

Deregulation of *Mexb* Gene in Ciprofloxacin Resistant Isolates of *Pseudomonas Aeruginosa* Treated with Silibinin-Encapsulated in Nanoparticles

Z. Ahmadi Roudbaraki (BSc)¹, N. Ranji (PhD)^{1*}, B. Soltani Tehrani (PhD)²

1.Department of Biology, Faculty of Basic Sciences, Rasht Branch, Islamic Azad University, Rasht, I.R.Iran

2.Cellular and Molecular Research Center, Gilan Islamic Azad University, Rasht, I.R.Iran

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ABSTRACT

BACKGROUND AND AIM: Silibinin (silybin) is an active component of silymarin with anti-bacterial activities. In drug resistant isolates of *Pseudomonas aeruginosa* has been reported overexpression of *mexB* gene. The aim of this study was evaluation of *mexB* gene expression in silibinin treated and untreated isolates of *Pseudomonas aeruginosa*.

METHODS: In this descriptive analytical study, *Pseudomonas aeruginosa* isolates were obtained from hospitals and laboratories of Guilan province. After determining several antibiotics susceptibility (disc diffusion and MIC), 5 ciprofloxacin resistant isolates of *Pseudomonas aeruginosa* were treated by ciprofloxacin (1/2MIC) only (group1) and in the combination with silibinin-encapsulated micelles (nanoparticles) (group2). After 24h, RNA extraction and cDNA synthesis was performed in group1 and group2 and *mexB* gene expression was evaluated by quantitative-realtime PCR (Q-RT-PCR) and $2^{-\Delta\Delta CT}$ equation.

FINDINGS: In this study from 69 isolates, 33.33% by disc diffusion test and 37.86% by MIC test were determined ciprofloxacin resistant. Our analysis showed that silibinin -encapsulated nanoparticles (400µg/ml) induced death up to 50% in ciprofloxacin (1/2MIC) treated resistant isolates during 24h. In treated cells with silibinin and ciprofloxacin revealed downregulation of *mexB* gene compared to treated cells with ciprofloxacin alone.

CONCLUSION: It seems that silibinin is cause of increasing ciprofloxacin effect on inhibition of growth of *Pseudomonas aeruginosa* through decreasing expression of genes implicated in efflux pump systems such as *mexB*.

KEYWORDS: Ciprofloxacin, *mexB*, Nanoparticles, *Pseudomonas aeruginosa*, Realtime PCR, Silybin.

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* Corresponding author: N. Ranji (PhD)

Address: Department of Biology, Rasht Branch, Islamic Azad University, Taleshan Bridge, Rasht, I.R.Iran

Tel: +98 13 33424080

E-mail: najmehranji@gmail.com

Introduction

Silibinin (silybin) is a polyphenolic flavonoids and an active silymarin compound derived from *Silybum marianum*. Silymarin is composed of several compounds, including silibinin (silybin), isosilybin, silicic acid and silindanin (1). Silybinin is known as antioxidant, anti-inflammatory, and liver protective (2), and has been shown to be ant carcinogenic indifferent studies (3).

Its antibacterial properties have also been reported in recent years in studies (4 and 5). *Pseudomonas aeruginosa* is an opportunistic pathogen in a hospital that is a threat to medical care due to the intrinsic and acquired resistance of a wide range of antibiotics, including fluoroquinolones. The arbitrary use of antibiotics in immunosuppressive individuals, such as severe burns in the treatment of *Pseudomonas aeruginosa*, has become problematic (7), and new strategies need to be taken to confront this group of pathogens. The use of fluoroquinolones, such as ciprofloxacin, in the wrong treatment of these drug resistant infections can result in no response to other antibiotics, including imipenem (8) (due to the similarity of some of the mechanisms of resistance). The need to use appropriate drugs is one of the most important issues for the treatment of infections with a high risk of death, such as severe burns and cancers. Different drug resistance *pseudomonas aeruginosa* isolates have different mechanisms, such as increasing the expression of efflux pump genes, changing the structure of the target enzymes of these drugs and the horizontal transmission of beta-lactamase genes (7). With the release of drugs, stains, detergents and toxins, cause bacteria to survive (9).

One of the most important systems of the efflux pump is the MexAB-oprM system, which is involved in the development of resistance to antibiotics in *Pseudomonas aeruginosa* (10). Mutations in the regulator genes of the efflux pump system increase the expression of these genes (11), resulting in more drug excretion from the cell and the need for higher drug concentrations for the effectiveness and degradation of the bacterium. One of the new drug strategies is the use of herbal remedies for the treatment of infections. The aim of this study was to examine the expression of the MexB gene from the MexAb-oprM of efflux system in Ciprofloxacin-resistant *pseudomonas aeruginosa* treated with encapsulated silibinin in micelle and ciprofloxacin nanoparticles with quantitative real-time-PCR.

Methods

Preparation of *Pseudomonas aeruginosa* isolates: In this descriptive-analytical study, 200 susceptible isolate to *Pseudomonas aeruginosa* were collected from different clinical specimens including burns, tissue necrosis, urine and respiratory secretions from Wilayat, Aria, Ghaem and Razi hospitals of Rasht and the Mehr and Razi Labs of Lahijan. Samples were cultured in Muller-Hinton Agar (Quelab, Canada) for detection of *Pseudomonas aeruginosa* and incubated for 24 hours at 37°C. Then the bacteria were identified based on the colony morphology (rod form) under a microscope, Gram staining, green pigment formation in Muller-Hinton Agar media, positive oxidase test and growth at 42°C on a nutrient agar.

Determination of antibiotic susceptibility: Antibiotic resistance by disc diffusion method was performed using antibacterial disks of amikacin (30 µg), gentamicin (10 µg) and ciprofloxacin (5 µg), (HiMedia India). After 18-24 hours incubation at 37°C, the diameter of the inhibition zone around each disc was measured.

Determination of the Minimum Concentration of Antibiotic Inhibitory: To determine the minimum inhibitory concentration (MIC), the broth dilution method was used. The bacterial dilutions were made at 0.5 McFarland and incubated with ciprofloxacin dilution (200mg / 120mL) (Ronak Drug Co., Iran) at 1-2048 µg/ml concentration for 24 hours at 37°C. The first tube with no visible opaque was considered as MIC.

Preparation of silibinins enclosed in mycelium nanoparticles: In this study, mycelium nanoparticles (oleic acid and polyethylene glycol composition in nano dimensions) were prepared by a research group and evaluated in terms of standard tests. Then, the silibinin powder (Sigma aldrich, Germany) was embedded in it (12).

Treatment of *Pseudomonas aeruginosa* isolates with silabbinin and ciprofloxacin: Several ciprofloxacin resistant isolates were treated with ciprofloxacin alone (control sample) or in combination with encapsulated silibinin in the mycelium nanoparticles (test sample) to investigate the antibacterial effect of silibinin. The aim of this study was to evaluate the effect of silibinin on the reduction of resistance to ciprofloxacin in resistant isolates. Due to the fact that MIC concentration of ciprofloxacin for each sample had growth inhibitory potentials, a concentration below the MIC (MIC1/2) plus silybinin was used to study the effect of silybinin

on inhibition of bacterial growth. The bacteria have the ability to grow in MIC 1/2, but with the presence of silybin, it was expected that the concentration of ciprofloxacin would inhibit the growth and cause the death of bacteria. For this purpose, 50 µl of microbial suspension with a concentration equivalent to half McFarland (108×1.5), ciprofloxacin and encapsulated silibinin in micelle nanoparticles were added to the wells at concentrations (0, 100, 200 and 400 µg/ml) and incubated at 37°C for 24 hours. The minimum bactericidal concentration (MBC) was determined to ensure the silybin fatal effect. For this purpose, 50 µl of each treated sample in tube was transferred to a muller hinton agar medium and was cultured. After 24 hours, samples of ciprofloxacin treated alone or in combination with silibinin were investigated for the formation of colony count in agar medium. Each treatment was repeated at least twice.

RNA Extraction: RNA extraction from ciprofloxacin-treated isolates (1.2 MIC) and effective concentrations of mycelia containing silibinin (test specimen) and untreated isolates with silibinin (treated with MIC1/2 concentration of ciprofloxacin as control sample). after 24 hours, the Total RNA Extraction Mini Kit (Yekta Taghiz Azma, Iran) was done under class II laminar hood and in sterile conditions. To determine the concentration of RNA extracted from c2000 nanotubes (USA).

Synthesis of cDNA and examination of MexB gene expression by Q-RT-PCR method: To synthesize cDNA from extracted RNAs the cDNA Synthesis Kit (Unique Equipments of Azma, Tehran) was used. For this purpose, RNA, Oligo dT and RNase free water were mixed for 5 minutes at 70°C. The enzyme and other components of the reaction were then added to the mixture at a temperature of 42°C (for reaction) for 60 minutes and at 70°C for 10 minutes (for deactivation of the RT enzyme) in an Analytik Jena Thermocycler were put (Germany). After cDNA synthesis, the Q-RT-PCR reaction was performed using the SYBR® Premix Ex Taq™ II Kit (TaKaRa, Japan) on the ABI StepOne (USA) device. The Q-RT-PCR reaction temperature program consisted of the following: initial denaturation phase (95 ° C for 30 seconds) and 40 cycles including 95 ° C for 5 seconds and 60 ° C for 30 seconds. Rpsl gene was used as reference gene. Primers used (Table 1) were synthesized by South Korea's Bioneer Company. Analysis of expression of genes was performed using the 2-ΔΔCt equation.

Statistical test: Chi-square test was used to determine the resistance to drugs and 2 x tests was used to determine the distribution of infection. To analyze the results of realtime PCR, T-test was used to compare the differences between the two groups. P <0.05 was considered significant.

Table1. Specifications of used primers

Reference	The length of the PCR product	Primer sequence	Primer	Gene name
(13)	244 bp	5'- GTGTTTCGGCTCGCAGTACTC -3'	F	<i>mexB</i>
		5'- AACCGTCGGGATTGACCTTG -3'	R	<i>mexB</i>
this study	249 bp	5'- GCTGCAAACTGCCCGCAAC-3'	F	<i>Rpsl</i>
		5'- CCCGAGGTGTCCAGCGAACC-3'	R	<i>Rpsl</i>

Results

In this study, out of 200 suspicious samples, 69 isolates of *Pseudomonas aeruginosa* were identified. Most of the cases were burn injuries (20.29%) and urine specimens (42.33%), respectively (Fig 1). The results of this study showed that the sensitivity of this bacterium to 3 antibiotics showed a significant difference ($p < 0.05$) between the resistant (40%), sensitive dose-dependent (10%) and sensitive (60%) (table 2). In this test, less than 40% of the samples were resistant to ciprofloxacin, gentamicin or amikacin. About 33.33% of the samples were resistant to ciprofloxacin by disc diffusion method (table 2). However, MIC results showed that 37.68% of the

samples were resistant to ciprofloxacin. In this study, the highest resistance to ciprofloxacin was 1024 MIC/µg/ml in 7 isolates and the lowest was 32MIC/µg/ml in 8 isolates (Fig 2).

Treatment with encapsulated silibinin in mycelium and ciprofloxacin nanoparticles: 5 resistant strains treated with different concentrations of ciprofloxacin and nanoparticles mycelium containing silibinin for 24 hours. An MBC test was used to confirm the 50% fatal effect of treated isolates. MBC results showed that MIC1/2 concentration of ciprofloxacin (which did not have the ability to inhibit bacterial growth), combined with silibinin nanoparticles, and could reduce the

growth of drug resistant bacteria by up to 50%. The results of this study showed that the ciprofloxacin fatal effect on resistant isolates increased by 400 µg/ml concentration of mycelia containing silabinin for 24 hours (Fig 3). Decrease of MexB gene expression in mycelium nanoparticles containing silibinin treated

isolates: After treatment with ciprofloxacin and mycelium nanoparticles containing silibinin, the mexB gene expression was compared with the control sample (treated with ciprofloxacin (MIC 1/2). Reduction MexB expression was evident in all samples treated with silibinin compared to the control sample (Fig 4).

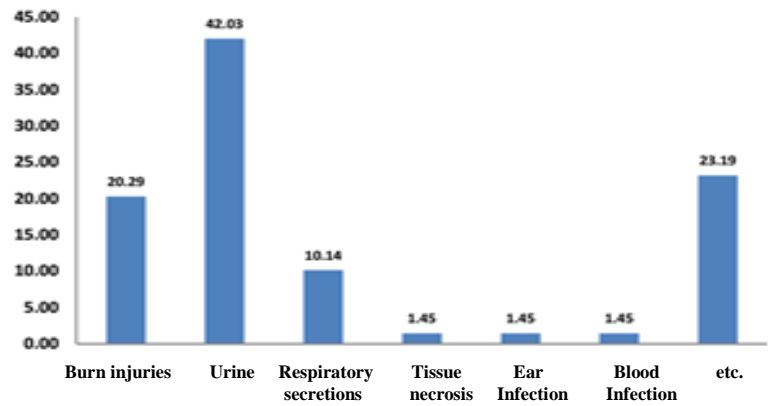


Figure 1. Distribution of ciprofloxacin-resistant pseudomonas aeruginosa infection in terms of infection position

Table2. Standard inhibition growth zone diameter of the Pseudomonas aeruginosa strains based on CLSI reference method

Antibiotics	Halo diameter (mm)			Number of isolates(%)		
	Sensitive	semi-sensitive	resistant	sensitive	semi-sensitive	resistant
Ciprofloxacin	≥21	20-16	≤15	41(59.42)	5(7.24)	23(33.33)
Gentamicin	≥15	13-14	≤12	35(50.72)	7(10.14)	27(39.13)
Amikacin	≥17	15-16	≤14	38(55.07)	5(7.24)	26(37.68)

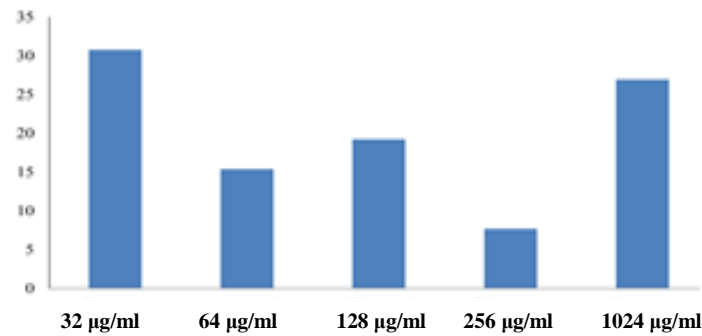


Figure2. Distribution of MIC in the isolates of Ciprofloxacin-resistant Pseudomonas aeruginosa

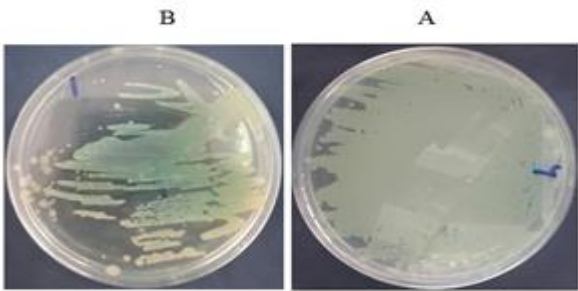


Figure 3. Treatment of number 64 of ciprofloxacin resistant pseudomonas aeruginosa (32 MIC / µg/ml). A: Ciprofloxacin treated sample (16 µg/ml) and B: Ciprofloxacin treated sample (16 µg/ml) and mycelium nanoparticles containing silibinin (400 µg/ml)

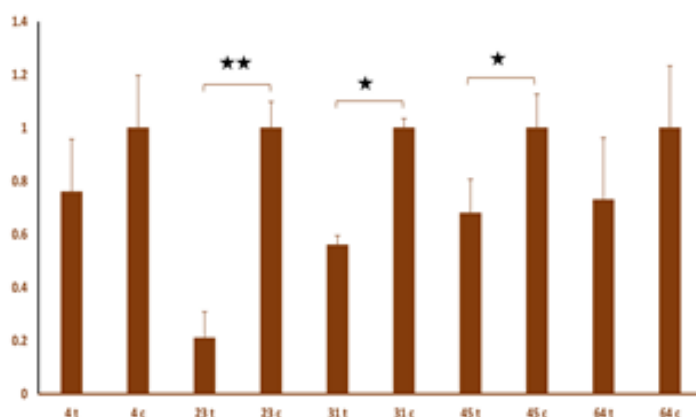


Figure 4. Study of gene expression in mex B of ciprofloxacin-treated and mycelium nanoparticles containing silibinin sample compared to ciprofloxacin treated control sample alone. The bacteria number 4, 23, 31, 45 and 64 were studied in this test.

Discussion

In this study, it was found that encapsulated silybinin in mycelium nanoparticles can increase the efficacy of ciprofloxacin in these isolates. In a study conducted by Nahaei et al. resistance to gentamycin, ciprofloxacin and amikacin was reported to be 51%, 22% and 15%, respectively (14). In the study of Taghvaei et al. among the isolates of *Pseudomonas aeruginosa*, resistance to ciprofloxacin, gentamicin was reported as 15.7%, 19.4%, 20.4%, respectively (15). In the study of Nikokar et al. the rate of resistance to ciprofloxacin, amikacin, and gentamicin was 63.3%, 48.8%, 37.2% respectively (16).

Low levels of resistance to antibiotics in the study of Nahaei et al. and Taghvaei et al. can be related to the year of reporting, and it is expected that in Arak and Tabriz, similar to Gilan, the increase in resistance has occurred over these years. The reason for the difference in the resistance to three antibiotics in the present study with the study of Nikokar et al. is due to the position of the infection. In the study of Nikokar et al. only burns have been reported, which, due to immune deficiency, are considered to be more favorable conditions for hospital infections and high levels of resistance. Based on the results of this study and other studies, it seems that resistance to antibiotics is increasing in different regions of the country and in different years.

Due to the high rate of these pathogenic bacteria in gaining resistance, appropriate methods have been used to identify resistant types in hospitals, so that treatment of patients is more successful with the selection of appropriate alternative drugs with fewer side effects. In the study of Hassanshahian et al. the antimicrobial effects of pomegranate extract on several bacteria, including *Pseudomonas aeruginosa*, were

confirmed (17). In the study of Ramezani et al. the antibacterial effect of aromatic violets on three pathogenic bacteria including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* was investigated and its greatest effect on *Staphylococcus aureus* and its lowest on *Pseudomonas aeruginosa* was seen (18).

In Roshani et al. study, Methanolic and Acetonic extract of nettle and *Zataria multiflora* have fatal effect on *pseudomonas aeruginosa* isolates (19). Based on previous studies, it is expected that plant compounds can be used to treat infections. In Molana et al. study it was found that garlic prevents the growth of *Pseudomonas aeruginosa* (20). In this study, the effect of silybinin on the active and effective combination of *Silybum marianum* plant was studied. In the study of Puri et al. the antibacterial effects of the aqueous and organic extracts of *Silybum marianum* were confirmed against several gram-positive bacteria such as *Staphylococcus aureus* and Gram-negative *E. coli* and *Pseudomonas aeruginosa* (21).

In the study of Evren et al. the effect of silymarin extract on gram-negative and gram-positive bacteria was found to have the potential to inhibit the growth of gram-positive bacteria (22). In the study of Lee et al. the synergistic effect of silibinin with ampicillin and gentamicin was observed on drug-sensitive bacteria such as streptococcal species (4). In the study of Oliveira et al. the synergistic effects of silbaine and silymarin on several antibiotics were shown in clinical and drug-resistant isolates (5). In two studies, the effect of this drug on several bacterial species sensitive to various antibiotics was studied. While the present study was carried out by providing different isolates from several hospitals and laboratories of the province, five isolates were selected for multidrug resistance and

examined for silibinin effect. The use of encapsulated silibinin in the mycelium nanoparticles increased the efficacy of the drug during transfer to the cell and thus increased its effectiveness on the cell.

So that silibinin nanoparticles with ciprofloxacin were able to reduce the growth of *Pseudomonas aeruginosa* that were resistant to the same dose of ciprofloxacin, so it could be said that silibinin contained nanoparticles have a synergistic effect on the ciprofloxacin and can be used for the treatment of *Pseudomonas* infections. Considering that in this study for the first time, the nanoparticles containing silibinin and ciprofloxacin were used to study the fatal effect of them on *Pseudomonas aeruginosa* and the appropriate fatal concentration of 400 µg/ml was determined in the shortest possible time (24 hours), while the lowest fatal concentration was determined by Lee and colleagues were effective after 48 hours (4).

Therefore, the results of this study showed that the use of nanoparticles in increasing the efficacy of the drug can be effective. One of the reasons for the resistance to antibiotics in *Pseudomonas aeruginosa* is the increased expression of the genes of the efflux pump system, such as *mexAB-oprM*. In the study of Riou et al. a statistically significant association was found between the increase in the expression of the *mexA* and *mexB* genes with the antibiotic resistance of *Pseudomonas aeruginosa* (23). In the study of Pourakbari et al. in several isolates of *Pseudomonas aeruginosa* in Iran were associated with increased expression of *mexA* and *mexB* genes (24). Considering that there is a correlation between resistance to ciprofloxacin and increased expression of genes such as *mexB* in *Pseudomonas aeruginosa*

specimens, and given that silibinin showed its antibacterial effects in previous studies, it was assumed that it was able to effect the efflux pump genes and reducing their expression reduces the release of antibiotics from the cell, resulting in a higher concentration of antibiotics in the cell. So that the lower concentration of ciprofloxacin can increase the lethality of the samples.

The results of this study suggest that silibinin is able to reduce *mexB* expression in treated isolates. Reducing the expression of this gene is associated with a reduction in the withdrawal of ciprofloxacin and the concentration of a higher concentration of this antibiotic in the cell, and thus, silibinin can increase the effectiveness of ciprofloxacin in isolates resistant to this antibiotic.

The results of this study showed that the concentration of 400 µg/ml of encapsulated silibinin in the mycelium nanoparticles, along with the ciprofloxacin by reducing the expression of the *mexB* gene, significantly improved the effect of ciprofloxacin and thus inhibited the growth of *Pseudomonas aeruginosa* after 24 hours. Therefore, it is expected that this herbal active compound may be used as a complement to treatment in antibiotic-resistant infections in the near future.

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