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# Inhibition of *Pseudomonas aeruginosa* biofilm formation by 2,2'-bipyridyl, lipoic, kojic and picolinic acids

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**ABSTRACT**

***Objective(s):*** The inhibitory effects of iron chelators, and $FeCl_3$ chelation on biofilm formation and swarming motility were investigated against an opportunistic human pathogen *Pseudomonas aeruginosa.*

***Materials and Methods:*** The inhibitory activity of 2,2'-bipyridyl, lipoic acid, kojic acid and picolinic acid on biofilm formation of *P. aeruginosa* strain PAO1 and three clinical isolates (*P. aeruginosa* PAK01, *P. aeruginosa* PAK02 and *P. aeruginosa* PAK03) were investigated, based on crystal violet assay, and swarming motility test.

***Results:*** The kojic, lipoic and picolinic acid inhibited biofilm formation by 5-33% in all tested *P. aeruginosa* isolates. When chelated iron was added, biofilm inhibition rates were determined to be 39-57%. Among the tested chelators against *P. aeruginosa*, lipoic acid (84%) and kojic acid (68%) presented the highest inhibition of swarming motility. This is the first study to report the inhibitory effect of lipoic acid on biofilm formation and swarming motility of *P. aeruginosa*.

***Conclusion:*** It is considered that lipoic and picolinic acids can serve as alternatives for the treatment of the *P. aeruginosa* infections by inhibiting biofilm formation.

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## Introduction

*Pseudomonas aeruginosa* induces serious infections in immunocompromised individuals (burnt and cystic fibrosis patients) (1-6). *P. aeruginosa* causes serious infections through tissue damages by producing virulence factors (biofilm formation, elastase, alkaline protease, pyocyanin, pyoverdine, phospholipase and exotoxin A) following the colonization of the host. The production of virulence factors in *P. aeruginosa* does not occur till high cell density is achieved in the media (7, 8), but once threshold cell density reached, the expression of virulence genes is triggered. Quorum sensing (QS) is known to be a mechanism regulating the expression of virulence genes in *P. aeruginosa*, as in many bacteria, depending on cell density (9). There are two hierarchically arranged quorum sensing systems working in *P. aeruginosa*; *las* and *rhl* systems. *lasI* regulates the synthesis of *N*-(3-oxo-dodecanoyl)-homoserine lactone (3-oxo-C12-HSL), (10) while *rhlI* regulates the synthesis of *N*-(butanoyl)-homoserine lactone (C4-HSL), (11). 3-oxo-C12-HSL and C4-HSL bind and activate the cognate response regulator LasR, RhlR resulting in the regulation of target gene expression.

These two QS systems are probably among the most studied bacteria, and studies showed that QS system plays an important role in biofilm formation and production of many virulence factors (12, 13). The fact that bacteria in biofilms are more resistant to antibiotics and disinfectants than other bacteria causes a critical problem in the eradication of biofilm infections (14). Further, considering that nearly 65% of the infectious diseases are caused by biofilm-forming bacteria (15), more effective strategies should be urgently produced to inhibit biofilm formation. Bacterial motility contributes to the biofilm formation (2); thus, impeding bacterial motility may prevent or disrupt the biofilm formation.

*P. aeruginosa* needs iron for basic cellular functions and metabolic activities during growth (16). Besides, iron serves as an environmental signal for biofilm growth in *P. aeruginosa* (4, 17). Thus, *P. aeruginosa* competes with the host to import iron from the environment, and to this end, they produce iron-chelating siderophores called pyoverdine and pyochelin when iron is scarce (18). However, proteins such as lactoferrin and transferrin provide a strong defence against bacterial infections by

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tightly binding iron and restricting the iron acquisition of bacteria in mammals. Singh reported in a study that lactoferrin inhibited biofilm formation on glass surfaces at a sub-inhibitory concentration (19). Moreover, it was revealed in another study that growth went on planktonically when iron concentration was lower than 1 µM in an aqueous medium, but when it was 100 µM, biofilm formation was occurred (20). In cases where iron concentration is quite low ($10^{-18}$ M) in human secretion (4), iron and ferritine concentration is higher in the lungs of cystic fibrosis patients (21). Hence, *P. aeruginosa* can easily form biofilm in lungs in these patients; therefore it is hard to treat associated infections. The significance of iron to control growth and biofilm formation in *P. aeruginosa* is that the reduction of iron to a level at which bacteria cannot import iron is an attractive option for antibacterial treatments. There are studies on the effects of some iron chelators on the production of various virulence factors in *P. aeruginosa* (22, 23).

This study is intended to investigate the effects of lipoic acid, 2,2'-bipyridyl, kojic acid and picolinic acid on biofilm formation and swarming motility in *P. aeruginosa* PAO1 and three clinical isolates (*P. aeruginosa* PAK01, *P. aeruginosa* PAK02 and *P. aeruginosa* PAK03) in the presence or absence of $FeCl_3$.

## Materials and Methods

### *Chemicals*

(±)-α-Lipoic acid, kojic acid, α -picolinic acid and 2,2'- bipyridyl were acquired from Sigma- Aldrich.

### *Bacterial strains*

*P. aeruginosa* strain PAO1 and three clinical isolates (*P. aeruginosa* PAK01, *P. aeruginosa* PAK02 and *P. aeruginosa* PAK03) were obtained from cultures of Department of Biology, Suleyman Demirel University. All *P. aeruginosa* strains were grown at 37 ˚C in Luria Bertani (LB) broth or LB agar (Difco).

### *Characterization of antibacterial properties of 2,2'-bipyridyl, Kojic, lipoic and picolinic acids*

The chelators added to LB broth by a two-fold dilution to reach the final concentration of 8 mM-0.125 mM. After the addition of *P. aeruginosa* culture (diluted to an $OD_{600}$ 0.1), they were incubated at 37 ˚C for 18 hr. During the incubation, absorbance of cultures at 600 nm was measured with a microplate reader(Biotek) every 30 min. Then, growth curves were prepared for each chelator.

### *Biofilm test*

The modified version of the method described by O'Toole ve Kolter (1998) (24) was used for biofilm testing. Overnight bacterial cultures (diluted to match a 0.5 McFarland turbidity standard) were added in plastic test tubes containing of LB broth and incubated at 37 ˚C overnight by adding 2,2'-bipyridyl 0,125 mM, kojic acid 2 mM, lipoic acid 4 mM, picolinic acid 2 mM or $FeCl_3$ 100 µM (as their final concentrations) in test tubes. Following the incubation, the culture was poured after the absorbance of culture measured at 600 nm. The tubes were washed with pure water 3 times, and incubated for 30 min by adding 0.1% crystal violet solution. At the end of the incubation, crystal violet solution was poured and the tubes were washed with pure water 3 times. Then, ethanol was added in the tubes, where it was left for 15 min. Finally, their absorbance was measured at 570 nm.

The study was performed in triplicate for each sample. The results were evaluated in consideration of their averages, and additive-free samples were used as control group.

### *Preparation of chelated iron solutions*

Stock solutions of chelated iron (50 mM) were prepared using 2,2'-bipyridyl, kojic, lipoic, and picolinic acids, and $FeCl_3$. Obtained solutions were mixed to prepare chelated iron solution at 3:1 (chelator:iron) ratio (23). Final concentrations of prepared solutions for biofilm testing, were added into the test tubes at 100 µM and 250 µM, and used for biofilm formation test.

### *Swarming motility test*

Swarm medium was prepared (Rashid and Kornberg, 2000) (25) and supplemented with glucose (0.5%), and solidified with noble agar (0.5%) Then chelators (2,2'-bipyridyl 0,125 mM, kojic acid 2 mM, lipoic acid 4 mM, picolinic acid 2 mM) and $FeCl_3$ (100 µM) were added in swarm agar. Bacteria were spot inoculated on swarm agar plates and incubated for 20 hr at 37 ˚C and swarming motility was measured as the diameter of the swarming zone. Swarming assays were repeated three times and chelator was not added during the experiment that used as a control sample.

## Results

### Characterization of antibacterial properties of 2,2'-bipyridyl, lipoic, kojic and picolinic acids for *P. aeruginosa*

To test the efficacy of chelators for the inhibition of biofilm formation and swarming motility of *P. aeruginosa*, first we investigated their effects on the growth of *P. aeruginosa* PAO1. And lipoic acid at 4 mM, kojic acid and picolinic acid at 2 mM and 2,2'-bipyridyl at 0,125 mM, which did not effect on the growth of the planktonic cultures (data not shown).

**Table 1**. The inhibitory effects of chelators on biofilm formation with or without $FeCl_3$ (100 µm)

| | Biofilm inhibition (%) *Pseudomonas aeruginosa* | | | |
|---|---|---|---|---|
| | PAO1 | PAK01 | PAK02 | PAK03 |
| Lipoic acid | 28 | 7 | 24 | 15 |
| Lipoic acid (+100 µM $FeCl_3$) | 25 | 5 | 18 | 20 |
| Picolinic acid | 24 | 30 | 28 | 33 |
| Picolinic acid (+100 µM $FeCl_3$) | 41 | 30 | 36 | 33 |
| Kojic acid | 18 | 5 | 11 | 27 |
| Kojic acid (+100 µM $FeCl_3$) | 20 | 18 | 24 | 31 |
| 2,2'-Bipyridyl | * | 14 | 2 | 25 |
| 2,2'-Bipyridyl (+100 µM $FeCl_3$) | 8 | 17 | 23 | 28 |

### *Effects of 2,2'-bipyridyl, lipoic, kojic and picolinic acids on biofilm formation*

Static biofilm quantification assay was performed to evaluate the effect of chelators on *P. aeruginosa* PAO1 and *P. aeruginosa* clinical isolates (*P. aeruginosa* PAK01, *P. aeruginosa* PAK02 and *P. aeruginosa* PAK03) biofilm formation. While 2, 2'-bipyridyl exhibited no inhibitory effect, significant decrease in biofilm formation was observed in tested strains when grown in the presence of (2-4 mM) kojic, lipoic and picolinic acids (Figure 1, Table 1 ).

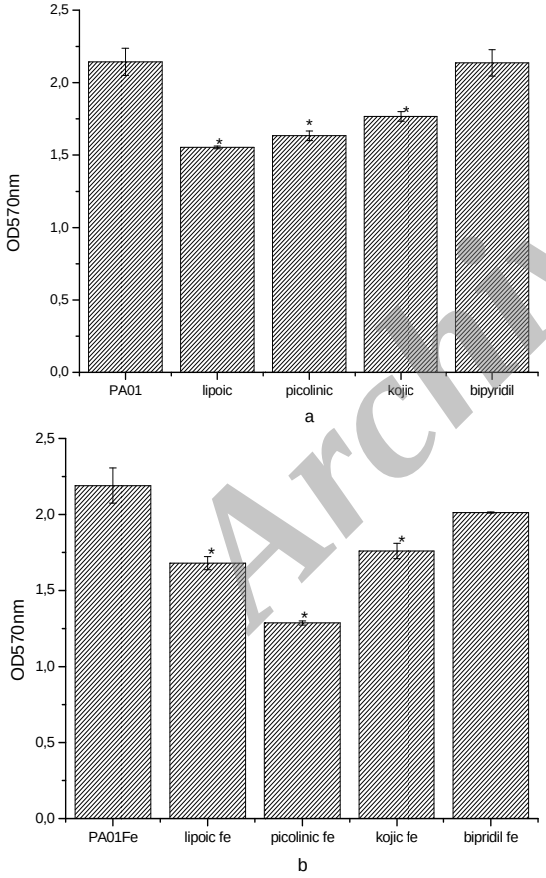



**Figure 1.** The inhibitory effects of 2,2'-bipyridyl (0,125 mM), kojic (2 mM), lipoic (4 mM), and picolinic (2 mM) acids on biofilm formation in *Pseudomonas aeruginosa* strain PAO1 (a) with 100 µM $FeCl_3$ (b). All experiments were done at least 3 times, and statistical significance was determined using one-way ANOVA. Asterix indicate data that statistically different ($P$<0.05) from control

In consideration of the effects of the chelators on biofilm formation in *P. aeruginosa* isolates, inhibition rates were found to range from 2% to 33%, and the most effective chelator to be picolinic acid on clinical isolates (Table 1). When $FeCl_3$ (100 µM) was added in testing media along with the chelator, the inhibition rates increased from 5% to 36%. Picolinic acid inhibited biofilm formation by 30% on *P. aeruginosa* PAK01 clinical isolate, by 28% on *P. aeruginosa* PAK02, and by 33% in *P. aeruginosa* PAK03, but the inhibition rates were revealed to be 30%, 36%, and 33%, respectively when $FeCl_3$ (100 µM) was added (Figure 1, Table 1).

Furthermore, at the concentrations of the chelators for biofilm testing, absorbance of culture of *P. aeruginosa* PAO1 culture were measured at 600 nm, and it was revealed that the chelators inhibited biofilm formation without decreasing the bacterial cells count.

### *Effects of chelated iron solutions on biofilm formation*

It is known that limited iron in growth medium adversely effect on biofilm formation. To this end, 2,2'-bipyridyl, lipoic, kojic or picolinic acid solutions prepared by adding $FeCl_3$ solution at the same concentration at 3:1 ratio and added to the testing medium through sequestration of iron in the testing medium. Later on, their effects on biofilm

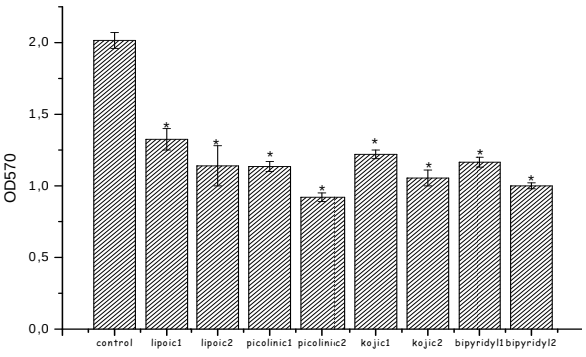



**Figure 2.** The inhibitory effects of chelated iron solutions on biofilm formation of *Pseudomonas aeruginosa* strain PAO1. (Final concentration of each chelator 1:100 µM; 2:250 µM). All experiments were done at least 3 times, and statistical significance was determined using one-way ANOVA. Asterix indicate data that statistically different ($P$<0.05) from control

**Table 2.** The inhibitory effects of chelated iron solutions at (3:1) ratio on *Pseudomonas aeruginosa* biofilm formation

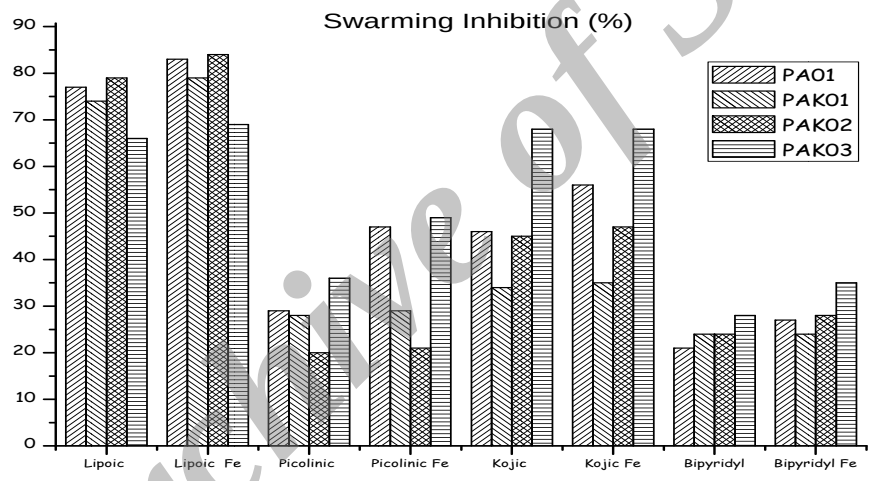
| | Biofilm inhibition (%) *P. aeruginosa* | | | |
|---|---|---|---|---|
| Chelated iron solutions at (3:1) | PAO1 | PAK01 | PAK02 | PAK03 |
| Lipoic acid+$FeCl_3$ (100 µM) | 33 | 35 | 27 | 49 |
| Lipoic acid+$FeCl_3$ (250 µM) | 43 | 46 | 40 | 55 |
| Picolinic acid+$FeCl_3$ (100 µM) | 44 | 45 | 41 | 45 |
| Picolinic acid+$FeCl_3$ (250 µM) | 54 | 51 | 50 | 57 |
| Kojic acid+$FeCl_3$ (100 µM) | 40 | 39 | 28 | 52 |
| Kojic acid+$FeCl_3$ (250 µM) | 48 | 45 | 39 | 57 |
| 2,2'-Bipyridyl+$FeCl_3$ (100 µM) | 42 | 34 | 21 | 36 |
| 2,2'-Bipyridyl+$FeCl_3$ (250 µM) | 50 | 44 | 41 | 55 |

formation on *P. aeruginosa* strain PAO1, *P. aeruginosa* PAK01, *P. aeruginosa* PAK02 and *P. aeruginosa* PAK03 clinical isolates were investigated.

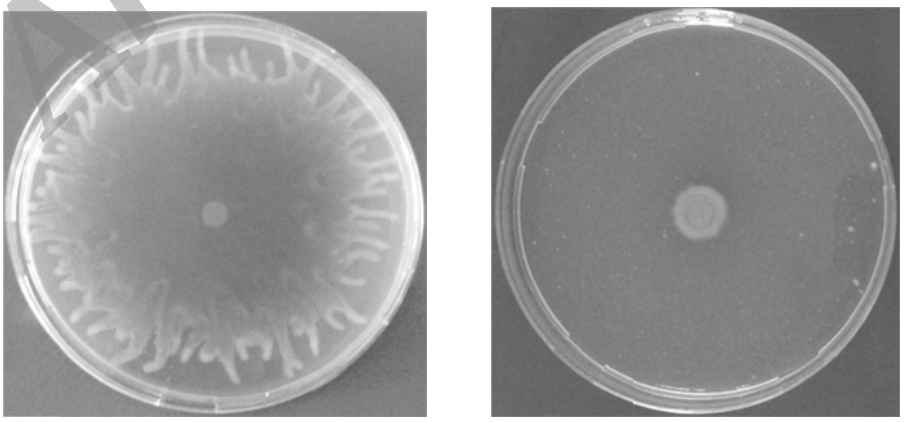
Inhibitory values were revealed to be 33-54% and 21-57% for biofilm formation of *P. aeruginosa* PAO1 and clinical isolates (Figure 2, Table 2), respectively. The most effective chelator was turned out to be picolinic acid.

### *Swarming motility*

Because of bacterial motility is crucial for biofilm formation in *P. aeruginosa* (24), we tested the impact of chelators on biofilm formation. 2,2'-bipyridyl, lipoic, kojic and picolinic acid significantly reduced the swarming ability of all *P. aeruginosa* strains, at sub-inhibitory concentrations (100 µM)




**a)**



**b1** **b2**

**Figure 4.** Inhibition of swarming motility with chelators in the presence or absence of $FeCl_3$ (a) and swarming motility of *Pseudomonas aeruginosa* PAO1 (b1) in the presence of lipoic acid (b2)

(Figure 4). This reduction of motility ranged from 20% to 84%, but varied by bacterial strain and chelator. Lipoic acid (66-84%) had the highest inhibitory activity, followed by kojic acid (34-68%) and picolinic acid (20-49%).

## Discussion

Iron is an important cation that serves for biofilm formation (4) and an integral cation for biofilm stability (26) in *P. aeruginosa*. Several studies reported that biofilm forming capability of *P. aeruginosa* cells on abiotic surfaces is reduced by iron chelators, including lactoferrin and EDTA (17, 25, 27). Moreau-Marquis *et al*, (28) showed that the combination of tobramycin with the FDA-approved iron chelators deferoxamine or deferasirox prevented the formation of *P. aeruginosa* biofilms on airway cells. Moreover, in a recent study on chelator–gallium complexes, biofilm formation was prevented in two different models of *P. aeruginosa* infection (29, 30).

However, there is no research on this property of lipoic acid which is commonly used as vitamin supplements thanks to its strong antioxidant property and has a chelating capability.

In the present study, it has been found that lipoic and kojic acids just like some of the previously reported chelators (22, 23) could inhibit biofilm formation of *P. aeruginosa* without disrupting bacterial growth. This is significant for the prevention of antibiotic resistance that could occur while developing drugs for the treatment of biofilm-induced infections (31). Inhibition rates of 2,2'-bipyridyl, kojic acid, picolinic acid and lipoic acid were 25%, 27%, 33% and 28%, and when $FeCl_3$ (100 µM) is added in the medium, the rates were found to be 28%, 31%, 33%, and 28%, respectively (Figure 1, Table 1). Swarming inhibition by 2,2'-bipyridyl and picolinic acid was more apparent than those by lipoic acid and kojic acid (Figure 4a).

The study by Musk and Hergenrother (23) revealed that picolinic acid: $FeCl_3$ mixture of 250 µmol l/l could prevent biofilm formation by 50% or more. Similarly, this study showed that picolinic acid: $FeCl_3$ mixture at the same concentration could inhibit biofilm formation in all test isolated and *P. aeruginosa* PA01 by 50-57%. Plus, the use of lipoic, kojic, and 2,2'-bipyridyl acids were revealed to result in 40-55%, 39-57%, and 41-55% inhibition, respectively. Rates concerning inhibited biofilm formation in clinical isolates are similar to those of *P. aeruginosa* PAO1 strain.

Iron chelators restricting the use of iron by sequestering the iron in the medium causes an inhibitory effect on biofilm formation in *P. aeruginosa*. This study revealed that iron chelators, including kojic, lipoic, and picolinic acids, remarkably inhibited swarming motility and biofilm formation of *P. aeruginosa* PAO1 and clinical isolates (Table 2). Picolinic acid is produced by human body as a natural by-product of tryptophan catabolism. It is non-toxic at high concentrations, and ferric picolinate is used for the treatment of iron deficiency in humans (23).

Lipoic acid is a FDA-approved, water- and fat-soluble vitamin and it is potentially important for further research that will aim to use lipoic acid as a chelator or together with other antibiotics to inhibit biofilm infections caused by *P. aeruginosa*. Moreover, its strong inhibitory effect on the production of some virulence factors in *P. aeruginosa* (unpublished data) makes the researcher regard it as a different alternative for upcoming studies in this field.

Iron is 400-fold higher in lungs of cystic fibrosis patients than other people (28), which make it easy for *P. aeruginosa* to form biofilms in such individuals. Therefore, it is a new approach to use chelators in the treatment of *P. aeruginosa*-associated biofilm infections.

## Conclusion

The data in the present study confirm that the 2,2'-bipyridyl, lipoic, kojic and picolinic acid may potentiate therapeutic approach to prevent or treat *P. aeruginosa* infections. Future research is needed to understand mechanisms of action and to determine whether these chelators could be used as therapeutic agents for iron chelation therapy with or without antibiotics.

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