Current Chemistry Letters 14 (2025) 271–278

Contents lists available at GrowingScience

Current Chemistry Letters

homepage: www.GrowingScience.com

In vitro evaluation of antibacterial and antibiofilm activity of new bis-quaternary ammonium compounds based on natural products

Liubov Muzychka[a*], Diana Hodyna[a], Larysa Metelytsia[a] and Oleg Smolii[a]

[a]*Department of Chemistry of Natural Compounds, V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry, NAS of Ukraine, 1, Academician Kukhar St., Kyiv, 02094, Ukraine*

C H R O N I C L E

Article history:
Received September 3, 2024
Received in revised form
October 10, 2024
Accepted November 25, 2024
Available online
November 25, 2024

Keywords:
Bis-quaternary ammonium compounds
Natural products
Antibiofilm
Antibacterial activity

A B S T R A C T

Continuing the search for new antimicrobial agents with antibiofilm activity, bis-quaternary ammonium compounds based on natural (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives were synthesized. Antibacterial and antibiofilm activity of ammonium salts was evaluated *in vitro* against *S. aureus*, *E. coli*, and *P. aeruginosa*, including antibiotic-resistant strains. Bis-quaternary ammonium salts **1**, **5**, and **6** showed antibacterial activity with MIC values ranging from 25.0 to 200.0 μg/mL. Notably, these compounds demonstrated a high level of antibiofilm activity. Ammonium salts **1** and **6** reduced biofilm formation against *S. aureus* ATCC 25923, *P. aeruginosa* PA01, *E. coli* ATCC 25922, and antibiotic-resistant *P. aeruginosa* by 92 - 97% at a concentration of 100.0 μg/mL. In addition, compounds **1** and **6** inhibited the biofilm formation of antibiotic-resistant *E. coli* isolate by 60% and 63%, respectively.

1. Introduction

The spread of antimicrobial resistance is a global public health problem caused by the overuse and misuse of antibiotics. The formation of a bacterial biofilm is considered the main reason for the ineffectiveness of antibiotic treatment. [1-3] Therefore, there is an urgent need to develop new effective antimicrobial agents with antibiofilm activity against gram-positive and gram-negative bacteria, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Quaternary ammonium salts exhibit a broad spectrum of antimicrobial activity against pathogenic microorganisms and have been used as effective disinfectants, biocides, and fungicides for many decades. [4-6] Their activity is based on the interaction of the positively charged ammonium group of the quaternized compounds with the negatively charged membrane of the bacterial cell, which leads to the destruction of the membrane and, ultimately, cell death. [7, 8] In addition, ammonium salts covering biotic and abiotic surfaces reduce the adhesion of microorganisms, preventing biofilm formation by pathogenic microorganisms. [9, 10] It is believed that the presence of two ammonium groups in the molecule leads to increased antimicrobial properties of bis-quaternary ammonium compounds. [7, 11-13] One of the promising strategies for the development of new quaternary ammonium salts is the quaternization of natural products. [14-16] Among marine natural products, bromotyrosine alkaloids have emerged as promising compounds with diverse biological properties, including antimicrobial and antibiofilm activities. [17-21] Our previous studies of bromotyrosine derivatives, in particular pulmonarin B analogues, demonstrated the antimicrobial efficacy of quaternary salts against both gram-positive and gram-negative bacteria. [22] Motivated by these results, we developed new bis-quaternary ammonium compounds inspired by natural bromotyrosine alkaloids. We synthesized a series of bis-quaternary ammonium salts based on natural (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives and evaluated their *in vitro* antibacterial and antibiofilm activity against *E. coli*, *S. aureus*, and *P. aeruginosa*, including antibiotic-resistant strains.

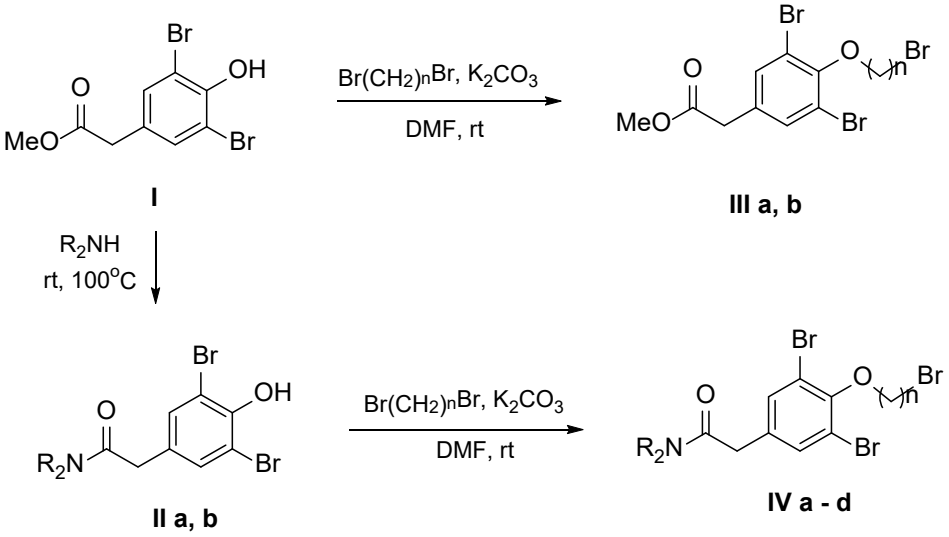
* Corresponding author
E-mail address liubovmuzychka@gmail.com (L. Muzychka)

doi: 10.5267/j.ccl.2024.11.008

2. Results and Discussion

2.1. Chemistry

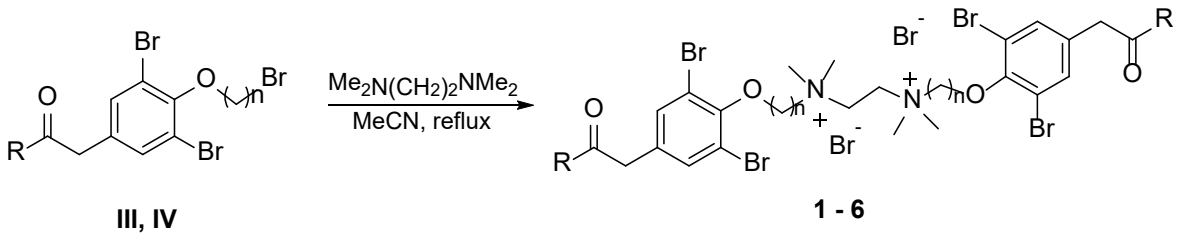
The target quaternary ammonium salts **1-6** were obtained by quaternization of commercially available *N,N,N′,N′*-tetramethylethylenediamine with bromoalkoxy-substituted (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives **III** and **IV**. The synthesis of key intermediates **III** and **IV** was carried out according to **Scheme 1**. The synthetically available methyl (3,5-dibromo-4-hydroxyphenyl)acetate **I**, isolated from the Marine Red Alga Symphyocladia latiuscula, was chosen as the starting material. [23] The details of the synthesis and characteristics of alkoxy-substituted derivatives **IIIa** and **IIIb** were described previously. [22] Four bromoalkoxy-substituted (3,5-dibromo-4-hydroxyphenyl)acetamides **IVa-d** were obtained in two steps. First, the aminolysis reaction of ester **1** using an excess of pyrrolidine or piperidine yielded acetamides **IIa** and **IIb**, respectively. Notably, a close analogue of acetamides **IIa** and **IIb** is a natural 2-(3,5-dibromo-4-hydroxyphenyl)acetamide that was isolated from marine sponges of the Verongiida order. [24, 25] In the second step, alkylation of **IIa** and **IIb** with 1,3-dibromopropane or 1,4-dibromobutane in the presence of K_2CO_3 in *N,N*-dimethylformamide gave alkoxy-substituted (3,5-dibromo-4-hydroxyphenyl)acetamides **IVa-d**.



R_2N = $(CH_2)_5N$ (**IIa**, **IVa**, **b**), $(CH_2)_6N$ (**IIb**, **IVc**, **d**); n = 3, 4.

Scheme 1. Synthesis of alkoxy-substituted (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives **III**, **IV**.

The final bis-quaternary ammonium compounds **1-6** were obtained according to a simple synthesis procedure by refluxing bromoalkoxy-substituted (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives **III** and **IV** with *N,N,N′,N′* -tetramethylethylenediamine in acetonitrile for 2-15 h (**Scheme 2**). Structures of the target bis-quaternary ammonium compounds **1-6** are presented in the **Fig. 1**.



R = MeO, $(CH_2)_5N$, $(CH_2)_6N$; n = 3, 4.

Scheme 2. Synthesis of bis-quaternary ammonium compounds **1-6**.

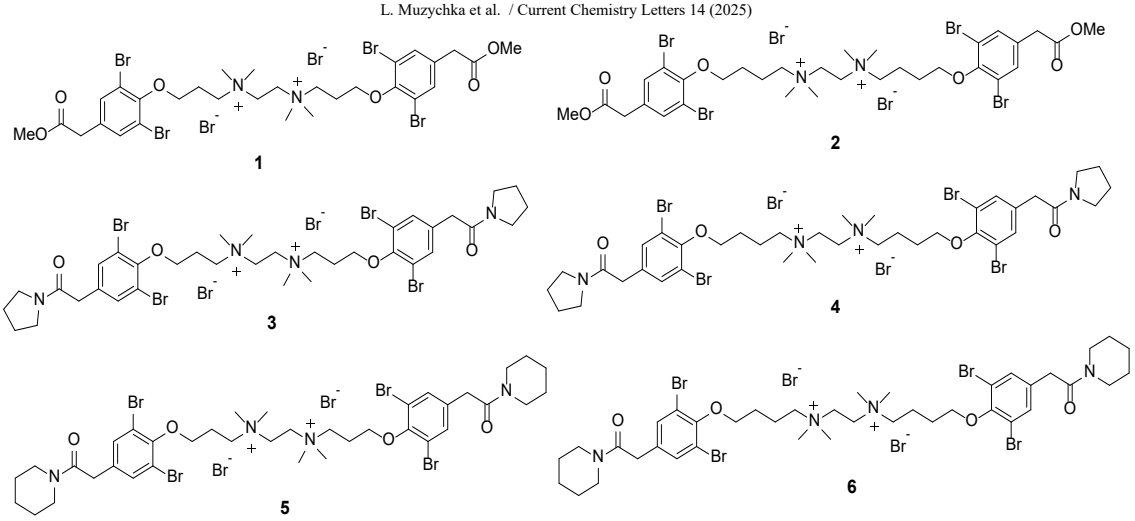


Fig. 1. Structures of the target bis-quaternary ammonium compounds **1-6**.

The structures of the synthesized compounds were confirmed by the elemental analysis, ^{1}H and ^{13}C NMR, and mass spectrometry.

2.2. Biology

2.2.1 Antibacterial activity

Table 1 presents the results of the preliminary evaluation of antibacterial activity of the synthesized bis-quaternary ammonium compounds **1-6** against a number of standard strains (typical ATCC bacterial cultures of *S. aureus* and *E. coli* and type PA01 *P. aeruginosa* culture), as well as their antibiotic-resistant (ABR) isolates (resistant to oxacillin, carbenicillin, ceftazidime, and colistin). The disk diffusion method, used for the evaluation, allowed to determine the most general trend of activity levels distribution among the compounds (**Table 1**). The data in **Table 1** indicate that compounds **1**, **5**, and **6** exhibit the highest antibacterial potential in the range from 12 mm to 21 mm, according to the measured diameters of bacterial growth inhibition zones. Bis-quaternary ammonium compounds **2**, **3**, and **4** demonstrate activity at an average level of 11 mm. The level of antibacterial effect of the most active compounds **1**, **5**, and **6** against standard strains was slightly higher than the action against antibiotic-resistant isolates (**Table 1**).

Table. 1. Antibacterial activity of ammonium compounds **1-6** by the disk diffusion method, mm.

Compounds	*S. aureus* ATCC 25923	*S. aureus* ABR	*P. aeruginosa* PA01	*P. aeruginosa* ABR	*E. coli* ATCC 25922	*E. coli* ABR
1	17	16	21	13	20	17
2	16	14	12	8	17	13
3	7	7	10	9	10	7
4	12	11	15	12	14	11
5	16	13	19	21	20	15
6	17	16	18	13	19	12

Antibacterial activity of bis-quaternary ammonium compounds **1-6** was also measured using the broth microdilution method, which allows the quantitative measurement of the minimum inhibitory concentration (MIC) of a potentially active compound. The results are presented in **Table 2**.

Table. 2. Antibacterial activity of bis-quaternary ammonium compounds **1-6** (MIC, μg/mL).

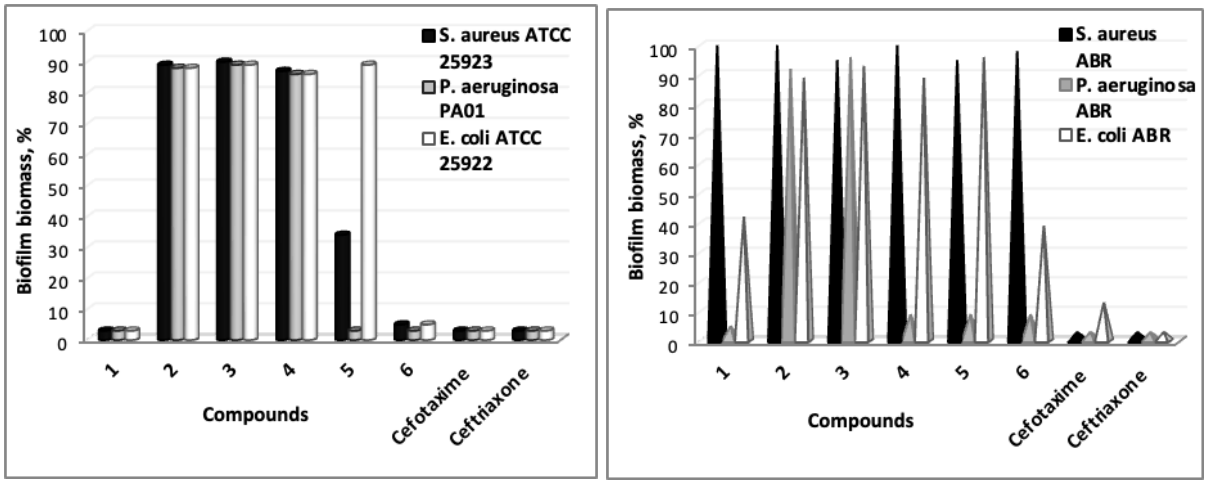
Compounds	*S. aureus* ATCC 25923	*S. aureus* ABR	*P. aeruginosa* PA01	*P. aeruginosa* ABR	*E. coli* ATCC 25922	*E. coli* ABR
1	25.0	>200.0	100.0	25.0	100.0	200.0
2	>200.0	>200.0	>200.0	200.0	>200.0	>200.0
3	>200.0	>200.0	>200.0	50.0	>200.0	>200.0
4	>200.0	>200.0	>200.0	200.0	>200.0	>200.0
5	50.0	>200.0	50.0	25.0	200.0	200.0
6	50.0	200.0	50.0	50.0	50.0	100.0
Cefotaxime[1]	≤0.8	12.5	6.3	3.1	25.0	50.0
Ceftriaxone[1]	1.6	25.0	6.3	3.1	12.5	25.0

[1]cefotaxime and ceftriaxone antibiotics were used as third-generation cephalosporins

The results presented in **Table 2** show that the range of the measured MIC of the compounds was on average from 25.0 μg/mL to 200.0 μg/mL and >200.0 μg/mL depending on the type of bacterial strain. Compounds **1**, **5**, and **6** demonstrated the highest antibacterial potential with MIC values ranging from 25.0 to 200.0 μg/mL. The MICs of these compounds were >200.0 μg/mL only in the case of *S. aureus* ABR.

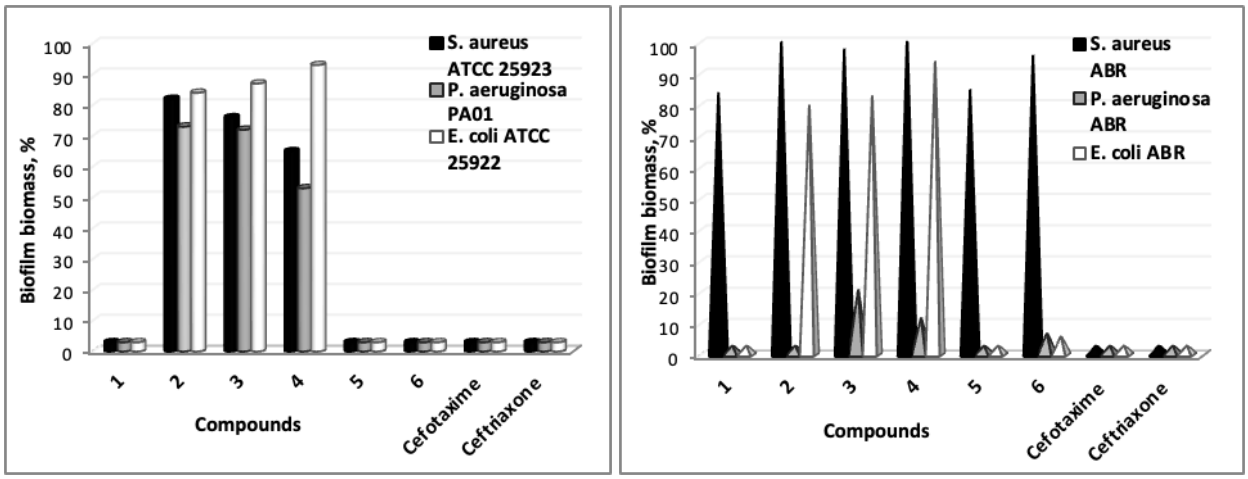
2.2.2 Antibiofilm activity

Fig. 2 and **Fig. 3** show the results of the antibiofilm activity assays detecting the level of sensitivity of standard strains and antibiotic-resistant isolates to the compounds **1**-**6**, taking into account 100.0 μg/mL and 200.0 μg/mL as MIC values.



A) **B)**

Fig. 2. Effect of ammonium compounds (100.0 μg/mL) on biofilm formation of *S. aureus* ATCC 25923, *P. aeruginosa* PA01 and *E. coli* ATCC 25922 (A) and *S. aureus*, *P. aeruginosa* and *E. coli* ABR isolates (B).



A) **B)**

Fig. 3. Effect of ammonium compounds (200.0 μg/mL) on biofilm formation of *S. aureus* ATCC 25923, *P. aeruginosa* PA01 and *E. coli* ATCC 25922 (A) and *S. aureus*, *P. aeruginosa* and *E. coli* ABR isolates (B).

The experiments showed that bis-ammonium salts **1** and **6** demonstrated the highest level of antibiofilm activity against *S. aureus* ATCC 25923, *P. aeruginosa* PA01, *E. coli* ATCC 25922, and *P. aeruginosa* ABR isolate. Biofilm biomass decreased by 92 - 97% at the compound concentration of 100.0 μg/mL (**Fig. 2**). Compound **5** at a concentration of 100.0 μg/mL showed significant inhibitory activity against *S. aureus* ATCC 25923, *P. aeruginosa* PA01, and *P. aeruginosa* ABR: biofilm biomass decreased by 66-97% (**Fig. 2**). In addition, compounds **1**, **5** and **6** at a concentration of 200.0 μg/mL inhibited the biofilm formation of *S. aureus* ATCC 25923, *P. aeruginosa* PA01, *E. coli* ATCC 25922, *P. aeruginosa* ABR and *E. coli* ABR by 95 - 97% (**Fig. 3**). Biofilm formation of the most resistant strain of *S. aureus* ABR was practically not disturbed (up to 16% inhibition) in the presence of the compounds at the highest tested concentration of 200.0 μg/mL.

Increasing the concentration of the most active compounds **1**, **5**, and **6** from 100.0 to 200.0 μg/mL enhanced their antibiofilm activity, and the percentage of biofilm biomass formed by the standard bacterial strains decreased even in the case of the less active compounds **2**, **3**, and **4** from 30% to 10% (**Fig. 2A** and **Fig. 3A**). The most sensitive to elevated concentrations were *P. aeruginosa* PA01 and *S. aureus* ATCC 25923. Biofilm formation by antibiotic-resistant isolates (**Fig. 2B** and **Fig. 3B**) was also dependent on the effective concentration of the compounds. The inhibitory effect (%) on

the biofilm biomass increased by an average of 20-25 % with an increase in their concentration from 100.0 to 200.0 μg/mL. The most resistant biofilm forming strain was *S. aureus* ABR, and the most sensitive was *E. coli* ABR.

3. Conclusions

In this study, six bis-quaternary ammonium salts based on natural (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives were synthesized and their antibacterial and antibiofilm activities against *S. aureus*, *E. coli*, and *P. aeruginosa,* including antibiotic-resistant strains, were evaluated. Bis-quaternary ammonium salts **1**, **5**, and **6** showed the best antibacterial activity with MIC values ranging from 25.0 to 200.0 μg/mL. In addition, these compounds demonstrated a high level of antibiofilm activity. Ammonium salts **1** and **6** reduced biofilm formation of *S. aureus* ATCC 25923, *P. aeruginosa* PA01, *E. coli* ATCC 25922, and antibiotic-resistant *P. aeruginosa* by 92-97% at a concentration of 100.0 μg/mL. In addition, compounds **1** and **6** inhibited the biofilm formation of an antibiotic-resistant *E. coli* isolate by 60% and 63%, respectively. Compound **5** showed significant inhibitory activity against *S. aureus* ATCC 25923, *P. aeruginosa* PA01, and antibiotic-resistant *P. aeruginosa*. Biofilm biomass decreased by 66-97% at a 100.0 μg/mL concentration of **5**. The study also found that increasing the concentration of the most active compounds from 100.0 to 200.0 μg/mL increases their antibiofilm activity.

The obtained results hold considerable potential to advance the research and development of effective antimicrobial and antibiofilm agents based on synthetic analogues of natural compounds.

Acknowledgements

This work was supported by the National Research Foundation of Ukraine (Grant number 2021.01/0022). The authors thank Inna Sokolenko for her help with the manuscript and all brave defenders of Ukraine, who allowed us to continue our scientific work and made this publication possible.

4. Experimental

4.1. Materials and Methods

4.1.1. Chemistry

All reagents and solvents were commercially available and were used without further purification. ^{1}H and ^{13}C NMR spectra were acquired on Varian Unity INOVA 400 (400 and 100 MHz for ^{1}H and ^{13}C nuclei, respectively) and Bruker Avance DRX-500 (500 and 125 MHz for ^{1}H and ^{13}C nuclei, respectively) instruments (TMS as internal reference) in DMSO-d_6 or $CDCl_3$. LCMS spectra were performed on Agilent 1100 Series HPLC equipped with diode array and Agilent LC/MSD SL mass selective detector, ionization method – chemical ionization at atmospheric pressure. Zorbax SB-C18 column was used, and gradient elution with 0.1% HCOOH in H_2O–MeCN was applied. Elemental analysis was performed at the Analytical laboratory of the V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry of the NAS of Ukraine. Melting points were determined on the Boetius hot stage apparatus. The progress of the reaction was monitored by the TLC.

*General procedure for the synthesis of acetamides **IIa** and **IIb**.* A mixture of methyl (3,5-dibromo-4-hydroxyphenyl)acetate **I** (10.0 mmol) and pyrrolidine/piperidine (30.0 mmol) was left for 12 h at room temperature. The reaction mixture was heated at 100 °C for 1-3 h, cooled to room temperature and 2-propanol was added. The precipitate was filtered, dissolved in 3M HCl and left for 20 min. The resulting precipitate was filtered and recrystallized from methanol.

*2,6-Dibromo-4-(2-oxo-2-pyrrolidin-1-ylethyl)phenol (**IIa**).* Yield 72%, white solid, m.p. 170-172 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.72-1.79 (m, 2H, CH_2), 1.84-1.90 (m, 2H, CH_2), 3.25-3.32 (m, 2H, CH_2), 3.43-3.46 (m, 2H, CH_2), 3.54 (s, 2H, CH_2), 7.39 (s, 2H, 2CH), 9.73 (br s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 24.5, 26.1, 46.0, 46.6, 112.0, 130.9, 133.7, 149.6, 168.4. LCMS $[M+H]^+$: 364.0. Anal. Calcd. for $C_{12}H_{13}Br_2NO_2$: C, 39.70; H, 3.61; Br, 44.02. Found: C, 39.73; H, 3.60; Br, 44.08.

*2,6-Dibromo-4-(2-oxo-2-piperidin-1-ylethyl)phenol (**IIb**).* Yield 65%, white solid, m.p. 135-137 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.37-1.42 (m, 4H, $2CH_2$), 1.52-1.56 (m, 2H, CH_2), 3.89-3.44 (m, 4H, $2CH_2$), 3.59 (s, 2H, CH_2), 7.36 (s, 2H, 2CH). ^{13}C NMR (100 MHz, DMSO-d_6): δ 25.1, 26.4, 27.1, 38.5, 43.3, 47.4, 112.9, 130.8, 133.9, 150.9, 169.2. LCMS $[M+H]^+$: 378.0. Anal. Calcd. for $C_{13}H_{15}Br_2NO_2$: C, 41.41; H, 4.01; Br, 42.38. Found: C, 41.36; H, 4.04; Br, 42.43.

*General procedure for the synthesis of bromoalkoxy-substituted derivatives **IVa-d**.* A mixture of acetamide **IIa** or **IIb** (5 mmol), 1,3-dibromopropane or 1,4-dibromobutane (15 mmol), and K_2CO_3 (15 mmol) in *N,N*-dimethylformamide (15 mL) was stirred at room temperature for 24 h. Cold water was added and CH_2Cl_2 was extracted. The organic layer was dried over Na_2SO_4, concentrated in vacuo and the residue was purified by crystallization from hexane/2-propanol.

*1-{[3,5-Dibromo-4-(3-bromopropoxy)phenyl]acetyl}pyrrolidine (**IVa**).* Yield 73%, white solid, m.p. 51-53 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.77-1.86 (m, 2H, CH_2), 1.87-1.92 (m, 2H, CH_2), 2.29-2.35 (m, 2H, CH_2), 3.26-3.29 (m, 2H, CH_2), 3.45-3.49 (m, 2H, CH_2), 3.62 (s, 2H, CH_2), 3.75 (t, J = 6.0 Hz, 2H, CH_2), 4.06 (t, J = 6.0 Hz, 2H, CH_2), 7.52 (s, 2H, 2CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.9, 25.6, 30.9, 32.9, 38.8, 45.5, 46.2, 71.0, 116.8, 133.9, 135.5, 150.6, 167.4. LCMS $[M+H]^+$: 485.2. Anal. Calcd. for $C_{15}H_{18}Br_3NO_2$: C, 37.22; H, 3.75; Br, 49.52. Found: C, 37.25; H, 3.79; Br, 49.45.

*1-{[3,5-Dibromo-4-(4-bromobutoxy)phenyl]acetyl}pyrrolidine (**IVb**).* Yield 86%, white solid, m.p. 74-76 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.85-1.91 (m, 2H, CH_2), 1.95-2.04 (m, 4H, $2CH_2$), 2.16-2.23 (m, 2H, CH_2), 3.44-3.51 (m, 4H, $2CH_2$), 3.54-3.57 (m, 4H, $2CH_2$), 4.02 (t, J = 6.0 Hz, 2H, CH_2), 7.44 (s, 2H, 2CH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 23.8, 25.7, 28.1, 28.9, 33.1, 39.9, 45.5, 46.4, 71.6, 117.6, 132.9, 133.1, 151.5, 167.6. LCMS $[M+H]^+$: 499.0. Anal. Calcd. for $C_{16}H_{20}Br_3NO_2$: C, 38.59; H, 4.05; Br, 48.13. Found: C, 38.53; H, 4.11; Br, 48.07.

*1-{[3,5-Dibromo-4-(3-bromopropoxy)phenyl]acetyl}piperidine (**IVc**).* Yield 65%, white solid, m.p. 57-59 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.41-1.44 (m, 4H, $2CH_2$), 1.52-1.57 (m, 2H, CH_2), 2.29-2.35 (m, 2H, CH_2), 3.32-3.45 (m, 4H, $2CH_2$), 3.69 (s, 2H, CH_2), 3.74 (t, J = 6.0 Hz, 2H, CH_2), 4.05 (t, J = 6.0 Hz, 2H, CH_2), 7.51 (s, 2H, 2CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.8, 25.3, 26.0, 30.9, 32.9, 37.5, 42.2, 46.2, 71.0, 116.9, 133.7, 135.9, 150.6, 167.6. LCMS $[M+H]^+$: 499.2. Anal. Calcd. for $C_{16}H_{20}Br_3NO_2$: C, 38.59; H, 4.05; Br, 48.13. Found: C, 38.64; H, 4.00; Br, 48.16.

*1-{[3,5-Dibromo-4-(4-bromobutoxy)phenyl]acetyl}piperidine (**IVd**).* Yield 78%, yellowish oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.47-1.58 (m, 4H, $2CH_2$), 1.61-1.65 (m, 2H, CH_2), 1.97-2.04 (m, 2H, CH_2), 2.16-2.23 (m, 2H, CH_2), 3.37-3.44 (m, 2H, CH_2), 3.54-3.57 (m, 4H, $2CH_2$), 3.61 (s, 2H, CH_2), 4.02 (t, J = 6.0 Hz, 2H, CH_2), 7.40 (s, 2H, 2CH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 23.9, 25.0, 25.9, 28.1, 28.9, 33.1, 38.6, 42.6, 46.7, 71.6, 117.7, 132.6, 133.5, 151.4, 167.4. LCMS $[M+H]^+$: 513.0. Anal. Calcd. for $C_{17}H_{22}Br_3NO_2$: C, 39.87; H, 4.33; Br, 46.81. Found: C, 39.79; H, 4.39; Br, 46.75.

*General procedure for the synthesis of bis-quaternary ammonium compounds **1-6**.* A mixture of *N,N,N′,N′*-tetramethylethylenediamine (0.3 mmol) and bromoalkoxy-substituted (3,5-dibromo-4-hydroxyphenyl)acetic acid derivatives **III** [22] or **IV** (0.63 mmol) in MeCN (10 mL) was refluxed for 2-15 h. The solvent was removed in vacuo and 2-propanol (10 mL) was added to the resulting residue. The resulting precipitate was filtered and dried.

N,N′-Bis{3-[2,6-dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]propyl}-N,N,N′,N′-tetramethylethane-1,2-diaminium dibromide (**1**). Yield 70%, white solid, m.p. 184-186 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 2.33-2.36 (m, 4H, $2CH_2$), 3.25 (s, 12H, $4CH_3$), 3.64 (s, 6H, $2OCH_3$), 3.72-3.78 (m, 8H, $4CH_2$), 4.04-4.08 (m, 8H, $4CH_2$), 7.60 (s, 4H, 4CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.2, 38.0, 50.5, 51.9, 55.1, 56.2, 62.6, 69.9, 117.1, 134.0, 134.3, 150.8, 171.0. LCMS $[M-2Br]^{2+}$: 423.0. Anal. Calcd. for $C_{30}H_{42}Br_6N_2O_6$: C, 35.81; H, 4.21; Br, 47.65. Found: C, 35.78; H, 4.17; Br, 47.70.

*N,N′-Bis{4-[2,6-dibromo-4-(2-methoxy-2-oxoethyl)phenoxy]butyl}-N,N,N′,N′-tetramethylethane-1,2-diaminium dibromide (**2**).* Yield 58%, white solid, m.p. 178-180 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.83-1.88 (m, 4H, $2CH_2$), 1.96-2.02 (m, 4H, $2CH_2$), 3.18 (s, 12H, $4CH_3$), 3.45-3.49 (m, 4H, $2CH_2$), 3.62 (s, 6H, $2OCH_3$), 3.72 (s, 4H, $2CH_2$), 3.91 (s, 4H, $2CH_2$), 4.00-4.03 (m, 4H, $2CH_2$), 7.60 (s, 4H, 4CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 19.4, 26.8, 38.5, 51.2, 52.4, 55.6, 64.5, 72.7, 117.7, 134.4, 151.7, 171.5. LCMS $[M-2Br]^{2+}$: 437.0. Anal. Calcd. for $C_{32}H_{46}Br_6N_2O_6$: C, 37.17; H, 4.48; Br, 46.36. Found: C, 37.22; H, 4.43; Br, 47.39.

*N,N′-Bis{3-[2,6-dibromo-4-(2-oxo-2-pyrrolidin-1-ylethyl)phenoxy]propyl}-N,N,N′,N′-tetramethylethane-1,2-diaminium dibromide (**3**).* Yield 64%, white solid, m.p. 208-210 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.76-1.77 (m, 4H, $2CH_2$), 1.87-1.89 (m, 4H, $2CH_2$), 2.34-2.36 (m, 4H, $2CH_2$), 3.23-3.28 (m, 16H, $4CH_3$, $2CH_2$), 3.47-3.49 (m, 4H, $2CH_2$), 3.61-3.64 (m, 4H, $2CH_2$), 3.75-3.77 (m, 4H, $2CH_2$), 4.05-4.09 (m, 8H, $4CH_2$), 7.54 (s, 4H, 4CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.6, 24.4, 26.0, 39.2, 46.0, 46.6, 51.0, 55.6, 63.0, 70.4, 117.3, 134.5, 136.4, 150.8, 168.1. LCMS $[M-2Br]^{2+}$: 462.2. Anal. Calcd. for $C_{36}H_{52}Br_6N_4O_4$: C, 39.88; H, 4.83; Br, 44.22. Found: C, 40.00; H, 4.79; Br, 44.28.

*N,N′-Bis{4-[2,6-dibromo-4-(2-oxo-2-pyrrolidin-1-ylethyl)phenoxy]butyl}-N,N,N′,N′-tetramethylethane-1,2-diaminium dibromide (**4**).* Yield 51%, white solid, m.p. 54-56 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.75-1.90 (m, 12H, $6CH_2$), 1.98-2.03 (m, 4H, $2CH_2$), 3.21-3.27 (m, 16H, $4CH_3$, $2CH_2$), 3.46-3.52 (m, 8H, $4CH_2$), 3.61-3.64 (m, 4H, $2CH_2$), 3.97-4.05 (m, 8H, $4CH_2$), 7.52 (s, 4H, 4CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 19.4, 24.4, 26.1, 26.8, 39.2, 46.0, 46.6, 51.1, 55.6, 64.4, 72.8, 117.5, 134.4, 135.9, 151.2, 168.1. LCMS $[M-2Br]^{2+}$: 476.0. Anal. Calcd. for $C_{38}H_{56}Br_6N_4O_4$: C, 41.03; H, 5.07; Br, 43.10. Found: C, 41.18; H, 4.93; Br, 43.16.

*N,N′-Bis{3-[2,6-dibromo-4-(2-oxo-2-piperidin-1-ylethyl)phenoxy]propyl}-N,N,N′,N′-tetramethylethane-1,2-diaminium dibromide (**5**).* Yield 56%, white solid, m.p. 194-196 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.40-1.45 (m, 8H, $4CH_2$), 1.54-1.57 (m, 4H, $2CH_2$), 2.33-2.37 (m, 4H, $2CH_2$), 3.26 (s, 12H, $4CH_3$), 3.41-3.43 (m, 8H, $4CH_2$), 3.70 (s, 4H, $2CH_2$), 3.72-3.77 (m, 4H, $2CH_2$), 4.05-4.07 (m, 8H, $4CH_2$), 7.52 (s, 4H, 4CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 23.6, 24.4, 25.7, 26.4, 37.9, 42.7, 46.6, 51.0, 55.6, 63.0, 70.4, 117.2, 134.3, 136.6, 150.7, 168.1. LCMS $[M-2Br]^{2+}$: 476.0. Anal. Calcd. for $C_{38}H_{56}Br_6N_4O_4$: C, 41.03; H, 5.07; Br, 43.10. Found: C, 40.97; H, 4.99; Br, 43.18.

*N,N'-Bis{4-[2,6-dibromo-4-(2-oxo-2-piperidin-1-ylethyl)phenoxy]butyl}-N,N,N',N'-tetramethylethane-1,2-diaminium dibromide (**6**).* Yield 48%, white solid, m.p. 62-65 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.39-1.42 (m, 8H, 4CH_2), 1.43-1.44 (m, 4H, 2CH_2), 1.55-1.56 (m, 4H, 2CH_2), 1.82-2.00 (m, 4H, 2CH_2), 3.24 (s, 12H, 4CH_3), 3.38-3.42 (m, 8H, 4CH_2), 3.51-3.54 (m, 4H, 2CH_2), 3.69 (s, 4H, 2CH_2), 3.98-4.00 (m, 4H, 2CH_2), 4.01-4.06 (m, 4H, 2CH_2), 7.50 (s, 4H, 4CH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 18.9, 23.9, 25.3, 26.0, 26.4, 37.4, 42.2, 46.2, 50.6, 55.2, 64.0, 72.4, 117.0, 133.6, 135.8, 150.7, 167.7. LCMS [M-2Br]$^{2+}$: 490.0. Anal. Calcd. for $C_{40}H_{60}Br_6N_4O_4$: C, 42.13; H, 5.30; Br, 42.04. Found: C, 42.21; H, 5.34; Br, 41.98.

4.1.2. Antibacterial activity by zone of inhibition

Initially the antibacterial activity of studied compounds was evaluated by disk diffusion method [26] against standard/collection *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* PA01, and oxacillin, carbenicillin, colistin, and ceftazidime resistant isolated strains of *S. aureus* (ABR), *E. coli* (ABR), *P. aeruginosa* (ABR). All bacterial cultures were received from culture collections of the Shupyk National Healthcare University of Ukraine, including *S. aureus* (ABR), *E. coli* (ABR), and *P. aeruginosa* (ABR), which are clinical isolates. The bacterial suspension was prepared from 24 h old culture strain and controlled by the optical turbidity standard (McFarland). The final microbial load of bacterial cultures was 1×10^5 colony-forming units in 1 mL of physiological solution (CFU/mL). The investigated compounds were dissolved in DMSO and tested in the content on the disk of 1.0 μM. The standard antibiotics such as oxacillin (0.003 μM on disk), carbenicillin (0.26 μM on disk), colistin (0.01 μM on disk), and ceftazidime (0.06 μM on disk) were selected to determine the resistance of the tested bacterial strains. The plates were incubated for 24 h at 37°C. The activity of the compounds was recorded by the diameters of zones of bacterial growth in mm.

4.1.3. Minimum inhibitory concentration (MIC) determination

Broth microdilution test [27] was used for MIC determination of studied compounds against Gram-positive and Gram-negative bacteria. Serial two-dilutions in Mueller Hinton broth (MHB) were carried out in sterile 96-well microplates over the concentration range 200.0-0.8 μg/mL. The bacterial cultures were grown over 18-24 h at 37 °C in MHB, and the inoculum equivalent to 1×10^8 CFU/mL (to McFarland standard) was further diluted to give the final inoculum density of 5×10^5 CFU/mL. The plates with controls and test concentrations were incubated at 37 °C for 24 h. The lowest concentration of compound at which bacterial growth is completely inhibited (measured by Microplate Reader MR-96A at a wavelength of 630 nm) was considered as the MIC (μg/mL). The compounds were dissolved in DMSO. Cefotaxime and ceftriaxone were used as reference drugs.

4.1.4. Inhibition of biofilm formation

Antibiofilm activity of synthesized compounds was performed on polystyrene sterile microplates as described by O'Toole. [28] The inoculum density was 1.5×10^8 CFU/mL (to 0.5 McFarland standard) culture media. A serial of two-fold dilutions (200.0, 100.0, 50.0, 25.0 μg/mL) of the tested compounds with the concentration of bacterial cultures grown in MHB was incubated at 37°C for 24 h. Positive (MHB) and negative (MHB with inoculum) controls (3 replicates) were included in each plate. The optical density (OD) in each well was measured by Microplate Reader MR-96A at a wavelength of 630 nm and compared with negative control (100% of biofilm biomass).

Statistical analyses of the obtained results were made using MS Excel for Windows and Origin Pro8. A p-value of <0.05 was considered as significant. The experiments were performed in triplicate.

References

1 Abdelhamid A.G. and Yousef A.E. (**2023**) Combating Bacterial Biofilms: Current and Emerging Antibiofilm Strategies for Treating Persistent Infections. *Antibiotics,* 12(6) 1005.

2 Gattu R., Ramesh S.S., and Ramesh S. (**2024**) Role of small molecules and nanoparticles in effective inhibition of microbial biofilms: A ray of hope in combating microbial resistance. *Microb. Pathog.,* 188 106543.

3 Galvez-Llompart M., Hierrezuelo J., Blasco M., Zanni R,, Galvez J., de Vicente A., Pérez-García A., and Romero D. (**2024**) Targeting bacterial growth in biofilm conditions: rational design of novel inhibitors to mitigate clinical and food contamination using QSAR. *J. Enzyme Inhib. Med. Chem.,* 39(1) 2330907.

4 Zhou Z., Zhou S., Zhang X., Zeng S., Xu Y., Nie W., Zhou Y., Xu T., and Chen P. (**2023**) Quaternary Ammonium Salts: Insights into Synthesis and New Directions in Antibacterial Applications. *Bioconjugate Chem.,* 34(2) 302-325.

5 Fedorowicz J., and Sączewski J. (**2024**) Advances in the Synthesis of Biologically Active Quaternary Ammonium Compounds. Int. J. Mol. Sci., 25 4649.

6 Kwaśniewska D., Chen Y.-L., and Wieczorek D. (**2020**) Biological Activity of Quaternary Ammonium Salts and Their Derivatives. *Pathogens,* 9(6) 459-470.

7 Mazurkiewicz E., Lamch Ł., Wilk K.A., and Obłąk E. (**2024**) Anti-adhesive, anti-biofilm and fungicidal action of newly synthesized gemini quaternary ammonium salts. *Sci. Rep.,* 14 14110.

8 Nadagouda M.N., Vijayasarathy P., Sin A., Nam H, Khan S., Parambath J.B.M., Mohamed A.A., and Han C. (**2022**) Antimicrobial activity of quaternary ammonium salts: structure-activity relationship. *Med. Chem. Res.,* 31 1663-1678.

9 Kula N., Lamch Ł., Futoma-Kołoch B., Wilk K.A., and Obłąk E. (**2022**) The effectiveness of newly synthesized quaternary ammonium salts differing in chain length and type of counterion against priority human pathogens. *Sci. Rep.,* 12(1) 21799.
10 Obłąk E., Futoma-Kołoch B., and Wieczyńska A. (**2021**) Biological activity of quaternary ammonium salts and resistance of microorganisms to these compounds. *World J. Microbiol. Biotechnol.,* 37(2) 22-32.
11 Schallenhammer S.A., Duggan S.M., Morrison K.R., Bentley B.S., Wuest W.M., and Minbiole K.P.C. (**2017**) Hybrid BisQACs: Potent Biscationic Quaternary Ammonium Compounds Merging the Structures of Two Commercial Antiseptics. *ChemMedChem*, 12(23) 1931-1934.
12 Obłąk E., Piecuch A., Rewak-Soroczyńska J., and Paluch E. (**2019**) Activity of gemini quaternary ammonium salts against microorganisms. *Appl. Microbiol. Biotechnol.,* 103(2) 625-632.
13 Black J.W., Jennings M.C., Azarewicz J., Paniak T.J., Grenier M.C., Wuest W.M., and Minbiole K.P.C. (**2014**). TMEDA-derived biscationic amphiphiles: An economical preparation of potent antibacterial agents. *Bioorg. Med. Chem. Lett.,* 24(1) 99-102.
14 Allen R.A., McCormack C.E.M., and Wuest W.M. (**2023**) Deriving Novel Quaternary Ammonium Compound Disinfectant Scaffolds from a Natural Product: Mechanistic Insights of the Quaternization of Ianthelliformisamine C. *ChemMedChem,* 18(22) e202300253.
15 Bazina L., Maravić A., Krce L., Soldo B., Odžak R., Popović V.B., Aviani I., Primožič I., and Šprung M. (**2019**) Discovery of novel quaternary ammonium compounds based on quinuclidine-3-ol as new potential antimicrobial candidates. *Eur. J. Med. Chem.,* 163 626-635.
16 Deng Y., Liu Y., Li J., Wang X., He S., Yan X., Shi Y., Zhang W., and Ding L. (**2022**) Marine natural products and their synthetic analogs as promising antibiofilm agents for antibiotics discovery and development. *Eur. J. Med. Chem.,* 239 114513.
17 Arrigoni R., Ballini A., Jirillo E., and Santacroce L. (**2024**) Current View on Major Natural Compounds Endowed with Antibacterial and Antiviral Effects. *Antibiotics,* 13(7) 603.
18 Wang K.L., Dou Z.R., Gong G.F., Li H.F., Jiang B., and Xu Y. (**2022**) Anti-Larval and Anti-Algal Natural Products from Marine Microorganisms as Sources of Anti-Biofilm Agents. *Mar. Drugs,* 20(2) 90-115.
19 Krishna M.S.A, Mohan S., Ashitha K.T., Chandramouli M., Kumaran A., Ningaiah S., Babu K.S., and Somappa S.B. (**2022**) Marine Based Natural Products: Exploring the Recent Developments in the Identification of Antimicrobial Agents. *Chem. Biodivers.,* 19(10) e202200513.
20 Lahiri D., Nag M., Dey A., Sarkar T., Pati S., Nirmal N.P., Ray R.R., Upadhye V.J., Pandit S., Moovendhan M., and Kavisri M. (**2023**) Marine bioactive compounds as antibiofilm agent: a metabolomic approach. *Arch. Microbiol.,* 205(1) 54.
21 Muzychka L., Voronkina A., Kovalchuk V., Smolii O.B., Wysokowski M., Petrenko I., Youssef D.T.A., Ehrlich I., and Ehrlich H. (**2021**) Marine biomimetics: bromotyrosines loaded chitinous skeleton as source of antibacterial agents. *Applied Physics A,* 127 15-26.
22 Muzychka L., Boiko I., Vrynchanu N., and Smolii O. (**2024**) Synthesis, antibacterial, and antibiofilm activities of pulmonarin B analogues. *Curr. Chem. Lett.,* 13 335-342.
23 Guopeng Z. and Chun-Li J. (**2023**). Bromophenols from the Marine Red Alga Symphyocladia latiuscula and Their Radical Scavenging Activity. *Rec. Nat. Prod.,* 17(4) 726-730.
24 Chib J.S., Stempien M.F.Jr., Mierzwa R.A., Ruggieri G.D., and Nigrelli R.F. (**1978**) Physiologically Active Substances from Marine Sponges V: Isolation of Physiologically Active Compounds from the Sponge Verongia archeri. *J. Pharm. Sci.,* 67 264-265.
25 Gallimore W.A. (**2013**) Bioactive brominated metabolites from the natural habitat and tank-maintained cuttings of the Jamaican sponge Aplysina fistularis. *Chem. Biodivers.,* 10(6) 1055-1060.
26 Bauer A.W., Kirby W.M., Sherris J.C., and Turck M. (**1966**) Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.,* 45(4) 493-496.
27 Kowalska-Krochmal B. and Dudek-Wicher R. (**2021**) The minimum inhibitory concentration of antibiotics: methods, interpretation, clinical relevance. *Pathogens,* 10(2) 165.
28 O'Toole G.A. (**2011**) Microtiter Dish Biofilm Formation Assay. *J. Vis. Exp.,* 47 2437-2438.