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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

Inhibition of *Pseudomonas aeruginosa* Biofilm Formation and Quorum Sensing System by Extracts of *Prunus avium* Stalk

Prunus avium Sapından Elde Edilen Özütlerle Pseudomonas aeruginosa'ya ait Quorum Sensing Sisteminin ve Biyofilm Oluşumunun İnhibisyonu

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Abstract
Recently, misuse or overuse of antibiotics has led to antibiotic resistance problem, a global healthcare problem. Most virulence factors and biofilm formation in *Pseudomonas aeruginosa* are controlled by quorum sensing (QS). The inhibition of QS system by inhibitor molecules has been suggested as a novel alternative antivirulence approach in which no need to kill the bacteria. In the present study, QS and biofilm inhibitory potentials of the methanol and acetone extracts of *Prunus avium* stalk against *P. aeruginosa* were evaluated. The extracts were tested at the concentrations of 240, 120, and 60 µg/ml. *lasB-gfp, rhlA-gfp, pqsA-gfp* biosensor strains and *P. aeruginosa* PAO1 were used to monitor QS and biofilm inhibition, respectively. Fluorescence and absorbance measurements were performed on Cytation 3 multimode microplate reader. QS inhibition ratios for *las, rhl,* and *pqs* systems and biofilm inhibition ratios of the acetone extracts were recorded as 70.43%, 47.25%, 76.31%, and 47.76% (±6,60) and of the methanol extracts as 74.96%, 40.10%, 71.89%, and 38.54% (±3,56) at a certain concentration of 240 µg/ml, respectively. As a result, anti-QS and anti-biofilm properties of acetone extracts were better than that of methanol extracts. Further investigations are needed to discover inhibitor compounds of *P. avium* and also their effects on human cells and then these compounds may be used in new drug discoveries.
Keywords: *Pseudomonas aeruginosa*, quorum sensing, biofilm, quorum quenching, *Prunus avium* stalk, sweet cherry.

Öz
Son zamanlarda, antibiyotiklerin yanlış veya aşırı kullanımı, küresel bir sağlık sorunu olan antibiyotik direnci sorununa yol açmıştır. *Pseudomonas aeruginosa*'da çoğu virülans faktörü ve biyofilm oluşumu, quorum sensing (QS) ile kontrol edilir. QS sisteminin inhibitör moleküller tarafından inhibisyonu, bakterileri öldürmeye gerek olmayan yeni bir alternatif antivirulens yaklaşımı olarak önerilmiştir. Bu çalışmada, *Prunus avium* sapından elde edilen metanol ve aseton özütlerinin *P. aeruginosa*'ya karşı QSI (QS inhibitörleri) ve anti-biyofilm potansiyellerini değerlendirdik. Ekstraktlar 240, 120 ve 60 µg/ml'lik konsantrasyonlarda test edilmiştir. QS ve biyofilm inhibisyonunu izlemek için *lasB-gfp, rhlA-gfp,* ve *pqsA* biyosensör suşları ve *P. aeruginosa PAO1* kullanıldı. Floresans ve absorbans ölçümleri Cytation 3 çok modlu mikroplaka okuyucu üzerinde gerçekleştirildi. 240 µg/ml konsantrasyonunda aseton özütlerinin *las, rhl* ve *pqs* sistemleri üzerine QS ve biyofilm inhibisyon oranları sırasıyla % 70.43, % 47.25, % 76.31 ve % 47.76 (± 6,60) ve metanol özütlerinin sırasıyla % 74.96, % 40.10, % 71.89 ve % 38.54 (± 3,56) olarak kaydedilmiştir. Sonuç olarak, aseton özütlerinin anti-QS ve anti-biyofilm özellikleri metanol özütlerinden daha başarılı olmuştur. *P. avium*'un inhibitör bileşikleri ve bu bileşiklerin insan hücreleri üzerindeki etkilerini keşfetmek için daha fazla araştırmaya ihtiyaç vardır ve daha sonra bu bileşikler yeni ilaç keşiflerinde kullanılabilir.
Anahtar Kelimeler: *Pseudomonas aeruginosa*, quorum sensing, biyofilm, quorum quenching, *Prunus avium* stalk, sweet cherry.

I. INTRODUCTION

Bacterial pathogenicity is defined as the potential of an organism to cause any disease. As known, the production of virulence factors by microorganisms has great importance in the clinical course of a disease. These factors may cause damages on the host immune system due to failure in the balance between bacterial pathogenicity and host resistance (1). Unfortunately, antibiotics are not sufficiently efficient in the treatment of bacterial infections. Therefore, an antivirulence approach has been proposed to treat infections. By this approach, expressions or

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activities of virulence properties can be prevented and bacteria cannot colonize the host. Furthermore, it has been assumed that there is probably less evolutionary pressure to develop resistant clones than conventional antibiotics because this strategy does not directly kill bacteria but prevent bacterial infections and damages to their host. These anti-virulent drugs can potentially be used in combination with synergistically established or new antimicrobials to prolong the life of these drugs (2, 3).

The World Health Organization (WHO) declared the priorities of antibiotics for pathogens as critical, high, and medium highlighting the need for new antibiotics. According to this list, *Pseudomonas aeruginosa* is categorized as critical for the discovery of new antibiotics (4). *P. aeruginosa* can cause nosocomial infections such as cystic fibrosis, especially in immunocompromised patients. In 2004, the U.S. Cystic Fibrosis Foundation Patient Registry reported that *P. aeruginosa* was identified in 57.3% of all respiratory cultures (5). The data from newborn screening programs showed that the total number of cystic fibrosis (CF) patients in sixteen European countries, CF adults and CF children in 2025 would rise by 50%, 75%, 20% respectively (6). The clinical importance of this opportunistic pathogen attributed to its resistance to multiple antimicrobial agents, its quorum sensing mediated virulence factors (exoproteases, siderophores, exotoxins and, rhamnolipids, etc.), and ability to form biofilm formation resulting in the community- or hospital-acquired infections (7-9).

Bacteria communicate via quorum sensing system (QS) which allows controlling their social behaviors. A high density of bacterial population in the surrounding environment trigger QS system for intra-species, inter-species, or inter-kingdom interactions (10, 11). Autoinducers (AIs), small molecules that can easily diffuse across inner and outer membranes, are secreted into bacterial local milieu. Gram-negative bacteria utilize homoserine lactones (HSLs). It is well documented that *P. aeruginosa* has las, rhl, pqs and iqs systems for interspecies communication (12). Approximately a tenth of the total *P. aeruginosa* genes are coordinated by QS. These genes are responsible for many virulence factors, antibiotic resistance, regulation of metabolic pathways under stress and biofilm structure of *P. aeruginosa* (13). The critical importance of biofilm structures in chronic infections has been emphasized in the literature. Biofilms are sessile community complexes in which bacterial cells attach onto various surfaces in an exopolysaccharide matrix. Biofilm forms are more resistant to antibiotics compared to planktonic forms (14).

Most researchers have focused on an alternative antivirulence approach to combat bacterial antibiotic resistance by disrupting the QS system, called quorum quenching (QQ) (15). In this way, several compounds and enzymes with quorum sensing inhibitory (QSI) potential have been identified to quench the QS mechanism. The criteria for QSI molecules are notified as high specificity, efficiency, stability, having low-molecular-weight (16).

Since ancient times, different parts of plants have traditionally been used in the treatment of various disorders. Nowadays, they are globally valuable resources of new drugs not only in developing countries but also in modern countries (17). For this reason, compounds with QSI properties are investigated especially in plants and anti-QS potentials of plant species collected from different localities are investigated as direct extracts or based on the substances they contain (18).

Prunus avium L., (sweet cherry) is a member of *Rosaceae* family and is distributed around the world with a temperate climate but especially in Europe, North Africa, South Australia, New Zealand, USA, Canada, Argentina and Chile (19, 20). *P. avium* has several beneficial effects on various illnesses such as cancer, cardiovascular disease, diabetes, Alzheimer's disease, neurodegenerative diseases and, other inflammatory diseases as well as being consumed as food (21, 22). Different parts of *P. avium* such as its fruit, stem, and bark are used for medicinal and therapeutic purposes (20). Furthermore, its antibacterial, antioxidant, and anti-inflammatory activities have been demonstrated (23- 29).

To our knowledge, there is no study investigating the QSI and anti-biofilm potentials of *P. avium* stalk against *P. aeruginosa.* In the view of an urgent need for new alternative approaches that can solve the global health problem due to the current antibiotic resistance, the potential impact of *P. avium* (sweet cherry) stalk was investigated to inhibit the QS system and biofilm formation of *P. aeruginosa*. For this purpose, QSI potentials of acetone ad methanol extracts of *P. avium* stalk samples were tested on the biosensor strains of *P. aeruginosa, lasB-gfp, rhlA-gfp* and *pqsA-gfp,* and anti-biofilm activities were tested on the *PAO1* wild type strain.

II. MATERIAL AND METHODS

2.1. Sample Collection and Extraction of P. avium Stalk Samples by Acetone and Methanol Solvents

Following the washing and drying of *P. avium* stalk samples, ten grams of each sample were weighed and pulverized. Acetone and methanol solvents were added into sterile bottles including the samples and stored in a dark place for 3 days. They were evaporated in a rotary evaporator at 40 °C and 100 rpm. The acetone and methanol extracts of *P. avium* stalk samples were weighed again to obtain the weight of crude extracts. To evaluate the anti-QS and anti-biofilm properties, a

stock concentration of these extracts was prepared as 16 mg/ml and then dissolved in 100% DMSO. Finally, these extracts were diluted with a physiological saline solution.

2.2. Monitor Strains

lasB-gfp, rhlA-gfp, and *pqsA-gfp* were used as QS monitor strains of *P. aeruginosa* (30-32). These monitor strains included *lasR, rhlR, pqsR* regulated promoters and a gene for an unstable green fluorescent protein (gfp). In the present study, M9 minimal media supplemented with 2.5 mg/l thiamine, 0.5% (wt/vol) glucose, and 0.5% (wt/vol) casamino acids were used for the growth of the test bacteria.

2.3. QSI Screening

QSI potentials of the acetone and methanol extracts of *P. avium* stalk samples were examined in 96-well black microplates (Nunc, Thermo Scientific) (33). 100 µl of the prepared growth medium given above was added to each well. The test extracts were then diluted three-fold to obtain final concentrations of tested extracts as 240, 120 and 60 µg/ml in 96-well black microplates. The total volume in each well was then adjusted to 200 µl by adding overnight cultures of the *lasB-gfp, rhlA-gfp* and *pqsA-gfp* monitor strains with an OD 450 nm of 0.1. The positive and negative control groups were also tested. The experiments were performed in three replicates. The bacterial growth and gfp expressions were measured every 15 minutes using Cytation 3 multimode microplate reader (Biotek) for 16 h. The measurements of fluorescence were recorded at 485 nm excitation and 535 nm emission wavelengths.

2.4. Biofilm Experiments

P. aeruginosa PAO1 strain was incubated overnight in a prepared M9 growth medium at 37°C. In 96-well microplates, the acetone and methanol extracts of *P. avium* stalk samples were tested at the concentrations of 240, 120 and 60 µg/ml, respectively. The experiments included positive and negative controls. Three replicates were made for the tests. The biofilm forms were stained with 0.1% crystal violet and measured at OD 590 nm in the microplate reader (Cytation 3-BioTek).

III. RESULTS

Three concentrations (240, 120 and 60 µg/ml) of acetone and methanol extracts of *P. avium* stalk samples were tested on *lasB-gfp, rhlA-gfp* and *pqsA-gfp* monitor strains of *P. aeruginosa.* Since azithromycin was reported to reduce the transcription of *lasI* by 80% and of *rhlI* by 50% in the literature (34), azithromycin was also tested as a positive control to inhibit tested QS systems (*las, rhl* and *pqs*). We determined that azithromycin was significantly able to inhibit gfp production of tested monitor strains (The data was not shown). In our experiments, maximum QS inhibition ratios on *las, rhl* and *pqs* systems were detected at a certain concentration of 240 µg/ml for acetone and methanol extracts of *P. avium* stalk samples. The acetone extracts of *P. avium* stalk at a concentration of 240 µg/ml inhibited *las, rhl* and *pqs* systems of *P. aeruginosa* in ratios of 70.43%, 47.25%, and 76.31% respectively. The related dose-response curves of *lasB-gfp, rhlA-gfp* and *pqsA-gfp* monitor strains of *P. aeruginosa* treated with the acetone extracts of *P. avium* stalk at certain concentrations of 240, 120 and 60 µg/ml were given in Figure 1A-C.

On the other hand, QS inhibitory potentials for *las, rhl* and *pqs* systems of methanol extracts of *P. avium* stalk were recorded as 74.96%, 40.10%, and 71.89%, respectively Figure 2.

Anti-biofilm properties of acetone and methanol extracts of *P. avium* stalk samples were tested against *PAO1* strain. The inhibition percentages for biofilm formation belonging to the extracts of *P. avium* stalk samples at a dose of 240 µg/ml were found to be slightly different and recorded as 47.76% (±6.60) and 38.54% (±3.56), respectively. Biofilm inhibition ratios of the acetone and methanol extracts of *P. avium* stalk at the concentrations of 240, 120, 60 µg/ml against *PAO1* strain were given in Figures 3-4. These results indicate that acetone extracts have a more pronounced effect in comparison to methanol extracts.

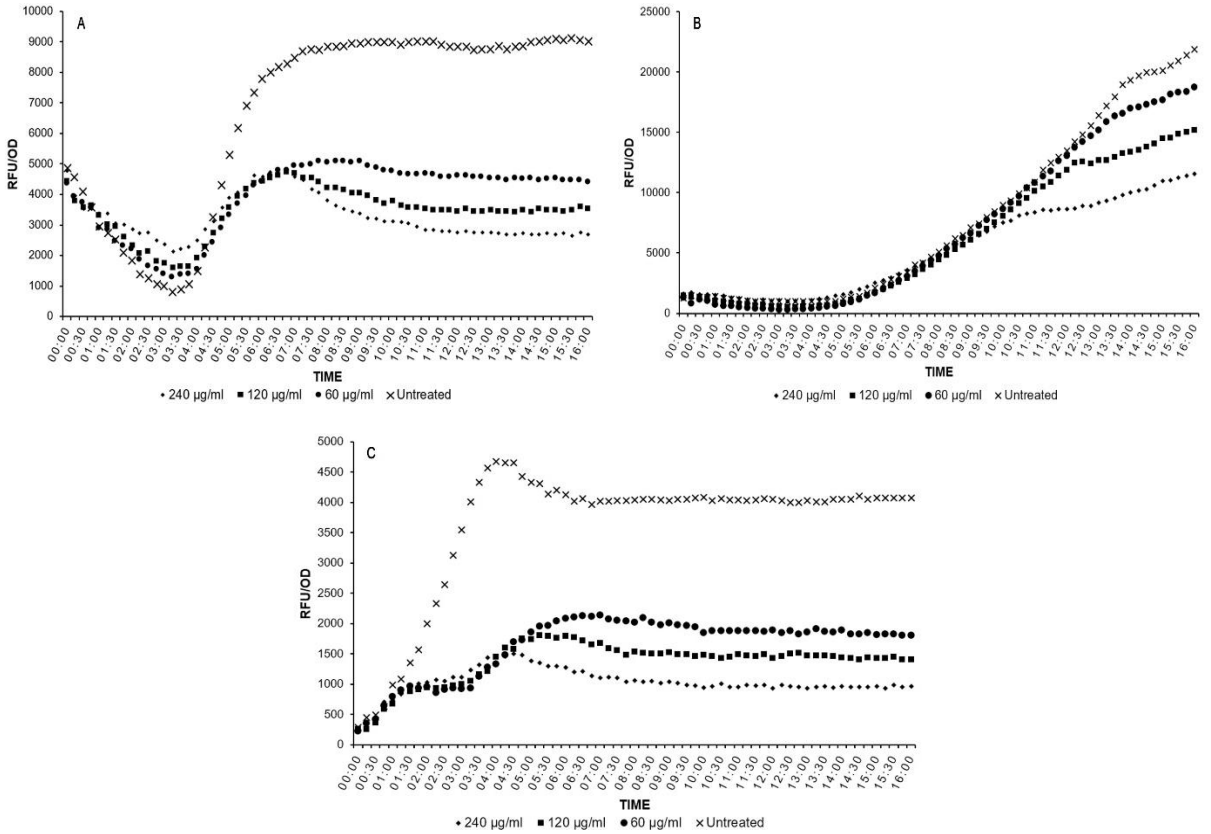

Figure 1. Dose-response curves of biomonitor strains of *P. aeruginosa* treated with the acetone extracts of *P.avium* stalk at certain concentrations of 240, 120 and 60 µg/ml. Data are shown as relative fluorescence unit over OD 450 nm.

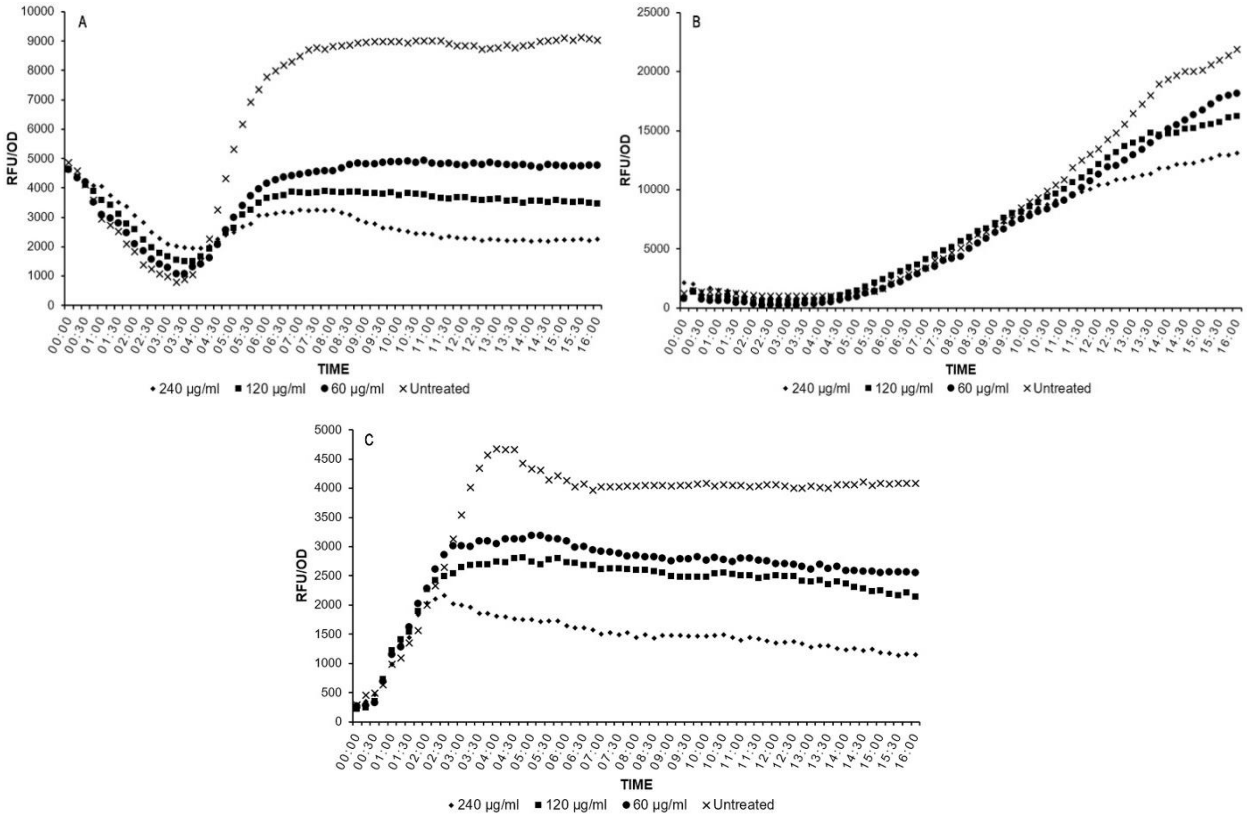

Figure 2. Dose-response curves of biomonitor strains of *P. aeruginosa* treated with the methanol extracts of *P.avium* stalk at the certain concentrations of 240, 120 and 60 µg/ml. Data are shown as relative fluorescence unit over OD 450 nm.

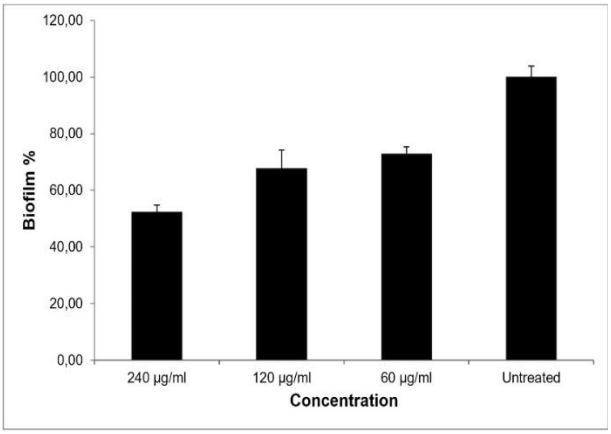

Figure 3. The percentage of anti-biofilm properties of the acetone extracts of *P.avium* stalk at concentrations of 240, 120, 60 µg/ml against *PAO1* strain.

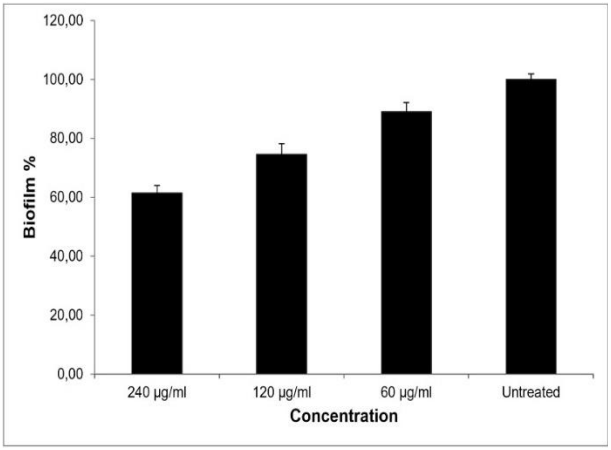

Figure 4. The percentage of anti-biofilm properties of the methanol extracts of *P.avium* stalk at the concentrations of 240, 120, 60 µg/ml against *PAO1* strain.

IV. DISCUSSION

As known, antibiotic misuse or overuse led to the problem of antibiotic resistance as a global healthcare problem. *P. aeruginosa* controls its' virulence factors and biofilm formation by QS mechanism. Alternatively, QS system and biofilm formation can be inhibited by inhibitor molecules as an antivirulence approach without killing bacteria. To the best of our knowledge, anti-QS and anti–biofilm effects of *P. avium* stalk samples against *P. aeruginosa* have not been studied yet. In this study, we demonstrated the potential inhibitory properties of acetone and methanol extracts of *P. avium* stalk samples on QS mechanisms and biofilm formation.

It has been well documented that the different parts (fruit, seed, stem bark and roots) of *P. avium* (sweet cherry) has several bioactive compounds (35-39). Several chemical compounds such as flavonoids, phenolics, polyphenols, alkaloids, tannins were reported in *P. avium* (37, 40-42). There are many studies in the literature focusing on the antibacterial activities of extracts or fractions of different parts of *P. avium* obtained by different solvents or of its' fruit juice. Rovčanin *et al.* (2015*)*, reported that ethanol extracts of *P. avium* petiole had an antibacterial effect against multiple antibiotic-resistant *Escherichia coli* and they detected also high concentrations of phenols and flavonoids in the ethanol extracts (26). Accordingly, the inhibitory activities of leaf and stem bark ethanol extracts of *P. avium* were found to be more effective against Gram-negative compared to the Gram-positive bacteria. In this study, researchers detected that the stem bark extracts were more potent when compared to the leaf extracts (39).

On the other hand, it was demonstrated that ethyl acetate and butanol fractions of *P. avium* had high antibacterial activity against *Listeria monocytogenes, Staphylococcus aureus,* and *Salmonella typhimurium* but the extracts and fractions had no inhibitory effect against *P. aeruginosa* (23). *Propionibacterium acnes*, acne-inducing bacteria, was also inhibited by the juice of *P. avium*. The juice and/or methanol extracts of *P. avium* inhibited the growth *of Streptococcus pyogenes* and *P. acnes* but not *Staphylococcus epidermidis* (24). The reason for differences in the sensitivity of Gram-positive and Gram-negative bacteria might be dependent on variables in the pattern including the cell wall such as peptidoglycan structure or high levels of lipopolysaccharides (39). Taken together, *P. avium* has noticeable antibacterial potentials against Gram-positive, or Gram-negative bacteria depending on the species. Nevertheless, studies about the antibacterial properties of *P. avium* against *P. aeruginosa* are scarce. To our knowledge, there is no study investigating the QSI and anti-biofilm potentials of *P. avium* stalk against *P. aeruginosa.* As mentioned before, quorum quenching, by other words quorum sensing inhibition, is a novel approach to overcome bacterial antibiotic resistance. In this meaning, plant-based QSIs with fewer side effects, considerable bioavailability, low costs and no toxicity may serve alternatively promising treatment strategies individually or along with conventional antibiotics. The stalk parts of *P. avium* are generally discarded. The evaluation of stalk parts of this plant that will be disposed may provide highly possible add value to the country's economy since *P. avium* stalk may ensure beneficial effects in terms of health without any cost. Therefore, there are many positive aspects of utilization of *P. avium* stalk in healthcare because they are plant-based material and have cost-effectiveness as well as their potential therapeutic effects. Besides the evaluation of the material to be discarded can be achieved.

Considering the extract concentrations that we applied in our biofilm experiments, we can suggest that all our three concentrations (240, 120, and 60 µg/ml) are considerably low according to other studies testing the anti-biofilm properties of extracts from various plants. For example, Ravichandiran *et al.* (2013) reported that *Melia dubia* bark extracts reduced biofilm formation by

84% and QS system by 75% in *E.coli* at a concentration of 30 mg/ml (43). In another study, it was shown that *Capparis spinosa* extract inhibited biofilm formation of some pathogen Gram-negative bacteria at a concentration of 2 mg/ml (44). Sandasi *et al.*, demonstrated that *Rosmarinus officinalis, Mentha piperita*, and *Melaleuca alternifolia* exhibited anti-biofilm activity against *Listeria monocytogenes* at a 1 mg/ml (45). Trentin *et al.* (2011) tested the antibiofilm properties of several medicinal plants from the Caatinga in Brazil at the concentration of 0.4 mg/mL and 4.0 mg/mL against *S. epidermidis* (46).

In other respects, QS inhibition is usually assessed by the detection of AHL-related inhibitory activity based on the violacein pigment production in *Chromobacterium violaceum* strain CV026, which is unable to synthesize its AHL in the literature. Fluorescence-based biosensor strains have more advantages to evaluate QS response of bacterial cells due to their ability for screening directly QS-related gene expressions. Because we tested our samples on biomonitor strains, we could easily observe the inhibition rates by gene expression levels. According to our results, both extracts of *P. avium* stalk inhibited the *las* and *pqs* system but they provided less inhibition on *rhl* system of *P. aeruginosa*. Anti-biofilm properties of *P. avium* stalk extracts were tested against *PAO1* strain. The inhibition percentages for biofilm formation belonging to the acetone and methanol extracts of *P. avium* stalk samples at a concentration of 240 µg/ml were found to be slightly different and recorded as 47.76% (±6,60) and 38.54% (±3,56), respectively. Accordingly, to the QS experiments, our acetone extracts were found to be more effective on biofilm inhibition in contrast to methanol extracts.

From this point, taken into consideration of ethnobotanical importance and bioactive potential of *P. avium* stalk, antibiotic resistance problems may be solved by interrupting the QS system as an alternative strategy. Further detailed studies about the bioactive compounds of this plant should be performed. These compounds may be used in the manufacture of new drugs or alternatively in combination with antibiotics in drug discovery.

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