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Effect of Chloroxylenol on Gene Expression of *pslA* and *pelA* in *Pseudomonas aeruginosa*

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Abstract

Chloroxylenol is widely used in hospitals and other healthcare facilities to reduce the growth or the microbial load of pathogens. What's more, biofilms are a major challenge in treating *P. aeruginosa* infections. The genes of *PslA* and *pelA* in two operons encode two polysaccharide polymers present in the biofilm of *P. aeruginosa*, *Psl* and *Pel*, respectively. Therefore, the present study aimed to evaluate the contribution of chloroxylenol to the inhibition of the *P. aeruginosa* biofilm. Here, the intensity of biofilms was determined by the crystal violet and microtiter plate method, whereas the RT-qPCR technique was adopted to illustrate the effect of chloroxylenol on the expression of the *pslA* and *pelA* genes. The results showed that chloroxylenol suppresses the formation of biofilms and reduces the expression of both studied genes. As a result, chloroxylenol had inhibitory activity over *P. aeruginosa* biofilm via downregulating the *pelA* and *pslA* genes.

Keywords: Biofilm, Chloroxylenol, *Pseudomonas aeruginosa*, *pslA* and *pelA*

تاثير الكلوروزايلينول على التعبير الجيني لـ *pslA* و *pelA* في *Pseudomonas aeruginosa*

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الخلاصة

يستعمل الكلوروزايلينول عل نطاق واسع في المستشفيات و بقية المنشات الصحية للتقليل من نمو الممرضات او من الحمل المايكروبي لها. علاوة على ذلك، تشكل الاغشية الحياتية تحديا كبيرا في علاج الاصابات الناجمة عن بكتريا *Pseudomonas aeruginosa*. جينات الـ *pslA* و *pelA* في اثنين من الاوبرونات المشفرة لاثنين من عديد السكريد الموجودة في الغشاء الحياتي لبكتريا *P. aeruginosa* متمثلا بـPsl و Pel على التتابع. ولهذا هدف هذا العمل الى مساهمة الكلوروزايلينول في تثبيط الغشاء الحياتي لبكتيريا *P. aeruginosa* . هنا في هذا البحث تم تحديد كثافة الغشاء الحياتي بطريقة البنفسج البلوري و طبق المعايرة الدقيقة في حين تم اعتماد تفاعل البلمرة اللحظي الكمي لاظهار تأثير الكلوروزايلينول في التعبير الجيني لكل من *pslA* و *pelA* أظهرت النتائج أن الكلوروزايلينول ثبط تكوين الغشاء الحياتي و خفض من

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التعبير الجيني للجينات المدروسة. كاستنتاج، يمكن القول ان الكلوروزايلينول يثبط الغشاء الحياتي عن طريق تثبيط جينات *pslA* و*pelA* .

1. Introduction

Markedly, it is well-known that chloroxylenol can govern the growth of many pathogens [1]; however, literature has failed to provide solid evidence on the underlying mechanism of its efficacy to evoke resistance [2]. The pathogen *Pseudomonas aeruginosa* is a frequent microbe implicated in many severe nosocomial infections [3].

One of the major problematic therapeutic issues in treating infections caused by *P. aeruginosa* is its capacity to develop robust biofilm. Nonetheless, this biofilm is a highly complicated community of bacterial cells adhered to a solid stratum enclosed with a profound matrix that generates strong resistance toward many antibacterial compounds. Despite the wide-spectrum antibacterial characteristics of chloroxylenol, its activity on the biofilm of *P. aeruginosa* upstretched a debatable argument among scientists [4]. Aka, et al. [5] stated that chloroxylenol is more effective against biofilm initiated by some Gram-negative bacteria compared to *P. aeruginosa*. Yet, it might be more effective once it is mixed with another antimicrobial agent.

In *P. aeruginosa* biofilm formation, Psl and Pel are two significant polysaccharides that participated in biofilm development and maintained its presence [6, 7]. Psl is a linear β-1,6 Nacetylglucosaine polysaccharide. Its synthesis is performed by the action of enzymes encoded by the *pls* gene; however, the regulation of its production is accomplished by the transcriptional regulator protein PelD. Biofilm stability is maintained by the participation of Psl by providing structural integrity and encouraging cell-surface and cell-cell interactions. Moreover, Psl participated significantly in surface attachment and the initial biofilm establishment [8].

The other polysaccharide of *P. aeruginosa* biofilm exoplymeric substance is Pel; which is a glucose-rich polysaccharide synthesized by the enzymes encoded by the *pel* gene. The transcriptional regulator protein PelA regulates the production of Pel polysaccharide. Pel shares Psl in biofilm structural integrity and adhesion to surfaces. Pel has a crucial contribution to the creation of mushroom-shaped towers inside the biofilm topography, stabilizing the three-dimensional configuration of the biofilm [9]. Thus, Psl and Pel are vital for the maturation of biofilm. Moreover, their interlay participates in the structural constancy of *P. aeruginosa* biofilm [3]. Both polysaccharides permit the attachment of microorganism to a scaffolding surface, co-aggregation, and eventually forming the intricate three-dimensional distinctive structure of biofilm. In addition, Psl and Pel enable the acquisition of nutrients and water, shielding the microorganism from environmental stresses and offering a defensive strategy against host immunity and antimicrobial agents [10].

2. Materials and Methods

2.1 Pseudomonas aeruginosa isolates

The *P. aeruginosa* isolates (30 isolates) were taken from the microbiology lab at the Department of Biology, College of Science, University of Baghdad. The obtained *P. aeruginosa* isolates were initially collected from 30 non-repeated specimens collected from patients who were admitted to three different hospitals in Baghdad, including Al-Yarmouk Teaching Hospital, Baghdad Medical City Hospitals, and Al-Kindy Teaching Hospital.

2.3 Estimation of minimal inhibitory concentration of chloroxylenol

The broth microdilution method was followed to estimate the minimum inhibitory concentration (MIC) of chloroxylenol on *P. aeruginosa* [11]. A pure and fresh culture of *P. aeruginosa* isolate was prepared, and the turbidity of the culture was adjusted to match the 0.5 McFarland standard (approximately 1-2 x 10^8 CFU/ml). Simultaneously, twofold serial dilutions of chloroxylenol (Dettol/ United Arab Emirates) ranging from 0.00 to 4.5% in Mueller-Hinton broth were prepared as well. In a sterile 96-well microtiter plate, 100 µl of sterile broth or Mueller-Hinton broth was added to all wells except for the first column. In the first column, 100 µl of the microbial suspension was added to each well. Starting from the second column, 100 µl of the chloroxylenol dilutions were added to the corresponding wells of the microtiter plate. The last column contained only a growth medium without any chloroxylenol or microorganisms. This serves as the sterility control. The microtiter plate was sealed to prevent evaporation and contamination and incubated at 37°C for 24 hours. After the incubation period, the wells were visually examined for the presence or absence of visible growth. The MIC is considered the lowest concentration of chloroxylenol, which completely inhibits the growth of *P. aeruginosa*.

2.4 Effect of chloroxylenol on biofilm of P. aeruginosa

Pseudomonas aeruginosa isolate was cultivated in tryptic soy broth according to standard laboratory protocols. The bacterial culture (compatible with 0.5 McFarland standard) was inoculated onto a surface of sterile polystyrene coupons (1 cm^2) and incubated at 37°C for 24 hours. Chloroxylenol solution at ½MIC was applied to the pre-formed *P. aeruginosa* biofilm for 24 hours, and complete coverage of the biofilm surface was ensured. The control group included untreated biofilms. After the specified contact time, the coupons were removed and rinsed gently with sterile water to remove any residual chloroxylenol and fixed with methanol for 10 minutes. Thereafter, the stain was solubilised by placing the coupons in a container filled with 33% glacial acetic acid for 10 minutes. The solubilised stain was measured at 600 nm with the aid of a microplate reader (Organon Teknika, Australia). Finally, the results of the chloroxylenol-treated biofilms were compared with the control groups to determine the antimicrobial effects of chloroxylenol on *P. aeruginosa* biofilms [12].

2.2 Detection of pslA and pelA in Pseudomonas aeruginosa isolates

The most affected isolates by the treatment with chloroxylenol were chosen for this study.

2.2.1 Extraction of DNA

As instructed by the manufacturer instructions, the genomic DNA extraction kit (Promega, USA) was used to extract DNA from all *P. aeruginosa*.

2.2.2 Primer Selection

Table 1 grouped the primers employed for the amplification of fragments of the genes under study represented by *pelA* (118 bp) and *pslA* (70 bp) [13]. A concentration of 100 pmol /µl was prepared from each primer to be included in the final reaction mixture. Subsequently, they were stored in a deep freezer until use.

Table 1: Oligonucleotides used in the current work [13]

Primer name	Sequence (5′ - 3′)	Amplicon Size (bp)
pelA	F- CCTTCAGCCATCCGTTCTTCT R- TCGCGTACGAAGTCGACCTT	118
pslA	F- ATAAGATCAAGAAACGCGTGGA R- TGTAGAGGTCGAACCACACCG	70

2.2.3 Gene amplification

The reaction mixture contained Promega Green Master Mix (12.5 µl), 10 µM primer (2 µl per each of the forward and reverse primers), and the DNA template (3 µl); the volume was completed up to 25 µl with free nuclease water. Thereafter, the program described by Colvin, et al. [13] was followed with some modifications and included in Table 2. Finally, the PCR amplicons were resolved in 1.5% agarose gel [13].

Table 2: PCR program for the amplification of *pslA* and *pelA* [13]

Step	Temperature (°C)	Time	Number of Cycles
Initial denaturation	95	5 minutes	1
Denaturation	95	25 seconds	35
Annealing	53	15 seconds	
Extension	72	45 seconds	
Final Extension	72	3 minutes	1

2.5 Effect of chloroxylenol on the expression of pslA and pelA genes

An identical approach assaying the biofilm mentioned earlier was adopted employing Muller Hinton broth containing ½MIC chloroxylenol. Following the manufacturing company, the RNA was extracted from biofilm cells using Trizol reagent (Promega, USA). cDNA was synthesized using ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs, UK). The primers listed in Table 1, in addition to the primer set for the housekeeping gene, *16SrRNA* (5′-ACCTGGACTGATACTGACACTGA-3′ and 5′-GTGGACTACCAGGGTATCTAATCCT-3′ is the forward and reverse primers, respectively) [14] and reaction mixture are summarized in Table 3. Furthermore, after many attempts, the thermocycling procedure was fine-tuned, and the resulting procedure is shown in Table 4. To obtain a melting curve, a temperature gradient was set up by incrementing the temperature by 1˚C per second, spanning from 60˚C to 95˚C.

Table 3: qPCR reactants

Reactant	Volume (µl)
Luna Universal qPCR Master Mix	10
Reverse primer (10 µM)	1
Forward primer (10 µM)	1
cDNA (50 ng/µl)	5
Nuclease-free Water	3

Table 4: Thermocyling conditions

Step	Temperature (°C)	Time (second)	Number of Cycles
Initial Denaturation	95	60	1
Denaturation	95	15	45
Extension	60	30	

The expression levels were measured using relative quantification and then normalised with those of the housekeeping gene (*16SrRNA*). The study assessed the disparity in cycle thresholds (ΔCt) and fold changes between the treatment groups and the calibrators of each gene [18]. A fold change below 2-fold was deemed insignificant [19].

2.6 Statistical analysis

A normality test was performed using the Shapiro-Wilk test. Related-Samples Wilcoxon Signed Rank Test was employed for means comparison using GraphPad Prism version 9.5.0.

3. Results

3.1 Estimation of minimal inhibitory concentration of chloroxylenol

The determined MIC value of chloroxylenol for all *P. aeruginosa* isolates in this study was 2.25%.

3.2 Effect of chloroxylenol on biofilm of P. aeruginosa

At a concentration of 2.25%, chloroxylenol significantly ($P< 0.05$) inhibited the biofilm of all 30 isolates (Figure 1).

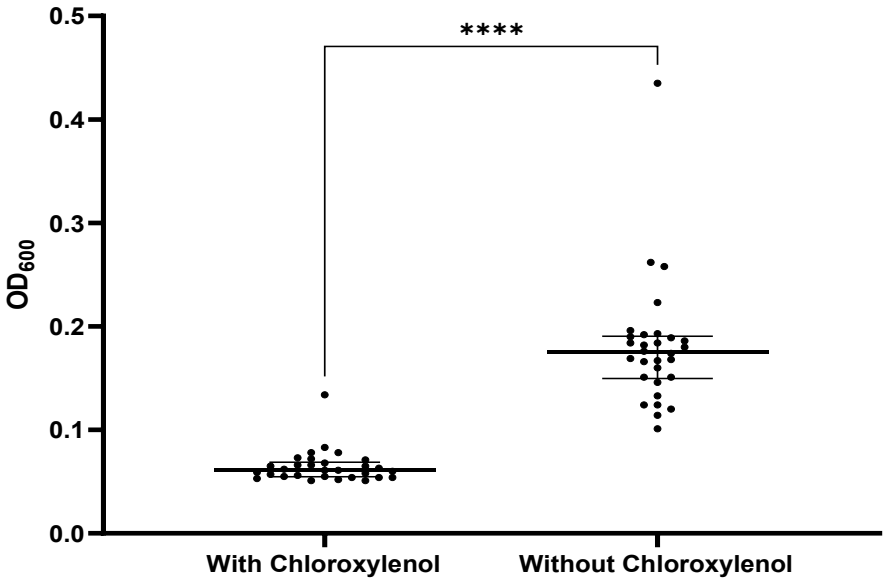

Figure 1: Scatter plot showing the effect of chloroxylenol on *Pseudomonas aeruginosa* biofilm (n= 30). Horizontal lines denote the median ± interquartile range

3.3 Detection of pslA and pelA

The results depicted in Figures 2 and 3 demonstrated that *pslA* and *pelA* have existed in all isolates of *P. aeruginosa* under study.

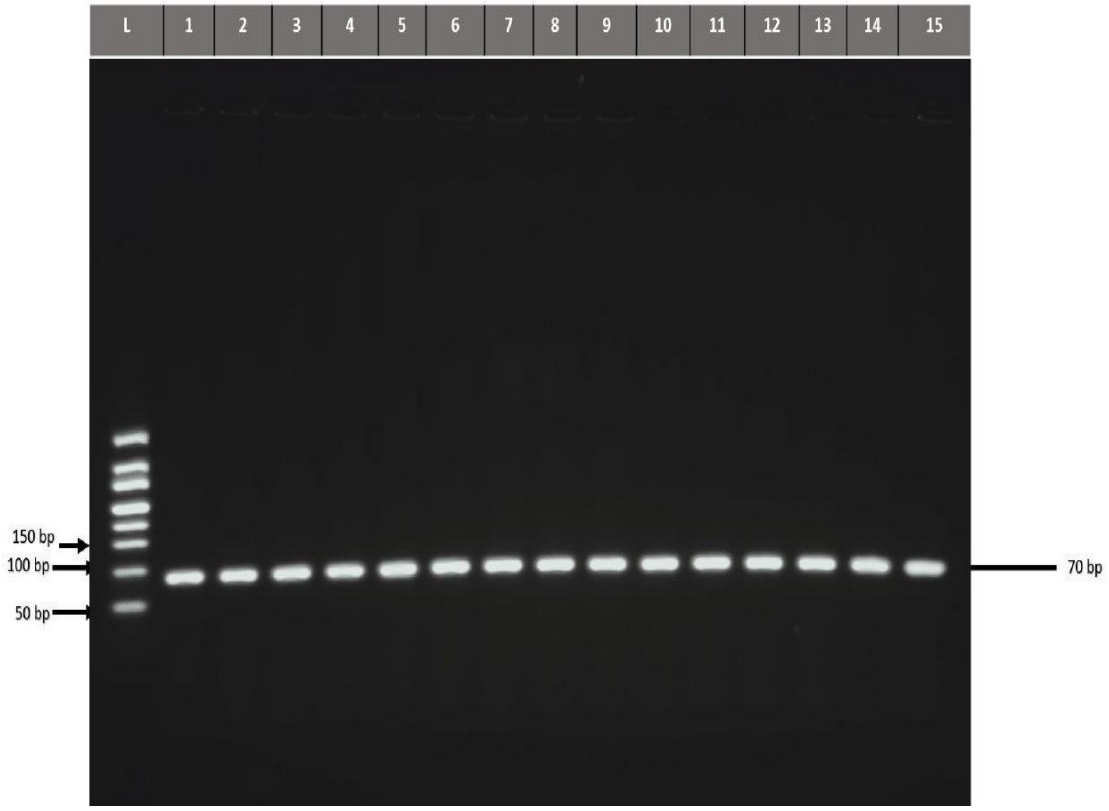

Figure 2: *pslA* amplicons (70 bp) resolved in 1.5% agarose gel at 5 V/cm. Lane L represents a 50 bp DNA ladder. Lanes 1-15 represent *Pseudomonas aeruginosa* isolates.

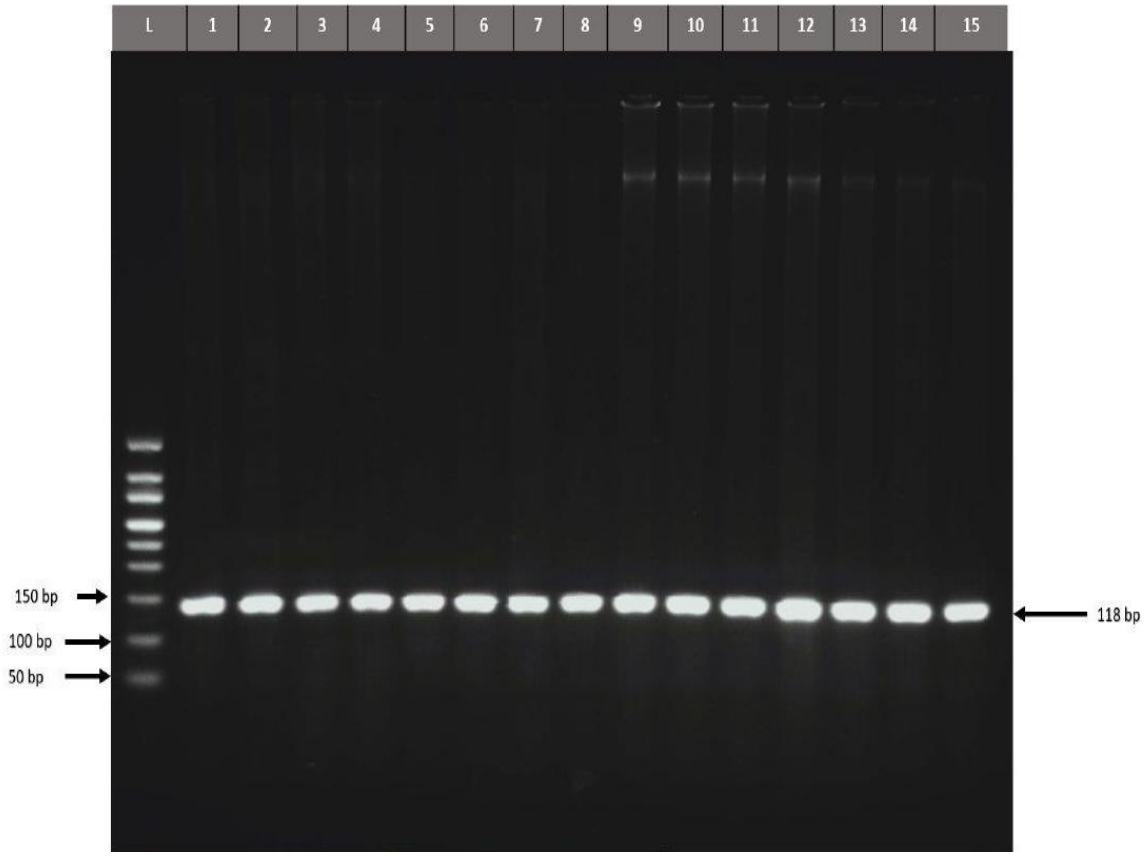

Figure 3: *pelA* amplicons (118 bp) resolved in 1.5% agarose gel at 5 V/cm. Lane L represents a 50 bp DNA ladder. Lanes 1-15 represent *Pseudomonas aeruginosa* isolates.

3.4 Effect of chloroxylenol on the expression of pslA and pelA

Results depicted in Figure 4 demonstrated that chloroxylenol downregulated the expression of *pslA* and *pelA genes*

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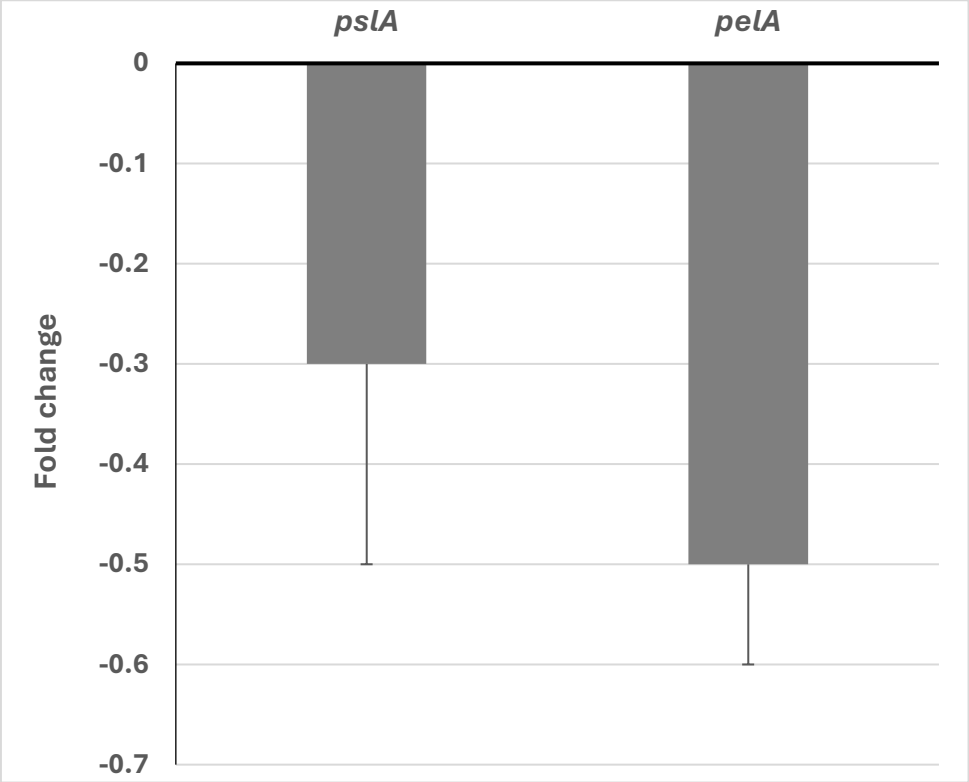

Figure 4: The data illustrates the decrease in expression levels of *pslA* and *pelA* genes in *P. aeruginosa* biofilm after exposure to chloroxylenol. Error bars represent standard deviation.

4. Discussion

The outer membrane of Gram-negative bacteria acts as a selective barrier, permitting certain materials to pass into the cytoplasm and simultaneously preventing others from getting in. Together with other mechanisms, such as efflux pumps, many substances like chloroxylenol will be prevented from entering the cell [15]. On the other hand, *P. aeruginosa* showed exquisite resisting mechanisms in comparison to other bacterial species, principally because of modifications in the structure of lipopolysaccharide (LPS) in addition to positively charged ions composition in the outer membrane. These differences lead to strong LPS-LPS interaction and selectively allow the diffusion of some particles [16]. Porins and efflux pumps of *P. aeruginosa* can cooperate to expel a wide array of antibiotics and biocides out of cells [2].

Regarding the existence of *pelA* and *pslA* genes in *P. aeruginosa* isolates, the current findings are parallel to those of many other studies. For instance, Al-Sheikhly, et al. [3] stated that all their isolates carried these genes; however, in the study of Nader, et al. [17], 91.9% of their isolates harboured the *pslA* gene. Moreover, pelA and pslA were located at 86.4% and 94.9%, respectively, in an investigation conducted by Farhan, et al. [18]. Markedly Grossich, et al. [19] indicated that both pelA and pslA polysaccharides participate in the protection of *P. aeruginosa* either in planktonic or biofilm states from ultraviolet radiation and hypochlorite effect.

In the context of this investigation, the impact of chloroxylenol on the biofilm of *P. aeruginosa* was weighed, and the outcomes revealed that chloroxylenol significantly

($P<0.05$) reduced the intensity of biofilm. Recalling the results presented in Figures 1 and 4, it can be perceived that the inhibitory action of chloroxylenol was achieved by lowering the gene expression of *pslA* and *pelA*.

Chloroxylenol is accountable for decreasing the microbial load when it is applied in hospitals and when it is used to disinfect prosthetics [20, 21]. An interesting aspect of the current investigation was the intentional selection of the biofilm-establishing clinical isolates to enhance the treatment applicability of the study outcomes a preceding study exposed the effectiveness of antiseptics, including the antiseptic tested in this work, in opposing planktonic bacteria. However, paying attention to the pathogens frequently found in biofilms is vital, given that this signifies their essential ecological conditions [22].

Conclusion

In conclusion, chloroxylenol established noteworthy effectiveness in combining the free swimming as well as the biofilm of *P. aeruginosa* through lowering the expression of biofilm-related genes, *pslA* and *pelA*.

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