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Pseudomonas aeruginosa ve Pyoverdinler: Apiterapi Uygulamalarında Gizli Bir Tehdit

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ÖZ

Gram negatif çubuk şeklinde bir bakteri olan *Pseudomonas aeruginosa*, hastane enfeksiyonlarında en sık görülen patojenlerden biridir ve bu patojenik tür, septisemili hastalarda yüksek prevalansı ile dikkat çekmektedir. Pyoverdinler, *P. aeruginosa* ve *Pseudomonas fluorescens* gibi bazı *Pseudomonas* türleri tarafından üretilen floresan sideroforlardır. Bu sideroforlar, bu mikroorganizmalarda biyofilm üretimini destekler ve aynı zamanda bir virülans faktörü olarak rol oynar. Bu çalışmada 10 farklı bal örneğinin anti-*Pseudomonas* aktivitesi agar kuyusu difüzyon (AWD) yöntemi ile değerlendirilmiştir. Ayrıca bu bal örneklerinin minimum inhibisyon konsantrasyonu (MIC) ve minimum bakterisidal konsantrasyon (MBC) değerleri mikrobroth seyreltme yöntemi ile belirlenmiştir. Elde edilen sonuçlar sadece meşe balı ve kestane balının ihmal edilebilir ölçekte düşük anti-*Pseudomonas* aktivitesine sahip olduğunu göstermiştir. Ayrıca diğer bal örneklerinin bu patojenlere karşı inhibitör etkisinin olmadığı gözlemlenmiştir. Son olarak, bu bal örneklerinin pyoverdin siderofor üretimini teşvik edici özelliği değerlendirilmiş ve tüm bal örneklerinin ½ w/v konsantrasyonda pyoverdin üretimini uyardığı sonucuna varılmıştır.

Pseudomonas aeruginosa and Pyoverdines: A Hidden Threat in Apitherapy Applications

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ABSTRACT

Pseudomonas aeruginosa, a Gram-negative rod-shaped bacterium, is one of the most common pathogens in nosocomial infections and this pathogenic species draws attention with its high prevalence rate in patients with septicemia. Pyoverdines are fluorescent siderophores produced by some *Pseudomonas* species such as *P. aeruginosa* and *Pseudomonas fluorescens*. These siderophores support biofilm production in these microorganisms and also play a role as a virulence factor. In this study, the anti-*Pseudomonas* activity of 10 different honey samples was evaluated by the agar well diffusion (AWD) method. In addition, minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) values of these honey samples were determined via the microbroth dilution method. The obtained results showed that only oak honey and chestnut honey have negligible scale low anti-*Pseudomonas* activity. In addition, it was observed that other honey samples did not have an inhibitory effect against these pathogens. Finally, the promoting property of pyoverdine siderophore production of these honey samples was evaluated and it was concluded that all honey samples stimulated pyoverdin production at a concentration ½ w/v.

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Introduction

Pseudomonas is a bactrerial genera consist of non-fermentative Gram-negative rod-shape bacteria. This genus distributes in many different regions from the tropics to the Antarctic. This ubiquitous genus was firstly described in 1894 by Walter Migula and so far, 272 different species of these microorganisms have been isolated from many different environments such as soil, water, air, sediments and clinical isolates. These opportunistic pathogens, which can cause serious infections in humans, have also been isolated from many different hosts such as animals, plants, fungi and algae (Silby et al., 2011; Peix et al., 2018).

P. aeruginosa is a Gram-negative rod-shaped bacterium, belonging to the Pseudomonadaceae family of the Gammaproteobacteria class (Casabona et al., 2013; Nikolaidis et al., 2020). Today, *P. aeruginosa* is known to be one of the most common pathogens in nosocomial infections and this opportunistic pathogen attracts attention with its high prevalence rate in patients with septicemia (Zavascki et al., 2006; Castañeda-Montes et al., 2018; Esparcia et al., 2019). In particular, Metallo-Beta-Lactamase (MBL) producing *P. aeruginosa* strains and multidrug-resistant *P. aeruginosa* (MDRPA) strains are the leading causes of nosocomial infections with high mortality rates. In recent years, many published articles have emphasized the increasing resistance of these microorganisms to antibiotics such as carbapenem and cephalosporin (Matos et al., 2018).

Siderophores are low molecular weight chelators produced by different living groups such as bacteria, fungi and even sometimes plants to scavenge iron. These secondary metabolites bind the low concentration of free iron in the environment and play a role in its transport into the cell via membrane receptor molecules for many different cellular processes (Albelda-Berenguer et al., 2019; Pecoraro et al., 2021). Pyoverdines are fluorescent siderophores produced by some *Pseudomonas* species such as *P. aeruginosa* and *P. fluorescens*. These siderophores support biofilm production in these microorganisms and also play a role as a virulence factor. These fluorescent siderophores contribute to the growth of microorganisms by binding this element in cases where the iron element is very limited (Ringel and Brüser, 2018). Until now, more than a hundred different types of pyoverdines have been isolated from different *Pseudomonas* strains and it has been observed that all of them have some structurally common features. All these siderophores have a dihydroxyquinoline molecule in their core. In all pyoverdine producer *Pseudomonas* species, the structure of this dihydroxyquinoline core molecules consists of (1S)-5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido [1,2-a] quinoline-1-carboxylic acid. This fluorescent dihydroxyquinoline core part gives pyoverdins their yellowish or greenish color and this part of the pyoverdine molecules is invariant for all *Pseudomonas* species. As the second part, there is a peptide chain integrated with this dihydroxyquinoline core and this chain consists of 6-14 amino acids. The number of amino acids in this peptide chain varies depending on the groups of microorganisms. The most important distinction of these strain specific amino acid chains is that they are not synthesized in ribosomes. This non-ribosomal amino acid chain is synthesized on the dihydroxyquinoline chromophore core. The third and last part of this dihydroxyquinoline core is 4-5

carbon α-ketoacid, and this organic acid is produced in the Krebs cycle or citric acid cycle (Moll et al., 2008; Cézard et al., 2019).

Honey is a sweet substance used widely by humankind as a functional food since ancient times. Mankind has been used honey not only as a food but also as a therapeutic agent for healing wounds and treating infectious disease for many years (Hadagali et al., 2014; Oryan et al., 2016; Anand et al., 2019; Ronsisvalle et al., 2019). The dehydrated structure of honey, its acidity feature and the components it contains such as hydrogen peroxide, phenolic acids and flavonoids prevent the development of microorganisms, and in addition to this feature, the nutritional content of honey accelerates the healing of wounds (Dryden et al., 2014; da Silva et al., 2016; Oryan et al., 2016; Karlıdağ et al., 2021).

In this study, the anti-*Pseudomonas* activity of 10 different honey samples was investigated against two different *P. aeruginosa* strains. *P. aeruginosa* strains were obtained from Bayburt State Hospital, Medical Microbiology Laboratory. In addition, the ability of honey samples to induce pyoverdin production in *Pseudomonas* strains was evaluated. The obtained results revealed that eight honey samples did not have an inhibitory effect against target pathogens, only oak and chestnut honey samples had inhibition effects at a negligible scale. In addition to these results, it was observed that all honey samples stimulated pyoverdine siderophore production in target pathogens.

Materials and Methods

Preparation of Honey Samples for Anti-*Pseudomonas* Activity Assays

Honey samples were purchased from beekeepers from different cities. The list of honey samples is given in Table 1. Initially, the honey samples were kept in a water bath at 40^{o}C for 30 minutes to become more fluid. After this process, honey samples weighing 1 gram using a sterile wooden stick were transferred to sterile 2 mL Eppendorf tubes. Immediately afterward, the total volume was adjusted to 2 mL using sterile distilled water. These prepared honey solutions (at a concentration ½ w/v) were used to determine the anti-*Pseudomonas* activities (Vică et al., 2021).

Microorganisms and Growth Conditions

In this study, target *P. aeruginosa* strains were kindly obtained from Bayburt State Hospital, Medical Microbiology laboratory. Two different *Pseudomonas* strains were used to determine *in-vitro* anti-*Pseudomonas* activities of ten different honey samples. First of all, these pathogenic *Pseudomonas* strains were incubated in a liquid medium (Mueller Hinton Broth = MHB) at 37°C for 24 hours. After this incubation period, these suspensions were adjusted to 0.5 McFarland standard turbidity of (10^6 CFU/mL) and used as inoculum (Bayram et al., 2019).

Agar Well Diffusion Method (AWD)

To determine the *in-vitro* anti-*Pseudomonas* effect of different honey samples, AWD assay was used. Initially, Mueller-Hinton Agar (MHA) medium was autoclaved at 121 ^{o}C for 15 minutes and cooled to 50 °C at room temperature. 25 mL MHA medium was transferred to each sterile petri dishes. After these petri dishes were cooled at room temperature, 8 mm diameter wells were cut into the solidified media using a sterile cork borer. And then, all these prepared MHA media were inoculated using a sterile cotton swab. After these inoculation procedures, 70 µL of the diluted honey samples (at a concentration ½ w/v) were transferred to these wells with the help of a micropipette and incubated at 36 °C for 24 hours. At the end of this incubation period, inhibition zones formed by honey samples around agar wells were measured with the help of a vernier caliper and recorded (Sherlock et al. 2010; Osés et al. 2016; Bayram et al., 2020). Each assay was carried out in duplicate.

Determination of Minimum Inhibitory Concentration (MIC)

For the purpose of determine minimum inhibition concentration (MIC) values of different honey samples against target *Pseudomonas* strains broth microdilution method was used. In this assay period, 96-well polystyrene microtiter plates were used. Initially, 96 µL of sterile MHB medium was added to all wells using a multichannel pipette. And then 4 µL of *Pseudomonas* inoculums were added to all wells. After that, 100 µL of prepared honey samples (at a concentration ½ w/v) were added to all the first wells of the microtiter plates and mixed thoroughly with the help of a multichannel pipette. After these procedures, 100 µL of the prepared sample was taken from the first wells of the 96 well plates and mixed into the second wells. This half-dilution procedure was repeated sequentially up to the 8th well. In this way, the concentration of honey samples in the wells was serially diluted. And then, these microtiter plates were measured at 600-nanometer wavelength using a microplate reader (Thermo, Multiskan Go). After these spectrophotometric measurements were recorded, the microplates were incubated at 36^{o}C for 24 hours. At the end of this 24 hours incubation period, the microplates were measured at 600-nanometer wavelength, and obtained results were again recorded. At the end of all these measurements, the lowest concentration inhibiting *Pseudomonas* strains was accepted as MIC (Sherlock et al. 2010; Huttunen et al., 2013).

Determination of Minimum Bactericidal Concentration (MBC)

Minimum bactericidal concentration (MBC) assays were performed with small modifications as previously described by Ecem-Bayram et al. (2021). For this purpose, at the end of the MIC assays, 7 microliter samples were taken from each well of the microplate using a micropipette and transferred to the MHA media in sequence. After that, these media were incubated at 36^{o}C for 24 hours. At the end of this 24-hour incubation period, the lowest concentration that did not form *Pseudomonas* colonies was recorded as MBC (Sherlock et al. 2010; Ecem-Bayram et al., 2021).

Table 1. Anti-*Pseudomonas* activity of different honey samples. [Inhibition zone diameter: IZD = mm; MIC= minimum inhibition concentration: w/v; MBC: minimum bactericidal concentration: w/v; PP: Pyoverdine production]

Honey Samples	Province	*P. aeruginosa* EPSB1				*P. aeruginosa* EPSB2			
		IZD	MIC	MBC	PP	IZD	MIC	MBC	PP
Acacia honey	Manisa	-	-	-	+	-	-	-	+
Cedar honey	Muğla	-	-	-	+	-	-	-	+
Chestnut honey	Zonguldak	12	½	-	+	11	½	-	+
Clover honey	Muğla	-	-	-	+	-	-	-	+
Heather	Muğla	-	-	-	+	-	-	-	+
Linden	Bursa	-	-	-	+	-	-	-	+
Oak honey	Edirne	11	½	-	+	11	½	-	+
Thyme honey	Amasya	-	-	-	+	-	-	-	+
Wildflower honey 1	Bayburt	-	-	-	+	-	-	-	+
Wildflower honey 2	Erzincan	-	-	-	+	-	-	-	+

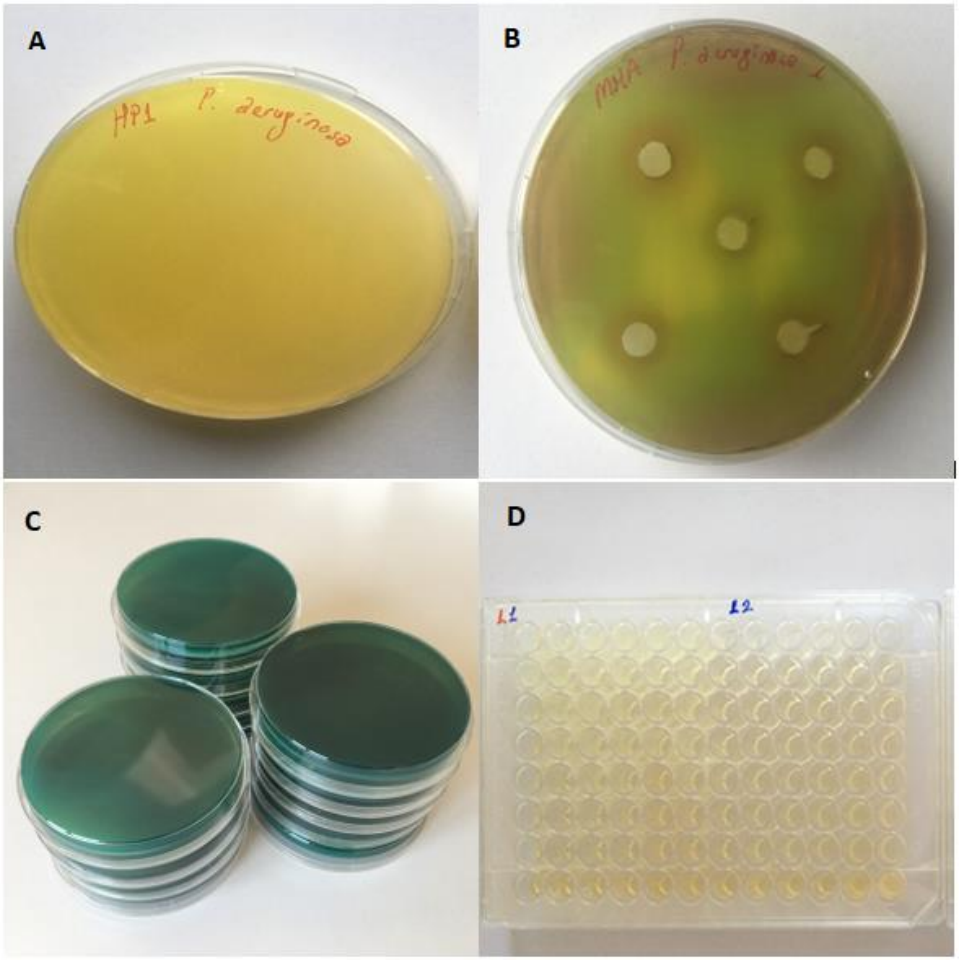

Figure 1. Determination of the anti-*Pseudomonas* effect of different honey samples. (A: *Pseudomonas* strain that does not produce pyoverdin on MHA. B: Agar well diffusion method. Stimulation of

pyoverdin production by honey sample. C: Increasing pyoverdin production after spreading one mL of honey suspension over the entire petri dish. D: Determination of MIC and MBC values

Results and Discussions

In this study, the inhibitory effect of ten different honey samples taken from different provinces of Turkey was investigated against two different pathogen *P. aeruginosa* strains (EPSB1 and EPSB2 strains) (Table 1). The antibacterial activity of these samples was evaluated by the agar well diffusion (AWD) method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined by the micro broth dilution method (Fig. 1). Obtained *in-vitro* anti-*Pseudomonas* activity results showed that eight of the honey samples had no inhibitory effect against target pathogens. It was observed that only oak and chestnut honey samples had negligible scale weak inhibition effects at ½ w/v concentration. In addition, it was observed when 70 µL volume and ½ w/v concentration chestnut honey transferred to wells in MHA medium (8 mm diameter) formed an inhibition zone of 12 mm against *P. aeruginosa* EPSB1 and 11 mm against *P. aeruginosa* EPSB2. Moreover, it was observed that oak honey formed an inhibition zone of 11 mm against both pathogens at the same concentration. When evaluated in terms of MIC and MBC activities, it was seen that the same honey samples had a bacteriostatic effect at a concentration of ½ w/v against target pathogens, but did not show a bactericidal effect. None of the honey samples except chestnut and oak honey showed an inhibition effect against target pathogens. It is observed that all of these honey samples are ineffective in MIC and MBC applications.

After these procedures, both target pathogens were seeded in ten different petri dishes with the help of a sterile swab, and 1 mL of honey solution was added to all petri dishes immediately after this process (½ w/v concentration). After that, honey solutions were spread to cover the entire surface of the petri dish with the help of a sterile swab. After these processes, inoculated petri dishes were incubated at 37^{o}C for 24 hours and it was observed that pyoverdine production reached the maximum level in these media (Fig 1C). This situation indicates the possibility that if honey is used as an antibacterial agent in *Pseudomonas* infections, it may stimulate the production of pyoverdine and increase the severity of the infection.

When we evaluate the previously published studies in the literature, it can be said that our obtained results are compatible with the literature. In a study performed by Leyva-Jimenez et al. (2019), the phenolic components of 33 different Iranian honey were purified by solid-phase extraction method and their antibacterial activity was investigated. In their study, two Gram-positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and two Gram-negative bacteria (*Escherichia coli* and *P. aeruginosa*) were used. In the results obtained in this study, it was seen that the most resistant bacteria against phenolic compounds purified from honey was *P. aeruginosa* (Leyva-Jimenez et al., 2019).

Additionally, in a study conducted by Oluwapelumi et al. (2017), the antibacterial activity of six different honey samples was investigated. In this study, researchers determined the inhibition effects

of honey samples against *Staphylococcus aureus, P. aeruginosa* and *Klebsiella pneumoniae* bacteria using the AWD method. In the obtained results, it was observed that *Staphylococcus aureus* and *Klebsiella pneumoniae* strains were sensitive to all honey samples. On the other hand, *P. aeruginosa* strain was resistant to all samples but sensitive to only one sample (Oluwapelumi et al., 2017).

In another study performed by Olatunji et al. (2018), researchers investigated the antimicrobial activities of five different honey samples. The researchers in this study used the agar well diffusion method. During the study, one *Candida albicans* strain and six different bacterial strains (*Staphylococcus aureus, Escherichia coli, Bacillus subtilis, P. aeruginosa, Salmonella paratyphi* and *Klebsiella pneumonia*) were used as the target pathogen. In the obtained results in the study, it was seen that *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Salmonella paratyphi* and *Candida albicans* strains were sensitive to all honey samples. In addition, it was stated that *Klebsiella pneumonia* strain was resistant to 2 different honey samples, and finally, it was emphasized that *P. aeruginosa* strain was resistant to 4 different honey samples and was sensitive to only one honey sample. In this study, it was seen that *P. aeruginosa* strain was the most resistant microorganism against honey samples among pathogenic samples (Olatunji et al., 2018).

In conclusion, the therapeutic properties of honey in apitherapy applications have been known for thousands of years. However in this study, based on these obtained results, in *Pseudomonas* infections, during the treatment with honey, if the necessary care is not taken and the wound area is moistened; it seems likely that pyoverdine production will be promoted and the severity of the infection will increase.

As a result, mankind has been benefiting from the therapeutic properties of honey in healing wounds since ancient Egyptian times. This traditional method both contributes to faster healing of wounds and prevents the risk of infection thanks to the antimicrobial properties of honey samples. However obtained results in this study, showed that only chestnut and oak honey samples had an inhibitory effect against target *Pseudomonas* strains at the highest concentration (½ w/v), whereas other honey samples did not have an inhibitory effect against these pathogenic bacteria. In addition to these results, it was revealed that all honey samples stimulated pyoverdine production in *P. aeruginosa* EPSB1 and *P. aeruginosa* EPSB2 strains. The obtained results revealed that honey samples may induce pyoverdin production and in this way may increase virulence and pathogenicity in *Pseudomonas* infections.

Statement of Conflict of Interest

The author has declared no conflict of interest.

Author's Contribution

The contribution of the author is 100%.

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