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Antibacterial Effects of *Phlomoides molucelloides* (Bunge) Salmaki

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ABSTRACT

The insufficiency of existing antibiotics in the combat against antibiotics-resistant bacteria has necessitated the discovery of new and effective antibacterial drugs. The discovery that bacteria synthesize various virulence factors by the quorum sensing system has suggested that quorum sensing inhibitors may be used in the fight against infectious diseases. This study aimed to determine the antibacterial and anti-quorum sensing activities of methanol and water extracts of aerial and root parts of *Phlomoides molucelloides* (Bunge) Salmaki. The minimal inhibitory concentration (MIC) values of the extracts were investigated against reference bacterial strains using the broth microdilution method. Anti-quorum sensing activities were examined by violacein and pyocyanin pigments inhibition and swarming motility inhibition assays against *Chromobacterium violaceum* ATCC 12472 and *Pseudomonas aeruginosa* PAO1 bioreporter strains. The antibiofilm activities of the extracts were tested against *P. aeruginosa* PAO1 using the crystal violet staining method. The MIC value (> 2000 µg/mL) of all extracts against the tested bacteria could not be determined at the concentrations studied. All extracts partially inhibited the swarming motility of *P. aeruginosa*. Methanol extract of the aerial part inhibited pyocyanin production by 81.7% without interfering with *P. aeruginosa* growth. The extracts had no significant inhibitory activity on biofilm formation and violacein pigment production. These results showed that the extracts of *P. molucelloides* may be good anti-quorum sensing agents. Further research can be done to elucidate the mechanisms underlying these biological activities.

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Phlomoides molucelloides (Bunge) Salmaki'nin Antibakteriyel Etkileri

ÖZET

Antibiyotiklere dirençli bakterilerle mücadelede mevcut antibiyotiklerin yetersizliği, yeni ve etkili antibakteriyel ilaçların keşfini zorunlu kılmıştır. Bakterilerin quorum sensing mekanizması ile çeşitli virülans faktörlerini sentezlediğinin keşfedilmesi, quorum sensing inhibitörlerinin enfeksiyon hastalıkları ile mücadelede kullanılabileceğini düşündürmüştür. Bu çalışmada, *Phlomoides molucelloides* (Bunge) Salmaki türünün toprak üstü ve kök kısımlarının metanol ve su özütlerinin antibakteriyel ve anti-quorum sensing aktivitelerinin araştırılması amaçlanmıştır. Özütlerin referans bakteri suşlarına karşı minimal inhibitör konsantrasyon (MİK) değerleri sıvı mikrodilüsyon yöntemi ile araştırılmıştır. Anti-quorum sensing aktivite, *Chromobacterium violaceum* ATCC 12472 ve *Pseudomonas aeruginosa* PAO1 biyoraportör suşlarda viyolasin ve piyosiyanin pigment üretiminin ve

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swarming (yayılma) hareketinin inhibisyonu üzerine belirlenmiştir. Antibiyofilm aktivite testi *P. aeruginosa* PAO1 suşuna karşı kristal viyole yöntemi ile değerlendirilmiştir. Tüm özütlerin test edilen bakterilere karşı çalışılan konsantrasyonlarda MİK değeri (> 2000 µg/mL) tespit edilmemiştir. Buna karşılık tüm özütlerin *P. aeruginosa*'nın swarming hareketini baskıladığı saptanmıştır. Ayrıca, bitkinin toprak üstü kısımlarının metanol özütlerinin bakteri üremesini baskılamadan piyosiyanin üretimini kontrole göre %81.7 oranında inhibe ettiği belirlenmiştir. Tüm özütlerin biyofilm oluşumu ve viyolasin pigment üretimi üzerine önemli bir inhibitör etkinliği saptanmamıştır. Sonuçlar *P. molucelloides* özütlerinin iyi bir anti-quorum sensing ajanı olabileceğini göstermiştir. Bu biyolojik aktivitelerin altında yatan mekanizmaları aydınlatmak amacıyla ileri çalışmaların yapılması gerekmektedir.

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INTRODUCTION

Antibiotic resistance, which has become widespread today, has necessitated the development of new antibacterials or alternative treatment methods in the combat against infectious diseases (Uddin et al., 2021). The fact that bacteria modulate the synthesis of virulence factors using the Quorum Sensing (QS) system suggested that QS inhibition could be an alternative antibacterial treatment method. QS is the regulation of some phenotypic and biochemical activities of bacteria reaching a certain population density with signal molecules called autoinducers (Jiang et al., 2019).

Some autoinducers as QS signal molecules have been identified in bacteria. These are autoinducing peptides (AIP) used by Gram-positive bacteria, N-acyl homoserine lactone (AHL) used by Gram-negative bacteria, and autoinducer-2 (AI-2) used by both Gram-positive and Gram-negative bacteria (Prazdnova et al., 2022). These signaling molecules regulate the expression of virulence-related genes such as biofilm formation, swarming motility, pigment, enzyme and toxin production in many bacteria strains (Eberl et al., 1996; McClean et al., 1997; Ohtani et al., 2002; Marketon et al., 2003; Rice et al. 2005).

QS inhibition in bacteria can occur such as i) by inhibiting autoinducer synthesis, ii) by cleaving autoinducers, iii) by preventing autoinducers from binding to the specific receptor through competition for the binding site, iv) by blocking the binding of the signal-receptor complex to the gene promoter and inhibiting gene expression (Prazdnova et al., 2022). There are many compounds known to have QS inhibitory activity in the literature. However, most of them are not suitable for human use (Nain et al., 2020). Therefore, it is significant to discover natural and reliable compounds.

Plants have the potential to develop new and effective antibacterial with their secondary metabolites (Gorlenko et al., 2020). *Phlomoides molucelloides* (Bunge) Salmaki (also known as *Eremostachys molucelloides* Bunge) is a plant species belonging to the Lamiaceae family. It is a perennial herbaceous plant. Limestone hills, schist and volcanic slopes are its natural habitats (Babaç, 2004). It is stated that *Eremostachys* species have ethnopharmacological activities and have essentials oils and secondary metabolites such as flavonoids, monoterpenes, irioid, and chrysoeriol glycosides (Mohammadhosseini et al., 2019). Previous studies have demonstrated the potential of *Eremostachys* genus to have various biological effects. For example, the iridoid glycosides of *Eremostachys laciniata* (L) Bunge have been reported to have antibacterial effects (Modaressi et al., 2009). It was shown that essential oils obtained from the root, stem, and flower parts of *Eremostachys laevigata* Bunge had high antibacterial effects in another study (Esmaeilli et al., 2012). It was emphasized that *Eremostachys labiosa* Bunge has a high potential in terms of anti-leishmanial, anticancer, and anti-inflammatory activity in a study (Taghizadeh Rabe et al., 2014). Dichloromethane, n-hexane and methanol extracts of rhizomes of *Eremostachys azerbaijanica* Rech.f. have been reported to have antibacterial and antioxidant activity, respectively. Moreover, it has been reported that all extracts exhibit cytotoxic activity against the A549 cell line. The presence of fatty acids and steroids as the main compounds in the extracts has been held responsible for those bioactivities (Asnaashari et al., 2017).

To the best of our knowledge, there is no previous

report about the antibacterial effects of *P. molucelloides* in the literature. Therefore, this study aimed to investigate the antibacterial and anti-QS activities of methanol and water extracts of aerial and root parts of *P. molucelloides*.

MATERIALS and METHODS

Plant material and preparation of extracts

P. molucelloides were collected from Konya province (Akbaş Village, Bozdağ Natural park around, 1020 m, 38° 02' 51" N, 32° 56' 20" E), Türkiye in June 2019. The plant picture from natural location is given in Figure 1. The plants were confirmed by a botanist at Selcuk University (Konya, Türkiye) and one voucher specimen has been deposited in Selcuk University (EY-3004). The aerial and root parts of the plant were kept in a shaded environment and room temperature for 10 days and dried until weighing constant. The samples were then pulverized (particle size about 2 mm) by using a laboratory mill (Retsch SM-200, Germany). The pulverized samples were stored in a dark environment and at room temperature. In the present work, it was used methanol and water as solvents. Maceration was used as an extraction method. In addition, infusion was prepared with water. In the maceration, the plant materials (10 g) were macerated with 200 mL methanol at room temperature for 24 h. Then, after the extracts were filtered, the methanol was removed by using a rotary evaporator. Regarding infusion, the plant materials (10 g) were kept with 200 mL boiled water for 15 min and then filtered (2 µm, Whatman 589/3). Water extracts were lyophilized and all extracts were held at 4°C until study.

Figure 1. *Phlomoides molucelloides*
Şekil 1. Phlomoides molucelloides

Bacterial strains and growth conditions

Antibacterial activities of the extracts were investigated against three Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633) and nine Gram-negative bacteria (*Escherichia coli* ATCC 25922, *E. coli* NCTC 13846, *Klebsiella pneumoniae* ATCC 13883, *K. pneumonia* ATCC 700603, *K. pneumonia* NCTC 13440, *Salmonella* Typhimurium ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* ATCC 13048, *Acinetobacter haemolyticus* ATCC 19002).

P. aeruginosa PAO1 and *C. violaceum* ATCC 12472 strains were used as bioreporters for the anti-QS activities of the extracts. These strains regulate the synthesis of various phenotypic behaviors using the QS mechanism, so they are widely used to investigate QS inhibition. For example, *C. violaceum* ATCC 12472 produces violacein pigment using *cvi* system via long-chain (C10-C16) AHL molecules (Morohoshi et al., 2008). *P. aeruginosa* PAO1 regulates the synthesis of pyocyanin pigment, swarming motility and biofilm formation using *rhI* system via short-chain C4-AHL molecules and *las*, *rhI*, *pqs*, and integrated QS systems via various signal molecules, respectively (Tapia-Rodriguez et al., 2019; Vetriel et al., 2021).

All bacteria were National Collection of Type Cultures (NCTC) and American Type Culture Collection (ATCC) reference strains. They were

obtained from the culture collection of the Department of Medical Microbiology, Faculty of Medicine, Karadeniz Technical University.

C. violaceum was grown in Luria-Bertani (LB) medium at 30ºC, and the others were grown in Mueller-Hinton medium at 37ºC aerobically.

Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) values of the extracts were determined by the broth microdilution method as previously described (Wiegand et al. 2008). Briefly, the test was carried out in 96-well plates every well containing 100 µL of Mueller Hinton broth (MHB; Merck, Darmstadt, Germany). The tested concentration range of the extracts was from 2000 to 62.5 µg mL^{-1}. Levofloxacin (Chemical Industry, Tokyo, Japan) was used as a standard antibiotic in the concentration range of 0.015-128 µg mL^{-1}. The inoculums of bacteria prepared from the fresh cultures were set to 0.5 McFarland (~1.0×10^8 CFU mL^{-1}) turbidity standards using a turbidimeter (DEN-1B, Biosan, Latvia) and then diluted 10-fold. Five µL of prepared inoculums were put into the wells. One well without the extracts was used as a growth control and one well without the bacteria was used as a sterility control. The plate was covered with a lid and incubated at 37ºC for 24 h. The minimal extract concentration without visible bacterial growth was determined as the MIC value.

Anti-quorum sensing inhibition assays

Violacein inhibition

The abilities of the extracts to inhibit violacein pigment production was investigated by the soft agar method on *C. violaceum* ATCC 12472 (McClean et al. 1997). Briefly, 50 µL of an overnight culture of *C. violaceum* was transferred into a 5 mL volume of molten soft LB agar (0.5% w v^{-1}) (NZYTech, Lisbon, Portugal). The agar-culture mixture was vortexed and directly spilled over the surface of prewarmed LB agar plates. After solidifying the agar, a set of blank discs in 6 mm diameter were on the seeded agar media and then impregnated with 20 µL from each extract (20 mg mL^{-1}) to obtain 400 µg extract per disc. The supernatant (20 µL) of an overnight culture of *P. aeruginosa* PAO1 strain was used as a positive control. The cultures were incubated at 30°C for 18 h. Violacein inhibition was determined by a colorless, opaque, but viable halo around the discs.

Pyocyanin inhibition

The MIC value of the extracts against *P. aeruginosa* PAO1 has been determined using the broth microdilution assay. For avoiding any antibacterial effect, the sub-MIC values (500 µg mL^{-1}) of the extract have been used for this assay. The pyocyanin production inhibitory activities of the extracts were performed using the pyocyanin extraction method described by Tüfekci et al. (2020). Briefly, the supernatants from 16 h cultures of *P. aeruginosa* PAO1 grown in broth with and without the extracts were obtained. The pigment in the supernatant was extracted by chloroform, then by 0.2 N HCl. The absorbance was determined at 520 nm against 0.2 N HCl using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific). Whether bacterial growth was interfered or not was determined by viable cell count. For this purpose, serial dilutions were prepared from the cultures and spread on MHA medium. After overnight incubation, colonies were counted on the media and compared with the control.

Swarming inhibition

Swarming inhibition was done based on a previously described assay (Saliha et al., 2020). Briefly, a small piece of the colony was taken from a fresh culture of *P. aeruginosa* PAO1 on MHA with a sterile toothpick and inoculated onto swarm agar plates. Swarm agar contained 1% glucose, 0.5% peptone, 0.5% bacteriological agar, 0.2% yeast extract and the test material (500 µg mL^{-1} of the extract). Sterile distilled water was added to the control plate instead of the extract. The plates were incubated in the upright position at 37°C for 24 hr. At the end of the incubation, the swarming motility was determined from the point of inoculation to the environment.

Biofilm inhibition

This experiment was done in 96-well flat-bottom polystyrene plates as described previously (O'Toole, 2011). In brief, a 16 h broth culture of *P. aeruginosa* PAO1 was diluted 100-fold in fresh LB broth and 180 µL was inoculated into each well. Then, 20 µL of the extract at a concentration of 5 mg mL^{-1} was added to provide a final concentration in the wells was 500 µg mL^{-1}. For control wells, 20 µL sterile distilled water was put to the wells as the solvent of the extracts. Typically, three replicate wells were used for each treatment. The plate was incubated at 37ºC without agitation for 24 h after the plate was covered with a lid. After incubation, it was determined whether the bacterial growth in the wells was suppressed by measuring the plate at 600 nm using the spectrophotometer. Then, the media from the wells was discarded and biofilm was washed three times by gently dipping into a container including sterile distilled water. The biofilm layer was stained with 200 µL 0.1% (w v^{-1}) crystal violet (Merck) solution for 5 min, and then the unbound stain was discarded. The washing process was repeated as described above. The crystal violet retained by the biofilm layer was dissolved with 100 µL absolute ethanol. The absorbance was determined at 570 nm against absolute ethanol using the spectrophotometer.

Statistical analysis

The tests were repeated three times and the data were given as mean ± standard deviation (SD) values. Wilks-Shapiro test (Shapiro and Wilk, 1965) has been used for normality of the data analysis. Mann-Whitney *U* test and independent samples t-test were done using IBM-SPSS statistics version 23.0 (IBM Inc., Armonk, NY, USA) for the analysis of nonparametric data and parametric data, respectively. A consider of $P < 0.05$ was statistically significant.

RESULTS and DISCUSSION

Anti-quorum sensing activity results

Pyocyanin is a secondary metabolite produced by *P. aeruginosa* strains and exhibits cytotoxicity by inducing oxidative stress in host cells (Gonçalves and Vasconcelos, 2021). Methanol extract of the aerial part inhibited pyocyanin production by 81.7 ± 0.2% ($P < 0.05$ versus control) without interfering with bacterial growth. However, other extracts could not inhibit pyocyanin production at the concentration tested (Figure 2). This means that there are compounds that inhibit the production of pyocyanin pigment in the aerial part of *P. molucelloides*. Since no inhibition was detected in water extracts, the compounds in question were probably extracted with methanol.

Swarming motility is a virulence factor that enables *P. aeruginosa* strains to attach to biotic or abiotic surfaces and form a biofilm. Also, bacteria adapt to the environment they are in with their swarming motility (Khan et al., 2020). As with pyocyanin, *P. aeruginosa* PAO1 swarming motility is regulated by the system of *rhI* using C4-AHL signal molecules (Tapia-Rodriguez et al., 2019). In the presented study, it was observed that all extracts suppressed the swarming motility of the *P. aeruginosa* PAO1 strain (Figure 3). Considering that pyocyanin pigment production is also regulated by the *rhI* system, it can be said that *P. molucelloides* interferes with the *rhI* system.

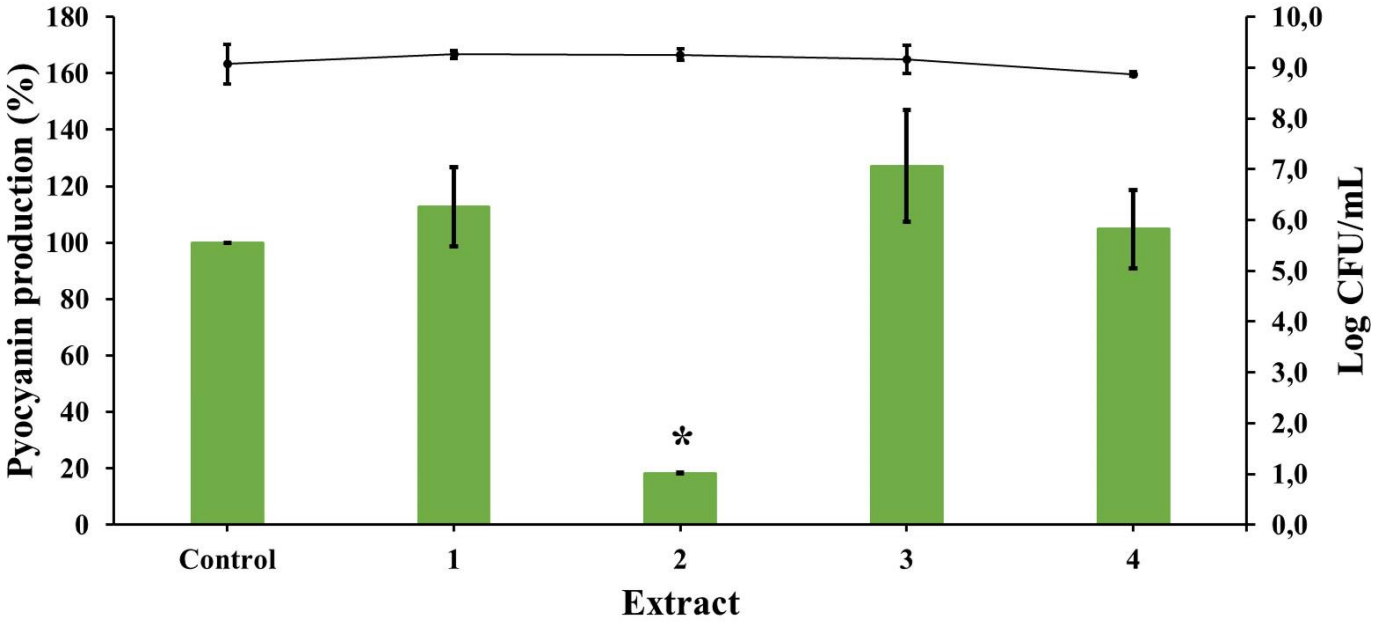

Figure 2. The result of the pyocyanin inhibition assay (1; water extract of aerial part, 2; methanol extract of aerial part, 3; water extract of root part, 4; methanol extracts of root part). Data are presented as the mean ± SD. *P < 0.05.

*Şekil 2. Piyosiyanin inhibisyon deneyinin sonucu (C; kontrol, 1; toprak üstü kısmın su özütü, 2; toprak üstü kısmın metanol özütü, 3; kök kısmın su özütü, 4; kök kısmın metanol özütü). Veriler ortalama ± standart sapma olarak sunulmuştur. *P < 0.05.*

Biofilms are cell communities formed by bacteria on biotic and abiotic surfaces. The biofilm layer protects bacterial cells against various environmental stresses such as disinfectants, antibiotics, and the host's immune system (Maurice et al., 2018). However, the extracts did not have any inhibitory activity on biofilm formation at the concentrations studied (Figure 4). This may be due to the inability of the extracts to inhibit the QS pathways for biofilm synthesis in *P. aeruginosa*. Moreover, none of the extracts could inhibit violacein pigment production in this study (Figure 5). This result showed that the extracts could not have any inhibitory effect on the *cvi* system.

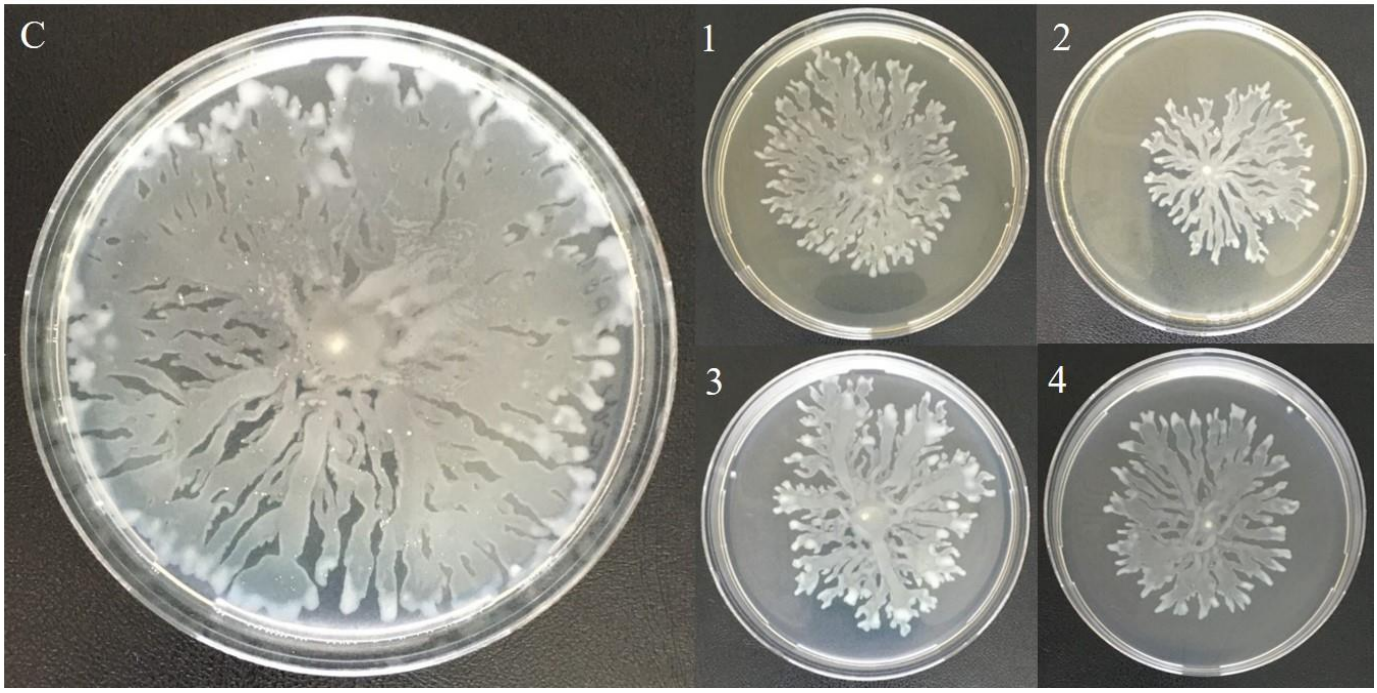

Figure 3. Swarming inhibition assay (C; control, 1; water extract of aerial part, 2; methanol extract of aerial part, 3; water extract of root part, 4; methanol extract of root part).

Şekil 3. Swarming inhibisyon deneyi (C; kontrol, 1; toprak üstü kısmın su özütü, 2; toprak üstü kısmın metanol özütü, 3; kök kısmın su özütü, 4; kök kısmın metanol özütü).

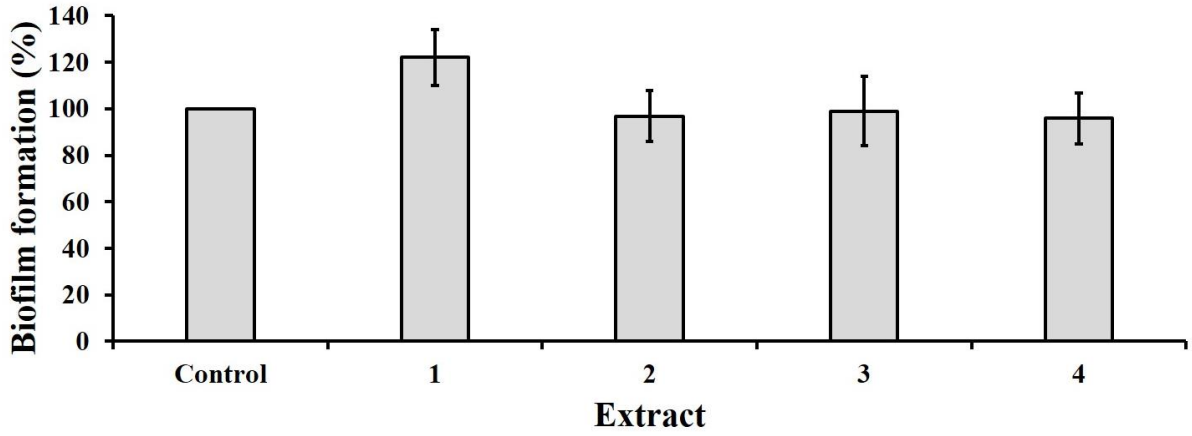

Figure 4. The result of antibiofilm activity (1; water extract of aerial part, 2; methanol extract of aerial part, 3; water extract of root part, 4; methanol extract of root part). Data are presented as the mean ± SD. P > 0.05.

Şekil 4. Biyofilm inhibisyon deneyinin sonucu (C; kontrol, 1; toprak üstü kısmın su özütü, 2; toprak üstü kısmın metanol özütü, 3; kök kısmın su özütü, 4; kök kısmın metanol özütü). Veriler ortalama ± standart sapma olarak sunulmuştur. P > 0.05.

Antibacterial activity results

The antibacterial activity of the extracts of aerial and root parts of *P. molucelloides* was investigated against some reference bacterial strains representing pathogens in the presented study. The broth microdilution method was done to test the antibacterial activity of the extracts at high concentrations in this study. However, MIC values of the extracts against all tested bacteria could not be determined (MIC > 2000 µg/mL). There are studies in the literature researching the antibacterial activity of various *Eremostachys* species. For example, it has been reported that n-hexane, dichloromethane, and methanol extracts of the aerial parts of *Eremostachys macrophylla* Montbret & Aucher ex Benth. and methanol extracts of the rhizomes of *E. azerbaijanica* had no antibacterial activity as in the current study (Asgharian et al., 2017; Asnaashari et al., 2017).

Figure 5. The result of violacein inhibition assay (1; positive control, 2 and 4; methanol extracts of aerial and root parts, respectively, 3 and 5; water extracts of aerial and root parts).

Şekil 5. Viyolasin inhibisyon deneyi sonucu (1; pozitif kontrol, 2 ve 4; sırasıyla toprak üstü ve kök kısımlarının metanol özütleri, 3 ve 5; sırasıyla toprak üstü ve kök kısımlarının su özütleri).

However, dichloromethane and n-hexane extracts of the rhizomes of *E. azerbaijanica* have been shown to exhibit antibacterial activity against *S. aureus* and *Staphylococcus epidermidis* (Gram-positive bacteria) (Asnaashari et al., 2017). Moreover, it has been stated that essential oils obtained from the root, stem, and flower parts of *E. laevigata* also exhibit high antibacterial activity (Esmaeilli et al., 2012). In addition, it was reported that iridoid glycosides purified from *E. laciniata* have a remarkable antibacterial activity especially against *Bacillus cereus*, *S. aureus* (Gram-positive bacteria) and *E. coli*, *Proteus mirabilis* (Gram-negative bacteria) (Modaressi et al., 2009). In the current study, it was determined that methanol and water extracts of the aerial and root parts of *P. molucellides* did not exhibit any antibacterial activity against the Gram-positive (*S. aureus*, *E. faecalis* and *B. subtilis*) and Gram-negative (*E. coli*, *K. pneumoniae*, *S.* Typhimurium, *P. aeruginosa*, *E. aerogenes*, *A. haemolyticus*) bacteria tested, even at high concentrations. This result may be due to the chemical content of the plant, the geographical region where it grows, the harvesting, or the extraction method.

CONCLUSION

The lack of determination of the chemical content of the plant and the lack of investigation of other biological activities such as cytotoxic and antioxidant activities limit this study. This research was reported as preliminary study based on the *in-vitro* antibacterial and anti-QS effects of methanol and water extracts of aerial and root parts of *P. moluccellides.* Based on the results, *P. molucelloides* had not been found antibacterial and antibiofilm activities. However, its inhibitory activities on swarming motility were noteworthy. In particular, methanol extract of the aerial part of *P. molucelloides* had strong inhibitory activity on pyocyanin production. These results showed that *P. molucelloides* is a good candidate for the development of anti-QS agents. Further research can be done to elucidate the mechanisms underlying these biological activities.

Author's Contributions

Authors declare the contribution of the authors is equal.

Statement of Conflict of Interest

The authors have declared no conflict of interest.

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