et al., 2002).

Existing samples were examined for their anti-inflammatory properties by a simple spectrophotometric assay. While there is more data in the literature regarding the presence of anti-inflammatory activity in propolis, pollen and royal jelly samples (Kolaylı et al., 2016), it is rare in the case of bee bread. Therefore, the anti-inflammatory activity values determined in the current study, although low value for bee bread samples, will be valuable in terms of literature.

The known anti-inflammatory activity of propolis is due to the components it contains such as phenolic acids and their esters, flavonoids, steroids, terpenoids and amino acids. Propolis reveals its anti-inflammatory activity by inhibiting cyclooxygenase (COX), prostaglandin and nitric oxide biosynthesis, reducing the cytokine level and thanks to its antioxidant and immunosuppressive activities (Braakhuis, 2019). Although it is reported in the literature that propolis samples may have anti-inflammatory activity due to the components they contain, there are hardly any studies on different propolis samples to confirm this information. Valenzuela-Barra et al. (2015), who investigated the anti-inflammatory effect of propolis samples obtained from two different locations in Región Metropolitana de Santiago, Chile, showed that the anti-inflammatory activity in the type with higher phenolic and flavonoid content was also more effective. The anti-inflammatory activities of honey and propolis samples collected from a Langstroth beehive in a mountain forest in north-central Portugal, especially in the Sabugal (Guarda) region, were tested with the same method and it was revealed that propolis samples prevented BSA denaturation more effectively than honey samples (Afonso et al, 2020). Protein denaturation occurs in health problems that develop as a result of an inflammatory reaction, such as rheumatoid arthritis. Prevention of this denaturation is possible with nonsteroidal antiinflammatory drugs (NSAIDs). The potential of the bee products we examined to inhibit protein denaturation due to heating may contribute to their anti-inflammatory properties and have a significant effect on anti-arthritic activity. Gallic acid, chrysin, Quercetin, phenethyl ester, caffeic acid, luteolin, kaempferol and hesperetin found in honey have been shown to have anti-inflammatory and immunomodulatory effects (Zaidi et al., 2019). The fact that the detected phenolic acids are higher in number and amount in propolis samples is the basis of our findings.

Soltani et al. (2020), in their study, investigated the

effectiveness of 5 different propolis species obtained from Algeria on various fish pathogens and showed that a minimum of 1000 µg/mL of all tested species could only inhibit *V. anguillarum.* Therefore, it is clear that the calculated MIC values for the propolis samples that you can see in Table 2 show that the propolis samples we have are much more effective. The smallest amount of *Apis mellifera anatoliaca* propolis sufficient to inhibit the growth of *V. anguillarum and L. garvieae* can be associated with the highest total phenolic and flavonoid content and total antioxidant activity of this sample.

It has already been described that the antimicrobial activity of propolis is mainly against Gram-positive bacteria (Marcucci et al., 2001).Thus, it is consistent with the literature that although propolis samples were more effective exhibiting small MIC values against gram-positive *L. garvieae*, they showed antimicrobial activity against gram-negative *V.anguillarum* only with larger MIC values, and no activity was observed against all other gram-negative species tested.While Burdock (1998) attributes this capacity to the presence of aromatic acids and esters, Takaisi-Kikuni and Schilcher (1994) and Cushnie and Lamb (2005) have suggested that it is due to the action of the flavonone pinocembrin and the flavonolgalangin and CAPE, which act on the inhibition of bacterial RNA polymerase. The effect is thought to involve disruption of the cytoplasmic membrane of bacteria causing damage which triggers cell autolysis through loss of potassium ions (Soltani et al., 2020).

Among the propolis samples we evaluated, those with favorable findings have the potential to function as natural antimicrobial agents, especially in the fish farming industry. Given the limited data on the antibacterial action of propolis in aquaculture, the significance of these results is increased.The small number of studies (Eswaran and Bhargava, 2014; Kieliszek et al., 2018) examining the antimicrobial activity of bee bread samples on selected microorganism species in the literature supports our inability to find activity in bee bread and shows that the study provides new data entry to the literature.

The presence of 25 phenolic compounds, including caffeic acid and CAPE, was examined in all samples to display the phenolic profiles. It is known that p-coumaric acid, which is detected at varying rates in all samples, including bee bread, and caffeic acid, whose presence is only detected in propolis extracts, reduces oxidative damage caused by hypoxia, thanks to their neuroprotective effects (Cruz et al., 2016).The anti-diabetic properties of caffeic acid found in propolis samples are known (Spilioti et al., 2014).

Phenolic acids and flavonoids detected in bee products tested are important antioxidant and anti-inflammatory molecules, and are responsible for the apitherapeutic properties of these products. It has been shown by various studies that these phenolic compounds have also medicinal properties. For example, pinocembrin, which was detected in almost all of the samples, but more in propolis samples, has been shown to be an effective flavonoid in neurodegenerative and cardiovascular diseases (Nyokat et al., 2017). The flavonoid of chrysin, whose presence in samples varies in parallel with pinosembrin, is known for its anti-tumoral properties (Kasala et al., 2015).

CONCLUSIONS

Based on the findings, the difference in antioxidant, antimicrobial and anti-inflammatory properties of two different bee products was revealed, depending only on the race and ecotype difference. It was concluded that propolis samples were significantly more effective in terms of the tested parameters. Propolis sample obtained from the *Apis mellifera anatoliaca* strain, especially colonized in Ordu province conditions, contains a higher level of secondary metabolites compared to the others. However, it should not be forgotten that the superiority in biological activity values, which we interpret as being due to race, may also depend on feeding and collecting habits, since no botanical origin analysis was performed. The results of the present study are indications that, it is possible to obtain bee products at the desired level by raising prominent breeds independently of environmental conditions in order to provide ingredients for a wide variety of sectors where bee products are used.

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CONFLICT OF INTEREST

The author declare no conflict of interest.

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