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INVESTIGATION OF ANTIMICROBIAL AND ANTI-QUORUM SENSING PROPERTIES OF SOME DERIVATIVES OF CEPHALOSPORANIC ACID, CIPROFLOXACIN, NORFLOXACIN, AND PENICILLANIC ACID

BAZI SEFALOSPORANİK ASİT, SİPROFLOKSASİN, NORFLOKSASİN VE PENİCİLLANİK ASİT TÜREVLERİNİN ANTİMİKROBİYAL VE ANTI-QUORUM SENSING ÖZELLİKLERİNİN ARAŞTIRILMASI

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Abstract

Objective: The rapid increase of antibiotic resistance among bacteria is a serious public health problem. Studies have focused on the discovery of new antibiotic molecules and the development of new therapeutic strategies to combat these resistant bacteria. Once it was known that pathogenic bacteria regulate synthesis of virulence factors by the quorum sensing (QS) mechanism, QS inhibition became an attractive target for antibacterial treatment. This study aimed to investigate the antimicrobial activities and anti-QS properties of 16 derivatives of cephalosporanic acid, ciprofloxacin, norfloxacin, and penicillanic acid.

Methods: Antimicrobial activity of the derivatives was tested by the agar well diffusion method against various microorganisms. The minimum inhibitory concentration (MIC) of effective derivatives was assessed by the broth microdilution method. Anti-QS properties were investigated using the soft agar method, observing inhibition of violacein production in *Chromobacterium violaceum*. The data were compared statistically.

Results: Six norfloxacin derivatives displayed antimicrobial activity against a number of organisms, three of which were more effective than control antibiotics (p<0.05) against some organisms. One ciprofloxacin derivative demonstrated antimicrobial activity against all tested bacteria and was more effective against some bacteria than control antibiotic (p<0.05). The MIC values of these six norfloxacin and one ciprofloxacin derivatives were between 0.04–6.25 µg/mL and 0.04–3.12 µg/mL, respectively. A cephalosporanic acid and a penicillanic acid derivative displayed anti-QS properties.

Conclusion: This study shows that some new derivatives of cephalosporanic acid, ciprofloxacin, norfloxacin, and penicillanic acid may have potential for development of new antibacterial and anti-QS agents.

Keywords: *Chromobacterium violaceum, antibiotic resistance, antimicrobial tests, derivatives*

Öz

Amaç: Bakteriler arasında antibiyotik direncinin hızla artışı halk sağlığı açısından ciddi bir problem oluşturmaktadır. Çalışmalar, yeni antimikrobiyal moleküllerin keşfi ve dirençli bakterilerle mücadelede yeni tedavi stratejilerinin geliştirilmesi üzerine yoğunlaşmıştır. Patojenik bakterilerin, quorum sensing (QS) mekanizmasını kullanarak virülans faktörlerinin sentezini düzenlediği bulunduktan sonra, QS inhibisyonu antibakteriyel tedavi için cazip bir hedef haline gelmiştir. Bu çalışmada sefalosporanik asit, siprofloksasin, norfloksasin ve penisillanik asit türevi 16 maddenin antimikrobiyal aktivitelerinin ve anti-QS özelliklerinin araştırılması amaçlandı.

Yöntem: Maddelerin antimikrobiyal aktiviteleri çeşitli mikroorganizmalara karşı agar kuyucuk diffüzyon yöntemi ile test edildi. Etki gösteren maddelerin minimum inhibisyon konsantrasyon (MİK) değerleri sıvı mikrodilüsyon yöntemi ile belirlendi. Maddelerin anti-QS özellikleri *Chromobacterium violaceum* raportör suşunda viyolasin inhibisyonu üzerine yarı-katı agar yöntemi ile araştırıldı. Veriler istatistiksel olarak karşılaştırıldı.

Bulgular: Altı norfloksasin türevi test edilen bakterilerin bir kısmına karşı antimikrobiyal aktivite göstermiş olup üç türevin bazı organizmalara karşı kontrol antibiyotiklerden daha etkili olduğu tespit edildi (p<0,05). Bir siprofloksasin türevi, test edilen tüm bakterilere karşı antimikrobiyal aktiviteye sahip olup bakterilerin bir kısmına karşı kontrol antibiyotiğinden daha etkili olduğu belirlendi (p<0,05). Altı norfloksasin ve bir siprofloksasin türevinin MİK değerleri, sırasıyla 0,04–6,25 µg/mL ve 0,04–3,12 µg/mL aralığında tespit edildi. Bir sefalosporanik asit ve bir penisillanik asit türevinin anti-QS özellik gösterdiği belirlendi.

Sonuç: Bu sonuçlar, bazı yeni sefalosporanik asit, siprofloksasin, norfloksasin ve penicillanik asit türevlerinin yeni antibakteriyel ve anti-QS ajanların geliştirilmesinde potansiyel moleküller olabileceğini göstermiştir.

Anahtar Kelimeler: *Chromobacterium violaceum, antibiyotik direnci, antimikrobiyal testler, türevler*

Introduction

Misuse and overuse of antibiotics worldwide have resulted in development of resistance in bacteria, and in particular, multidrug-resistant strains pose a serious public health problem.[1, 2] Since existing antibiotics are often ineffective against multi-drug resistant bacteria and the discovery of new antibiotics in recent years has not met the need, research has focused on finding alternative methods to combat resistant strains.[2, 3]

Quorum sensing (QS) is a phenomenon by which bacteria communicate with each other to regulate most of their physiological and biochemical activities via several signal molecules that they secrete into the environment. These signaling molecules modulate the expression of genes related to functions such as biofilm formation, swarming, pigment productions, and synthesis of virulence factors.[4-7]

Inhibition of bacterial QS provides an alternative method to combat infectious diseases, because it inhibits bacterial pathogenicity by blocking the ability to synthesize and secrete virulence factors. Furthermore, unlike antibiotics, QS inhibitors do not stress the bacteria. Therefore, resistance development is not expected with the use of QS inhibitors.[8] The mechanism of QS inhibition in bacteria is divided into three main groups: prevention of signal molecule production; destruction of signal molecules upon release; and prevention of signal molecule uptake into the cell.[9]

Chromobacterium violaceum is a gram-negative bacterium found abundantly in nature. This bacterium forms a blue-violet and a water-insoluble pigment called violacein, which is commonly used as an indicator to test the anti-QS property of an agent.[10] One of these strains, *C. violaceum* ATCC 12472, produces violacein using long–chain acyl homoserine lactone (AHL) molecules (C10-C16) and is a commonly used reporter strain for anti-QS testing.[11]

The aim of this study was to investigate the antimicrobial activities and anti-QS properties of 16 newly synthesized derivatives of cephalosporanic acid, ciprofloxacin, norfloxacin, and penicillanic acid. In this study a range of gram-positive and gram-negative organisms and two *Candida* strains were used for antimicrobial activity tests as representative pathogens. These included, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 7002, *Salmonella typhimurium* ATCC 10708, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter haemolyticus* ATCC 19002, *Candida albicans* ATCC 10231, and *C. parapsilosis* ATCC 22019. *C. violaceum* ATCC 12472 was used for the QS inhibition test.

Methods

Microorganism Strains, Culture Conditions and Reagents

The microorganism used to determine antimicrobial activities in this study are listed in Table 1. They were obtained from the Department of Medical Microbiology, Faculty of Medicine, Karadeniz Technical University's strain collection. All of the tested microorganisms were American Type Culture Collection (ATCC) reference strains. The *C. violaceum* strain ATCC 12472 was obtained from Suleyman Demirel University, Faculty of Sciences, Department of Biology, Isparta, Turkey to determine the QS inhibitory activity of the derivatives.

Table 1. The microbial strains used to determine antimicrobial activities in this study

Microorganisms
Staphylococcus aureus ATCC 25923
Enterococcus faecalis ATCC 29212
Bacillus subtilis ATCC 6633
Escherichia coli ATCC 25922
Enterobacter aerogenes ATCC 13048
Klebsiella pneumoniae ATCC 13883
Proteus mirabilis ATCC 7002
Salmonella typhimurium ATCC 10708
Pseudomonas aeruginosa ATCC 27853
Acinetobacter haemolyticus ATCC 19002
Candida albicans ATCC 10231
Candida parapsilosis ATCC 22019

C. violaceum strain was cultured in Luria Bertani (LB) media (Lab M, Bury, England) at 30°C. The other reference strains were cultured on nutrient agar (NA; Merck, Darmstadt, Germany), Mueller Hinton agar (MHA; Merck, Darmstadt, Germany) and Saboraud Dextrose agar (SDA; Merck, Darmstadt, Germany) at 37°C.

Derivatives

A total of 16 newly synthesized derivatives of cephalosporanic acid, ciprofloxacin, norfloxacin, and penicillanic acid (Table 2) were evaluated for their antimicrobial activities and anti-QS properties for the first time in the present study. All derivatives were dissolved in dimethylsulfoxide (DMSO; Sigma, USA) at a final concentration of 100 µg/mL.

Table 2. The derivatives of cephalosporanic acid, ciprofloxacin, norfloxacin, and penicillanic acid used in this study (n =16)*

Derivative Code*	Molecular Weight (MW)	Chemical Formula	Derivative of
B21	645.75	$C_{28}H_{35}N_7O_7S_2$	Cephalosporinic acid
B29	555.61	$C_{21}H_{28}N_6O_6S_2$	Cephalosporinic acid
B36	631.72	$C_{27}H_{33}N_7O_7S_2$	Cephalosporinic acid
B30	603.61	$C_{27}H_{34}FN_7O_6S$	Norfloxacin
B49	621.73	$C_{31}H_{36}FN_7O_4S$	Norfloxacin
N16	504.91	$C_{24}H_{23}ClF_2N_4O_4$	Norfloxacin
N19	522.59	$C_{26}H_{27}FN_6O_3S$	Norfloxacin
Y32	381.79	$C_{17}H_{17}N_3O_4FCl$	Norfloxacin
Y33	665.74	$C_{32}H_{36}N_7O_6FS$	Norfloxacin
Y34	623.68	$C_{31}H_{38}N_7O_6F$	Norfloxacin
Y35	757.82	$C_{39}H_{44}N_7O_8F$	Norfloxacin
Y37	660.72	$C_{35}H_{38}N_6O_5F_2$	Norfloxacin
B28	500.59	$C_{19}H_{28}N_6O_6S_2$	Penicillanic acid
B35	575.20	$C_{25}H_{33}N_7O_5S_2$	Penicillanic acid
B47	518.65	$C_{23}H_{30}N_6O_4S_2$	Penicillanic acid
B31	615.68	$C_{28}H_{34}FN_7O_6S$	Ciprofloxacin

* All derivatives were synthesized at the Department of Chemistry, Faculty of Science, Karadeniz Technical University

Antimicrobial activity

Antimicrobial activity of the derivatives were screened using the agar well diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) procedures with minor modifications.[12] Briefly, fresh cultures of each strain grown on NA plates were adjusted to 0.5 McFarland turbidity standard (1.0 – 2.0

× 10^8 CFU/mL for bacteria and 1.0 – 2.0 × 10^6 CFU/mL for *Candida* spp.) using the direct colony suspension method. Then, a sterile cotton swab was dipped into the suspension and inoculated by swabbing in three directions on the agar media (MHA for bacteria, SDA for *Candida* spp). Fifty microliters of each derivative were dropped into wells punched in the agar with a sterile cork borer. Ampicillin (10 µg/well; for *S. aureus*, *E. faecalis*, and *B. subtilis*), gentamicin (10 µg/well; for *E. coli*, *K. pneumoniae*, *E. aerogenes*, *S. typhimurium*, *P. aeruginosa*, and *A. haemolyticus*), cefotaxime (30 µg/well; for *P. mirabilis*), and amphotericin B (20 µg/well; for *C. albicans* and *C. parapsilosis*) were used as positive control. The control antibiotics were selected according to EUCAST.[13] DMSO was used as negative control. The plates were incubated at 18±2 hours for bacteria, 48 hours for *Candida* strains. After the incubation, zones of inhibition (ZOI) surrounding the wells were measured using a ruler (mm). The experiments were performed in triplicate and the results were given as ZOI ± standard deviations (SD) in millimeters.

Minimal Inhibitory Concentration (MIC) values (µg/mL) were determined by the broth microdilution method according to the Clinical Laboratory Standards Institute.[14] Briefly, the method was carried out using 96-well plates comprising 100 µL/well of Mueller Hinton broth-II (MHB-II; BBL, Becton Dickinson, USA). A stock solution of the derivatives was transferred to the first well and serially diluted to obtain concentrations ranging from 50 to 0.01 µg/mL. Then, five microliters of cell suspensions (approximately 5.0 × 10^5 CFU/mL) were added to each well of the microtiter plate. One well without the bacterial suspension was used as sterility control and one well without the derivative to be tested was used as growth control. The plate was covered with a sterile lid and incubated for 18±2 hours. The MICs were determined as the minimum concentration of derivatives at which there was no visible growth of the test strain.

Quorum Sensing Inhibition

The anti-QS potential of each derivative was tested using the reporter strain of *C. violaceum*. Fifty microliters of *C. violaceum* overnight culture in LB broth was inoculated to five milliliters of molten soft LB agar (0.6% w/v), vortexed, and poured over the surface of prewarmed LB agar plates.[10] Fifty microliters of each derivative were dropped into the wells punched in the solidified agar with a sterile cork borer. Vanillin (500 µg/mL; Merck, Darmstadt, Germany) and DMSO were used as positive and negative control, respectively. The cultures were incubated for 18±2 hours and violacein inhibition surrounding the wells was examined. QS inhibition was determined by a colorless, opaque, but viable, halo around the wells.[11]

The data were tested for normality using the Wilks-Shapiro test. Non-parametric data was analyzed with Mann-Whitney *U* test using IBM-SPSS statistics version 23.0 (IBM Inc., Armonk, NY, USA), and $p<0.05$ was considered statistically significant.

Results

The antimicrobial activity of 16 novel derivatives were tested against various microorganisms by the agar well diffusion method and results are shown in Table 3.

Six norfloxacin derivatives (B30, B49, N16, N19, Y32, and Y33) had a degree of antimicrobial activity against several bacteria tested (Figure 1.). B31, a ciprofloxacin derivative, exhibited significantly higher antibacterial activity than control antibiotics against *B. subtilis*, *P. aeruginosa*, *E. coli*, *E. aerogenes*, and *S. typhimurium* ($p<0.05$). Furthermore, the antibacterial activity of some of the other derivatives was more effective than control antibiotics: B30, and Y32 against *B. subtilis*; B49 versus *E. aerogenes*; and B30 versus *S. typhimurium* ($p<0.05$).

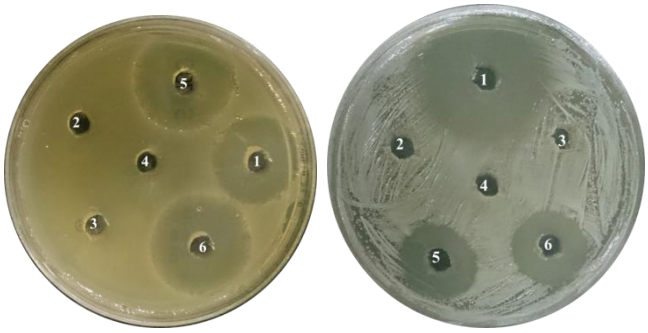

Figure 1. Examples of antimicrobial activitiy of norfloxacin derivatives (B30 and B49) against *Enterobacter aerogenes* ATCC 13048 (left) and *Staphylococcus aureus* ATCC 25923 (right), 1; Positive control, 2; Negative control, 3; N16, 4; N19, 5; B30, 6; B49

While N16 and N19 displayed antibacterial activity towards *B. subtilis*, Y32 and Y33 demonstrated antibacterial activity against *E. coli* and *S. typhimurium*. However, the antibacterial activity of these derivatives was no greater than the control antibiotics ($p>0.05$).

No antimicrobial activity of cephalosporinic acid and penicillanic acid derivatives was evident against any of the tested bacteria. Similarly, no antifungal activity against *Candida* species was evident with any of the derivatives tested.

The MIC values of the six norfloxacin and one ciprofloxacin derivatives which showed antibacterial activity at concentrations ranging from 0.04–6.25 µg/mL and 0.04–3.12 µg/mL, respectively, was shown in Table 4. The lowest MIC value was determined in norfloxacin (B30 and B49) and ciprofloxacin (B31) derivatives against *E. coli* (MIC= 0.04 µg/mL). Also, B31 had a antibacterial activity with a MIC value of 0.04 µg/mL against *E. aerogenes* and *S. typhimurium* in addition to *E. coli*.

Loss of pigmentation of *C. violaceum* surrounding agar wells containing tested derivatives were indicative of QS inhibition. One cephalosporanic acid (B29) and one penicillanic acid (B28) derivative inhibited the production of violacein in *C. violaceum* (Figure 2.).

Figure 2. Inhibition of violacein production in *C. violaceum* ATCC 12472. 1; B30, 2; B49, 3; N16, 4, N19; 5; Y32, 6; B28, 7; B29, 8; Vanillin, 9; DMSO

Discussion

Increased antibiotic resistance among bacteria has led to the search for new antimicrobial agents and alternative treatment methods such as phage therapy or inhibition of bacterial quorum sensing.[8-11, 15] The formation of new and more potent derivatives through chemical or biological modification of existing antibiotics is one of the strategies adopted in the search for agents to combat antibiotic resistance.[16] Therefore, in this study, the antimicrobial activity of 16 derivatives of cephalosporanic acid, ciprofloxacin, norfloxacin, and penicillanic acid against three gram-positive and seven gram-negative bacteria, and two yeast strains was examined. The results showed that six norfloxacin and one ciprofloxacin derivative displayed antibacterial activity against various pathogenic bacteria.

It has been shown that synthesized quinolone derivatives have more antibacterial activity than standard drugs. Chen *et al*.[17] investigated the antibacterial activity of 7-substituted quinolone derivatives against methicillin-resistant *S. aureus* (MRSA), erythromycin- and ampicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant *E. faecalis*. Significantly increased antimicrobial avtivity of most of the derivatives was observed against MRSA when compared to norfloxacin.

Antibacterial activities of piperazinyl-quinolone derivatives synthesized by Foroumadi *et al.*[18] was tested against gram-positive and gram-negative bacteria. It was found that most of the derivatives were more effective against *S. aureus* ATCC 6538p and *S. epidermidis* ATCC 12228 than enoxacin, norfloxacin, and ciprofloxacin. In another study,[19] the ciprofloxacin derivative containing an N-chloro-substituted phenylethyl residue was found to be more effective against gram-positive and gram-negative bacteria than the quinolone group reference drugs. Wang *et al.*[20] demonstrated that new ciprofloxacin derivatives have

Table 3. Antimicrobial activity of derivatives*

Derivative code and antibiotics	*S. aureus* ATCC 25923	*E. faecalis* ATCC 29212	*B. subtilis* ATCC 6633	*P. aeruginosa* ATCC 27853	*A. haemolyticus* ATCC 19002	*E. coli* ATCC 25922	*E. aerogenes* ATCC 13048	*K. pneumoniae* ATCC 13883	*P. mirabilis* ATCC 7002	*S. typhimurium* ATCC 10708	*C. albicans* ATCC 10231	*C. parapsilosis* ATCC 22019
B30	20 ± 0.6	0	**26 ± 0.6**	20 ± 1.5	12 ± 1.5	31 ± 1.0	31 ± 1.5	28 ± 1.5	0	**30 ± 0.6**	0	0
B49	23 ± 0.6	0	23 ± 2.0	21 ± 1.0	17 ± 0.6	33 ± 1.0	**29 ± 0.6**	29 ± 1.5	0	20 ± 0.6	0	0
N16	0	0	18 ± 0.6	0	0	0	0	0	0	0	0	0
N19	0	0	16 ± 0.6	0	0	0	0	0	0	0	0	0
Y32	0	0	**22 ± 0.6**	0	0	18 ± 1.0	0	0	0	18 ± 2.0	0	0
Y33	0	0	0	0	0	14 ± 0.6	0	0	0	16 ± 1.0	0	0
B31	30 ± 0.0	20 ± 0.0	**32 ± 0.0**	**27 ± 0.6**	24 ± 0.6	**39 ± 0.6**	**34 ± 0.6**	32 ± 2.0	28 ± 0.0	**36 ± 0.6**	0	0
Ampicillin	36 ± 1.5	29 ± 1.5	20 ± 1.5	-	-	-	-	-	-	-	-	-
Gentamicin	-	-	-	22 ± 1.5	24 ± 1.0	25 ± 1.5	24 ± 1.0	24 ± 1.0	-	22 ± 1.5	-	-
Cefotaxime	-	-	-	-	-	-	-	-	35 ± 0.5	-	-	-
Amphotericin B	-	-	-	-	-	-	-	-	-	-	22 ± 1.0	21 ± 0.5

*Data are are presented as the zones of inhibitors (in mm) ± standard deviations. "-" indicates Not tested. Significant results are shown in bold.

Table 4. MIC values (µg/mL) of derivatives against bacteria

Derivative Code	*S. aureus* ATCC 25923	*E. faecalis* ATCC 29212	*B. subtilis* ATCC 6633	*P. aeruginosa* ATCC 27853	*A. haemolyticus* ATCC 19002	*E. coli* ATCC 25922	*E. aerogenes* ATCC 13048	*K. pneumoniae* ATCC 13883	*P. mirabilis* ATCC 7002	*S. typhimurium* ATCC 10708
B30	0.78	-	0.78	3.12	3.12	0.04	0.19	0.39	-	0.19
B49	0.78	-	0.78	1.56	1.56	0.04	0.19	0.39	-	0.09
N16	-	-	0.78	-	-	-	-	-	-	-
N19	-	-	1.56	-	-	-	-	-	-	-
Y32	-	-	0.39	-	-	1.56	-	-	-	3.12
Y33	-	-	-	-	-	3.12	-	-	-	6.25
B31	0.78	3.12	0.09	0.39	0.39	0.04	0.04	0.09	0.19	0.04

-: No antimicrobial activity was detected in agar well diffusion method.

significantly higher antibacterial activity against all of the tested gram-positive bacteria including MRSA and methicillin-resistant *S. epidermidis* compared with ciprofloxacin. It was shown by Abdelrahman *et al.* [21] that some of the quinolone hydrazide derivatives and standard drugs like ciprofloxacin and ampicillin have similar antimicrobial activity. Also, aminothiazolyl norfloxacin analogs synthesized by Wang *et al*.[22] demonstrated more efficient antimicrobial activity against *K. pneumoniae* and *C. albicans* than chloromycin and ciprofloxacin.

In the current study, B31 showed antibacterial activity against all tested bacterial strains. The antibacterial activity of B31 against *B. subtilis*, *P. aeruginosa*, *E. coli*, *E. aerogenes*, and *S. typhimurium* was found to be statistically significantly better than control antibiotics. The antibacterial activity of B30 against *B. subtilis* and *S. typhimurium*; the antibacterial activity of B49 against *E. aerogenes*; the antibacterial activity of Y32 against *B. subtilis* were statistically significantly more potent than control antibiotics. It has been shown in this study that a range of ciprofloxacin and norfloxacin derivatives, which were more effective against bacteria than control antibiotics, may be considered as potential candidates in developing new antimicrobial molecules.

Since QS signals play a key role in the pathogenesis of microorganisms, the elimination or inhibition of QS signals can be evaluated as an alternative approach to combat pathogens.[23] Therefore, the anti-QS properties of derivatives of cephalosporanic acid, ciprofloxacin, norfloxacin, and penicillanic acid were also investigated. Although several compounds have been identified as QS inhibitors, the most widely known QS inhibitors are halogenated furanones. However, most of them have limited medical use due to their high toxicity.[24] Hence, research has been undertaken to look for new candidates that have QS inhibitory activity and are suitable for human use.[8, 25, 26] In the present study, one of the cephalosporinic acid derivatives, B29, and one penicillanic acid derivative, B28, were found to inhibit the violacein production in *C. violaceum* via QS inhibition, as reported by other groups.[27, 28]

Conclusion

In conclusion, six norfloxacin derivatives and one ciprofloxacin derivative were shown to have antibacterial activity against some of the bacteria tested in this study. All the data obtained in this study are preliminary results. Further development of the promising derivatives is required and should be compared to reference antibiotics of the quinolone group in further studies. Moreover, derivatives inhibiting violacein production in *C. violaceum* should be tested against a greater range of QS reporter strains, and their cytotoxic properties should be further investigated.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Author Contributions

AOK: Design; SH, EFT, AOK: Project development; SH, EFT, AOK, ND, YU: Data collection; SH, EFT, AOK, ND, YU: Analysis; SH, EFT, AOK: Literature search; SH, EFT, ND, YU, AOK: Manuscript writing

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