# Comparison of the Effect of Phenylalanine Arginine Beta Naphthylamide (PAβN) and Curcumin on Minimum Inhibitory Concentration of Aminoglycosides on *Pseudomonas aeruginosa* Clinical Isolates

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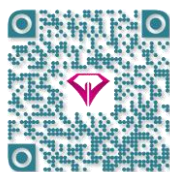
## Article Info

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## ABSTRACT

**Background & Objective:** Efflux pump inhibitors (EPIs) can block efflux pumps and are helpful in potentiating the activity of aminoglycosides against *Pseudomonas aeruginosa*. The present study compared the effects of phenylalanine-arginine beta naphthylamide (PAβN) and curcumin on aminoglycoside minimum inhibitory concentration (MIC) on *Pseudomonas aeruginosa* clinical isolates.

**Materials & Methods:** For this descriptive-analytical study, 100 clinical isolates of *Pseudomonas aeruginosa* were collected and identified by differential diagnostic tests. The MICs of amikacin, gentamicin, and tobramycin were evaluated before and after adding EPIs using a micro-broth dilution test.

**Results:** The bacteria were isolated from different types of samples, including urine (26 isolates), sputum (37 isolates), ulcers (20 isolates), catheters (eight isolates), blood (five isolates), feces (two isolates), and eyes (two isolates). Overall, 60% of the isolates were obtained from males (mean age = 47.85), and 40% from females (mean age = 44.76). In the MIC test, 11 (25.5%), 15 (34.8%), and 18 (41.8%) isolates were resistant to amikacin, gentamicin, and tobramycin, respectively. Significant reductions in the MICs of amikacin, gentamicin, and tobramycin were observed after adding curcumin in 54-100% of aminoglycoside-resistant isolates, while fewer changes in the MICs of aminoglycosides were seen against these clinical isolates after adding PAβN (36-55%).

**Conclusion:** Curcumin and PAβN can potentiate the effect of aminoglycosides on clinical isolates of *Pseudomonas aeruginosa* and change their susceptibility pattern due to efflux pump inhibition. However, our outcomes detected that curcumin was more effective than the PAβN against the aminoglycoside-resistant isolates of *P. aeruginosa*.

**Keywords:** Curcumin, Efflux pump, Inhibitor, Phenylalanine arginine beta naphthylamide, *Pseudomonas aeruginosa*

## Introduction

*Pseudomonas aeruginosa* is an opportunistic Gram-negative rod that causes nosocomial infections such as leukemia and cystic fibrosis (CF) in susceptible individuals (1) and accounts for nearly 30% of antibiotic-resistant nosocomial infections (1,2). The ability to survive under adverse environmental conditions (including drought)—along with the acquired and intrinsic factors of antibiotic resistance, such as receiving antibiotic resistance genes, altering membrane permeability, enhancing expression of efflux pumps, achieving secondary metabolic pathways, the production of enzymes that destroy and deactivate antibiotics, the acquisition of integrons carrying resistance genes, etc.—make this bacterium a lethal pathogen (3,4). Efflux pumps are one of the most significant mechanisms of resistance in *P. aeruginosa*. They are capable of excreting toxic substances such as antibiotics, drugs, and chemicals, and they can secrete cellular products from the bacterial cell (4,5). These systems prevent the formation of appropriate concentrations of toxic substances that inhibit bacteria and act as a defense mechanism against environmentally harmful substances, thus promoting bacterial survival in different environments (5,6). Based on the similarity and sequence of amino acids, efflux systems are divided into five large families from

which the clinically important resistance nodulation cell division (RND) family (which contains multidrug resistance efflux pumps) utilizes ATP energy to move the substrates (5,7). These efflux systems can extrude a wide range of antibiotics, including fluoroquinolones, aminoglycosides, tetracyclines, and chloramphenicol (7). Aminoglycosides play important roles in the treatment of infections caused by *P. aeruginosa*, especially in CF patients (8,9). A combination of aminoglycosides and beta-lactam is used to treat the severe infections caused by this bacterium, but unfortunately, resistance to these antibiotics is increasing (10). Studies have shown that by blocking efflux pumps in resistant strains of bacteria, the efficacy of antibiotics and biocides is significantly increased (11), while various materials have been identified as inhibitors of the performance of efflux pumps (12). Phenylalanine-arginine beta-naphthylamide (PAβN) is one of the most significant identified inhibitors of RND efflux pumps in Gram-negative bacteria (11,12). The mechanism of action of this substance is competitive inhibition, as efflux pumps in the bacteria detect and remove it instead of the target antibiotics (fluoroquinolones, mainly ciprofloxacin and levofloxacin) from the cells. As a result, antibiotics remain in the cell and increase in concentration (13). Curcumin is also a natural inhibitor derived from turmeric and has been proven to have anti-cancer, anti-inflammatory, and antimicrobial effects against viruses, bacteria, and pathogenic fungi (14). It also has a blocking effect on efflux pumps (15). The mechanism of its effect on efflux pumps is not yet fully understood, but in bacteria such as *Escherichia coli* and *Bacillus subtilis*, it inhibits cell division by preventing FtsZ polymerization and its assembly. It is also known to block *Helicobacter pylori* by inhibiting enzymes called matrix metalloproteinase 3 and matrix metalloproteinase 9, thereby preventing the inflammatory responses induced by this bacterium (14). Curcumin is not very toxic, and its pharmacological effects are well known (15).

Studies on non-toxic inhibitors and their effects on the minimum inhibitory concentration (MIC) of aminoglycoside against *P. aeruginosa* are very important in the treatment of infections caused by multidrug-resistant strains of this bacterium. Therefore, in this study, we compared the MICs of PAβN and curcumin on clinical isolates of *P. aeruginosa* and t investigated their synergistic effects in combination with aminoglycosides.

## Materials and Methods

### Cultivation and Isolation of Bacteria

In this descriptive-analytical study, 100 clinical isolates of *P. aeruginosa* were collected from patients hospitalized from September 2018 to September 2019 in different teaching and therapeutic hospitals of Mazandaran province, which is in northern Iran. The studied bacteria were isolated from sputum (37 isolates), urine (26 isolates), ulcers (19 isolates), intravenous catheters (eight isolates), blood (five isolates), feces (two isolates), ocular secretions (two isolates), and diabetic foot ulcer secretions (one isolate). The 100 clinical samples were collected from patients hospitalized in intensive care units (ICUs) (n=48), emergency rooms (n=13), burn wards (n=9), cardiac care units (CCUs) (n=6), pediatric units (n=6), internal medicine units (n=4), operating rooms (n=3), men's sections (n=3), surgical sections (n=3), women's sections (n=2), neurology departments (n=2), and oncology departments (n=1). All clinical isolates of *P. aeruginosa* were identified on the basis of morphology and colony odor, Gram staining, pigment production, biochemical test results, positive oxidase, and catalase tests, O/F test results, and the ability to grow at 42°C (16). Finally, pure isolates were cultured in a Trypticase soy broth (TSB) medium (Merck, Germany) containing 10% glycerol and were transferred to a -20°C freezer for subsequent work.

### Determination of Minimum Inhibitory Concentration (MIC) of Aminoglycosides by the Micro-Broth Dilution Method

The MIC of amikacin, gentamicin, and tobramycin (Sigma, Germany) against the clinical isolates of *P. aeruginosa* was determined based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (17) using a micro-broth dilution method. Then, the MIC tests of these aminoglycosides were repeated after adding 50 μg/mL of each efflux pump inhibitor (i.e., PAβN (Sigma, Germany) and curcumin (Sigma, Germany)) to the Müller-Hinton broth medium (Merck, Germany). After adding these inhibitors either separately or in combination with the Müller-Hinton broth medium (which contained definite concentrations of the antibiotics), the MICs were measured and recorded. The difference between the two MICs (i.e., with and without the inhibitors) was considered an indicator of the function of the efflux pump inhibitors. The reduction of the MIC by adding the inhibitors indicates their effect on efflux pumps and vice versa.

## Results

### Clinical Information

In this study, 100 clinical isolates of *Pseudomonas aeruginosa* were collected from patients hospitalized in Imam Khomeini (a general hospital) (40 isolates), Zare (a burn center) (11 isolates), BuAli Sina (a pediatric hospital) (17 isolates), Fatemeh al-Zahra (a heart diseases center) (10 isolates), and Razi (an infectious hospital) (22 isolates). The bacteria were isolated from different types of samples, including urine (26 isolates), sputum (37 isolates), ulcers (20 isolates), catheters (eight isolates), blood (five isolates), feces

**Table 1.** MIC values of aminoglycosides in the absence of efflux pump inhibitors

| | Number of isolates with different MIC ranges (µg/mL) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **MIC ranges** | 1024 | 512 | 256 | 128 | 64 | 32 | 16 | 8 | 4 | 2 | <0.5 |
| **Gentamicin** | 4 | 2 | 3 | 2 | 3 | 1 | - | - | - | - | - |
| **Tobramycin** | 2 | 3 | 3 | 3 | 3 | 3 | 1 | - | - | - | - |
| **Amikacin** | 4 | 2 | - | 2 | 3 | - | - | - | - | - | - |

**Table 2.** MIC values of aminoglycosides in the presence of efflux pump inhibitors

| | No. (%) of isolates with fold change of aminoglycosides' MIC with | | | | | |
|---|---|---|---|---|---|---|
| **MIC Fold change** | PAβN | | | Curcumin | | |
| | Gentamicin | Tobramycin | Amikacin | Gentamicin | Tobramycin | Amikacin |
| Unchanged | 9 (60%) | 8 (44.4%) | 7 (63.6%) | - | - | 5 (45.4%) |
| one-fold reduction | 4 (26.6%) | 8 (44.4%) | 3 (27.2%) | 1 (6.6%) | 7 (38.8%) | 2 (18.1%) |
| two-fold reduction | 1 (6.6%) | - | - | 5 (33.3%) | 5 (27.7%) | 1 (9.09%) |
| three-fold reduction | - | 1 (5.5%) | - | 5 (33.3%) | 3 (16.6%) | - |
| > three-fold reduction | 1 (6.6%) | 1 (5.5%) | 1 (9.09%) | 4 (26.6%) | 3 (16.6%) | 3 (27.2%) |
| Total resistant isolates | 15 | 18 | 11 | 15 | 18 | 11 |

(two isolates), and eyes (two isolates). Overall, 60% of the isolates were obtained from males (mean age = 47.85 years), and 40% were obtained from females (mean age = 44.76 years).

**The Minimum Inhibitory Concentration of Aminoglycosides**

In the MIC test, 11 (25.5%), 15 (34.8%), and 18 (41.8%) isolates were resistant to amikacin, gentamicin, and tobramycin, respectively (Table 1). The MIC results after adding efflux pump inhibitors (PAβN and curcumin) are shown in Table 2.

The results are as follows:

All 18 tobramycin-resistant isolates showed MIC reductions with curcumin, while seven isolates (38.8%) showed a one-fold reduction in MIC, five isolates (27.7%) exhibited a two-fold reduction, three isolates (16.6%) showed a three-fold reduction, and another three isolates (16.6%) showed more than a three-fold MIC reduction. Among the 18 isolates that were resistant to tobramycin, MIC decreased with phenylalanine arginine beta naphthylamide in 10 isolates (55.5%). Only one isolate (10%) exhibited more than a three-fold MIC reduction with this EPI. PAβN was completely ineffective on eight isolates and did not change the MIC of tobramycin in these isolates.

A similar situation was observed for gentamicin, in that out of 15 resistant isolates, all showed MIC reductions with curcumin, while five (33.3%) isolates showed a three-fold reduction and four (62.6%)

showed agreater-than-three-fold reduction in gentamicin MIC in combination with curcumin. However, only six (40%) gentamicin-resistant isolates exhibited an MIC reduction after phenylalanine arginine beta naphthylamide was added, and this EPI was ineffective in gentamicin MIC against nine (60%) resistant isolates.

The effect of curcumin on amikacin MIC depletion was less than that of the other two aminoglycosides, which were effective on six (54.5%) resistant isolates, while three isolates (50%) exhibited more than a three-fold reduction in amikacin MIC with curcumin. However, the inhibitory potency of PAβN in combination with amikacin was relatively weak, and four isolates (36.3%) that were resistant to amikacin, thus causing a reduction in antibiotic MIC, showed a one-fold decrease in MIC.

Out of 15 gentamicin-resistant isolates, four (26.6%) isolates changed as the sensitive isolates after adding curcumin, while one isolate (6.6%) was within the range of intermediate-resistant in combination with this EPI. Also, after the addition of curcumin to the MIC tests of 11 amikacin-resistant isolates, one isolate (9.09%) was changed to become susceptible to this antibiotic, and two others (18.18%) were reformed into intermediate resistant isolates. Moreover, after adding curcumin to 18 tobramycin resistant isolates, two isolates (11.11%) were transformed into susceptible

isolates, and another two (11.11%) changed into intermediate resistant isolates.

Also, after the addition of PAβN to the MIC tests of tobramycin resistant isolates, just one isolate (5.5%) was found to be susceptible, and one other (5.5%) changed into an intermediate resistant isolate. However, PAβN had no significant effect on the gentamicin- and amikacin-resistant isolates in the present study.

## Discussion

*Pseudomonas aeruginosa* is an important pathogen in nosocomial infections, especially in ICU and burn ward patients (18). This organism increases resistance to aminoglycosides by employing various mechanisms such as the production of drug-modifying enzymes, increased expression of efflux pumps, and uptake of aminoglycoside resistance genes (4). In the present study, the number of isolates resistant to aminoglycosides (gentamicin, tobramycin, and amikacin) was estimated at 41%, which is consistent with some other studies in Iran (19,20). Amikacin resistance was significantly lower than gentamicin and tobramycin in this study. One possible reason for the low rate of amikacin resistance is the widespread use of gentamicin and tobramycin in this region (21). Efflux pumps are important for the development of aminoglycoside resistance in *P. aeruginosa*, and it is necessary to apply a suitable strategy to combat them. One such strategy is the use of efflux pump inhibitors with antibiotics (22). The MIC results of the tested antibiotics showed that after the addition of efflux pump inhibitors (curcumin and PAβN), the efflux pump inhibited 36-100% of the aminoglycoside-resistant isolates, and the MIC of aminoglycosides decreased in these isolates (Table 2). We found that curcumin reduced the MIC in 100% of gentamicin- and tobramycin-resistant isolates. The MIC results after adding phenylalanine-arginine beta naphthylamide also showed that it had a beneficial effect on the reduction of tobramycin MIC, although this was only observed in 55% of the resistant isolates.

To date, several studies have examined efflux pump inhibitors and their effect on increasing the MIC of different antibiotics. Many of these studies have emphasized the increased antimicrobial activity of various antibiotics in addition to efflux pump inhibitors (11,23-25). Sonnet *et al.* showed that PAβN can reduce the MIC of ciprofloxacin in *P. aeruginosa* (26). However, most of the aminoglycoside-resistant isolates in our study did not show a significant change in the MIC values of gentamicin, tobramycin, or amikacin after adding a PAβN inhibitor. Our results showed that PAβN did not significantly enhance the antimicrobial activity of the tested antibiotics. Another study in Turkey on *Acinetobacter baumannii* isolates confirmed our results and showed that despite the inhibitory effect of PAβN on enhancing the activity of some antibiotics, this inhibitor does not have a significant effect on

aminoglycosides' action against the clinical isolates of *P. aeruginosa* (27).

Increasing drug resistance has led researchers to try to find alternative or complementary herbal medicines to inhibit the growth of these pathogens, and many studies have been conducted in this area. A study by Sisay *et al.* (2019) showed that the extract of *Myrtus* significantly reduces the growth inhibitory zone diameter of *P. aeruginosa* in laboratory (28). The results of another study carried out by Chakotiya *et al.* (2016) on *Glycyrrhiza glabra* exhibited the effective role of this plant's extract on inhibiting the growth of *P. aeruginosa* clinical isolates (29). Additionally, Morita *et al.* found that barberry extract specifically inhibits aminoglycoside-resistant *P. aeruginosa* strains (30). Similar to other researchers' findings, we observed a significant antibacterial effect of curcumin against clinical isolates of *P. aeruginosa* in the present study.

Ballard *et al.* showed that the use of natural curcumin in combination with antibiotics can decrease the MICs of antibiotics (31). Another study showed that as the concentration of curcumin rises, its effectiveness as an inhibitor increases (13). In our study, 54 to 100% of aminoglycoside-resistant isolates showed a decrease in the MICs of these antibiotics after curcumin addition. This suggests that this substance has a greater effect than PAβN as an inhibitor, which has been confirmed by other studies (13,15).

On the other hand, Eshra *et al.* found a significant difference in the reduction of the gentamicin MIC after adding PAβN and curcumin (13). Similar to the above-mentioned study, 100% of gentamicin-resistant isolates showed a significant decline in MIC after adding curcumin in the present study, while PAβN had no significant effect. One possible reason for the observed differences in the effect of inhibitors on the efficiency of different antibiotics is the presence of highly diverse drug resistance mechanisms in *Pseudomonas aeruginosa*, of which efflux pumps are only one.

## Conclusion

A phenotypic study on the expression of efflux systems using its inhibitors and reductions of the MIC of *Pseudomonas aeruginosa* clinical isolates in the present study showed that as a chemical inhibitor, natural curcumin had a better inhibitory effect on aminoglycoside-resistant clinical isolates than PAβN. However, other studies have reported that PAβN has a strong effect on decreasing the MICs of other antibiotics. In addition, the inhibitory effect of curcumin with gentamicin was better than that of tobramycin and amikacin, and it seems that gentamicin combined with curcumin (as an adjuvant) can be used to treat *Pseudomonas aeruginosa* infections and reduce the gentamicin resistance rate. However, further in vivo investigations are required to confirm this result. Since

curcumin is a natural substance, it can be substituted for chemicals that inhibit efflux pumps.

## Acknowledgments

## Conflict of Interest

Authors declared no conflict of interest.

## Ethical Approval

This research was approved by ethics committee of Mazandaran University of Medical Sciences, Sari, Iran, with ethical code IR.MAZUMS.REC.1397.1431.

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