Archives of Razi Institute, Vol. 78, No. 3 (2023) 899-905

DOI: 10.22092/ARI.2022.360019.2531

Original Article

Anti-Bacterial Activity of Four Distinct Propolis Extracts against *P. larvae* and *M. plutonius;* Etiological Agent of American and European Foulbrood Disease of Honeybees

Toutiaee, S[1], Mojgani, N[2]*, Harzandi, N[1], Moharrami, M[2], Mokhberalsafa, L[2]

1. Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran
2. Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Received 18 September 2022; Accepted 8 October 2022
Corresponding Author: dnmoj@yahoo.com

Abstract

Four different propolis samples obtained from different regions of Iran were evaluated for their antibacterial effects against the bacterial agents responsible for two important honeybee diseases. *Paenibacillus larvae* (*P. larvae*) and *Melissococcus plutonius* (*M. plutonius*), as the etiological agents of American foulbrood (AFB) and European foulbrood (EFB) diseases, were subjected to propolis ethanolic extracts in the agar well diffusion assay. The minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC) of the antibacterial effects of the samples against the two indicator organisms were determined by the microdilution technique using different concentrations of the propolis extracts. Finally, the synergistic antibacterial actions of the mixed propolis samples were determined, and their MIC and MBC values were recorded. A two-way analysis of variance was used to evaluate correlations among the diameters of the inhibition zones, the bacterial agents, and the propolis extracts. Based on our results, three of the propolis samples showed significant antibacterial effects against *P. larvae* and *M. plutonius* during the agar well diffusion assay. Furthermore, the antibacterial capacity of the propolis samples, when mixed in equal proportions, was significantly enhanced, as indicated by the obtained MIC and MBC values. Approximately, 0.02 mg/mL of mixed propolis samples was required for inhibiting the growth of both pathogens. A direct correlation was observed between propolis concentrations and their antibacterial activity. The results of the study are conclusive of the significant antibacterial actions of Iranian propolis samples against the etiological agents of the mentioned honeybee diseases, suggesting their probable use as a safe biological agent to control AFB and EFB diseases.

Keywords: American foulbrood disease, Antibacterial activity, Ethanol extraction, European foulbrood disease, Propolis

1. Introduction

Honeybees, *Apis mellifera* (*A. mellifera*), are ecologically and economically important for their critical role as plant pollinators. However, their survival is endangered by diverse and potentially virulent pathogens that threaten hive health. A decline in the number of native bees, such as *A. mellifera*, due to the spread of the American foulbrood (AFB) disease caused by *Paenibacillus larvae* (*P. larvae*) and the European foulbrood (EFB) diseases caused by *Meliscoccus plutonius* (*M. plutonius*), has had devastating effects on bees' biodiversity (1). The mentioned bacterial agents are responsible for adverse consequences especially affecting bees' survival and population, which alternatively affects honeybee products, such as honey and royal jelly. Disease

management strategies employed to control the spread of these diseases are of immense importance to insure bees' survival and colony health. In most developed countries, antibiotic therapy is not recommended, and its use in many animals has been banned owing to the emergence of drug-resistant strains. Ultimately, the attention of scientists and researchers has been diverted towards the use of different biologically active and natural products that could control the spread of several diseases while being safe and economical for use. Some biologically active antibacterial ingredients studied to date include probiotics, essential oils, phytochemicals, specific immunoglobulin Y antibodies, and propolis (2-4).

Propolis is a resinous, waxy substance that honeybees (*A. melllifera*) create by mixing their salivary secretions and some enzymes with beeswax, along with compounds from various plants and trees, including poplar, palm, pine, conifer secretions, gums, resins, mucilage, and leaf buds (5). Several *in vitro* and *in vivo* studies analyzing the properties and components of different propolis extracts showed the significant effect of this honeybee-related compound on the health of man and animals. Over the past couple of years, chemical constituents present in propolis have been consistently updated, and several new chemical compounds have been discovered. The substances that make up propolis can be complex, and to date, more than 420 compounds with variable biological activities have been identified in different propolis samples (6, 7).

The effective role of propolis obtained from different regions against several honeybee diseases, including AFB and EFB, has been reported. The antibacterial activity of propolis against AFB was illustrated by Lindenfelser as early as the 1960s. This researcher screened 15 different propolis samples from the USA against *P. larvae* and concluded that all 15 samples were able to inhibit the growth of this bacteria at the concentrations of 100 µg/mL (8). However, in this study, for the very first time, we report the antibacterial efficacy of Iranian propolis extracts obtained from geographically different areas of Iran against AFB and EFB diseases under *in vitro* conditions. Additionally, to the best of our knowledge, this is the first report which describes the synergistic antibacterial actions of propolis extracts against the mentioned honeybee diseases.

2. Materials and Methods

2.1. Bacterial Cultures and Growth Conditions

Bacterial cultures, including *P. larvae* (CIP 104052) and *M. plutonius* (CIP 104618), were obtained from the Pasteur Institute, France. *P. larvae* was cultivated in MYPGP medium at 37ºC with the addition of 5% CO_2, while *M. plutonius* was cultivated in the modified basal agar MBA (9) under micro-aerobic conditions using an atmosphere generation system (Oxoid Anaerobic Jar, Thermo Fisher Scientific, USA). For long-term preservation, the pure bacteria culture suspension was frozen in microtubes at -80ºC with 20% v/v glycerol.

2.2. Propolis Sampling

Four different raw propolis samples obtained from honeybees belonging to common *A. mellifera* species were studied during the present study. The samples were collected from local beekeepers in four geographically distinct regions in Iran (including Hamedan, Khorram Abad, Karaj, and Damavand) during April and May 2020. All samples were transported to the laboratory, wrapped tightly in aluminum foils, and kept at -20ºC until use.

2.3. Extraction of Propolis Samples

The frozen raw propolis samples were initially ground, and 10 g of powdered samples from each propolis was poured into 500 mL glass flasks containing 100 mL of 70% ethanol. The samples were stirred on a shaker for 48 h at room temperature in a dark place. The suspension was filtered using the Whatman No. 4 filter paper and later evaporated using the Rotary evaporator (Heidolph, Laborota 4000, Germany) at 40ºC for approximately one to two hours. All samples were stored in the dark at -20ºC until use.

2.4. Antibacterial Activity of Phenolic Extracts

The antibacterial activity of the prepared propolis extracts against *P. larvae* and *M. plutonius* was

determined by the agar well diffusion assay. An overnight bacterial suspension of the pathogens (10^6 cfu/mL) was spread evenly on the surface of agar plates with a sterile cotton swab. The wells were punched into the plates using a sterile cork-borer and were then filled with 50 µL of propolis extracts (at concentrations of 0.1 g/mL), and all plates were incubated at 37ºC for 48 h. The halo zones appearing around the wells were measured and recorded in millimeters.

2.5. Minimum Inhibitory Concentrations

The microdilution method (10) was used to determine the minimum inhibitory concentration (MIC) values of the prepared propolis extracts against *P. larvae* and *M. plutonius*. Different concentrations of the ethanolic propolis extracts (0.05, 0.10, 0.20, 0.40, 0.60, 0.80, and 1.0 mg/mL) were prepared in microtiter plates in respective broth mediums. A total of 10 uL of the pathogens (10^6 cfu/mL) was added to individual wells, and the plates were incubated at 37ºC for 48 h. The MIC values were defined as the lowest concentration that showed no growth. Bacterial cultures in their respective broth medium (without propolis extracts) were used as the control.

2.6. Minimum Bactericidal Concentrations

To determine the minimum bacterial concentration (MBC) values of the ethanolic extracts of the propolis, the wells in the microtiter plates during the MIC determinations, which showed no bacterial growth, were plated on MYPGP and then MBA plates. After incubation at 37ºC for 48 h, the plates were observed for growth. The MBC was defined as the lowest concentration of the propolis extracts required to kill microorganisms.

2.7. MIC and MBC of Mixed Propolis Extracts

Synergistic antibacterial effects of the propolis were determined by mixing the selected propolis extracts in equal proportions (1:1:1:1) and determining the MIC and MBC against the mentioned bacterial strains by microdilution methods described above. All tests were run in triplicate to ensure the reproducibility of the results.

2.8. Statistical Analysis

A two-way analysis of variance was used to evaluate the relationship between the diameters of the inhibition zones against the used bacterial pathogens and the propolis samples. The significance level was set at $P<0.05$. The analysis of variance was performed using the Origin software (version 8.0, OriginLab Corp, USA).

3. Results

The prepared propolis extracts showed variable limits of antibacterial actions against the honeybee pathogens used in this study. As seen in figure 1, both bacterial pathogens were inhibited by the used propolis extracts. Although the effects were more pronounced against *M. plutonius*, compared to *P. larvae*, the differences were not statistically significant ($P>0.05$). Figure 2 also shows the antibacterial action of propolis extracts against *M. plutonius* by the agar well diffusion assay.

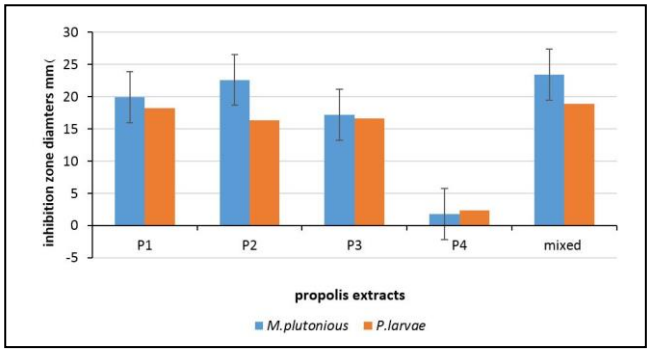

Figure 1. Antibacterial effects of four different propolis extracts (individually and mixed) against *P. larvae* and *M. plutonius* as evaluated by their inhibitory zone diameters by agar well diffusion assay

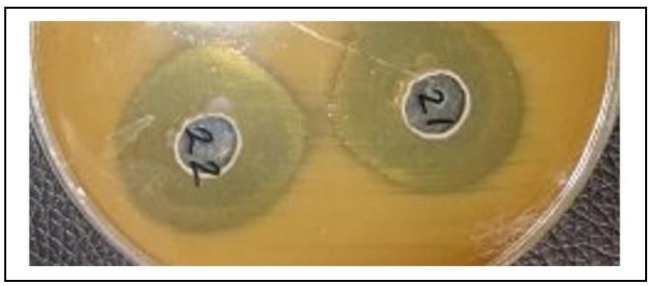

Figure 2. Antibacterial action of propolis extract against *M. plutonius* by agar well diffusion assay

Among the tested propolis samples, the P4 samples obtained from Damavand, Iran, did not show significant antibacterial effects against the indicator organisms and were thus omitted from further analysis.

Table 1 illustrates the MIC values of the propolis extracts against the bacterial indicator organisms used in this study. According to the obtained results, the P3 propolis samples obtained from Karaj, Iran, were most effective against the two pathogens, compared to the other two propolis samples. The MIC values for P3 against *P. larvae* and *M. plutonius* were 0.20 and 0.10 mg/mL, respectively ($P>0.05$), while these values were slightly higher in the other two propolis samples. The MBC values for the propolis extracts used in this study are shown in table 2. Based on the results, higher concentrations of propolis were required to kill the pathogens as their MBC values were higher than their MIC values ($P<0.05$). In the P3 samples, the MBC values were lower than those in the other two propolis samples. The P1 samples appeared to be least effective in killing these pathogens, and their MBC values were significantly higher (0.80 mg/mL) than P2 (0.60 mg/mL) and P3 (0.20 mg/mL) samples, respectively.

As can be seen in table 3, when the propolis extracts were mixed in equal proportions, the MIC and MBC values against both pathogens decreased to 0.10 mg/mL ($P<0.05$), showing the synergistic antibacterial actions of the three different propolis extracts.

Table 1. MIC of propolis extracts against *P. larvae* and *M. plutonius*

Propolis extract (mg/mL)	P1		P2		P3	
	P. larvae	*M. plutonious*	*P. larvae*	*M. plutonious*	*P. larvae*	*M. plutonious*
0.05	+ve	+ve	+ve	+ve	+ve	+ve
0.10	+ve	+ve	+ve	+ve	+ve	+ve
0.20	+ve	+ve	+ve	+ve	+ve	+ve
0.40	+ve	-ve	+ve	-ve	-ve	-ve
0.60	-ve	-ve	-ve	-ve	-ve	-ve
0.80	-ve	-ve	-ve	-ve	-ve	-ve
1.00	-ve	-ve	-ve	-ve	-ve	-ve

P1: propolis extract from Khoramabad; P2: propolis from Hamedan and P3: propolis extract from Karaj. +ve: growth recoded; -ve: no-growth recorded

Table 2. MBC of propolis extracts against *P. larvae* and *M. plutonius*

Propolis extract (mg/mL)	P1		P2		P3	
	P. larvae	*M. plutonious*	*P. larvae*	*M. plutonious*	*P. larvae*	*M. plutonious*
0.05	+ve	+ve	+ve	+ve	+ve	+ve
0.10	+ve	+ve	+ve	+ve	+ve	+ve
0.20	+ve	+ve	+ve	+ve	+ve	+ve
0.40	+ve	+ve	+ve	+ve	-ve	-ve
0.60	+ve	+ve	+ve	+ve	-ve	-ve
0.80	+ve	+ve	-ve	-ve	-ve	-ve
1.00	-ve	-ve	-ve	-ve	-ve	-ve

P1: propolis extract from Khoramabad; P2: propolis from Hamedan and P3: propolis extract from Karaj. +ve: growth recoded; -ve: no-growth recorded

Table 3. MIC and MBC values of mixed propolis extracts against *P. larvae* and *M. plutonius*

Mixed Propolis (mg/mL)	*P. larvae*		*M. plutonious*	
	MIC	MBC	MIC	MBC
0.05	+ve	+ve	+ve	+ve
0.10	+ve	+ve	+ve	+ve
0.20	-ve	-ve	-ve	-ve
0.40	-ve	-ve	-ve	-ve
0.60	-ve	-ve	-ve	-ve
0.80	-ve	-ve	-ve	-ve
1.00	-ve	-ve	-ve	-ve

P1: propolis extract from Khoramabad; P2: propolis from Hamedan and P3: propolis extract from Karaj. +ve: growth recoded; -ve: no-growth recorded

4. Discussion

An essential property of propolis, regardless of its origin, is its marked antibacterial, antiviral, antiparasitic, and antifungal activity (11, 12). In a study conducted by Veiga, De Mendonca (13), poplar propolis was shown to possess antibacterial effects against both Gram-positive and Gram-negative microorganisms. The antibacterial mechanisms of propolis include the inhibition of cell division, the collapse of microbial cytoplasm cell membranes and cell walls, the inhibition of bacterial motility, enzyme inactivation, bacteriolysis, and protein synthesis inhibition (14, 15). The multi-target antibacterial actions of propolis have made it a naturally active biological agent of great interest to researchers who search for alternative approaches to avoid the use of antibiotics and overcome drug resistance in microorganisms. Several extraction procedures have been used to evaluate the active chemical and biological components of propolis extracts, including ethanolic or aqueous extracts (8, 16). However, ethanolic extraction has been reported to be a more suitable procedure for the extraction of the majority of active components in propolis samples (14, 17). Therefore, in this study, we utilized ethanolic extraction procedures and evaluated the antibacterial activity of the samples against the two most important bacterial agents responsible for economically devastating honeybee diseases.

Several pathogens are known to weaken or lead to the collapse of honeybee colonies (18), among which AFB caused by the spore-forming Gram-positive bacterium *P. larvae* and EFB caused by Gram-positive bacterium *M. plutonius* are the main bacterial infections that affect honeybees' health by impairing their larval development (19, 20). In the present study, the selected propolis samples showed significant antibacterial effects against the growth of *P. larvae* and *M. plutonius*, with significant differences in the strength of activity in P1, P2, and P3, compared to P4 samples. The lower biological activity seen in the P4 samples, compared to other samples in the study, could be due to the low concentrations of active compounds present in the P4 propolis extracts. Moreover, the findings have shown that the strength of the antimicrobial activity of different propolis samples could differ based on the nature of the specific substances in each sample (17). The antibacterial actions of different propolis extracts against *P. larvae* and *M. plutonius* have been reported earlier by several other researchers (21, 22).

The antibacterial activity of the Iranian propolis samples against the mentioned pathogens was determined by the MIC assay. Different propolis extracts are reported to have different MIC and MBC values against pathogens depending on the procedure of extraction, geographical regions from which they were collected, and most importantly, the cell membrane of the respective pathogen. Variations in the biological activity of different propolis indirectly reflect the concentrations of the extracted biologically active substances, such as phenolic acid esters and flavonoids (pinocembrin and galangin) (23, 24). However, a synergistic action among the various active ingredients in propolis samples is believed to be the main factor in achieving the complex antimicrobial activity in this natural honeybee product (25). The results of our study indicated that concentrations of lower than 1 mg/mL are effective to inhibit and kill the studied pathogens and thus might be economical for use as biological agents to control AFB and EFB diseases in honeybees. The lower MIC and MBC values recorded mainly in P3 samples procured from Karaj, Iran, might indicate the higher percentage of galangin in these samples, compared to the other three propolis samples. Similarly, other researchers have also shown that low doses of propolis samples are effective against various Gram-positive and Gram-negative bacteria. Seidel, Peyfoon (26) showed that the MICs of propolis collected from North America, South America, and Europe range from 0.125 to 0.5 mg/mL, while the MICs of the samples of African and Asian origin range from 0.08 to >0.5 mg/mL.

In this study, for the very first time, we showed the synergistic antibacterial actions of propolis samples in combinations. The observed synergistic antibacterial effects might be due to the variable concentrations of different biologically active components present in each sample and the synergistic interactions among these active components, which result in enhanced activity (27). The mixed propolis samples showed that 0.1 mg/mL of the sample is effective enough to kill the respective pathogens.

In conclusion, our study is indicative of the antibacterial actions of the ethanol propolis extracts against *P. larvae* and *M. plutonius*. The synergistically enhanced activity of the propolis extracts was observed when all four propolis extracts were used in equal proportions. To the best of our knowledge, this is the first report that states the synergistic antibacterial actions of three propolis samples active against the bacterial agents responsible for AFB and EFB diseases in honeybees. Further investigations are in progress for evaluating the chemical components in these propolis samples and determining their role in antibacterial actions.

Authors' Contribution

Study concept and design: S. T. and N. M.
Collection of data: S. T. and N. M.
Analysis and interpretation of data: S. T.
Drafting of the manuscript: N. M.
Critical revision of the manuscript: N. M, N. H, and M. M.
Intellectual content: N. M.
Administrative, technical, and material support: S. T., N. M., N. H, M. M., and L. M.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Cuthbertson AGS, Brown MA. Issues affecting British honey bee biodiversity and the need for conservation of this important ecological component. Int J Environ Sci Technol. 2009;6(4):695-9.
2. Moharrami M, Mojgani N, Bagheri M, Toutiaee S. Role of Honey Bee Gut Microbiota in the Control of American Foulbrood and European Foulbrood Diseases. Arch Razi Inst. 2022;77(4):1331-9.
3. Tootiaie S, Moharrami M, Mojgani N. Honeybee Gut: Reservoir of Probiotic Bacteria .In: Mojgani N, Dadar M, editors. Probiotic Bacteria and Postbiotic Metabolites: Role in Animal and Human Health. Singapore: Springer Singapore; 2021. p. 221-36.
4. Toutiaee S, Mojgani N, Harzandi N, Moharrami M, Mokhberosafa L. In vitro probiotic and safety attributes of Bacillus spp. isolated from beebread, honey samples and digestive tract of honeybees Apis mellifera. Lett Appl Microbiol. 2022;74(5):656-65.
5. Anjum SI, Ullah A, Khan KA, Attaullah M, Khan H, Ali H, et al. Composition and functional properties of propolis (bee glue): A review. Saudi J Biol Sci. 2019;26(7):1695-703.
6. Bankova V, Galabov AS, Antonova D, Vilhelmova N, Di Perri B. Chemical composition of Propolis Extract ACF(R) and activity against herpes simplex virus. Phytomedicine. 2014;21(11):1432-8.
7. Milojkovic Opsenica D, Ristivojevic P, Trifkovic J, Vovk I, Lusic D, Tesic Z. TLC Fingerprinting and Pattern Recognition Methods in the Assessment of Authenticity of Poplar-Type Propolis. J Chromatogr Sci. 2016;54(7):1077-83.
8. Lindenfelser LA. In vivo activity of propolis against Bacillus larvae. J Invertebr Pathol. 1968;12(1):129-31.
9. Forsgren E, Budge GE, Charrière J-D, Hornitzky MAZ. Standard methods for European foulbrood research. J Apic Res. 2013;52(1):1-14.
10. Andrews JM. Determination of minimum inhibitory concentrations. J Antimicrob Chemother. 2001;48(1):5-16.
11. Agüero MB, Svetaz L, Baroni V, Lima B, Luna L, Zacchino S, et al. Urban propolis from San Juan province (Argentina): Ethnopharmacological uses and antifungal activity against Candida and dermatophytes. Ind Crops Prod. 2014;57:166-73.
12. Coelho GR, Senna Villar Kd, Figueiredo CA, Badari JC, Zucatelli Mendonça RM, Oliveira MI ,et al. Antiviral effects of Scaptotrigona postica propolis and their fractions. BMC Proc. 2014;8(4):P63.
13. Veiga RS, De Mendonca S, Mendes PB, Paulino N, Mimica MJ, Lagareiro Netto AA, et al. Artepillin C and phenolic compounds responsible for antimicrobial and

antioxidant activity of green propolis and Baccharis dracunculifolia DC. J Appl Microbiol. 2017;122(4):911-20.
14. Al-Ani I, Zimmermann S, Reichling J, Wink M. Antimicrobial Activities of European Propolis Collected from Various Geographic Origins Alone and in Combination with Antibiotics. Medicines (Basel). 2018;5(1).
15. Srinivas S, Gujjari AK, Kenganora M, Rudraswamy S, Ravi MB, Manjula S. Analysis of antimicrobial activity of Karnataka propolis against oral pathogens - An in vitro study. J Oral Maxillofac Pathol. 2021;25(3):449-56.
16. Chen YW, Ye SR, Ting C, Yu YH. Antibacterial activity of propolins from Taiwanese green propolis. J Food Drug Anal. 2018;26(2):761-8.
17. Garedew A, Schmolz E, Lamprecht I. Effect of the bee glue (propolis) on the calorimetrically measured metabolic rate and metamorphosis of the greater wax moth Galleria mellonella. Thermochimica Acta. 2004;413(1):63-72.
18. Goulson D, Nicholls E, Botias C, Rotheray EL. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 2015;347(6229):1255957.
19. de Graaf DC, Alippi AM, Antúnez K, Aronstein KA, Budge G, De Koker D, et al. Standard methods for American foulbrood research. J Apic Res. 2013;52(1):1-28.
20. Forsgren E. European foulbrood in honey bees. J Invertebr Pathol. 2010;103(l):5-9.
21. Fangio MF, Orallo DE, Gende LB, Churio MS. Chemical characterization and antimicrobial activity against Paenibacillus larvae of propolis from Buenos Aires province, Argentina. J Apic Res. 2019;58(4):626-38.
22. Isidorov VA, Buczek K, Zambrowski G, Miastkowski K, Swiecicka I. In vitro study of the antimicrobial activity of European propolis against Paenibacillus larvae. Apidologie. 2017;48(3):411-22.
23. Jug M, Končić MZ, Kosalec I. Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures. LWT - Food Sci Technol. 2014;57(2):530-7.
24. Kasote D, Bankova V, Viljoen AM. Propolis: chemical diversity and challenges in quality control. Phytochem Rev. 2022;21(6):1887-911.
25. Mihai CM, Marghitas LA, Dezmirean DS, Chirila F, Moritz RF, Schluns H. Interactions among flavonoids of propolis affect antibacterial activity against the honeybee pathogen Paenibacillus larvae. J Invertebr Pathol. 2012;110(1):68-72.
26. Seidel V, Peyfoon E, Watson DG, Fearnley J. Comparative study of the antibacterial activity of propolis from different geographical and climatic zones. Phytother Res. 2008;2(9):1256-63.
27. Caesar LK, Cech NB. Synergy and antagonism in natural product extracts: when 1 + 1 does not equal 2. Nat Prod Rep. 2019;36(6):869-88.