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In-Vitro Evaluation of Biological Activity of New Series of 1, 3, 4-Oxadiazole Derivatives Against *Bap* Gene Expression in *Acinetobacter baumannii* Biofilm

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Abstract

Background: *Acinetobacter baumannii* is one of the most common opportunistic pathogens in health centers that is resistant to many antibiotics due to biofilm production. 1, 3, 4-oxadiazoles have a wide range of biological activities.
Objectives: The aim of this research was to examine the impact of new 1, 3, 4-oxadiazole derivatives on the expression of biofilm-associated surface protein (*Bap*), playing an important role in promoting the biofilm formation ability of *A. baumannii* strains.
Methods: Derivatives of 1, 3, 4-oxadiazole were synthesized through a one-step synthesis. *Acinetobacter baumannii* strains were identified and isolated in the laboratory. The antimicrobial properties of the synthesized materials against the isolated strains were investigated. DNA, RNA, and cDNA were extracted, and the relative expression of *BAP* gene in *A. baumannii* isolates was evaluated by real-time polymerase chain reaction.
Results: The compound with methoxyphenyl functional group with MIC = 62.50 mg/mL had the best inhibitory performance among all derivatives. Also, the combination of 4i reduced the expression of the *Bap* gene by about 24 times, but it had no effect on the expression of the *16srRNA* housekeeping gene.
Conclusions: 1, 3, 4-oxadiazole derivatives, especially the methoxyphenyl functional group, act as an inhibitor of bacterial biofilm formation and have the potential to be used in the pharmaceutical and biological industries.

Keywords: Oxadiazole, *Acinetobacter baumannii*, Biofilm-Associated Surface Protein, Real-time PCR

1. Background

Acinetobacter baumannii (*A. baumannii*) is an opportunistic pathogen that has always been identified as one of the most important pathogens of nosocomial infections (1). One of the major problems in treating and preventing infections caused by *A. baumannii* is its antibiotic resistance (2). *Acinetobacter baumannii* develops multidrug resistance, which has been demonstrated by a variety of antibiotic resistance mechanisms, including the activation of efflux pumps, production of beta-lactamase enzymes, reduction of foreign membrane permeability, and synthesis of enzymes such as phosphoryl transferases and acetyltransferases that cause resistance to aminoglycosides (3).

In addition to the above, there is another mechanism in the antibiotic resistance of *A. baumannii* called biofilm (4). Biofilms are complex bacterial communities attached to surfaces that are created by an extracellular matrix produced by bacteria. This matrix is composed of polysaccharides, DNA, and proteins. Bacterial biofilms cause many problems in medical, industrial, and environmental fields. Adherence of bacteria to surfaces is an important issue in ecology, biotechnology, bio-pollution, and wastewater treatment. Various proteins are involved in the development of this resistance, including proteins that create and strengthen the biofilm structure (5). The first member of this group of proteins, called *BAP*, has been identified in the bacterium *A. baumannii* (6). These proteins are located on the outer surface of bacteria. They consist of a central nucleus consisting of iterations of similar sequences. Bacteria are able to form biofilms, thus playing an important role in infectious pathogens. The structural features of these proteins include their presence on the outer surface of bacteria, high molecular weight, and the presence of a central nucleus consisting of repetitive sequences of similar sequences. These proteins give the bacterium the ability to form biofilms, and thus, play an important role

in infectious pathogens (7).

Today, researchers are looking for new solutions to treat drug-resistant bacteria (8). One area of application, in this case, is the use of newly synthesized drug structures in the treatment of microbial infections. So far, numerous studies have shown that various compounds of oxadiazole derivatives have antibacterial properties against *A. baumannii* strains (9). Among these compounds, we can mention fluorophenyl compounds with thiol (10), fluorophenyl with chlorophenyl group (11), etc. Due to the antibiotic resistance of *A. baumannii*, few classes of antibiotics can be used to treat infections caused by this bacterium.

In this study, the effect of the synthesized materials was investigated for the first time on the expression of biofilm-inducing *Bap* gene in *A. baumannii* isolates. The effect of synthesized derivatives on the bacteria present in the biofilm, especially in deeper layers, was comparable to antibiotic treatment and phagocytic function of immune cells through inhibiting the formation of biofilm and regulating the development of the degenerative environment. Just as it is important to identify genes involved in the biofilm formation and pathogenesis of this bacterium, it is also important to recognize the environmental factors playing a role in regulating the expression of these genes (12).

2. Objectives

The aim of this study was to investigate the impact of new derivatives of 1, 3, 4-oxadiazole based on the 1, 3, 4-oxadiazole-2-yl-piridin-2-yl-methanol group on the expression of *A. baumannii Bap* gene, which is effective in promoting the biofilm formation ability of drug-resistant specimens.

3. Methods

This study was conducted in the genetic laboratory of Islamic Azad University, Tehran branch, with code number 10130553971011 in 2019. Starting materials, solvents, and culture environments were obtained from Merck, Germany, and used moving forward without any more filtration.

3.1. Preparation of Samples

In this study, 100 urine samples suspected of *Acinetobacter* infection were cultured on plates (Mueller Hinton agar). The samples were from patients with severe urinary tract infections and were delivered from the research department of Shariati and Sarem hospitals in Tehran. In the microbiology laboratory of Islamic Azad University, Central Tehran Branch, by using biochemical methods, 10 strains of *A. baumannii* were identified. A pure culture of all isolates of *A. baumannii* strains was prepared on blood agar medium, and then the cultures were incubated for 24 h at 37°C. To preserve the bacteria, they were inoculated in liquid culture medium containing glycerol and then stored at -70°C. The presence of *Bap* gene was confirmed by the PCR technique. In addition to clinical specimens, the standard strain and reference *A. baumannii* ATCC25923 were used, which makes the test conditions under quality control. The standard strain was obtained from the Iranian Research Organization for Science and Technology (IROST).

3.2. PCR to Confirm the Bap Gene

The presence of *Bap* gene was confirmed by molecular PCR using CinnaGen fermentase kit. The reaction mixture for PCR of the *Bap* gene was as follows: (1) MasterMix (1x): 12.5 μL; (2) primer F (0.1 - 1 μM) 1 μ; (3) primer R (0.1 - 1 μM): 1μ; (4) template DNA: 5 μ (20 pg), (5) sterile deionized water: 5.5 μ; the final volume was 25 μL, and PCR was set up to detect the *Bap* gene according to the following method. Initial denaturation: 95°C, 120 s, 1 cycle, denaturation: 93°C, 45 s, 35 cycles, annealing: 57°C, 60 s, 35 cycles, extension: 72°C, 30 s, 35 cycles, and final extension: 72°C, 90 s, 1 cycle. The primers used for the *Bap* and *16srRNA* genes are listed in Table 1. Finally, six strains of *A. baumannii* with the *Bap* gene were selected for molecular studies.

3.3. Synthesis of Derivatives

1, 3, 4-oxadiazole compounds were synthesized using a single-stage, high-yield method. Single-stage: (1) N-isocyan-imino-triphenyl-phosphoran (1 mmol) + 2-pyridinecarbaldehyde (1 mmoL) were dissolved in CH_3COCH_3 (7 mL); (2) in the next step, the carboxylic acid (1 mmoL) in CH_3COCH_3 (10 mL) was added to the previous solution. The final solution was stirred for 12 h by a magnetic stirrer at room temperature. The solvent was removed by evaporation, and the viscous residue was purified by flash column chromatography [silica gel powder: petroleum ether-ethyl acetate (4: 1)] (13).

3.4. Preparation of Derivatives Suspension

To prepare the stock solution, 1 g of each synthetic material was dissolved in 100 mL of dimethyl sulfoxide (99% DMSO) to prepare 0.1 mg/mL of each compound.

3.5. Broth Microdilution Test to Determine the Minimum Inhibitory Concentration (MIC)

To determine the MIC, the serial dilution method was performed in three replications in the culture medium.

Table 1. Primers Used to Target and Control Gene Amplification

Gene Name	Primers	Product Size
BAP	Forward: 5'- TAGACGCAATGGATAACG -3'	127 bp
	Reverse: 5'- TTAGAACCGATAACGATACC -3'	
16s rRNA	Forward: 5'- TATCAGGACCATCTGGAGTAGG -3'	110 bp
	Reverse: 5'- CATCAACTTCACCTTCACGC -3'	

Thus, different dilutions from 1000 to 31.25 μL/mL of synthetic materials were added to 96-well plate wells. Then, 100 μL of microbial suspension was added to each well, and the plates were treated overnight at 37°C in a greenhouse. Finally, the turbidity of all wells was visually investigated and reported as MIC in each sample.

3.6. Treatment of Acinetobacter baumannii Samples with Synthesized Derivatives

To investigate the effects of synthesized derivatives (4a-4i) on the expression of the desired genes, *A. baumannii* samples were treated with the treated compounds, and then RNA extraction was performed. To do this, samples of *A. baumannii*, equivalent to half a McFarland, were cultured in a test tube containing 5 mL of broth nutrient medium. The MIC equivalent of the compounds was then added to each tube. After mixing, it was incubated for 42 h at 39°C. After incubation, the samples were used to extract RNA.

3.7. RNA Extraction

To extract RNA from CinnaPure-RNA kit with No. cat PR891620 of Cinnagen Company was used. Then, the accuracy of RNAs was measured by a NanoDrop, and the samples were placed at -80°C.

3.8. Total RNA Treatment of Acinetobacter baumannii Samples Before and After Proximity with Derivatives by DNase Enzyme

To convert RNA to cDNA and perform real-time PCR, RNA samples must be free of genomic DNA contamination; thus, before cDNA synthesis, all RNA samples were treated with DNase enzyme. RNA treatment was performed using the Fermentas kit during a two-step process. Then, 1 μg of RNA was added to a sterile nuclease-free microtube, and 1 μL of reaction buffer 10x DNase1 and 1 μL of RNase-free were added to the microtubes, and the microtubes were incubated for 30 min at 39°C. Finally, 1 μL of EDTA (25 mM) was added to each microtube, and the microtubes were placed at 65°C for 10 min. RNA concentration of the extracted samples was measured after treatment with DNase by a nanodrop device. After confirming the accuracy of RNA and removing the extra DNA, all RNA samples were quickly converted to cDNA, and its conversion was confirmed by electrophoresis.

3.9. Investigation of Bap Gene Expression

RNA samples of pathogenic strains were prepared immediately after quality assay with a nanodrop device. The accuracy of the cDNA reaction was confirmed by PCR on the agarose gel. The result of electrophoresis of the PCR product of cDNA samples is shown in Figure 1.

3.10. cDNA Synthesis

To make cDNA, Cinnagen Company RT PCR kit with the product code RTPL12 was used. The samples were then used for real-time PCR.

3.11. Real-time PCR

Real-time PCR technique was used to determine the expression of *Bap* and *16srRNA* genes. The efficiency and melting temperature (TM) of these primers were evaluated and confirmed by ID Allele software. Requirements for real-time PCR were performed according to the protocol of Fermentase SYBR Green MasterMix kit and according to Table 2 in 36-house plates for Corbett machine. The amplification fragment contains 127 nucleotides for the *Bap* gene and 110 nucleotides for the *16srRNA* gene. The annealing temperature was 57°C for the *Bap* gene and 51.8°C for *16srRNA* gene. The preparation of real-time PCR samples and the conditions for real-time PCR are shown in Table 2. It should be noted that the real-time PCR was performed according to the MIC results and for 4i combination.

3.12. Quantitative Determination of Bap Gene Expression Before and After Exposure with a Concentration of 1 Mg/Ml of 4a, 4d, and 4i Derivatives Using Real-time PCR Technique

After real-time PCR, relative quantification can be performed in several ways, including using the standard curve of one gene and using the standard curve of both genes and the Δ_{CT} method. The default of the Δ_{CT} method is the relative parity of the cDNA amplification reaction efficiency with these two primers. Due to the equality of amplification efficiency of the two primers in the present study, the relative quantification of the cDNA of the studied genes in comparison with the cDNA of the *16srRNA* gene (internal reference gene) was performed by the Δ_{CT} method.

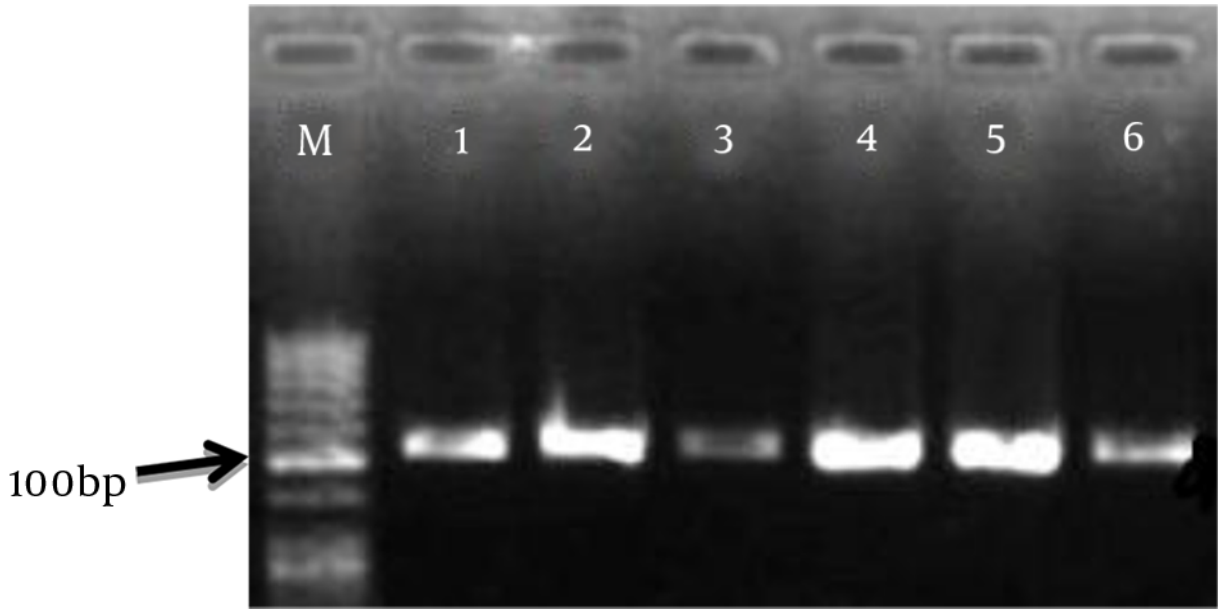


Figure 1. Columns 1, 2, 3 and 4 of the *Bap* gene PCR product, column 5 of the 16s rRNA gene PCR product, column 6 of the standard strain PCR product and column M (100 bp marker) are shown. The amplified fragment is 127 bp for the *Bap* gene and 110 bp for the 16s rRNA gene.

Table 2. Preparation of Samples and Conditions for Performing Real-time PCR

Material		Final Concentration		Volume	
qPCR SYBR green master mix		1x		12.5 μL	
ROX dye 50x		1x		0.05 μL	
F primer		(0.1 - 1 μM)		1 μL	
R primer		(0.1 - 1 μM)		1 μL	
Template		1 μg		1 μL	
D.W		-		9.45 μL	
Total volume		-		25 μL	
Initial Activation	**Denaturation**	**Annealing**	**Extension**	**Repeat**	**Final Extension**
95°	95°	-	72°	40 cycle	72°
10 min	20 sec	20 sec	20 sec	-	1 min

4. Results

4.1. Isolation and Diagnosis of Isolates from Clinical Samples

Four isolates were identified as *A. baumannii* using diagnostic and confirmatory microbiological/biochemical tests.

4.2. PCR to Confirm the Bap Gene

The results of electrophoresis of the *Bap* gene PCR products on agarose gel are shown in Figure 1.

4.3. Preparation of Derivatives

4a: (5-(phenyl)-1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol; 4b: (5-(3-(bromophenyl))-1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol; 4c: (5-(3-(chlorophenyl))-1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol, 4d: (5-((naphthalene)-2-il)-1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol; 4e: (5-(3-(fluorophenyl))-1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol; 4f: (5-(4-(fluorophenyl))-1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol; 4g: (5-(3,4-(difluorophenyl))-1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol; 4h: (5-(4-(methoxyphenyl))-1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol; 4i: (5-(3-(methoxyphenyl))-

1,3,4-oxadiazole-2-il)(pyridine-2-il)methanol (Figure 2).

4.4. MIC Results

The results showed that the combinations of 4a, 4d, and 4i, respectively, have the minimum inhibitory concentrations of 500, 250, and 62.50. In this regard, compound 4i has the highest inhibitory effect among the synthesized compounds. Among the compounds, this compound (4i) was used to continue experiments (Figure 2).

4.5. Real-time PCR

Figure 3 shows the *Bap* gene amplification diagram before and after treatment with compound 4i and the *16srRNA* gene progression diagram as the housekeeping gene, along with a table of calculated CT values. A1 shows the Bap gene before treatment with compound 4i, A2 exhibits the Bap gene after treatment with compound 4i, S1 displays the housekeeping gene before treatment with compound 4i, and S2 shows the housekeeping gene after treatment with compound 4i. Controls template No (NTCS) did not have a reaction progression graph, but the samples had an exponential multiplication graph, and multiplication was performed. The *16srRNA* gene was obtained before and after treatment at 14.51 and 14.03, respectively, considering that the difference in Ct before and after treatment with the combination of 4i was as small as 0.48; thus, the *16srRNA* gene was used as a home gene and an internal standard. The results of the melting curve of the Bap gene in the clinical strain of *A .baumannii* treated with 4i showed that the main peak for the Bap gene occurs at 79 to 80°C. The Δ_{Ct} results of the Bap gene in the presence of the 4i combination are given below. The PFAFFL method was used to determine the expression of the *Bap* gene. In this method, it is assumed that the sample yield and internal control are equal to 100%, and the $2^{-\Delta\Delta Ct}$ formula was used to investigate gene expression.

$$Ratio = \frac{(Etarget)^{\Delta CT\ target\ (control-sample)}}{(Eref.)^{\Delta CT\ ref\ (Control-Sample)}}$$

$$Ratio = \frac{10.35 - 12.59}{14.51 - 14.03} = \frac{2.24}{0.48} = 4.6$$

$CT = 2^{4.6} = 24.25$

As can be noted, the combination of 4i reduced the expression of the *Bap* gene by about 24 times, but it had no effect on the expression of the *16srRNA* housekeeping gene.

5. Discussion

Acinetobacter baumannii is the most common human pathogen in the genus *Acinetobacter* and is considered as one of the most important causes of nosocomial infections. One of the major problems in the treatment and prevention of infections caused by *A. baumannii* is antibiotic resistance, and carbapenems are the last line of treatment in infections caused by Gram-negative bacteria, including *A. baumannii*. The main factor of resistance can be biofilm production. There have been many studies on the resistance of *Acinetobacter*, the results of which have varied according to time and place (14-17). Therefore, one of the central goals of this research was to synthetize new structures for the treatment and elimination of this bacterium.

In recent years, the resistance and stability of bacteria to antibiotics has been a good reason to research the gene expressing biofilm (*Bap*) in various bacteria. For example, De Gregorio et al. conducted a study on proteins related to *Acinetobacter* biofilm. They stated that a large protein called *Bap* was involved in the formation of biofilms and the attachment of *A. baumannii* to host cells. The results of this group showed that several proteins with Ig-like repeats appear to be involved in biofilm formation (18). It can be said that several researchers have been interested in studying the resistance of *A. baumannii* to various antibiotics.

In 2016, Qi et al. conducted a study on the relationship between antibiotic resistance, biofilm formation, and biofilm specific resistance in *A. baumannii*. In their study, the relationship between antibiotic resistance, biofilm formation, and biofilm specific resistance in clinical isolates of *A. baumannii* was investigated (19). Other researchers have tried to show the effect of biofilm on the stability and survival of bacteria in the environment, especially in hospitals, which is a factor in causing nosocomial infections. Regarding biofilm structure, Fernández-Cuenca et al. demonstrated that in *A. baumannii*, adhesive cells that are coated with various coatings form an intermediate biofilm structure in the absence of nutrients. The findings indicate a rapid increase in the number of biofilm cells and a relative increase in the number of cells containing resistance (20).

This study proved that the presence of a biofilm in *A. baumannii* protects this microorganism against various antibacterial factors. The extraordinary ability of numerous structures containing the 1, 3, 4-oxadiazole ring as an antimicrobial agent has led to the use of derivatives of these structures in several studies. Various studies have shown that structures containing 1, 3, 4-oxadiazole have a significant ability to inhibit bacterial infectious diseases. In our previous study (14), we tested synthesis derivatives at a concentration of 1 mg/mL on a standard sample of *A.*

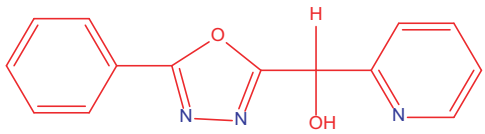
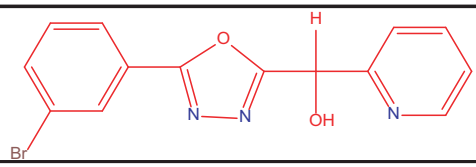
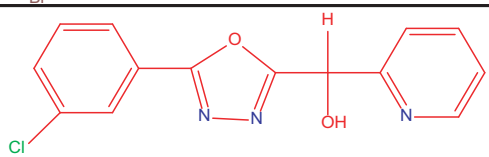
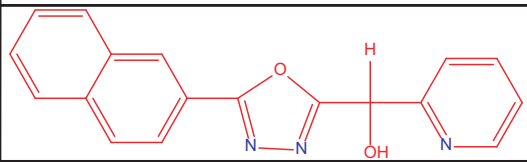
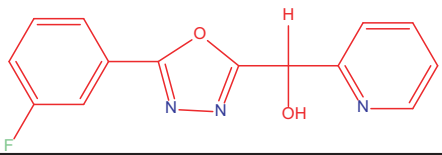
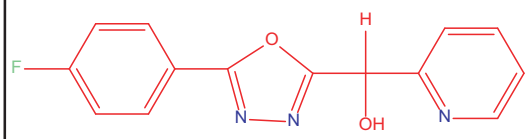
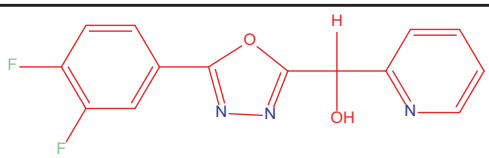
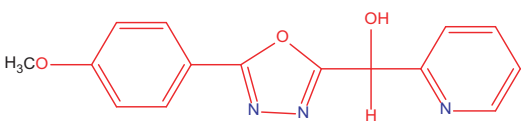
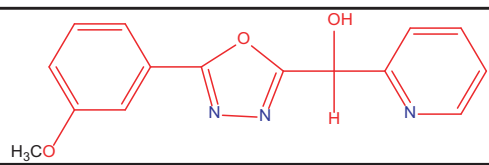
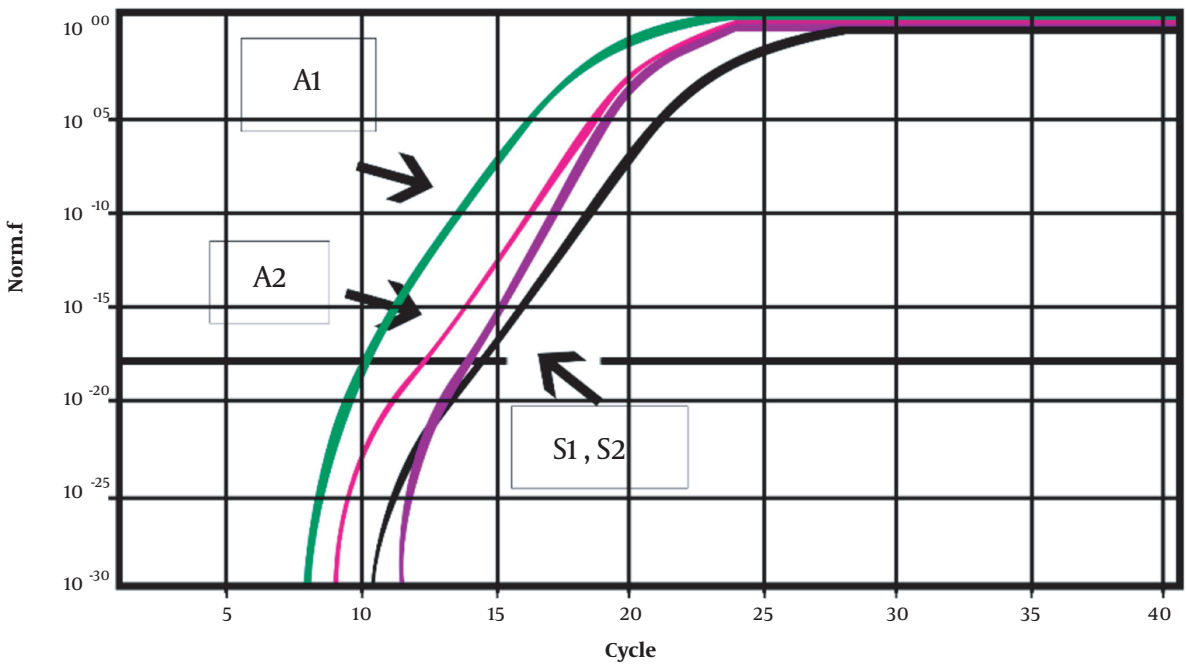
Compound	Structure	MIC
4a		≥ 500
4b		≥ 1000
4c		≥ 1000
4d		≥ 250
4e		≥ 1000
4f		≤ 1000
4g		≤ 1000
4h		≤ 1000
4i		≤ 62.50

Figure 2. Structures of new derivatives of 1, 3, 4-oxadiazole (14) and MIC results



No	Colour	Name	Type	Ct
6		A1	Unknown	10.35
5		A2	Unknown	12.59
25		S1	Unknown	14.51
27		S2	Unknown	14.03

Figure 3. *Bap* gene replication diagram

baumannii, but in this study, despite a concentration 10 times lower and the use of a clinical sample, amazing results were obtained.

The 4i derivative in the presence of methoxyphenyl attached to the main structure had acceptable anti-biofilm properties against *A. baumannii*. Methoxyphenyl is one of the most effective compounds in various structures of 1, 3, 4-oxadiazoles, whose antibacterial and anti-cancer properties have already been proven. This compound (methoxyphenyl) has the ability to affect the cytoplasmic membrane, electron transport chain, metabolic activity, and gene synthesis and inhibit protein synthesis. By studying these compounds on other virulence genes of *A. baumannii*, we can study these compounds and other derivatives of these structures in laboratory and animal studies as a treatment or adjunctive therapy in the form of tablets or additives used in patients' food as well as hand and environment disinfectants to prevent the spread of infection caused by this bacterium in hospital settings.

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Footnotes

Authors' Contribution: Study concept and design, N. Z. , Y. S.; Acquisition of data, N. Z., Y. S., and S. A.; Analysis and interpretation of data, N. Z., Y. S., and S. A.; Drafting of the manuscript, N. Z. and Y. S.; Critical revision of the manuscript for important intellectual content, N. Z. and Y. S.; Statistical analysis, N. Z„ Y. S„ and S. A.; Administrative, technical, and material support, N. Z. and Y. S.; Study supervision, N. Z. and Y. S.

Conflict of Interests: The authors declare no conflicts of interest.

Ethical Approval: This Research study was conducted in Genetic laboratory of Islamic Azad University, Tehran branch, with code number, 10130553971011 in 2019.

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