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In vitro antimicrobial, antioxidant, antibiofilm and quorum sensing inhibitory activities of *Bellis perennis* L.

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ABSTRACT

The aqueous and ethanolic extracts of the aerial parts of *Bellis perennis*, widely used for edible vegetable in southwest Anatolia, were isolated and its antimicrobial, antioxidant, anti-biofilm, and quorum sensing inhibitory activities were investigated. Its antimicrobial activity was evaluated against 15 bacterial strains and *Candida albicans* using the disc diffusion assay and broth microdilution assay. Among the microorganisms tested, the most susceptible strains were *Staphylococcus epidermidis* MU 30 and *Staphylococcus aureus* MU 38. The antioxidant capacity was determined by the ferric thiocyanate (FTC) and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assays. Extracts showed weak radical scavenging activity with the DPPH method. IC_{50} values were found as 37.85 mg/ml for ethanolic extract and 96.98 mg/ml for aqueous extract, respectively. Results obtained from FTC assay showed 16.98% inhibition for ethanolic extract and 58.14% inhibition for aqueous extract compared with BHT (63.36% inhibition) and ascorbic acid (77.67% inhibition). The antibiofilm effect of the extracts was measured by microplate biofilm method. Ethanolic extract of *B. perennis* did not inhibit biofilm formations of the tested microorganisms, however the aqueous extract inhibited limited anti-biofilm activity against *P. aeruginosa* ATCC 27853, *P. fluorescens* MU 181 and *S.epidermidis* MU 30 at 10 mg/ml concentration. Anti-Quorum Sensing (QS) activity of extracts was determined using biosensor bioassay with *Chromobacterium violaceum* CV026. At the concentration of 100 mg/ml of aqueous extract of *B. perennis* showed promising anti-QS activity on *Chromobacterium violaceum* CV026 with zone of pigment inhibition 10 mm. Inhibition of QS-regulated violacein production in *Chromobacterium violaceum* ATCC 12472 and swarming motility in *Pseudomonas aeruginosa* PA01 were carried out using standard methods. Aqueous and ethanol extracts of *B. perennis* inhibited swarming by 9.5% and 38.1%, respectively. The results suggest that *B. perennis* could be an alternative source to explore for useful contents in the fight against bacterial infections.

Key words: *Bellis perennis*, antimicrobial, antibiofilm, antioxidant, Quorum sensing

Introduction

Biofilm is a community of cells attached to either a biotic or abiotic surface enclosed in a complex exopolymeric substance (EPS)(Flemming et al., 2000). The biofilm forming bacteria are resistant to antimicrobial agents due to the lack of penetration of antimicrobial agents (Frank & Koffi, 1990). The biofilm have been found to cause a wide variety of microbial infections in the body, such as urinary tract infections, catheter infections, middle-ear infections,

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formation of dental plaques, gingivitis, coating contact lenses (Costerton et al., 1999).

The discovery of anti-infective agents, which are active not only against planktonic microorganisms but also against microbial biofilms, represents an important goal (Projan & Youngman, 2002). At present, biofilm-mediated antibiotic resistance has become a major cause of anxiety for many clinical and device associated infections (Murugan et al., 2013). The biofilm-forming bacterial cells are able to communicate by the density-dependent cell-to-cell communication mechanism which is called quorum sensing (QS). This plays an important role in biofilm development, resistance, virulence and the production of EPS. Researchers believe that by controlling QS mechanism and biofilm formation, the resistance of these structures could be influenced (Wang et al. 2007). Recently, antivirulence therapies have drawn attention as a new strategy to combat microbial infections, and in that context bacterial QS represents a promising therapeutic target (Kaufmann et al., 2008).

Many pharmacognostical and pharmacological investigations are carried out to identify new drugs or to find new lead structures for the development of novel therapeutic agents in the antibiotic treatments (Newman et al., 2003). Inhibition of QS is therefore being considered as a new target for antimicrobial chemotherapy with the current quest on discovering non-toxic QS inhibitors from natural sources (Vattern et al., 2007).

Bellis perennis L. (Asteraceae) is a perennial native herbaceous plant in Brazil, Europe, Turkey, Cyprus, Syria, and Azerbaijan (Avato & Tava, 1995). This plant is traditionally used in the treatment of rheumatisms and as an expectorant (Hansel et al., 1992), as well as due to its anti-inflammatory (Schöpke & Hiller, 1992; Grabias et al., 1995; Nazaruk & Gudej, 2001), diuretic (Nazaruk & Gudej, 2001), antihyperlipidemic (Morikawa et al., 2008), antiechymotic (Avato & Tava, 1995), anxiolytic and antidepressant-like (Marques et al., 2012), antifungal (Desevedavy et al., 1989), antimicrobial (Avato et al., 1997; Kavalcioglu et al., 2010) and antioxidant (Kavalcioglu et al., 2010; Siatka & Kašparová, 2010; Marques et al., 2012; Marques et al., 2013) activities. To our knowledge, this is the first study of the antibiofilm and quorum sensing inhibitory activities of *B. perennis.* These activities are an important topic in the medical field as well as in the food safety and industry.

Materials and Methods

Preparation of the aqueous and ethanolic extracts

The whole plants of wild-growing *Bellis perennis* were collected in May-July, 2012 from natural populations in the vicinity of Kotekli (Mugla), Southwest Turkey. The air-dried and powdered plant material (30 g) were extracted with ethanol (Merck) (300 ml) using the Soxhlet apparatus. An aqueous extract was prepared by boiling 10% wt/wt of the air-dried powdered plant part in sterile distilled water for 30 min and then cooled to room temperature overnight. The aqueous extracts were passed through a membrane filter (Sartorius, Ø 0.20µm) to remove particulate matter. The ethanol extract was evaporated and the aqueous extract was lyophilized and then kept in small sterile opac bottles under refrigerated conditions until used.

Microbial strains

The in vitro antimicrobial and antibiofilm activity of the extracts of *B. perennis* was tested against three Gram-positive standard test bacteria *(Staphylococcus aureus* ATCC 25923, *Micrococcus luteus* NRLL B-4375 and *Bacillus subtilis* ATCC 6633), the two Gram-negative standard test bacteria *(Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853), one yeast *(Candida albicans* ATCC 10239), and multiresistant strains of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* and various species of Staphylococcus . The strains coded MU (the multiresistant bacteria) were obtained from the Mugla University Culture Collection.

The *Staphylococcus* sp., *Micrococcus luteus* and *Bacillus subtilis* strains were incubated at 37±0.1°C for 24–48 h, *Escherichia coli, P. aeruginosa* and *P.fluorescens* strains were incubated at 30±0.1°C for 18-24 h, and *C. albicans* were incubated at 30±0.1°C for 24-48 h. Inocula were prepared adjusting the turbidity of the medium to match the 0.5 McFarland standard dilutions. The strains were maintained in their appropriate agar slants at 4°C throughout the study and used as stock cultures.

Determination of antimicrobial activity by the disk diffusion method

The antimicrobial activity was assayed by the disc diffusion method (Bauer et al., 1966; Collins et al., 1995; Murray et al., 1995) using bacterial cell suspensions whose

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concentration was equilibrated to a 0.5 McFarland standard. One hundred microliters of each bacterial suspension was spread on a Mueller-Hinton agar plate. Sterile paper discs (6mm in diameter) were impregnated with 20 µL of each extract dissolved in the solvent used for extraction at 25 mg/mL. The discs were allowed to dry and then placed on the inoculated agar. The plates were incubated at the appropriate temperature and time for microorganisms. Discs of ethyl alcohol and distilled water were used as controls. After incubation time, the zone of inhibition was measured. The experiment was performed in triplicate.

Determination of minimal inhibitory concentrations

The minimal inhibitory concentration (MIC) was determined by a broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006). The test medium was Müeller Hinton Broth (MHB) and the density of bacteria was 5×10^5 colony-forming units (CFU)/mL. Cell suspensions (200µL) were inoculated into the wells of 96-well microtitre plates (Nunc F96 MİKroWell™ plates; NunclonTM Δ, Denmark) in the presence of extract with different final concentrations (1.25, 2.5, 5, 10, 20, mg/ml). The MIC was defined as the lowest concentration of extract at which no visible growth was observed. The optical density at 550 nm (OD_{550}) of each well content was recorded using an microplate reader (Thermo Scientific Multiskan FC, Vantaa, Finland), as a measure of microbial growth. Each assay was performed in triplicate for all bacteria.

Effect of extracts on bacterial biofilm formation

The effect of different concentrations of extract (ranging from 1 to 0.125 MIC) on biofilm-forming ability was tested on polystyrene flat-bottomed microtitre plates as described by Merritt et al. (2005). Briefly, 1% of overnight cultures (OD adjusted to 0.4 at 600 nm) of test pathogens were added into 200 µl of fresh TSB medium and cultivated in the presence and absence of extract without agitation for 48 h at 37ºC. The wells containing only TSB served as control. Wells containing only broth and different concentrations of extract (MIC, MIC/2, MIC/4 and MIC/8) were also observed. After incubation, the wells were washed with water to remove planktonic bacteria. The remaining bacteria were subsequently stained with 0.1% crystal violet solution for 10 min at room temperature. Wells were washed once again to remove the crystal violet solution that is not specifically staining the adherent bacteria. Microplates inverted and vigorously tap on paper towels to remove any excess liquid and air dried. 200 µl of 95% ethanol and 33% glacial acetic acid (Sigma Chemical Co) poured in Gram-negative bacteria/*C.albicans* and Gram-positive bacteria wells respectively. Biofilm stains solubilized at room temperature. The stained biofilms were resuspended in 200 µl PBS and OD_{550} was measured by spectrophotometry using an microplate reader (Thermo Scientific Multiskan FC, Vantaa, Finland). Percentage of inhibition of the tested extracts was calculated using the formula [1-(OD_{550} sample/OD_{550} control)]x 100%. All tests were done as triplicates.

Determination of DPPH radical scavenging activity

Antioxidant activity of the extracts were determined based on its ability to react with the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical (Yamasaki et al., 1994). Fifty µl of the extract (1.25, 2.5, 5 and 10 mg/ml in ethanol/water (1:1, v/v)) was added to 5 ml DPPH solution (0.004%) in ethanol. After incubation at room temperature for 30 min, the absorbance of each solution was determined at 517 nm. Percentage of inhibition and the concentration of sample required for 50% scavenging of the DPPH free radical (IC_{50}) were determined. BHT and ascorbic acid were used as a positive control.

Ferric thiocyanate (FTC) method

A screw-cap vial (φ 38mm × 75 mm) containing a mixture of 4 mg of sample in 4ml of 99.5% ethanol, 4.1 ml of 2.51% linoleic acid in 99.5% ethanol, 8.0 ml of 0.02M phosphate buffer (pH 7.0) and 3.9 ml of water (final concentration 0.02%, w/v) was placed in an oven at 40°C in the dark (Kikuzaki & Nakatani, 1993). To 0.1 ml of this mixture in a test tube (φ 1.5mm×14.5 cm), 9.7 ml of 75% (v/v) ethanol, 0.1 ml 30% ammonium thiocyanate and finally, 0.1 ml of 2×10^{-2}M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture. Three minutes after the addition of ferrous chloride, the absorbance was measured at 500 nm. This step was repeated every 24 h until the control reached its maximum absorbance value.

Bacterial strains and culture medium

The strains of *C. violaceum* CV12472, CVO26 and *Pseudomonas aeruginosa* PAO1 were obtained from the

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Spanish Type Culture Collection (CECT)(Valencia, Spain). Unless otherwise stated, all strains were grown in or on LB (Luria–Bertani) broth (15 g tryptone, 0.5% yeast extract, 0.5% NaCl) solidified with 1.5% agar (Hi-media) at 28°C when required and supplemented with appropriate antibiotic (kanamycin 20 µg ml^{-1} for *C. violaceum* CVO26).

Determination of MIC

MIC of extracts was determined against biosensor strains (CV12472 and CVO26) by broth macrodilution method. MIC is defined as the minimum concentration of extracts at which there was no visible growth of test strain. Sub-MICs were selected for the assessment of anti-QS activity in the above-mentioned strains.

Violacein Inhibition Assay

C. violaceum (ATCC 12472) was used in qualitative violacein inhibition screening. 10 µL of overnight culture of *C. violaceum* ATCC 12472 (OD adjusted to 0.4 at 600 nm) was added into wells of sterile microtiter plates (MTPs) containing 200 µL of LB broth and incubated in the presence and absence of various concentrations of test extracts (0.5-25 mg/mL) at 30°C for 16 h and observed for inhibition in violacein pigment production.

Bioassay for QSI activity using CV026

Five milliliters of warm molten Soft Top Agar (1.3 g agar, 2.0 g tryptone, 1.0 g sodium chloride, 200 mL deionized water) was seeded with 100 µL of an O/N CV026 culture, and 20 µL of 100 µg/mL C6HSL was added as exogenous AHL source. This was gently mixed and poured immediately over the surface of a solidified LBA plate as an overlay. Wells of 5mm in diameter were made on each plate after the overlay had solidified. Each well was filled with 50 µL of filter sterilized herb extract. A white or cream colored halo around this well against a purple lawn of activated CV026 bacteria was an indication of QSI (Fig. 1). A clear halo indicated antimicrobial (AM) activity. The limit of detection of activity was also determined by applying serial dilutions of the extracts (1:1 to 1:16, using LB broth as diluent). Endpoints were estimated as the lowest dilution of extract giving discernible inhibition of violacein synthesis. Each experiment was repeated and the assay plates were incubated at 30°C for 3 days.

Anti-swarming in PA01

A loopful of O/N PA01 culture on LBA was resuspended in 4 mL of sterile LB broth. Fifty microliters of sterile plant extract (100 mg/ml) was mixed with 3 mL of molten Soft Top Agar and poured immediately over a plate. Five microliters of the PA01 suspension was inoculated in the center of the plate when the agar had solidified, and the surface was air-dried for 10-15 minutes. All experiments were repeated and the plates were incubated at 37°C for 3 days. Extent of swarming was determined by measuring the area of the colony in square millimetre using graph paper.

Results

The antimicrobial activity of *Bellis perennis* extracts were determined by the disc diffusion method and a broth microdilution method. The results obtained after evaluation of the antimicrobial activity of the extracts are shown in Table 1. In this study, eight Gram-positive test bacteria, seven Gram-negative bacteria, and one yeast were used. In disc diffusion assay, the aqueous extract had antimicrobial effect on two Gram-positive bacteria (*B. subtilis* and *S. epidermidis* MU 30) and two Gram-negative bacteria (*P. aeruginosa* ATCC 27853 and *P. fluorescens* MU 181), and the inhibition zones ranged between 7 and 10 mm. Ethanol extract inhibited the multiresitant strains of *S. epidermidis* MU 30 and *S. aureus* MU 38 with 9 mm inhibition zones. As a result of MIC test, the aqueous extract inhibited *S. epidermidis* MU 30, *P. aeruginosa* ATCC 27853 and *P. fluorescens* MU 181 at 10 mg/ml concentration. *S. aureus* MU 38 was found the most sensitive bacteria against ethanol extract with 10 mg/ml concentration (Table 1).

The percentage inhibition of biofilm formation against the test bacteria are given in Table 1. The aqueous extract of *B. perennis* were found to have different proportions of antibiofilm activity against three tested bacteria at MIC's. The highest antibiofilm activity was determined at the 10 mg/ml concentration of aqueous extract with the reduction rate of 22.89% against *S. epidermidis* MU 30 biofilm production. In the presence of 10 mg/ml aqueous extract (MIC), the mean biofilm formation values were equal to 19.57% for *P. aeruginosa* ATCC 27852, and 18.12% for *P. fluorescens* MU 181.

Antioxidant activity of ethanol and aqueous extracts from *B. perennis* was evaluated using FTC and DPPH free radical scavenging methods.

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Table 1. *Antimicrobial and antibiofilm activities of B.perennis extracts*

Microorganism	Extract	Planktonic		% inhibition on biofilms			
		Disc diffusion (mm)	MIC (mg/ml)	MIC	MIC/2	MIC/4	MIC/8
B.subtilis ATCC	Aqueous	7	>20	-	-	-	-
6633	Ethanol	-	>20	-	-	-	-
M.luteus NRRL	Aqueous	-	>20	-	-	-	-
B-4375	Ethanol	-	>20	-	-	-	-
S.aureus ATCC	Aqueous	-	>20	-	-	-	-
25923	Ethanol	-	20	-	-	-	-
S.aureus	Aqueous	-	>20	-	-	-	-
MU 38	Ethanol	9	10	-	-	-	-
S.aureus	Aqueous	-	>20	-	-	-	-
MU 40	Ethanol	-	20	-	-	-	-
S.aureus	Aqueous	-	>20	-	-	-	-
MU 46	Ethanol	-	20	-	-	-	-
S.aureus	Aqueous	-	>20	-	-	-	-
MU 47	Ethanol	-	>20	-	-	-	-
S.epidermidis MU	Aqueous	10	10	22.89	-	-	-
30	Ethanol	9	20	-	-	-	-
E.coli ATCC	Aqueous	-	>20	-	-	-	-
25922	Ethanol	-	>20	-	-	-	-
P.aeruginosa	Aqueous	9	10	19.57	-	-	-
ATCC 27853	Ethanol	-	20	-	-	-	-
P.aeruginosa MU	Aqueous	-	>20	-	-	-	-
187	Ethanol	-	20	-	-	-	-
P.aeruginosa MU	Aqueous	-	>20	-	-	-	-
188	Ethanol	-	20	-	-	-	-
P.aeruginosa MU	Aqueous	-	>20	-	-	-	-
189	Ethanol	-	20	-	-	-	-
P.fluorescens MU	Aqueous	-	>20	-	-	-	-
180	Ethanol	-	>20	-	-	-	-
P.fluorescens MU	Aqueous	8	10	18.12	-	-	-
181	Ethanol	-	>20	-	-	-	-
C. albicans	Aqueous	-	>20	-	-	-	-
ATCC 10239	Ethanol	-	20	-	-	-	-

As far as DPPH scavenging assay results in concerned, the ethanol extract was more effective in the DPPH assay, as it reduced the stable free radical DPPH with lower IC_{50} value (37.85 mg/ml) than the aqueous extract (96.98 mg/ml). Nevertheless, both extracts had weaker antioxidative capacity than the positive control, BHT and α-tocopherol, which had a very low IC_{50} values (0.487 mg/ml and 1.75 mg/ml) in the DPPH assay. As shown in Table 2, The FTC method measures the amount of peroxide in the initial stages of lipid peroxidation. Low absorbance value in the FTC method

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indicate high level of antioxidant activity. The absorbance was measured on the final day (4th day) of FTC assay. Results obtained from FTC assay revealed that aqueous and ethanol extracts of *B.perennis* carry the antioxidative potential for chain-breaking inhibition of lipid peroxidation and for free radical scavenging as extract has shown 58.14 and 16.98% inhibition.

Table 2. *Results of extracts of B.perennis on the in vitro free radical (DPPH) scavenging assay and Ferric thiocyanate assay (FTC)* [a]

Sample	DPPH[b]	FTC[c]
Aqueous extract	96.98±1.52	58.14±2.27
Ethanol extract	37.85±0.76	16.98±1.75
BHT	0.487±0.014	63.36±2.52
α-tocopherol	1.75±0.04	39.96±1.65
Ascorbic acid	NT	77.67±2.81

[a] Results are presented as mean± standard deviation, [b]IC50 values of DPPH assay (as mg/ml), [c]Given as percentage inhibition of the final day (4th day)

MIC of the test extracts against bio-reporter strains (CV 12472 and CV026) ranged from 0.5 to >100 mg/ml (Table 3). A range of sub-MICs were selected for anti-QS screening using *C.violaceum* CV 026 strain, agar well diffusion method was adopted in the presence of C_6-AHL. Inhibition of pigment production was detected in the aqueous extract of *B. perennis* with zone of pigment inhibition with 10 mm against CV 026 at 100 mg/ml concentration (Figure 1).

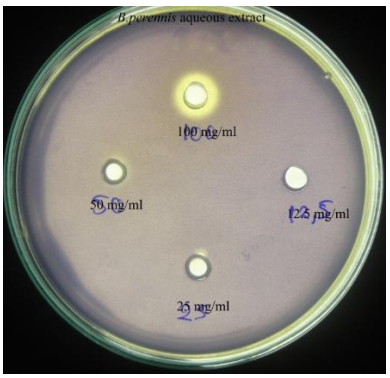

Figure 1. *Anti-quorum sensing (anti-QS) activity by B.perennis aqueous extract against bioreporter strain CV 026, using agar well diffusion method. Antibacterial activity is observed in addition to anti-QS by aqueous extract at 100 mg/ml.*

No effect on pigment inhibition was observed by ethanol extract at tested concentrations. In the qualitative analysis, extracts of *B.perennis* exhibited no inhibitory activity in violacein production of *C.violaceum* CV 12472.

With PA01 as a reporter organism of QSI, both of extract manifested a definite effect on swarming motility, a QS related phenotype (Table 3, Figure 2). Compared to control in which no extract was present, ethanol extract from *B.perennis* reduced swarming in PA01 by 38.1%. The aqueous extract of *B.perennis* inhibited swarming by 9.5%. Interestingly, of the aqueous extract that had marked QSI in the CV 026 assay, extract also showed antimicrobial activity.

Table 3. *Screening of B. perennis extracts for anti-QS activity, inhibition of violacein production, and swarming motility*

Plant extract	MIC against CV 12472 (mg/ml)	Inhibition of violacein production against CV 12472	Inhibition of swarming against PA01[a]	MIC against CV 026	Antimicrobial activity against CV 026[b] (inhibition zone size in mm)	Anti- QS activity against CV026 in presence of C6-AHL (inhibition zone size in mm)
Aqueous	25	No	Yes(9.5)	>100	Yes(8)	Yes(10)
Ethanol	25	No	Yes(38.1)	1	No	No
Tetracycline	NT	NT	NT	NT	Yes(32)	No
Gentamycin	NT	NT	NT	NT	Yes(11)	Yes (6)
Chloramphenicol	NT	NT	NT	NT	Yes (13)	No
Kanamycine	NT	NT	No	NT	NT	NT
Ampicilline	NT	NT	Yes (14.3)	NT	NT	NT

[a] Percentage reduction of area of colony (indicating inhibition of swarming) in *Pseudomonas aeruginosa* compared with control in parentheses. The percentage of reduction was calculated by comparing the area of the swarming colonies on medium supplemented with *B.perennis* extracts with reference to that of the control on medium without extract. [b]Presence of antimicrobial inhibition observed as a clear halo. Diameter of zone of antimicrobial inhibition in millimeter.

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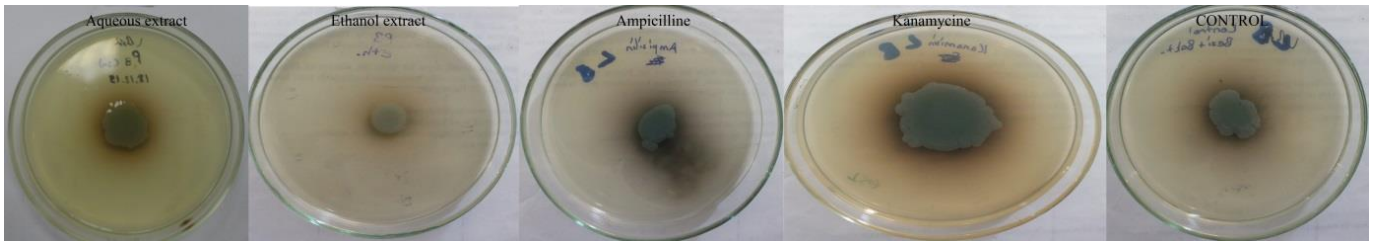

Figure 2. *Effect of extracts of B.perennis on swarming in PAO1. PAO1 colonies on LB media with aqueous and ethanol extracts of B.perennis showed swarming inhibition compared to the control without B.perennis extracts. In contrast to kanamycin, ampicillin showed swarming inhibition.*

Discussion

Several bacterial phenotypes such as virulence, biofilm maturation, secondary metabolite production, plasmid transfer, bioluminescence and motility are under control of QS circuitries (Di Cagno et al., 2011). Although several biological properties such as antioxidant (Kavalcioglu et al., 2010; Siatka & Kašparová, 2010; Marques et al., 2012; Marques et al., 2013), antifungal (Desevedavy et al., 1989), antimicrobial (Avato et al., 1997; Kavalcioglu et al., 2010) of *B. perennis* have been investigated so far, its ability to prevent antibiofilm and QS have not yet been reported. In the present study, the results indicate that the aqueous extract of the aerial parts of *B. perennis* possessed anti-QS activity. This is the first report on anti-QS activity of the tested extracts. Considering the fact that *B. perennis* is widely used locally as edible vegetable obtained anti-QS results are important. However, inhibition of violacein production by *C. violaceum* CV 12472 could not be detected both extract of *B. perennis*. Obviously, in the present study, the aqueous and ethanol extracts of *B. perennis* reduced the swarming motility of target pathogens. 38.1% reduction in swarming motility has been recorded by ethanol extract of *B. perennis*. This ratio was determined to be 9.5% for aqueous extract. Swarming phenomenon in bacteria is considered to be a virulence factor as it is involved in biofilm formation because of mass translocation of cells, and this relies on expression of biosurfactants molecules, the expression of which is under QS control in PA01 (Daniels et al., 2004).

The antimicrobial and antibiofilm activity assays indicated that *B. perennis* aqueous extract had moderately antimicrobial and antibiofilm activities against *S.epidermidis* MU30, *P. aeruginosa* ATCC 27853 and *P. fluorescens* MU 181. These activities are important in the treatment of the infections associated from biofilm-mediated antibiotic resistance bacteria.

This study also demonstrated that aqueous extract of *B. perennis* produced significant removal of lipid peroxidation, which may be capable of inhibiting cell damage caused by this radical. The obtained results are in accordance with the popular use in traditional medicine and food, further studies are necessary to confirm and extend these results.

Acknowledgement

The authors acknowledge the Mugla Sitki Kocman University Research Funds for financial support (Project 12/68).

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