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# The Strain-Dependent Antimicrobial and Antibiofilm effect of *Cis* and *Trans*-Vaccenic Acid against *Pseudomonas Aeruginosa*

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**ABSTRACT**

This study, it was aimed to investigate the antibacterial and antibiofilm activity of *cis* and *trans*-vaccenic acid against *Pseudomonas aeruginosa*. In the study, four different *P. aeruginosa* strains were used. Antibacterial activity was determined by microdilution and growth curve. The antibiofilm activity was determined by crystal violet assay. In addition, the effect of vaccenic acids on pyocyanin production was investigated. The minimum inhibitory concentration (MIC) of *cis* and *trans*-vaccenic acid against all strains was determined as 128-256 µg/mL, and the minimum biofilm inhibitory concentration (MBIC) value was 8-512 µg/mL. While vaccenic acids reduced cell growth in three strains, they also significantly inhibited pyocyanin production. In one strain, it inhibited biofilm formation without affecting cell growth. As a result, the presence of antibacterial and antibiofilm activity of *cis* and *trans*-vaccenic acid against *P. aeruginosa* was determined as potential agents in the fight against this bacteria.

***Keywords:*** Vaccenic acid, *Pseudomonas aeruginosa*, Antibiotic resistance, Biofilm, Pyocyanin

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## Introduction

Nowadays, antibiotic resistance is a major public health threat all over the world. Although resistance is a natural evolutionary process of bacteria, many factors contribute to its acceleration. Misuse and unnecessary usage of antibiotics by humans and their excessive usage in agriculture can be given as examples [1,2]. Biofilms, on the other hand, are a type of bacterial life. It consists of polysaccharides, e-DNA and proteins produced by bacteria [3]. Biofilms formed by quorum sensing (QS) mechanisms prevent antibiotics from penetrating into cells. It is 10-1000 folds more resistant to antibiotics compared to planktonic cells [4]. It is estimated that more than 80% of infections are caused by biofilm [5]. Therefore, the discovery of molecules with antibiofilm properties is quite significant.

*P. aeruginosa* is an opportunistic pathogen classified by the World Health Organization (WHO) as critical [6]. *P. aeruginosa* is a Gram-negative, non-spore-forming bacteria. It is the main cause of many diseases, especially cystic fibrosis, wound and urinary tract infections [7]. *P. aeruginosa* is highly resistant to antibiotics due to its biofilm-forming properties. Nowadays, its incidence is also increasing rapidly. Due to the rapid development of resistance, the number of effective antibiotics is also decreasing. Therefore, the development of new antibiotics or synergy studies is urgently needed [8].

Vaccenic acid, naturally found in human milk and yogurt, is a natural omega-7 fatty acid [9]. Previous studies have suggested that trans-vaccenic acid has a significant effect in reducing the incidence of cancer and obesity [10]. However, there are limited studies on the antimicrobial and antibiofilm activities of fatty acids. To the best of my knowledge, its effectiveness on *P. aeruginosa* is unknown.

For the first time, the efficacy of cis and trans-vaccenic acid against *P. aeruginosa* was assessed in this study. A reference strain (PAO1) and three different clinical isolates of *P. aeruginosa* were used in the study. As a result, cis and trans-vaccenic acid showed antibacterial and antibiofilm properties in *P. aeruginosa* and inhibited pyocyanin production.

## Material and Methods

### *Reagents*

Cis-vaccenic acid (Cas number: 506-17-2) and trans-vaccenic acid (Cas number: 693-72-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Both of them were dissolved in dimethyl sulfoxide (DMSO, Isolab, Cas number: 67-68-5). All other chemicals used in this study were analytical grade.

### *P. aeruginosa Strains and Culture Conditions*

In this study, four *P. aeruginosa* (PAO1, PA21, PA23 and PA41) were used. PA21, PA23 and PA41 are clinical isolates from our previous studies. (ETU, BAP: 2021/021). These isolates are in stocks of -80 ℃ in the Molecular Microbiology Laboratory, High Technology Research and Application Centre (YUTAM). Bacterial strains from the frozen stocks were inoculated on Mueller Hinton Agar (MHA, Across Bio) medium at 37 ℃. Liquid cultures were

made in Mueller Hinton Broth (MHB, Across Bio) medium at 37 ℃ and 150 rpm.

### Microdilution Assay

The microdilution test was performed to determine the minimum inhibitory concentration (MIC) value [11,12]. The amounts of the cis and trans-vaccenic acid were used in 1-512 µg/mL concentrations. Briefly, the overnight growing bacterial culture in MHB medium was diluted until the optical density at 600 nm was between 0,08 and 0,1. This value was accepted as 0,5 McFarland concentration in further experiments and 100 µL of these cells were dispensed into the wells. Subsequently, increasing concentrations of cis and trans-vaccenic acids were dispersed and the total volume was made up to 200 µL with MHB medium. The plates were incubated at 37℃ for 24 hours. The concentrations without growth after 24 hours were recorded as the MIC value.

### Growth Curve Pattern

The effect of cis and trans-vaccenic acids on the growth of *P. aeruginosa* was observed [13]. Briefly, the overnight growing bacterial culture was diluted to 0,5 McFarland concentration into 20 mL MHB medium containing ½ MIC and ¼ MIC concentrations of cis and trans-vaccenic acids. These tubes were incubated at 37℃ and 150 rpm. Then, the growth curve was measured for each *P. aeruginosa* strain at 600 nm optical density. Measurements were taken at 1, 2, 4, 6, 12 and 24 hours. Bacterial culture without cis and trans-vaccenic acids was used as a control.

### Biofilm Inhibition Assay

The effect of cis and trans-vaccenic acids on biofilm formation in *P. aeruginosa* was determined by the crystal violet test [14,15]. First, bacterial cells grown overnight were adjusted to a 0,5 Mcfarland concentration. A medium containing cis and trans-vaccenic acid at a concentration of 1-512 µg/mL and 100 µL of diluted cells were then added to the 96-well plates. The plate was statically incubated at 37℃ for 48 hours. At the end of the incubation, non-adherent cells were discarded and all wells were washed with sterile water. After, wells were stained with 0,5% crystal violet for 20 minutes in the dark. At the end of the period, the dye was removed and the biofilm layers were dissolved with 30% acetic acid. Measurements were taken at 590 nm optical density with the spectrophotometer (Multiscan Go, Thermo Scientific, USA).

### Pyocyanin Detection Assay

Pyocyanin is one of the virulence compounds produced by *P. aeruginosa*. It is secreted out of the cell. The effect of vaccenic acid on pyocyanin production was evaluated [16,17]. Briefly, a single colony of *P. aeruginosa* was inoculated in a 4 mL MHB medium containing ½ MIC and ¼ MIC concentrations of cis and trans-vaccenic acids into the 12-well plate. Medium without vaccenic acid was used to control. All tube was incubated at 37℃ for 24 hours. After that, culture was obtained with a centrifuge at 12.000 rpm for 5 min. Then, 500 µL chloroform was added and mixed vigorously. The chloroform layer was taken with a centrifuge at 12.000 rpm for 2 min and transferred new tube and 200 µL 0,2 M HCl was added and mixed. The obtained supernatant was taken and measured at 520 nm optical density in the spectrophotometer.

### Statistical Analysis

All experiments were performed in three or four replicates. All data were evaluated using the program GraphPad Prism, version 8.4. ANOVA was used for the comparison of data obtained from the pyocyanin inhibition assay.

## Results

### Antimicrobial Activity of Vaccenic Acid

The antimicrobial effect of vaccenic acids was determined against four different *P. aeruginosa* strains by microdilution assay. The results indicated that cis and trans-vaccenic acids have antimicrobial activity in increasing concentrations against *P. aeruginosa* tested references and clinic strains. Table 1 shows MIC values of cis and trans-vaccenic acids. The MIC values were similar between isolates.

Table 1. MIC value of *cis* and *trans*-vaccenic acid against *P. aeruginosa* (The results are an average of three independent experiments).

| Strains | MIC (µg/mL) | | MBIC (µg/mL) | |
|---|---|---|---|---|
| | *cis*-vaccenic acid | *trans*-vaccenic acid | *cis*-vaccenic acid | *trans*-vaccenic acid |
| PAO1 | 128 | 256 | 256 | 256 |
| PA21 | 256 | >256 | 256 | 256 |
| PA23 | 256 | 256 | 512 | 512 |
| PA41 | 256 | 256 | 8 | 32 |

### Growth Curve Pattern

Understanding the interaction between planktonic cells and vaccenic acid is definitely important. Therefore, 24-hour growth curves of bacteria were determined in the presence of vaccenic acids. Figure 1 describes the growth curve of *P. aeruginosa* with *cis* and *trans*-vaccenic acids. In PAO1, PA21 and PA23 strains, 1/2 MIC value decreased cell growth compared to the control group. However, in the PA41 strain, there was no significant difference between 1/2 MIC, 1/4MIC treatments and control groups.

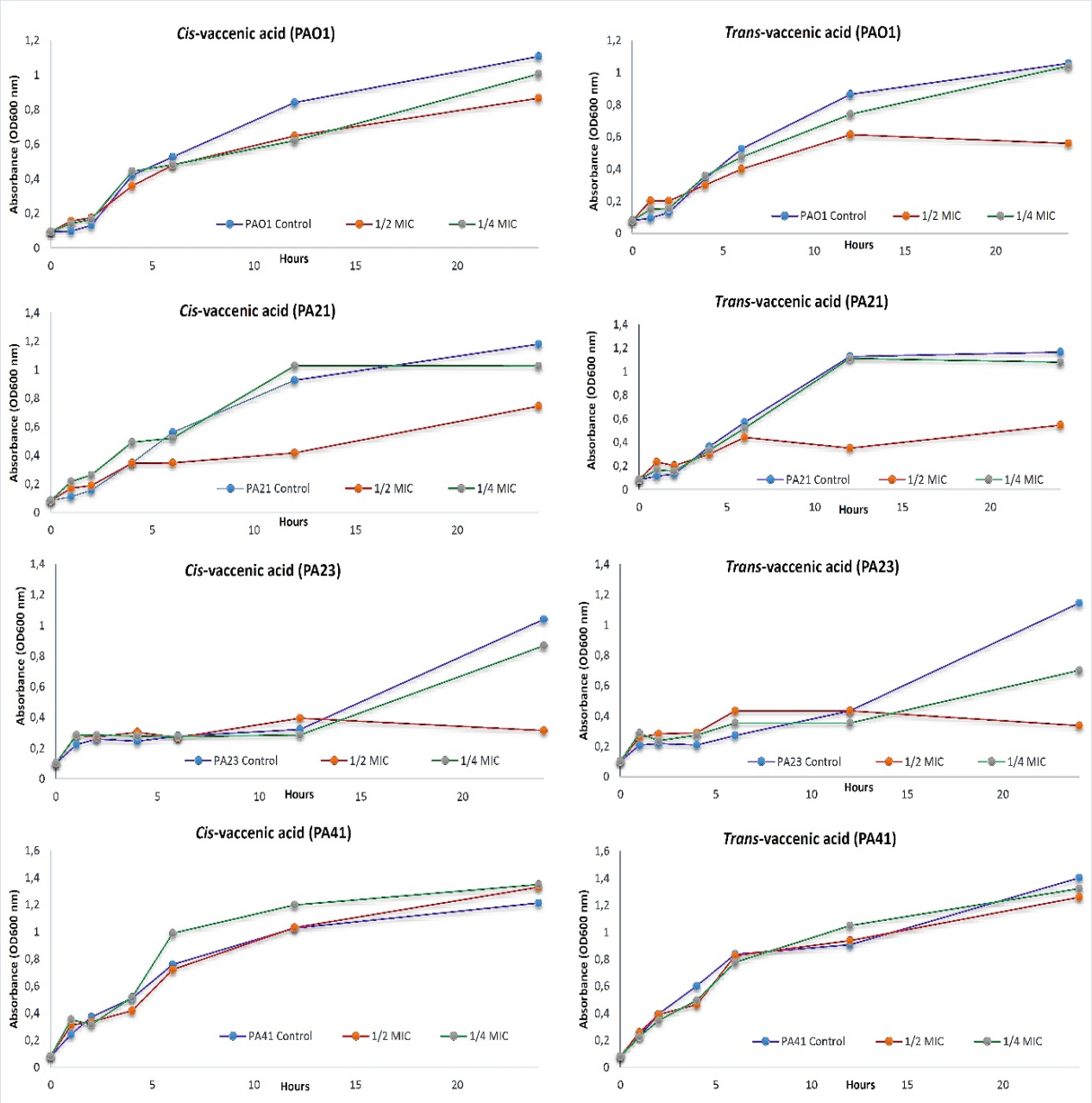


Figure 1. Growth curves of four P. aeruginosa strains in the presence of cis and trans-vaccenic acid at ½ MIC and ¼ MIC concentrations.

The antibacterial compounds may be inhibiting cell growth or killing cells [18]. Antibacterial compounds that inhibit growth may contribute to biofilm formation. Biofilms, on the other hand, are more robust assemblages than free-growing cells due to the complexity of their structures. They also create a tremendous environment for the spread of antibiotic resistance genes [18,19,20]. Therefore, inhibition of biofilm formation should be performed without affecting cell growth [21]. Synergistic effective antibiofilm molecules and antibiotics should be used together as a different approach. In a second approach, newly developed antibiotics should have dual effects, antibacterial and antibiofilm.

In the current study, the relationship between antibiofilm activity and cell growth of *cis* and *trans*-vaccenic acid was revealed by comparing the results of the crystal violet.

### *Antibiofilm Activity of Vaccenic Acids*

The effect of *cis* and *trans*-vaccenic acid on biofilm formation was investigated by the crystal violet test. *Cis* and *trans*-vaccenic acid showed antibiofilm activity at increasing concentrations (Figure 2). MIC and MBIC values were found to be similar for PAO1, PA21 and PA23 strains On the other hand, in PA41 isolate, the MBIC value was found to be much smaller than the MIC value (Table 1).

According to these results, *cis* and *trans*-vaccenic acids showed both antibacterial and antibiofilm activity. However, this activity altered depending on the strain. The higher antibiofilm activity was recorded in PA41 isolates.

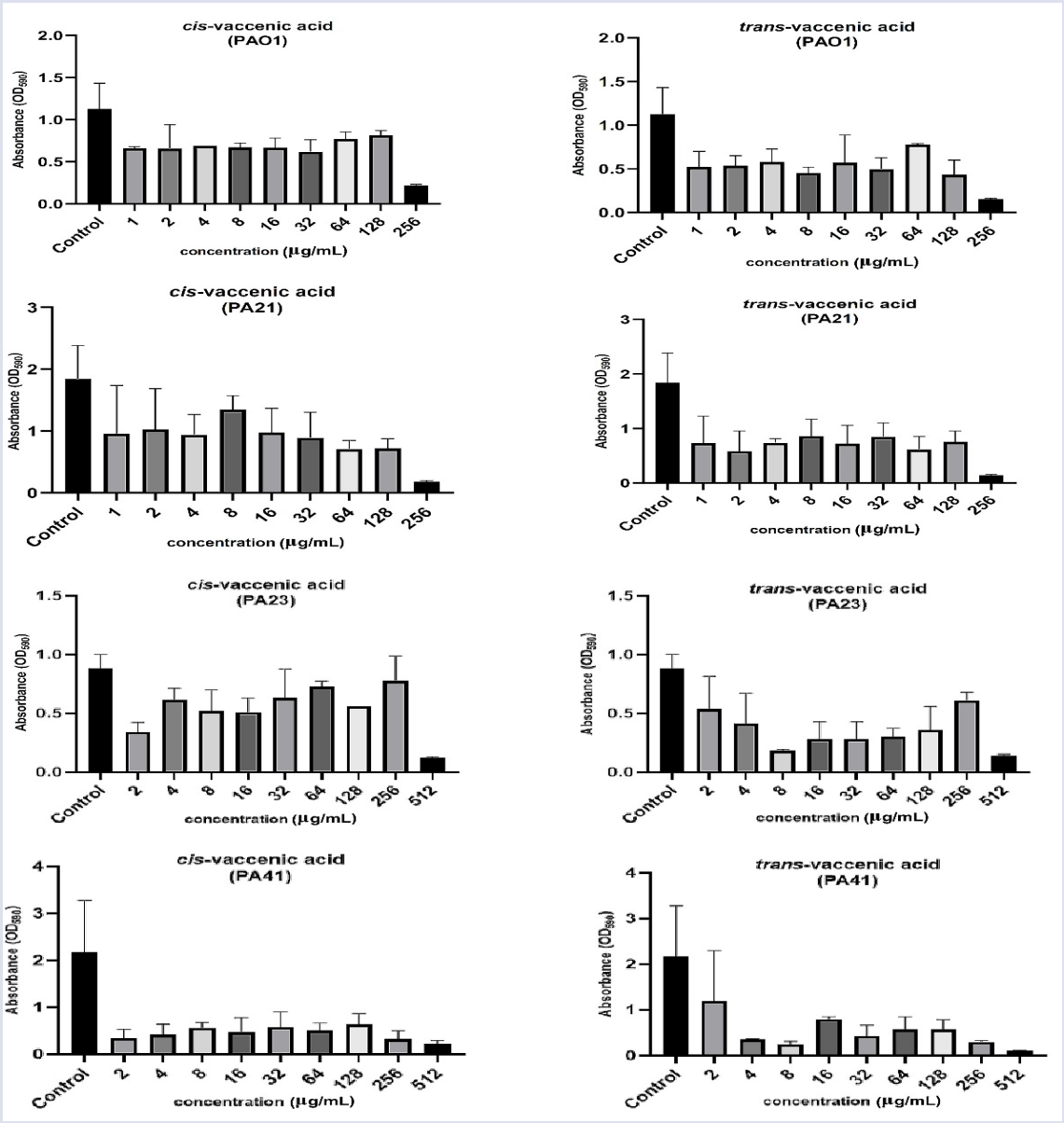


Figure 2. Crystal violet assay for the effect of cis and trans-vaccenic acid on biofilm formation in four P. aeruginosa strains. (The results are an average of four independent experiments).

### *Pyocyanin Detection Assay*

Pyocyanin is a blue-greenish color pigment produced by *P. aeruginosa*. This pigment contributes to the virulence properties of *P. aeruginosa* [16]. Pyocyanin, which is strongly blue in neutral and basic pH values, turns red color under acidic conditions [17]. In this study, the change in pyocyanin production in the presence of *cis* and *trans*-vaccenic acid was measured (Figure 3). *Trans*-vaccenic acid was found to inhibit pyocyanin production in all studied isolates. *Cis*-vaccenic acid inhibited pyocyanin production in clinical isolates except in PAO1 and PA41 isolates. PA41 isolate, on the other hand, produces pyocyanin at a very low rate. *Cis*-vaccenic acid had no effect on PA41 pyocyanin production like PAO1.

Pyocyanin helps QS mechanisms, biofilm formation and bacteria survival in an oxygen-free environment [35]. Inhibiting the production of this pigment, which contributes to virulence characteristics, is quite significant in reducing the ability of *P. aeruginosa* to cause infection.

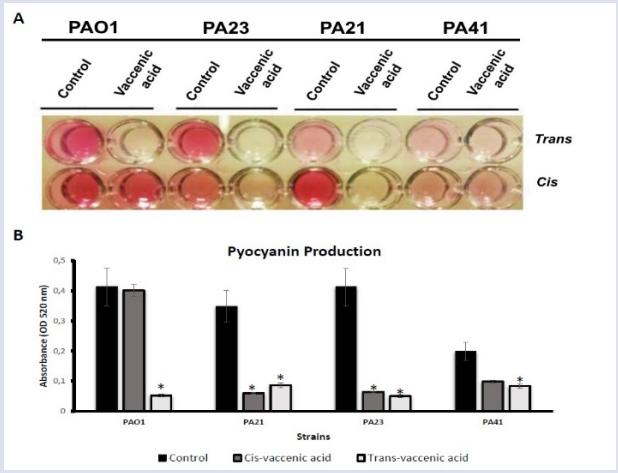


Figure 3. Pyocyanin detection assay plate images (A) of cis and trans-vaccenic acid on four P. aeruginosa strains. (B) Optical density readings at 520 nm. "*" indicates statistically significant differences at $p < 0,05$.

*Trans*-vaccenic acid inhibited pyocyanin production in all isolates studied. *Cis*-vaccenic acid, on the other hand, showed an isolate-dependent inhibition effect. As a result, inhibition of pyocyanin production gave more effective results in *trans*-vaccenic acid.

## Discussion

The spread of antibiotic resistance has been assessed as a severe hazard to public health globally [22]. Drug resistance is seen in almost all known bacteria. Not only the inaccurate and excessive use of antibiotics by people but also the use of antibiotics in agriculture and animal husbandry causes the development of resistance [23].

In the current investigation, *cis* and *trans*-vaccenic acid was assessed for antimicrobial and antibiofilm activity against *P. aeruginosa.* This bacteria has been classified among critical pathogens by the WHO [6,24]. Moreover, according to the Centers for Disease Control and Prevention's 2022 report, an increase in multi-drug-resistant (MDR) *P. aeruginosa* infections was observed in hospitals in 2020 [25]. The most important reason for this may be secondary infections developing after Covid-19 disease [39]. Other reasons may be that MDR *P. aeruginosa* infections are prevalent in people with lung disease and weakened immunity. Notwithstanding, the rate of discovery of new antibiotics and antimicrobial agents against MDR strains of *P. aeruginosa* is relatively slow [1,2].

Fatty acids have many biological properties, such as joining the membrane structure, forming phospholipids, and storing energy [26,27,28]. In addition to these features, they have properties such as antibacterial, antifungal, antiviral, antialgal and anti-protozoan. It performs these activities by inhibiting growth or killing the pathogen [29]. On the other hand, these properties may vary depending on the natural structure of fatty acids [30]. Furthermore, some studies have reported that fatty acids are more effective against Gram-positive bacteria [31]. There are a lot of studies about fatty acids of antibacterial activity against Gram-positive; even so, few studies have shown their effect against Gram-negative bacteria such as *Helicobacter pylori* and *Escherichia coli* [32,33].

Vaccenic acid is a type of fatty acid naturally found in human milk [9]. *Trans*-vaccenic acid (11-*trans*-Octadecenoic acid) is a positional and geometric isomer of oleic acid [10]. The anticancer effect of them and down-regulating the protein expression of Bcl-2 and procaspase-9 has been shown [10]. In addition, it has a beneficial effect on heart disease and obesity [10].

The antibacterial activity against Gram-positive and negative of the methanolic extract of *Quercus leucotrichophora*, which was found to contain *cis*-vaccenic acid by GC-MS [34], shows that further studies on vaccenic acid are needed. In addition, a recently published study demonstrated that *cis*-vaccenic acid has anti-QS activity against *Chromobacterium violaceum* and methicillin-resistant *Staphylococcus aureus* [9]. Similarly, in the current study, the both antimicrobial, and antibiofilm activity of vaccenic acid was demonstrated against four *P. aeruginosa* strains. The *cis* and *trans* isomers of vaccenic acid were used separately. It was determined that both isomers showed strain-dependent activity.

The antimicrobial activity of amphipathic fatty acids is intended to disrupt cell membrane integrity. It also blocks energy metabolism and nutrient intake of cells [29]. On the other hand, Kumar et al. suggested that fatty acids have an antibiofilm effect at sub-MIC values, and show non-specific antimicrobial effects at high concentrations [36]. The reason why concentration is so important is not obvious. However, in recent studies, it was understood that fatty acids behave as diffusible signal factor [37]. For instance, *cis*-11-metil-2-dodecenoic asid and *cis*-2-decenoic asid act as a diffusible signal factor and disrupted preformed biofilm [37,38].

In the current study, when the studied *P. aeruginosa* isolates were compared, it was observed that *cis* and *trans* vaccenic acids had different effects. For instance, in the PA41 isolate, both *cis* and *trans*-vaccenic acid inhibited biofilm formation at 32 and 8-fold lower MIC, respectively. Comparing this result with a growth curve test for PA41 isolate, it seems that antibiofilm activity does not occur by inhibiting cell growth. One possible reason may be that the antibiofilm activity disrupts the QS mechanisms. Other reasons may be reducing extracellular polymeric matrix (EPS) production, decreasing mobility, acting as a diffusible signal factor, and changing cell membrane fluidity [36]. In light of these results, the mechanism of action and molecular response of the vaccenic acid should be investigated.

## Conclusion

Fatty acids are being studied as potential antimicrobial agents. One of them is vaccenic acid which is a natural omega-7 fatty acid. In this study, the antibacterial activity of *cis* and *trans*-vaccenic acid on *P. aeruginosa* was shown, and it was also shown that it has an isolate-dependent strong antibiofilm activity. It is thought to inhibit the formation of virulence properties by suppressing the production of pyocyanin. Therefore, it may be suitable for *P. aeruginosa* treatment. However, further genetic analysis is needed.

## Conflict of interest

The author declare no competing interests.

## Acknowledgment

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