

1 **ACCEPTED ARTICLE**

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

4  
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14  
15 **ABSTRACT**

16 One of the most damaging diseases in beekeeping worldwide is American Foulbrood. The  
17 causative agent of the disease is *Paenibacillus larvae*, which can remain in spore form in the  
18 environment for decades and does not lose its virulence. In the management of this disease, it  
19 is inevitable to find an alternative method to the use of antibiotics and burning the hives. In this  
20 study, after determining the total phenolic (TPC) and total flavonoid content (TFC) of seven  
21 different Anatolian honey bee products (bee venom, bee bread, pollen, royal jelly, propolis,  
22 queen bee larvae, drone brood larvae), in vitro antimicrobial activities of these products against  
23 two different *P. larvae* strains were tested. As a result of Folin-Ciocalteu and AlCl<sub>3</sub> colorimetric  
24 methods, there were significant differences between the samples, and the highest content values  
25 were obtained from the propolis samples. The antimicrobial activity results showed that, *P.*  
26 *larvae* strains were susceptible to all bee products except queen bee larvae and drone brood  
27 larvae. The most significant inhibition was obtained from Anatolian bee venom with the lowest  
28 MIC dose 6.25 µg/mL. Bacterial strains showed susceptibility to Anatolian bee bread with an  
29 effective dose of 7.81 µg/mL following bee venom. This study is an important first step in  
30 identifying new active compounds for the use of in-hive natural products in the development of  
31 new preventive treatments against AFB disease, alternative to conventional antibiotic  
32 treatments.

33 **Key words:** Bee products, *Paenibacillus larvae*, American Foulbrood, Antimicrobial activity.

34  
35 **INTRODUCTION**

36 One of the reasons why the expected yield from beekeeping is not always achieved at the  
37 desired level is the bacterial diseases that bee colonies are exposed to. These diseases affect  
38 honey bees in their larval and adult stages and cause significant economic losses. Among the  
39 bacterial diseases seen in honey bee larvae, American Foulbrood (AFB) and European

40 Foulbrood (EFB) are highly contagious and dangerous (Forsgren, 2010; Moharrami *et al.*,  
41 2022). The World Organization for Animal Health (WOAH) has accepted these diseases in the  
42 list of notifiable diseases and affect veterinary public health worldwide, posing a serious threat  
43 to the safe international trade of honey bees and their products (Genersch, 2010). These diseases  
44 are the most important causes of colony losses and low yields. The disease is highly virulent  
45 and dangerous not only for individual larvae but also for the entire colony (Morse and Calderon,  
46 2000). Disease agents can be encountered at any stage of the bee's life cycle, but are most  
47 commonly encountered during the egg stage (Rauch *et al.*, 2009).

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

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

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

71 Bee products such as propolis, bee venom, honey and royal jelly are used in "Apitherapy" in  
72 many countries. Due to the role of bees in pollinating flowers, beekeeping is one of the  
73 indispensable agricultural activities all over the world (Etxegarai-Legarreta and Sanchez-

74 Famoso, 2022). It is thought that apitherapy products will be useful against bee diseases for the  
75 sustainability of beekeeping activities with a healthier and higher yield (Sevim *et al.*, 2021;  
76 Šedivá *et al.*, 2018; Naglaa *et al.*, 2020). Propolis is known as a strong antimicrobial substance,  
77 consisting of a mixture of different pollen, oils, special resins, and waxy collected by honey  
78 bees from the buds and sprouts of plants. It is used to close holes and cracks in the hive, repair  
79 honeycombs, glue honeycombs together, polish honeycomb eyes, narrow the hive entrance,  
80 protect from bee diseases, and prevent their development by neutralizing disease agents (Wagh,  
81 2013). The effect of propolis against microorganisms is its most important biological feature.  
82 It ensures that fungi and bacteria remain at a lower level in the hive. Propolis is a natural bee  
83 product that has been used by humans since ancient times due to its pharmacological properties  
84 (Wagh, 2013; Bogdanov, 2012). Pollen is the male reproductive unit that forms on the antennae  
85 of flowering plants and is involved in fertilization.

86 Honey bees collect pollen from flowers with their feet and deposit it on their hind legs. It  
87 mixes the pollen with digestive enzymes and some nectar and stores it in the honeycomb cells  
88 (Bogdanov, 2011a). Depending on the source, pollen has biological effects such as being  
89 antimicrobial, antitumoral (prostate and breast cancers), antioxidant, antiaging, anti-  
90 osteoporosis, anti-anemia, anti-diarrhea, memory enhancer, probiotic, regenerative,  
91 performance-enhancing, and aphrodisiac (Bogdanov, 2011a).

92 Drone brood larvae are obtained by collecting between 3-7 days of age of larvae (Bărnuțiu *et*  
93 *al.*, 2013). There are many androgenic hormones, sugars, amino acids, fatty acids, and a small  
94 amount of minerals in its content (Altan *et al.*, 2013). Due to the androgenic hormones, it  
95 contains, it is used to increase sperm count, as an aphrodisiac, and in bodybuilding (Mărgăoan  
96 *et al.*, 2017). Bee venom is produced in the venom glands of worker bees and stored in the  
97 venom bag (Bogdanov, 2011b). Newly emerged bees from the honeycomb cells have very little  
98 ability to produce venom and reach their highest capacity when they are 12 days old. Melittin  
99 is a peptide consisting of 26 amino acids that is the most abundant in bee venom (Rady *et al.*,  
100 2017). Melittin is a cytolytic peptide that is nonspecific and can attack the lipid bilayer, thus  
101 leading to toxicity. This peptide is a powerful agent that increases membrane permeability, and  
102 with this feature, it causes antibacterial, antifungal, antiviral and anticancer activity (Kohno *et*  
103 *al.*, 2014; Pandidan and Mechler, 2019).

104 Until now, there is limited information available regarding antimicrobial properties of  
105 Anatolian bee products against *P. larvae*, even though it is well known for its strong inhibitory  
106 effects against other Gram (+) bacteria (Sonmez *et al.*, 2023, 2022; Kekecoglu *et al.*, 2021;  
107 2022; Popova *et al.*, 2005; Erkmen and Ozcan 2008). Owing to these reason the aim of the

108 present study was to test the antimicrobial activity of seven different bee products obtained  
109 from Anatolian honey bees (*A. mellifera anatoliaca*, Yığılca ecotype) against the pathogen *P.*  
110 *larvae*, which causes serious economic losses in the beekeeping industry.

111

## 112 MATERIALS AND METHOD

### 113 Sample Preparation

114 All bee products (royal jelly (RJ), drone brood larvae (DBL), queen bee larvae (QBL), bee  
115 venom (BV), bee pollen (BP), bee bread (BB), and propolis samples) used in the study were  
116 produced and analyzed at Düzce University Beekeeping Research Development and  
117 Application Center (DAGEM), Düzce, Turkey. All samples obtained from three randomly  
118 selected healthy, similar conditions and free of pesticides colony. The hive type is wooden  
119 Langstroth, and the bee species forming the colony was Yığılca ecotype belonging to the *A.*  
120 *mellifera anatoliaca*. Raw propolis samples were pulverized using a laboratory type blender  
121 (Waring, commercial blender). These samples were weighted as 50 gr and 500 mL of 96%  
122 ethanol (Sigma-Aldrich) was transferred into the samples. The resulting mixture was shaken at  
123 150 rpm for 72 h and then filtered using filter paper. In order to remove the ethanol in the  
124 filtrate, the samples were kept in the evaporator (IKA RV10) at 50-60 °C for 10 minutes. The  
125 amount was determined by weighing the remaining resinous part, and stock solutions were  
126 obtained using 70% ethyl alcohol with each sample containing 10% propolis content (0.1 g/mL)  
127 (Kekecoglu *et al.*, 2021).

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

141 maximum amount of bioactive components. Finally, maximum dissolution and sterile  
142 homogenates were obtained and used in further studies.

143

#### 144 **Bacterial culture and Growth conditions**

145 The bacterial samples used in the study (*P. larvae* ATCC 9545 (ERIC I) and *P. larvae* DSM  
146 25430 (ERIC II)) were commercially purchased. *P. larvae* strains were revived from the culture  
147 collection in the microbiology research laboratory of Recep Tayyip Erdoğan University. The  
148 chemicals and bacteria growth media used in the study were purchased commercially.

149 Bacterial strains were inoculated on MYPGP agar (Mueller-Hinton broth (10 g.L<sup>-1</sup>), yeast  
150 extract (15 g.L<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub> (3 g.L<sup>-1</sup>), sodium pyruvate (1 g.L<sup>-1</sup>) (Fisher), glucose (2%) (Merck),  
151 and agar 14 g.L<sup>-1</sup>) and incubated at 37 °C for 3-4 days in a 5% CO<sub>2</sub> incubator. After the bacteria  
152 were revived, single colonies were taken and pure cultures were cultured on MYPGP agar and  
153 then overnight cultures were prepared from pure cultures (Sevim *et al.*, 2021).

154

#### 155 **Determination of Antimicrobial Activity**

156 The antibacterial activities of the samples used in the study were tested against *P. larvae*  
157 ATCC 9545 (ERIC I) and *P. larvae* DSM 25430 (ERIC II) strains using the agar-well diffusion  
158 method (Fünfhaus *et al.*, 2018). Bacterial density was prepared as McFarland 0.5 (10<sup>8</sup> CFU/mL)  
159 and spread over the entire surface of the MYPGP agar medium with a sterile cotton swab. Five  
160 millimetre wells were made/prepared at 2 cm intervals with the help of a sterile cork borer in  
161 the agar plates. 50 microliters of the test samples were poured into the wells in the overlaid  
162 plates and the plates incubated at 37 °C for 48 h in 5% CO<sub>2</sub>. Antimicrobial activity was  
163 evaluated by calculating the net inhibition zone, diameters in millimeters (Sevim *et al.*, 2021).

164 Minimal inhibition concentration values (MIC) were determined using the microdilution  
165 technique (CLSI, 2015; Alpay Karaoğlu *et al.*, 2022). Test samples were serially diluted in  
166 microplate wells containing MYPGP liquid medium. Turbidity suspensions of 0.5 McFarland  
167 (10<sup>8</sup> CFU/mL), were prepared from overnight cultures of *P. larvae* strains. After 10 µL of the  
168 bacterial suspensions were poured into each well containing the test samples, microplates were  
169 incubated in a 5% CO<sub>2</sub> incubator at 37 °C for 48 h. Ampicillin (10 µg/mL) was used as standard  
170 control, ethanol (99%) and methanol as solvent control. The wells at the lowest concentration  
171 without bacterial growth were determined as the MIC values (CLSI, 2015) and the antimicrobial  
172 effect of each bee product was tested in triplicate.

173

#### 174 **Determination of Total Phenolic Content**

175 The total phenolic content of honey bee products was determined by using the Folin Ciocalteu  
176 method according to the published protocols with minor changes (Singleton *et al.*, 1999). After  
177 20 mL of methanol extract from each sample was mixed with 680 mL of dH<sub>2</sub>O, 0.5 mol/L  
178 Folin-Ciocalteu reagent was added to this mixture. In the next step, the mixture was vortexed  
179 for 2 min and after 400 mL of 10% Na<sub>2</sub>CO<sub>3</sub> was added, it was kept at room temperature for 2  
180 h. The absorbance of the samples was measured at 760 nm, and the results were given in mg  
181 gallic acid equivalents (GAE) per gram of sample.

182  
183

#### 184 **Determination of Total Flavonoid Content**

185 Total flavonoid amounts of propolis, BP and BB were determined by making minor changes  
186 in the AlCl<sub>3</sub> colorimetric method described in Fukumoto and Mazza, (2000). Each sample was  
187 taken into volumetric bottles of 2 mL and 20 mL of methanol and 1 mL of 5% AlCl<sub>3</sub> were  
188 added. After the mixture was incubated for 30 min at room temperature, the absorbance value  
189 was measured at 420 nm. Each sample value was expressed as mg quercetin equivalent/g (mg  
190 QE/g).

191  
192

#### 192 **Statistical analysis**

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

200  
201

#### 201 **RESULTS**

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

208 Among the honey bee products, the lowest MIC values of 3.125 µg/mL were recorded for BV  
 209 while, DBL and QBL samples, that were not able to inhibit the growth of the pathogens showed  
 210 no activity during the MIC test either.

211 In present study we detected an important antimicrobial effect from Anatolian BB samples  
 212 and the MIC results of BB varied according to the solvent used. The obtained MIC values were  
 213 7.81 µg/mL for ethanolic extract against both *P. larvae* strains. The effectiveness values  
 214 obtained from the methanolic extract were 15.62 and 31.25 µg/mL for ATCC 9545 and DMG  
 215 9820 strains, respectively.

216

217

218 **Table 1.** Agar well diffusion and MIC values of the Anatolian honey bee products  
 219 against *P. larvae* strains.

Bee products	<i>Paenibacillus larvae</i> ATCC 9545		<i>Paenibacillus larvae</i> DMG 9820	
	Inhibition zone (mm)	MIC (µg/mL)	Inhibition Zone (mm)	MIC (µg/mL)
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	-	-	-	-

220

221 The MIC values of RJ was highest (250 µg/mL) compared to other tested honeybee products.

222 The MIC values of samples A and B of the propolis were different and sample A (7.81 µg/mL)  
 223 had lower MIC values than sample B (15.62 µg/mL). **Inhibition zone and MIC values were**

224 **not significantly different according to bacterial strains (U=.000; p=1.000). Inhibition zone**

225 **and MIC values differed significantly according to bee products (U=14.955; p<.037 and**

226 **U=15; p=.036, respectively). The inhibition zone obtained from BV was higher than RJ,**

227 **and the MIC value was lower and significant (Table 2).**

228

229

**Table 2:** Correlation Analysis of the variables.

	Inhibition Zones	MIC	Total Phenolic	Total Flavonoid
Inhibition Zones	-	.206	.562	.299
MIC	.445	-	-.410	.554
Total Phenolic	<b>.023</b>	.115	-	.262
Total Flavonoid	.261	<b>.026</b>	.327	-

230 \* The above-diagonal Spearman correlation coefficient is the p value for the below-diagonal correlation  
 231 coefficient.  
 232

233 In Table 3, the total phenolic (TPC) and flavonoid content (TFC) of honey bee products are  
 234 presented. According to the results of seven different samples analyzed with the Folin–  
 235 Ciocalteu method, the sample with the highest total phenolic content was Propolis A with a  
 236 value of 166.30 mg GAE/g. The lowest amount of phenolic substance was determined from the  
 237 BV sample. Determination of total phenolic content was done for BB, pollen and propolis  
 238 samples. The highest total phenolic substance content was detected in the propolis A sample,  
 239 as was the total phenolic content. The honey bee product containing the lowest flavonoid  
 240 component was determined as BV with a value of 0.03 mg QE/g.

241 **Moreover according to the statistical analysis results a significant positive correlation was**  
 242 **obtained between inhibition zones and total phenolic (r<sup>562</sup>; p=.023) and between MIC**  
 243 **and total flavonoids (r=.554; p=.026) (Table 2).**

244  
 245 **Table 3.** Total phenolic and flavonoid content of Anatolian honey bee products.

	Total Phenolic content ( mg GAE/g )	Total flavonoids (mg QE/g)
Bee venom	.82 ± .08 [ .79 (.76-.91)]	.03 ± .01 [.03 (.02-.04)]
Royal jelly	3.87 ± 0.16 [ 3. 94 (3.69-3.98)]	0.89 ± 0.11 [.90 (.78-.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)]	.08 ± .085 [.04 (.03-.21)]
Queen bee larvae	11.05 ± .06 [11.05 (11.01-11.09)]	.15 ± .06 [ .15 (.11-.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)]

246 \*  $\bar{x} \pm sd$  [Median (Min – Max)]

247  
 248 **DISCUSSION**

249 Honey bees are an important part of the food supply chain for both pollination and commercial  
 250 beekeeping activities. Although honey bees are among the most important pollinators, their  
 251 lives are under threat because they are infected with various pathogens. The most important of  
 252 these pathogens is *P. larvae* that causes AFB (Dickel *et al.*, 2022). The management of this  
 253 disease is the burning of the diseased hives today, or prophylactic feeding of antibiotics to the  
 254 hives practiced in some countries (Genersch, 2010). However, the resistance developed by  
 255 bacteria against the use of antibiotics and the residues in foods has become an increasing global  
 256 problem worldwide. Also the use of antibiotics is not effective against these bacterial spores

257 and their use is related to the alteration of gut microbiota and the modification of the  
258 development of bee behavior (Raymann and Moran, 2018; Ortiz-Alvarado *et al.*, 2020). In order  
259 to prevent this disease in honey bees, it is necessary to develop sustainable and non-chemical  
260 solutions, and alternatives to the use of antibiotics and burn the hives. Antimicrobial peptides  
261 are thought to be one of the mechanisms that affect the resistance of honey bees to AFB  
262 infection of colonies (Evans, 2004; Decanini *et al.*, 2007; Chan *et al.*, 2009). These natural  
263 antimicrobial peptides found in snake, scorpion, and BV cause inhibit the pathogens by  
264 breaking their membranes, moreover the bacteria do not develop resistance to these peptides  
265 (Ventola, 2015). In addition to these natural peptides, many researchers reported that the  
266 resistance of colonies to AFB was associated with larval feeding (Šedivá *et al.*, 2018). In line  
267 with these data, this study, it was aimed to test the effectiveness of bee products, which are  
268 known to be natural antimicrobial agents, against *P. larvae*. All tested bee products except DBL  
269 and QBL significantly inhibited the growth of two different strains of *P. larvae* at rates ranging  
270 from 6.25 to 62.5 µg/mL. Among these important bee products, BV was the most effective  
271 against both bacterial strains at the lowest dose. Studies about the antimicrobial activity of BV  
272 against bacterial strains that cause AFB are very limited. Lee *et al.* (2016) investigated the  
273 antimicrobial effect of one of the BV peptides, secapin (AcSecapin-1) against *P. larvae* and  
274 reported the MIC<sub>50</sub> value as 11.13 µM. Fernández *et al.* (2014) tested the efficacy of BV against  
275 five different strains of *P. larvae* and they obtained MIC values between 3.12 to 8.33 µg/mL. It  
276 was reported in a previous study that Anatolian BV is highly effective against yeast like fungi,  
277 Gram (+) and Gram (-) bacteria (Sonmez *et al.*, 2022). The present study, Anatolian BV  
278 significantly affected the growth and development of *P. larvae* strains and were effective  
279 against the pathogen at very low MIC dose (for both strains 6.25 µg/mL).

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

289 Like all insects, honeybees produce antimicrobial peptides to defend themselves against  
290 pathogens (Ilyasov *et al.*, 2013). The most important of these antimicrobial peptides are low

291 molecular weight proteins and peptides in RJ (Ramanathan *et al.*, 2018). Bíliková *et al.* (2001)  
292 tested the efficacy of one of these peptides, royalicin, against *P. larvae* and other Gram (+)  
293 bacteria using disk diffusion method and reported that this peptide inhibits the growth of this  
294 pathogenic bacteria. In a similar study, Bachanová *et al.* (2002) suggested that royalicin and  
295 other peptides are responsible for activity against *P. larvae* and other Gram (+) bacteria. In  
296 another study, Hornitzky, (1998) reported that RJ had a bactericidal effect against the vegetative  
297 form of *P. larvae* after application of 5 min. Šedivá *et al.* (2018) investigated the antibacterial  
298 effects of trans-10-hydroxy-2-desenoic acid (10-HDA), an important fatty acid of RJ, against  
299 *P. larvae* strains, including all Enterobacterial Repetitive Intergenic Consensus (ERIC)  
300 genotypes and they reported that 10-HDA showed higher activity against these genotype with  
301 decreasing pH. 10-HDA is an important component of RJ responsible for antimicrobial activity,  
302 and it has been reported in previous studies that this fatty acid derivative was found at a high  
303 level in Anatolian RJ (Sonmez *et al.*, 2023). Anatolian RJ, whose effectiveness was tested in  
304 this study, was also found to be effective against two different *P. larvae* strains. This high  
305 inhibition activity was thought to be due to its 10-HDA content, and it can be suggested that  
306 this bee product may have a broad-spectrum protective effect in microbial infections occurring  
307 in the hive.

308 Propolis has been used for many years due to its high biological activity. However, this high  
309 efficiency could not be evaluated to form a useful model about honey bee diseases that damage  
310 the beekeeping industry. Özkırım *et al.* (2014) investigated the antimicrobial activity of 18  
311 ethanolic extracts of propolis samples against 10 different *P. larvae* isolates and they reported  
312 that the bacterial strains were susceptible to all tested samples. Chen *et al.* (2018) tested the  
313 efficacy of Taiwan green propolis on some Gram (+) bacteria and *P. larvae* using different  
314 extraction methods and showed that the average MIC value was 20 µg/mL. Fangio *et al.* (2019)  
315 and Antunez *et al.* (2008) reported that ethanolic extracts of propolis samples formed different  
316 inhibition zones with values varying between 20-30 mm against *P. larvae* by disk diffusion  
317 method. Sevim *et al.* (2021) tested the potential antimicrobial activity of Anatolian propolis  
318 against *P. larvae* PB35 and SV35 strains and determined the MIC value as 74.87 µg/mL. It has  
319 been reported in previous studies that Anatolian propolis is effective against both Gram (+) and  
320 Gram (-) bacteria because of its high phenolic and flavonoid content (Kekecoglu *et al.*, 2021,  
321 2022; Velikova *et al.*, 2000; Uzel *et al.*, 2005; Katircioglu and Mercan, 2006). In present study,  
322 two different Anatolian propolis samples (A-B), which were tested for their effectiveness  
323 against the pathogen that causes severe honey bee and crop loss in hives, also caused high  
324 inhibition with low rates of MIC values (7.81 and 15.62 µg/mL respectively). Considering the

325 total phenolic and flavonoid content of Anatolian propolis examined in this study, it is not  
326 surprising that a very low effective dose was obtained. For this reason Anatolian propolis  
327 samples may have the potential to be used as an alternative disinfectant solution to the use of  
328 antibiotics in hives.

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

338 The total phenolic and flavonoid content and amounts of honey bee products vary according  
339 to the collected geographical region, collection time, vegetation cover, climate and bee race  
340 (Campos *et al.*, 2015; Arruda *et al.*, 2013). It is known that these bioactive components, which  
341 differ in each product, are also responsible for antimicrobial activity (Fatima *et al.*, 2014, Al-  
342 Juhaimi *et al.*, 2022, Kekecoglu *et al.*, 2021). In previous studies, it was reported that there is a  
343 positive correlation between total phenolic substance and antimicrobial activity (Pereira *et al.*,  
344 2007; Estevinho *et al.*, 2008; Nazzaro *et al.*, 2013). Soares de Arruda *et al.* (2021) reported that  
345 they observed moderate and weak correlations between total phenolics, total flavonols and  
346 antibacterial activity parameters. However, Morais *et al.* (2011) in their study, showed that there  
347 was no relationship between total phenolic substance and antimicrobial activity, and the extract  
348 containing a lower percentage of phenolic substances was more effective against  
349 microorganisms. In this study, although a positive correlation was obtained between total  
350 phenolic substance and antimicrobial activity among propolis samples, no correlation was  
351 found between antimicrobial activity with RJ, BB, pollen, QBL, and DBL.

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

358

359 **CONCLUSION**

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

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