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In Vitro Research on Antimicrobial Activity of Native Anatolian Honey Bee Products against *Paenibacillus larvae* Strains

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ABSTRACT

Worldwide, one of the most damaging diseases in beekeeping is American Foulbrood (AFB). The causative agent of the disease is *Paenibacillus larvae*, which can remain in spore form in the environment for decades and does not lose its virulence. In the management of this disease, it is inevitable to find an alternative method to the use of antibiotics and burning the hives. In this study, after determining the Total Phenolic (TPC) and Total Flavonoid Contents (TFC) of seven different Anatolian honey bee products (bee venom, bee bread, pollen, royal jelly, propolis, queen bee larvae, drone brood larvae), in vitro antimicrobial activities of these products against two different *P. larvae* strains were tested. As a result of Folin-Ciocalteu and $AlCl_3$ colorimetric methods, there were significant differences between the samples, and the highest content values were obtained from the propolis samples. The antimicrobial activity results showed that, *P. larvae* strains were susceptible to all bee products, except queen bee larvae and drone brood larvae. The most significant inhibition was obtained from Anatolian bee venom with the lowest MIC dose 6.25 µg mL^{-1}, Bacterial strains showed susceptibility to Anatolian beebread with an effective dose of 7.81 µg mL^{-1} following bee venom. This study is an important first step in identifying new active compounds for the use of in-hive natural products in the development of new preventive treatments against AFB disease, alternative to conventional antibiotic treatments.

Keywords: Bee venom, American Foulbrood, Anatolian beebread, Treatments against AFB.

INTRODUCTION

One of the reasons why the expected yield from beekeeping is not always achieved at the desired level is the bacterial diseases that bee colonies are exposed to. These diseases affect honey bees in their larval and adult stages and cause significant economic losses. Among the bacterial diseases seen in honey bee larvae, American Foulbrood (AFB) and European Foulbrood (EFB) are highly contagious and dangerous (Forsgren, 2010; Moharrami *et al*., 2022). The World Organization for Animal Health (WOAH) has accepted these diseases in the list of notifiable diseases that affect veterinary public health worldwide, posing a serious threat to the safe international trade of honey bees and their products (Genersch, 2010). These diseases are the most important causes of colony losses and low yields. The disease is highly virulent and dangerous not only for individual larvae but also for the entire colony (Morse and Calderon, 2000). Disease agents can be encountered at any stage of the bee's life cycle, but they are most commonly encountered during the egg stage (Rauch *et al.,* 2009).

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The AFB disease agent *P. larvae* is a Gram (+) and spore-forming bacterium. *P. larvae* spores are highly resistant to heating, adverse conditions, and chemical agents. These spores contaminate both honey and pollen, and are transmitted to larvae through contaminated food (Genersch, 2010). Some *Paenibacillus* species have been reported to be opportunistic human infections and can cause spoilage in pasteurized dairy products (Grady *et al.,* 2016). *P. lentimorbus* and *P.popilliae* cause infection in scarab beetle grubs, while *P. larvae* can cause infection in honey bee (*Apis mellifera*) larvae.

Although the use of antibiotics in beekeeping in European countries is prohibited, the European Food Safety Authority (EFSA) reported that there were antibiotic residues in honey samples (Chung *et al*., 2017; Savarino *et al*. 2020). The use of Tylovet and Lincomix has been approved in the USA to control this disease while Pennox 50 (oxytetracycline) and Terramycin (oxytetracycline hydrochloride) are present for controlling either foulbrood diseases (Mosca *et al*., 2023). In order to control the bacteria that cause the disease, it has become necessary to search for new drugs with different mechanisms of action against the development of resistance resulting from the use of inappropriate chemicals (Alpay Karaoğlu, 2014). Antibiotics are only effective on the vegetative form of the *P. larvae*. Antibiotic administration may temporarily hide or suppress symptoms, but the disease may reappear more severely (Borum, 2014).

Natural products such as plant extracts, plant essential oils, antimicrobial peptides, and propolis are shown as alternative options (Raut and Karuppayil 2014; Alvarenga *et al.,* 2021; Wang 2021). Cases in the advanced stages of the disease are difficult to treat. However, if the disease has just started and is diagnosed early, there is a chance of prevention of transmission and spread. The hive with suspected disease should be removed from the apiary urgently and quickly (Borum, 2014).

Bee products such as propolis, bee venom, honey and royal jelly are used in "Apitherapy" in many countries. Due to the role of bees in pollinating flowers, beekeeping is one of the indispensable agricultural activities all over the world (Etxegarai-Legarreta and Sanchez-Famoso, 2022). It is thought that apitherapy products will be useful against bee diseases for the sustainability of beekeeping activities with a healthier and higher yield (Sevim *et al*., 2021; Šedivá *et al*., 2018; Naglaa *et al*., 2020). Propolis is known as a strong antimicrobial substance, consisting of a mixture of different pollen, oils, special resins, and waxy collected by honey bees from the buds and sprouts of plants. It is used to close holes and cracks in the hive, repair honeycombs, glue honeycombs together, polish honeycomb eyes, narrow the hive entrance, protect from bee diseases, and prevent their development by neutralizing disease agents (Wagh, 2013). The effect of propolis against microorganisms is its most important biological feature. It ensures that fungi and bacteria remain at a lower level in the hive. Propolis is a natural bee product that has been used by humans since ancient times due to its pharmacological properties (Wagh, 2013; Bogdanov, 2017). Pollen is the male reproductive unit that forms on the antennae of flowering plants and is involved in fertilization.

Honey bees collect pollen from flowers with their feet and deposit it on their hind legs. It mixes the pollen with digestive enzymes and some nectar and stores it in the honeycomb cells (Bogdanov, 2015a). Depending on the source, pollen has biological effects such as being antimicrobial, antitumoral (prostate and breast cancers), antioxidant, antiaging, anti-osteoporosis, anti-anemia, anti-diarrhea, memory enhancer, probiotic, regenerative, performance-enhancing, and aphrodisiac (Bogdanov, 2015a).

Drone brood larvae are obtained by collecting larvae between 3-7 days of age (Bărnuţiu *et al.,* 2013). There are many androgenic hormones, sugars, amino acids,

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fatty acids, and a small amount of minerals in its content (Altan *et al.,* 2013). Due to the androgenic hormones, it contains, it is used to increase sperm count, as an aphrodisiac, and in bodybuilding (Mărgăoan *et al.,* 2017). Bee venom is produced in the venom glands of worker bees and stored in the venom bag (Bogdanov, 2015b). Newly emerged bees from the honeycomb cells have very little ability to produce venom and reach their highest capacity when they are 12 days old. Melittin is a peptide consisting of 26 amino acids that is the most abundant in bee venom (Rady *et al.,* 2017). Melittin is a cytolytic peptide that is nonspecific and can attack the lipid bilayer, thus leading to toxicity. This peptide is a powerful agent that increases membrane permeability, and with this feature, it causes antibacterial, antifungal, antiviral and anticancer activity (Kohno *et al.,* 2014; Pandidan and Mechler, 2019).

Until now, there is limited information available regarding antimicrobial properties of Anatolian bee products against *P. larvae*, even though it is well known for its strong inhibitory effects against other Gram (+) bacteria (Sonmez *et al.,* 2023, 2022; Kekecoglu *et al.,* 2021, 2022; Popova *et al.,* 2005; Erkmen and Ozcan, 2008). Owing to these reasons, the aim of the present study was to test the antimicrobial activity of seven different bee products obtained from Anatolian honey bees (*A. mellifera anatoliaca*, Yığılca ecotype) against the pathogen *P. larvae* that causes serious economic losses in the beekeeping industry.

MATERIALS AND METHOD

Sample Preparation

All bee products [Royal Jelly (RJ), Drone Brood Larvae (DBL), Queen Bee Larvae (QBL), Bee Venom (BV), Bee Pollen (BP), Bee Bread (BB), and propolis samples] used in the study were produced and analyzed at Düzce University Beekeeping Research Development and Application Center (DAGEM), Düzce, Turkey. All samples were obtained from three randomly selected healthy colonies in similar conditions and free of pesticides. The hive type was wooden Langstroth, and the bee species forming the colony was Yığılca ecotype belonging to the *A. mellifera anatoliaca*. Raw propolis samples were pulverized using a laboratory type blender (Waring, commercial blender). These samples were weighted as 50 g, and 500 mL of 96% ethanol (Sigma-Aldrich) was transferred into the samples. The resulting mixture was shaken at 150 rpm for 72 hours and then filtered using filter paper. In order to remove the ethanol in the filtrate, the samples were kept in the evaporator (IKA RV10) at 50-60°C for 10 minutes. The amount was determined by weighing the remaining resinous part, and stock solutions were obtained using 70% ethyl alcohol with each sample containing 10% propolis content (0.1 g mL^{-1}) (Kekecoglu *et al*., 2021).

To collect RJ sample, 3-day-old larvae in the queen bee cells were pulled out of the cells with the help of tweezers. Fresh royal jelly remaining in the cells was collected into opaque bottles using a spatula and, immediately, stored at −18°C. The obtained royal jelly samples were diluted with distilled water in sterile Eppendorf tubes. DBL and QBL were obtained directly from the opened or unsealed eyes of the honeycomb on 4-9 and 5-7 days after hatching, respectively. Each sample was homogenized with a tissue homogenizer and then freeze-dried at -70°C. For dehydration, the samples were kept at 0.1 bar at -55°C for 72 hours (Sonmez *et al.,* 2023). The obtained lyophilized samples were stored at -20ºC until further experiments. To dissolve the homogenates, 70% ethyl alcohol was transferred into 5 mg of the sample and this mixture was vortexed for 15 min, then, shaken at room temperature for 8 hours. The BV sample was obtained by the method previously mentioned by Sonmez *et al.* (2022). For BB and BP samples, 0.4 g of each bee product was weighted and dissolved in the same volume of 70% ethanol and methanol. Samples were shaken for 2 hours at room temperature to obtain the maximum amount of bioactive components. Finally, maximum dissolution and sterile homogenates were obtained and used in further studies.

Bacterial Culture and Growth Conditions

The bacterial samples used in the study (*P. larvae* ATCC 9545 (ERIC I) and *P. larvae* DSM 25430 (ERIC II)) were commercially purchased. *P. larvae* strains were revived from the culture collection in the microbiology research laboratory of Recep Tayyip Erdoğan University. The chemicals and bacteria growth media used in the study were purchased commercially.

Bacterial strains were inoculated on MYPGP agar [Mueller-Hinton broth (10 g L^{-1}), yeast extract (15 g L^{-1}), K_2HPO_4 (3 g L^{-1}), sodium pyruvate (1 g L^{-1}) (Fisher), glucose (2%) (Merck), and agar 14 g L^{-1}] and incubated at 37°C for 3-4 days in a 5% CO_2 incubator. After the bacteria were revived, single colonies were taken and pure cultures were cultured on MYPGP agar, then, overnight cultures were prepared from pure cultures (Sevim *et al.,* 2021).

Determination of Antimicrobial Activity

The antibacterial activities of the samples used in the study were tested against *P. larvae* ATCC 9545 (ERIC I) and *P. larvae* DSM 25430 (ERIC II) strains using the agar-well diffusion method (Fünfhaus *et al*., 2018). Bacterial density was prepared as McFarland 0.5 (10^8 CFU mL^{-1}) and spread over the entire surface of the MYPGP agar medium with a sterile cotton swab. Five mm wells were made/prepared at 2 cm intervals with the help of a sterile cork borer in the agar plates. Fifty µL of the test samples were poured into the wells in the overlaid plates and the plates were incubated at 37°C for 48 hours in 5% CO_2. Antimicrobial activity was evaluated by calculating the net inhibition zone, diameters in mm (Sevim *et al*., 2021).

Minimal Inhibition Concentration values (MIC) were determined using the microdilution technique (CLSI, 2015; Alpay Karaoğlu *et al.,* 2022). Test samples were serially diluted in microplate wells containing MYPGP liquid medium. Turbidity suspensions of 0.5 McFarland (10^8 CFU mL^{-1}) were prepared from overnight cultures of *P. larvae* strains. After 10 µL of the bacterial suspensions were poured into each well containing the test samples, micro-plates were incubated in a 5% CO_2 incubator at 37°C for 48 hours. Ampicillin (10 µg mL^{-1}) was used as standard control, and ethanol (99%) and methanol as solvent control. The wells at the lowest concentration without bacterial growth were determined as the MIC values (CLSI, 2015) and the antimicrobial effect of each bee product was tested in triplicate.

Determination of Total Phenolic Content

The total phenolic content of honeybee products was determined by using the Folin Ciocalteu method according to the published protocols with minor changes (Singleton *et al.,* 1999). After 20 mL of methanol extract from each sample was mixed with 680 mL of dH_2O, 0.5 mol L^{-1} Folin-Ciocalteu reagent was added to this mixture. In the next step, the mixture was vortexed for 2 min and after 400 mL of 10% Na2CO3 was added, it was kept at room temperature for 2 hours. The absorbance of the samples was measured at 760 nm, and the results were given in mg Gallic Acid Equivalents (GAE) per gram of sample.

Determination of Total Flavonoid Content

Total flavonoid amounts of propolis, BP and BB were determined by making minor changes in the $AlCl_3$ colorimetric method described in Fukumoto and Mazza, (2000). Each sample was taken into volumetric bottles of 2 mL and 20 mL of methanol and 1 mL of 5% $AlCl_3$ were added. After the mixture was incubated for 30 min at room

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temperature, the absorbance value was measured at 420 nm. Each sample value was expressed as mg Quercetin Equivalent g^{-1} (mg QE g^{-1}).

Statistical analysis

Each tested parameter for each sample was done in triplicate and as descriptive statistics, mean, standard deviation, median, minimum and maximum values were obtained. Mann-Whitney U test was performed to determine the variation of inhibition zone and MIC values according to bacterial strains, and Kruskal-Wallis H test was performed to determine the variation according to bee products. Spearman correlation coefficient was used for the relationship between variables. The significance level was taken as .05. Data were analyzed with SPSS 26.

RESULTS

Bee products obtained from DAGEM significantly inhibited the growth of *P. larvae* strains in cultures with different MIC doses. The obtained results are summarized in Table 1. The zones of inhibition varied between 0-28 mm, demonstrating that many of the samples inhibited the bacterial strains on the agar medium. In the agar well method, the largest inhibition zone was obtained from BV and propolis A with a diameter of 28 and 26 mm, respectively. DBL and QBL did not create any inhibitory zones against the tested pathogens.

Among the honeybee products, the lowest MIC values of 3.125 µg mL^{-1} were recorded for BV, while DBL and QBL samples that were not able to inhibit the growth of the pathogens showed no activity during the MIC test either.

In present study, we detected an important antimicrobial effect from Anatolian BB samples and the MIC results of BB varied according to the solvent used. The obtained MIC values were 7.81 µg mL^{-1} for ethanol extract against both *P. larvae* strains. The effectiveness values obtained from the methanol extract were 15.62 and 31.25 µg mL^{-1} for ATCC 9545 and DMG 9820 strains, respectively.

The MIC values of RJ was highest (250 µg mL^{-1}) compared to other tested honeybee products. The MIC values of samples A and B of the propolis were different and sample A (7.81 µg mL^{-1}) had lower MIC values than sample B (15.62 µg mL^{-1}). Inhibition zone and MIC values were not significantly different according to bacterial strains ($U= 0.000$; $P= 1.000$). Inhibition zone and MIC values differed significantly according to bee products ($U= 14.955$; $P< 0.037$ and $U= 15$; $P= 0.036$, respectively). The inhibition zone obtained from BV was higher than RJ, and the MIC value was lower and significant (Table 2).

Table 1. Agar well diffusion and MIC values of the Anatolian honeybee products against *P. larvae* strains.

Bee products	*Paenibacillus larvae* ATCC 9545		*Paenibacillus larvae* DMG 9820	
	Inhibition zone (mm)	MIC (µg mL^{-1})	Inhibition Zone (mm)	MIC (µg mL^{-1})
Bee venom	28	3.125	28	3.125
Royal jelly	8	250	8	250
Bee bread (Ethanol)	22	7.81	20	7.81
Bee bread (Methanol)	18	15.62	16	31.25
Pollen (Ethanol)	14	31.25	15	31.25
Pollen (Methanol)	15	31.25	15	31.25
Propolis A	26	7.81	26	7.81
Propolis B	24	15.62	24	15.62
Drone brood larvae	-	-	-	-
Queen bee larvae	-	-	-	-

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Table 2. Correlation analysis of the variables. [a]

	Inhibitation Zones	MIC	Total Phenolic	Total Flavonoid
Inhibitation Zones	-	0.206	0.562	0.299
MIC	0.445	-	0.410	0.554
Total Phenolic	**0.**023	0.115	-	0.262
Total Flavonoid	0.261	0**.**026	0.327	-

[a] The above-diagonal Spearman correlation coefficient is the P value for the below-diagonal correlation coefficient.

Table 3. Total phenolic and flavonoid content of Anatolian honeybee products. [a]

	Total Phenolic content (mg GAE g^{-1})	Total flavonoids (mg QE g^{-1})
Bee venom	0.82 ± 0.08 [0.79 (0.76-0.91)]	0.03 ± 0.01 [0.03 (0.02-0.04)]
Royal jelly	3.87 ± 0.16 [3. 94 (3.69-3.98)]	0.89 ± 0.11 [0.90 (0.78-0.99)]
Bee bread	9.06 ± 0.18 [9 (8.92-9.26)]	2.11 ± 0.21 [2.01 (1.97-2.35)]
Pollen	8.82 ± 0.89 [8.67 (8.01-9.78)]	3.90 ± 0.11 [3.90 (3.79-4.01)]
Drone brood larvae	10.86 ± .18 [10.84 (10.65-11.08)]	0.08 ± 0.085 [0.04 (0.03-0.21]
Queen bee larvae	11.05 ± 0.06 [11.05 (11.01-11.09)]	0.15 ± 0.06 [0.15 (0.11-0.19)]
Propolis A	166.30 ± 1.50 [165.94 (165.01-167.95)]	83.01± 0.18 [82.92 (82.89-83.22)]
Propolis B	152.76 ± 0.59[152.68 (152.21-153.39)]	81.70 ± 0.55 [81.64 (81.18-82.28)]

[a] $\bar{x} \pm \mathrm{sd}$ [Median (Min − Max)]

In Table 3, the Total Phenolic (TPC) and Flavonoid Contents (TFC) of honeybee products are presented. According to the results of seven different samples analyzed with the Folin–Ciocalteu method, the sample with the highest total phenolic content was Propolis A with a value of 166.30 mg GAE g^{-1}. The lowest amount of phenolic substance was determined from the BV sample. Determination of total phenolic content was done for BB, pollen and propolis samples. The highest total phenolic substance content was detected in the propolis A sample, as was the total phenolic content. The honeybee product containing the lowest flavonoid component was determined as BV with a value of 0.03 mg QE g^{-1}.

Moreover, according to the statistical analysis results, a significant positive correlation was obtained between inhibition zones and total phenolic (r= 0.562; P= 0.023) and between MIC and total flavonoids (r= 0.554; P= 0.026) (Table 2).

DISCUSSION

Honey bees are an important part of the food supply chain for both pollination and commercial beekeeping activities. Although honey bees are among the most important pollinators, their lives are under threat because they are infected with various pathogens. The most important of these pathogens is *P. larvae* that causes AFB (Dickel *et al.,* 2022). Today, the management of this disease is the burning of the diseased hives, or prophylactic feeding of antibiotics to the hives practiced in some countries (Genersch, 2010). However, the resistance developed by bacteria against the use of antibiotics and the residues in foods has become an increasing global problem. Also, the use of antibiotics is not effective against these bacterial spores, and their use is related to the alteration of gut microbiota and the modification of the development of bee behavior (Raymann and Moran, 2018; Ortiz-Alvarado *et al.,* 2020). In order to prevent this disease in honey bees, it is necessary to develop sustainable and non-chemical solutions, alternative to the use of antibiotics, and burn the hives. Antimicrobial peptides are thought to be one

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of the mechanisms that affect the resistance of honey bees to AFB infection of colonies (Evans, 2004; Decanini *et al.,* 2007; Chan *et al.,* 2009). These natural antimicrobial peptides found in snake, scorpion, and BV inhibits the pathogens by breaking their membranes, moreover, the bacteria do not develop resistance to these peptides (Ventola, 2015). In addition to these natural peptides, many researchers reported that the resistance of colonies to AFB was associated with larval feeding (Šedivá *et al.,* 2018). In line with these data, this study aimed to test the effectiveness of bee products, which are known to be natural antimicrobial agents against *P. larvae*. All tested bee products, except DBL and QBL, significantly inhibited the growth of two different strains of *P. larvae* at rates ranging from 6.25 to 62.5 μg mL^{-1}. Among these important bee products, BV was the most effective against both bacterial strains at the lowest dose. Studies about the antimicrobial activity of BV against bacterial strains that cause AFB are very limited. Lee *et al.* (2016) investigated the antimicrobial effect of one of the BV peptides, secapin (AcSecapin-1) against *P. larvae* and reported the MIC_{50} value as 11.13 μM. Fernández *et al.* (2014) tested the efficacy of BV against five different strains of *P. larvae* and they obtained MIC values between 3.12 to 8.33 μg/mL. A previous study reported that Anatolian BV was highly effective against yeast like fungi, Gram (+) and Gram (-) bacteria (Sonmez *et al.,* 2022). In the present study, Anatolian BV significantly affected the growth and development of *P. larvae* strains and were effective against the pathogen at very low MIC dose (for both strains 6.25 μg mL^{-1}).

In this study, another bee product that is significantly effective against *P. larvae* was BB. To our knowledge, no such study were present in the literature that tests the effectiveness of BB against this honeybee pathogen. Hence, our studies could be significant, highlighting the efficiency of BB against this pathogenic bacteria. However, Iorizzo *et al.* (2020) isolated *Lactobacillus plantarum* strains from BB and investigated its antimicrobial effect against *P. larvae*. They reported that isolated *Lactobacillus* strains were able to inhibit *P. larvae* growth. Considering the compatibility with the previous study (Iorizzo *et al.*, 2020), the low MIC values obtained from this study may be an indication that the probiotic bacteria in the content of BB play an active role in the defense of the immune system of the honey bees against these bacteria.

Like all insects, honeybees produce antimicrobial peptides to defend themselves against pathogens (Ilyasov *et al.,* 2013). The most important of these antimicrobial peptides are low molecular weight proteins and peptides in RJ (Ramanathan *et al*., 2018). Bílıková *et al.* (2001) tested the efficacy of one of these peptides, i.e. royalicin, against *P. larvae* and other Gram (+) bacteria using disk diffusion method and reported that this peptide inhibits the growth of this pathogenic bacteria. In a similar study, Bachanová *et al.* (2002) suggested that royalicin and other peptides are responsible for activity against *P. larvae* and other Gram (+) bacteria. In another study, Hornitzky, (1998) reported that RJ had a bactericidal effect against the vegetative form of *P. larvae* after application of 5 minutes. Šedivá *et al.* (2018) investigated the antibacterial effects of trans-10-Hydroxy-2-Desenoic Acid (10-HDA), an important fatty acid of RJ, against *P. larvae* strains, including all Enterobacterial Repetitive Intergenic Consensus (ERIC) genotypes and reported that 10-HDA showed higher activity against these genotypes with decreasing pH. 10-HDA is an important component of RJ responsible for antimicrobial activity, and it has been reported in previous studies that this fatty acid derivative was found at a high level in Anatolian RJ (Sonmez *et al.,* 2023). Anatolian RJ, whose effectiveness was tested in this study, was also found to be effective against two different *P. larvae* strains. This high inhibition activity was thought to be due to its 10-HDA content, and it can be suggested that this bee product

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may have a broad-spectrum of protective effect in microbial infections occurring in the hive.

Propolis has been used for many years due to its high biological activity. However, this high efficiency could not be evaluated to form a useful model about honey bee diseases that damage the beekeeping industry. Özkırım *et al.* (2014) investigated the antimicrobial activity of 18 ethanol extracts of propolis samples against 10 different *P. larvae* isolates and reported that the bacterial strains were susceptible to all tested samples. Chen *et al.* (2018) tested the efficacy of Taiwan green propolis on some Gram (+) bacteria and *P. larvae* using different extraction methods and showed that the average MIC value was 20 $\mu g\ mL^{-1}$. Fangio *et al.* (2019) and Antunez *et al.* (2008) reported that ethanol extracts of propolis samples formed different inhibition zones with values varying between 20-30 mm against *P. larvae* by disk diffusion method. Sevim *et al.* (2021) tested the potential antimicrobial activity of Anatolian propolis against *P. larvae* PB35 and SV35 strains and determined the MIC value as 74.87 µg/mL. It has been reported in previous studies that Anatolian propolis is effective against both Gram (+) and Gram (-) bacteria because of its high phenolic and flavonoid content (Kekecoglu *et al.,* 2021, 2022; Velikova *et al.,* 2000; Uzel *et al.,* 2005; Katırcıoglu and Mercan, 2006). In the present study, two different Anatolian propolis samples (A-B), which were tested for their effectiveness against the pathogen that causes severe honey bee and crop loss in hives, also caused high inhibition with low rates of MIC values (7.81 and 15.62 ug mL^{-1} respectively). Considering the total phenolic and flavonoid content of Anatolian propolis examined in this study, it is not surprising that a very low effective dose was obtained. For this reason, Anatolian propolis samples may have the potential to be used as an alternative disinfectant solution for the use as antibiotics in hives.

For many years, besides its nutritional properties, the biological properties of BP and the therapeutic effects resulting from this activity have been known worldwide (Soares de Arruda *et al.,* 2021). However, no study tested the effectiveness of this protein and lipid-rich product against *P. larvae*. Grubbs *et al.* (2021) reported that the Actinobacteria strain of the genus *Streptomyces* isolated from pollen stores exhibited significant inhibitory activity against *P. larvae*. In this study, BP samples, whose antimicrobial effect was evaluated by using two different solvents, were also effective against this pathogen with low MIC doses (31.25 $\mu g\ mL^{-1}$). Hence, for the very first time, we show that BP well known for its high nutritional value, acts as a strong antimicrobial control agent against the *P.larvae* that causes bee larval disease.

The total phenolic and flavonoid content and amounts of honey bee products vary according to the collected geographical region, collection time, vegetation cover, climate and bee race (Campos *et al.,* 2015; Arruda *et al.,* 2013). It is known that these bioactive components, which differ in each product, are also responsible for antimicrobial activity (Fatima *et al.,* 2014; Al-Juhaimi *et al.,* 2022; Kekecoglu *et al.,* 2021). In previous studies, it was reported that there was a positive correlation between total phenolic substance and antimicrobial activity (Pereira *et al.,* 2007; Estevinho *et al.,* 2008; Nazzaro *et al.,* 2013). Soares de Arruda *et al.* (2021) reported that they observed moderate and weak correlations between total phenolics, total flavonols, and antibacterial activity parameters. However, Morais *et al.* (2011) showed that there was no relationship between total phenolic substance and antimicrobial activity, and the extract containing a lower percentage of phenolic substances was more effective against microorganisms. In our study, although a positive correlation was obtained between total phenolic substance and antimicrobial activity among propolis samples, no correlation was found between antimicrobial activity with RJ, BB, pollen, QBL, and DBL.

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AL-Ani *et al.* (2018) reported that the bioactivities obtained from propolis and other bee products are not only due to the content of phenolic-flavonoid substances, but due to the synergistic effect between these biologically active substances. With these results, it can be concluded that the antimicrobial activity is not only due to the total phenolic and flavonoid substances, but also to the synergistic effect of the different components in these natural products.

CONCLUSIONS

In this study, very effective antimicrobial activity results were obtained from different bee products against pathogenic bacteria that cause serious damage to honey bee colonies. In particular, bee venom had a good potential to inhibit AFB destruction in colonies. The obtained MIC values were evaluated as an important result showing that these natural products have the potential to be used in the control of AFB disease. It is recommended that these products should be used as a preventative in larval feeding or hives before disease transmission.

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تحقیقات آزمایشگاهی در مورد فعالیت ضد میکروبی محصولات زنبورعسل بومی آناتولی در برابر لارو سویه های لارو پانی باسیلوس (*Paenibacillus*)

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چکیده

در سراسر جهان، یکی از ضرربارترین بیماری ها در زنبورداری، بیماری فولبرود آمریکایی (AFB) است. عامل بیماری لارو پانی باسیلوس است که می تواند ده ها سال به صورت هاگ (spore form) در محیط باقی بماند و قدرت بیماری زایی خود را از دست ندهد. در مدیریت این بیماری، یافتن روشی جایگزین برای مصرف آنتی بیوتیک و سوزاندن کندو اجتناب ناپذیر است. در این پژوهش، پس از تعیین میزان فنول کل (TPC) و کل فلاونوئید (TFC) هفت محصول مختلف زنبور عسل آناتولی (زهر زنبور عسل، نان زنبور عسل (bee bread)، گرده، ژل رویال، بره موم(propolis) ، لارو ملکه زنبور عسل، لارو نوزادان (drone brood larvae)، فعالیت ضد میکروبی این محصولات در برابر دو سویه مختلف *P. larvae* در شرایط آزمایشگاهی آزمایش شد. در اثر روش های رنگ سنجی Folin–Ciocalteu و AlCl3 بین نمونه ها تفاوت معنی داری وجود داشت و بیشترین مقدار محتوایی از نمونه های برهموم به دست آمد. نتایج فعالیت ضد میکروبی نشان داد که تمام محصولات زنبور عسل به سویه‌های *P. larvae* حساس بودند، به جز لارو ملکه زنبور عسل و لارو بچه‌ها. بیشترین میزان بازدارندگی از زهر زنبور عسل آناتولی با کمترین دوز MIC برابر ۶/۲۵ میکروگرم بر میلی لیتر بدست آمد. بعد از زهر زنبور عسل، سویه های باکتریایی به نان زنبورعسل آناتولی با دوز موثر ۷.۸۱ میکروگرم در میلی لیتر حساسیت نشان دادند. این بررسی اولین گام مهم در شناسایی ترکیبات موثر جدید برای استفاده از محصولات طبیعی درون کندو برای جایگزینی درمان‌های آنتی‌بیوتیک معمولی است و برای توسعه درمان‌های پیشگیرانه جدید علیه بیماری AFB.

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