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Original Article

Investigation of synergic activity of ceftazidime and colistin, and the effect of baicalin on biofilms

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Abstract

Introduction: The objectives of this study were to determine the rates of biofilm formation by *Pseudomonas aeruginosa* (n: 136) isolates from different samples collected from intensive care patients; and to determine the synergistic effects of the combination of ceftazidime and colistin, and the inhibitory effect of baicalin on biofilm formation in strong biofilm-producing bacteria (3+).

Methodology: Previous studies have performed biofilm grading based on microplate absorbance measurement to phenotype the biofilm formation rate. The in vitro synergistic efficacy of the combination of colistin and ceftazidime was evaluated using the checkerboard method for strains with 3+ biofilm test results. In addition, sub-minimum inhibitory concentration (sub-MIC; MIC/2, MIC/4, MIC/8) values of the biofilm inhibitory effect of baicalin were determined.

Results: The biofilm microplate method identified 5.15% of the isolates producing strong (3+) biofilms. Baicalin inhibited biofilm formation by 67.00–90.64% at sub-MIC concentration of 512 µg/mL, in 7 strong biofilm-producing isolates. These findings suggest that baicalin is a potential adjunctive therapy for disrupting biofilms, although the combination of ceftazidime and colistin may not be effective in this context.

Conclusions: No synergistic effect of ceftazidime and colistin antibiotics was detected in high biofilm-producing *P. aeruginosa* isolates from an intensive care unit, and it was determined that certain concentrations of baicalin were effective in biofilm formation.

Key words: *Pseudomonas aeruginosa*; baicalin; synergy; ceftazidime; colistin; sub-MIC.

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Introduction

Pseudomonas aeruginosa, a Gram-negative, aerobic, rod-shaped bacterium; is an opportunistic pathogen that can cause serious infections such as respiratory, urinary, circulatory, external ear, ocular, and wound infections; especially in immunocompromised patients [1]. These isolates are common causes of urinary tract, surgical wound, and respiratory tract infections in intensive care units [2]. Biofilms formed on medical devices and biomaterials such as intravenous catheters, implants, heart valves, and contact lenses reduce the chance of successful treatment of infections [3]. A Centers for Disease Control and Prevention (CDC) report states that *P. aeruginosa* strains pose a serious threat because they have the ability to acquire resistance during treatment and can cause multidrug resistance [4].

Baicalin (a major constituent of the roots of *Scutellaria baicalensis*) is widely used clinically to treat fever, bronchitis, and upper respiratory tract infections. Baicalin also has antifungal activity against *Candida albicans*, antiviral activity against enteroviruses, and antibacterial activity against methicillin-resistant *Staphylococcus aureus*. It has been reported to inhibit acyl homoserine lactone (AHL)-based quorum sensing (QS)-regulated gene expression in *Burkholderia cenocepacia* [5]. Baicalin interferes with QS pathways, which regulate biofilm formation, and has shown bacteriostatic effects on *P. aeruginosa* at high concentrations [6].

Inhibitory antibiotics, made from natural or chemical substances, inhibit the growth of other living microorganisms. The macromolecular targets of all antibiotics functionally interact with many other cellular elements. The minimum inhibitory concentration (MIC) is the lowest concentration of an antibiotic that inhibits bacterial growth [7]. Sub-minimal inhibitory concentrations (sub-MIC) are defined as concentrations that do not stop bacterial growth but have effects on various biochemical and surface structures of bacteria [8]. These doses can cause phenotypic and genotypic changes; in particular, changes in bacterial virulence, biofilm formation, gene expression, QS, and gene transfer. They can also accelerate the emergence of antibiotic-resistant bacterial strains by mimicking the role of autoinducers as signaling molecules [9,10]. Many antibiotics at sub-MIC levels increase or decrease the production of

numerous transcripts in different bacteria. Stress responses have been shown to occur at high antibiotic concentrations, and different responses have been shown at sub-MIC concentrations [11]. Therefore, it is important to determine the effects of sub-MICs on bacterial virulence in order to develop new treatment strategies against the relevant pathogen. In this context, more effective and precise methods of using antibiotics against the target bacteria can be determined, and perhaps antimicrobial resistance can be reduced [10].

Treatment with a single antibiotic is not recommended due to the possibility of rapid development of resistance during the treatment of the bacterial infection and insufficient success in treatment [12]. The target site of colistin is the bacterial membrane. Anionic lipopolysaccharide molecules in the cell membrane of bacteria create a high binding site for polymyxin, a cationic peptide. The competitive divergent cationic exchange in the cell membrane structure due to an electrostatic interaction, causes disruptions in the cell membrane structure. The mechanism causing the disruption is that polymyxin disrupts the calcium and magnesium ion balance that stabilizes the cell membrane structure. This disrupts membrane permeability, causing leakage of cell contents and bacterial death [13,14].

Ceftazidime is a cephalosporin that inhibits cell wall synthesis and supports bactericidal effects through cell lysis [15]. It has a long half-life and is bactericidal. It penetrates well into the cerebrospinal fluid in meningitis [16]. Ceftazidime and colistin are commonly used against multidrug-resistant *P. aeruginosa*; however, their combined efficacy against biofilm-producing strains is poorly understood.

This study aimed to fill the gap by exploring the anti-biofilm potential of baicalin at sub-MIC levels, and evaluating the synergy between ceftazidime and colistin; focusing on strong biofilm-producing *P. aeruginosa* isolates.

Methodology

In this study, 136 *P. aeruginosa* isolates from intensive care unit patients were tested between October 2019 and November 2020. Conventional methods (Gram stain, oxidase test) and the automated VITEK-MS (bioMerieux, Marcy-l'Étoile, France) system were used to identify the strains. Antibiotic susceptibility was tested using the VITEK 2 compact automated system (bioMérieux, Marcy l'Etoile, France). The in vitro synergistic efficacy of the combination of colistin and ceftazidime was evaluated by the checkerboard method in strains with 3+ biofilm test results, previously studied by the microplate method. In addition, the anti-biofilm effect of baicalin was determined by sub-MIC.

The biofilm grades were determined using the microplate biofilm assay method, as described in previous literature, by calculating optical density (OD) values, and the strong biofilm formation (3+) [4(control OD) < microorganism biofilm OD] value was determined [17].

The biofilm formation in treated and untreated groups were compared using analysis of variance (ANOVA), and $p < 0.05$ was considered statistically significant. Seven isolates were selected based on their strong biofilm formation.

Determination of MICs

The MIC values of the antibiotics colistin (solvent: sterile water), ceftazidime (solvent: sterile water), and baicalin (solvent: dimethylsulfoxide (DMSO)) for each isolate were obtained by calculating the antibiotic stocks according to the formula provided below in the following concentration ranges: 512–1 µg/mL, 512–0.25 µg/mL, and 4096–4 µg/mL for ceftazidime, colistin and baicalin respectively. The MIC ranges were determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2022 guidelines and data in literature [6].

$$\text{Volume (mL)} = \frac{\text{weight (mg) x potency (µg/mg)}}{\text{Concentration (µg/mL)}}$$

Cation-adjusted Müller-Hinton broth (CAMHB) medium was added to all the wells of the 96-well U plate at a volume of 100 µL. 100 µL of the prepared antibiotic solution was pipetted into the 2nd well, and 100 µL of the serial dilution was pipetted into the 11th well. Then 100 µL was discarded from the 11th well. A positive control (well 1) was used to determine if there was a problem with the growth of the strains, and a negative control (well 12) was used to determine contamination in the medium. After ensuring that the colonies were pure, 5 µL of the inoculum was diluted 1/10 with CAMHB by adjusting the inoculum density with 0.1% sterile saline to a turbidity equivalent of 0.5 McFarland (~10^8 CFU/mL) and added to all wells except the negative control. Positive and negative controls were added to each plate. Separate plates were prepared for both antibiotics and stored at – 20 °C until further analysis. The results were interpreted according to the EUCAST [18].

Determination of sub-MIC

The inhibition of biofilm formation in 7 isolates with strong biofilm-forming ability (+3) in media

containing baicalin at sub-MIC levels was investigated for the determination of antibiofilm activity. For this purpose, tryptic soy broth (TSB) media containing 3% glucose and antibiotics at MIC/2, MIC/4, and MIC/8 concentrations were prepared without baicalin for the 7 isolates whose MIC values were determined and studied in 3 replicates. Baicalin stocks were prepared using TSB, and 180 µL of baicalin-TSB stock were added to 3 wells for each strain. 20 µL of bacterial suspension adjusted to 0.5 McFarland ($\sim 10^8$ CFU/mL) was added to the well and incubated at 37 ºC for 24 hours. After incubation, the liquid medium was aspirated, taking care not to damage the biofilm. The plates were gently washed with sterile saline and dried inverted on blotting paper. Bacteria not adhering to the surface were removed. To ensure fixation of the biofilm layer, 99% methanol was added and left for 1 minute. The methanol was pipetted out of the wells and the plate was allowed to dry. Then, 200 µL of 1% crystal violet was added and incubated for 5 minutes at room temperature. This ensured staining of the biofilm-formed cells. At the end of the incubation, the wells were emptied without damaging the biofilm layer, washed 3 times with sterile serum fizyolojik (SF), inverted onto blotting paper, and dried at room temperature for 30 minutes. 200 µL of 96% ethanol was then added to the wells and left for 15 minutes to dissolve the stained biofilm layer. The plates were read using an optical enzyme-linked immunosorbent assay (ELISA) reader at 492 nm (Chromate, Awareness Technology Inc., Palm City, FL, USA). OD values were recorded for interpretation. The results were recorded by taking the difference in OD values between *P. aeruginosa* ATCC 15692 as a positive control and sterile saline as a negative control strain; and if this difference was less than the OD value determined, it was considered that the dose of baicalin used was effective, and if it was greater, it was considered that the dose used was ineffective [6]. Solvent-only controls were included to ensure that DMSO did not affect bacterial growth or biofilm formation.

Synergy testing

Seven isolates with strong biofilm-forming ability (+3) were tested using the checkerboard method. Serial dilutions were made starting from twice the concentration (2 MIC) of colistin and ceftazidime MIC values determined for *P. aeruginosa*, to 4 dilutions below the MIC value (MIC/32). The resulting solutions were applied to a 96-well U-bottom microplate from top to bottom for one antibiotic and from right to left for the other to obtain different concentrations of the two antibiotics in combination. After ensuring that the colonies were pure, the inoculum density was adjusted with 0.1% sterile saline to a turbidity equivalent to 0.5 McFarland ($\sim 10^8$ CFU/mL), diluted 1/10 with CAMHB, and 5 µL was added to each well, except the negative control. The plate was incubated at 37 ºC for 18–20 hours. One strain was tested in each plate, and ceftazidime MIC, colistin MIC, positive control, and negative control were added. The antibiotic concentrations in the non-growing wells were determined at the end of the incubation period, and the fractional inhibition concentration (FIC) values were calculated. The FIC index was used to determine the efficacy of the combinations according to the following formula [19].

Calculation of the FIC index:

$$FICA = \frac{\text{Numerical value of MIC of A in the presence of B}}{\text{Numerical value of MIC of A alone}}$$

$$FICB = \frac{\text{Numerical value of MIC of B in the presence of A}}{\text{Numerical value of MIC of B alone}}$$

where, A is the first antibiotic used in the combination, and B is the second antibiotic used in the combination.

The efficiency of combinations was determined based on:

$\Sigma FIC \leq 0.5$ synergy,

$0.5 < \Sigma FIC > 1$ partial synergy

$\Sigma FIC = 1$ additive

$1 < \Sigma FIC > 4$ ineffective

$\Sigma FIC \geq 4$ was considered as antagonism.

In the case of the n:7 *P. aeruginosa* isolates included in this study, FIC values were calculated for two antibiotic combinations, and the interactions were recorded as synergy, partial synergy, additive, no effect, and antagonism.

Results

In the previous study, 136 *P. aeruginosa* biofilm-forming isolates were classified using the microtiter plate method (0, 1, 2, 3), of which 51 (37.5%), 61 (44.85%), 17 (12.5%), and 7 (5.14%) were categorized as non-adherent, weak, moderate, and strong biofilm producers, respectively. The relationship between the biofilm grades and sample types was analyzed using the Chi square test, and no significant difference was found ($p > 0.05$).

The highest antibiotic resistance rate of the isolates was 60.29 (n = 82) against imipenem, and the lowest

Table 1. MIC values (in µg/mL) of baicalin, ceftazidime, colistin in biofilm microplaque grade 3 (n = 7).

Strain number	Baicalin MIC	Ceftazidime MIC	Colistin MIC
73	> 1024	8	0.5
99	> 1024	4	1
105	> 1024	4	0.5
107	> 1024	4	0.5
111	> 1024	2	0.5
113	> 1024	64	1
135	> 1024	8	0.5

MIC: minimum inhibitory concentration.

was 27.20 (n = 37) against amikacin. Based on the biofilm formation test, baicalin MIC values of 3+ (73, 99, 105, 107, 111, 113, 135) isolates were > 1024 µg/mL. The MIC values for ceftazidime and colistin are shown in Table 1. A line graph showing the MIC decreases at different baicalin concentrations is provided in Figure 1. Sub-MIC values were determined to be 512 µg/mL. The sub-MIC plate used in the study is shown in Figure 2. Biofilm inhibition varied significantly across isolates, ranging from 67% (isolate 73) to 90.64% (isolate 99). The biofilm inhibition of each isolate at sub-MIC concentrations is provided in Figure 3. Baicalin at sub-MIC concentrations significantly inhibited bio-film formation compared to untreated controls (mean inhibition 81.24%, $p < 0.01$).

Figure 1. A line graph showing minimum inhibitory concentration (MIC) reductions at different baicalin concentrations.

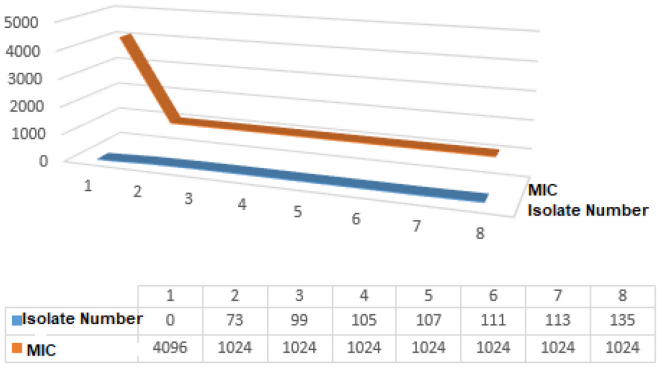


Figure 2. The baicalin sub-minimum inhibitory concentration (sub-MIC) plate used in the study.

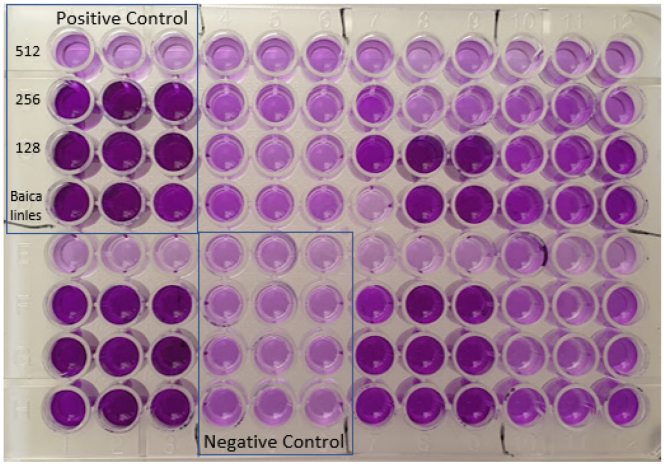


Figure 3. A bar chart showing biofilm inhibition for each isolate at sub-minimum inhibitory concentrations (sub-MIC).

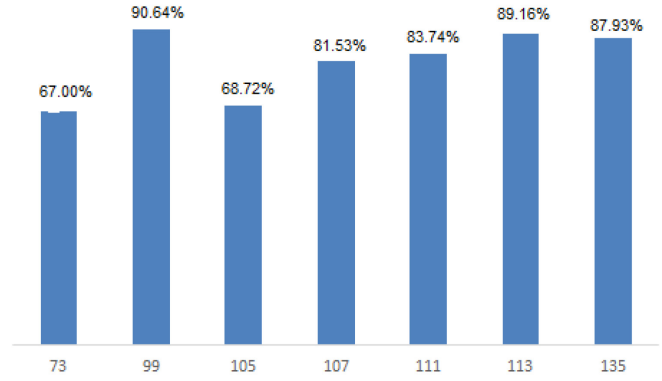


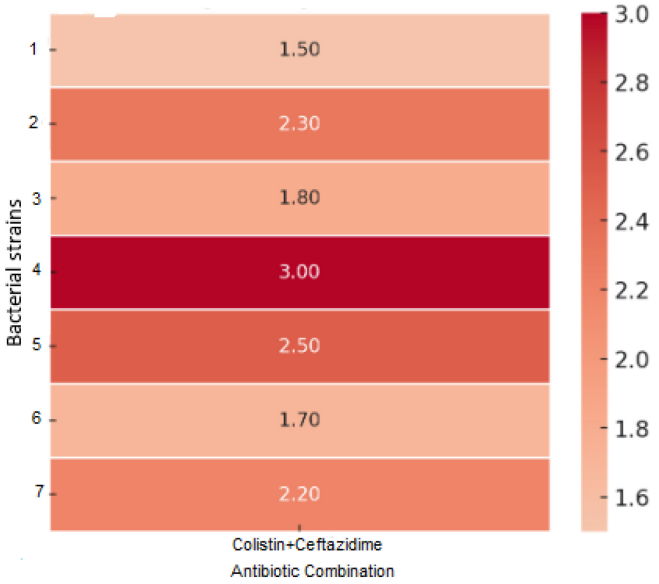
Table 2. A schematic representation of the experimental workflow for synergy testing.

Decreasing concentration of colistin	Decreasing concentration of ceftazidime (S)								SM	KM	P	N
	1	2	3	4	5	6	7	8	9	10	11	12
A	S128 + K128	S64 + K128	S32 + K128	S16 + K128	S8 + K128	S4 + K128	S2 + K128	S1 + K128	S128	K128	UC	BC
B	S128 + K64	S64 + K64	S32 + K64	S16 + K64	S8 + K64	S4 + K64	S2 + K64	S1 + K64	S64	K64	UC	BC
C	S128 + K32	S64 + K32	S32 + K32	S16 + K32	S8 + K32	S4 + K32	S2 + K32	S1 + K32	S32	K32	UC	BC
D	S128 + K16	S64 + K16	S32 + K16	S16 + K16	S8 + K16	S4 + K16	S2 + K16	S1 + K16	S16	K16	UC	BC
E	S128 + K8	S64 + K8	S32 + K8	S16 + K8	S8 + K8	S4 + K8	S2 + K8	S1 + K8	S8	K8	UC	BC
F	S128 + K4	S64 + K4	S32 + K4	S16 + K4	S8 + K4	S4 + K4	S2 + K4	S1 + K4	S4	K4	UC	BC
G	S128 + K2	S64 + K2	S32 + K2	S16 + K2	S8 + K2	S4 + K2	S2 + K2	S1 + K2	S2	K2	UC	BC
H	S128 + K1	S64 + K1	S32 + K1	S16 + K1	S8 + K1	S4 + K1	S2 + K1	S1 + K1	S1	K1	UC	BC

SM: determination of ceftazidime minimum inhibitory concentration (MIC) value; KM: determination of colistin MIC value; P: positive control; N: negative control; BC: sterility control; UC: growth control; S: ceftazidime; K: colistin.

Ceftazidime and colistin MIC values of the 7 isolates were determined, and synergy was investigated using the checkerboard method (Table 2). The study synergy plaque assessment results of *P. aeruginosa* (strain number 111) are summarized in Table 3. Based on the plate evaluations, 0% synergy and 100% ineffectiveness were determined according to the interaction result of the ceftazidime/colistin combination. The lack of synergy (FIC index ≥ 4) suggested that ceftazidime and colistin may act independently without enhancing each other's efficacy in biofilm-producing isolates. A heatmap summarizing the FIC index results for synergy testing is provided in Figure 4. The strains are shown in different shades of red because all the strains were in the ineffective range ($1 < \Sigma FIC < 4$).

Figure 4. A heat map summarizing the fractional inhibition concentration (FIC) index results for synergy testing



Discussion

The opportunistic pathogen *P. aeruginosa* is the most commonly isolated Gram-negative bacterium in nosocomial infections. Opportunistic infections, especially in immunocompromised patients; antibiotic resistance; and high mortality rates have necessitated the study of this bacterium [20].

Intensive use of antibiotics leads to the emergence of resistant strains, and the hospital flora is replaced by these strains. In order to prevent infections caused by biofilm-forming bacteria of these strains, it is important to plan antimicrobial treatment that can kill the bacteria in the biofilm to eradicate the bacteria. Luo *et al*. found that the sub-MIC value of baicalin was 256 µg/mL, and reported that baicalin had no direct bactericidal effect and was completely bacteriostatic on planktonic *P. aeruginosa* PAO1 cells with both minimum bactericidal concentration (MBC) and MIC > 1024 µg/mL [6].

In order to evaluate the potential anti-biofilm activity of baicalin, we first tested whether baicalin inhibited *P. aeruginosa* PAO1 biofilms at sub-MICs (16, 32, 64, 128, 256 µg/mL). Baicalin sub-MIC 256 µg/mL was found to significantly reduce biofilm formation, cell adhesion, and biofilm biomass growth in a dose-dependent manner [6, 21]. The sub-MIC in our study was set at 512 µg/mL. While baicalin alone showed limited efficacy, its ability to disrupt biofilm formation suggests its potential as an adjunct to conventional antibiotics, particularly in early-stage infections.

Baicalin inhibits biofilm formation and QS controlled virulence factors of *P. aeruginosa*. Baicalin treatment at sub-MIC levels suppresses the expression of QS regulatory genes (*lasI*, *lasR*, *rhlI*, *rhlR*, *pqsR*, and *pqsA*) and reduces the production of QS signaling molecules (3-oxo C12-HSL and C4-HSL). This leads to a reduction in biofilm formation and virulence [22].

Table 3. Synergy plaque assessment results of *P. aeruginosa* strain 111. FIC indices are stated in parentheses.

	1	2	3	4	5	6	7	8	9	10	11	12
A											Growth control	Sterility control
B											Growth control	Sterility control
C							Antagonism (4.25)	Antagonism (4.125)			Growth control	Sterility control
D					İneffectiveness (1.5)	İneffectiveness (2.5)					Growth control	Sterility control
E				İneffectiveness (3)					Ceftazidime MIC (1)	Colistin MIC (0.5)	Growth control	Sterility control
F				İneffectiveness (2.5)							Growth control	Sterility control
G				İneffectiveness (2.25)							Growth control	Sterility control
H				İneffectiveness (2.12)							Growth control	Sterility control

FIC index ≤ 0.5 indicates synergy, while FIC ≥ 4 indicates antagonism. FIC: fractional inhibition concentration; MIC: minimum inhibitory concentration.

Baicalin (the aglycone form of baicalein) enhances the efficacy of doxycycline against multidrug-resistant Gram-negative bacteria. This combination offered a potential approach to the treatment of bacterial infections and showed synergistic effects in both in vitro and in vivo models [23].

Synergy testing was applied only to strong biofilm-forming isolates due to financial constraints (limited availability of antibiotics), as their treatment was expected to be more challenging. In a study conducted with *P. aeruginosa* strains (n = 11), the in vitro synergistic activity of ceftazidime-colistin combinations was found to be 12.5% synergistic and 87.5% indifferent, based to the results of the checkerboard method [24]. No synergistic effect was observed in our study.

The sub-MIC and synergy analyses in our study were carried out with a limited number of microorganisms; therefore, new studies should be carried out by increasing the number of isolates. This is the only way to develop protocols to prevent biofilm formation. The lack of synergy may result from colistin's limited penetration into biofilms or the rapid efflux of ceftazidime by biofilm-embedded cells. The small sample size (n = 7) limits the generalizability of these findings. Additionally, in vitro conditions may not accurately reflect the complexity of biofilm formation in vivo.

Multi-drug-resistant bacteria are a serious and deadly problem in intensive care units. Strains that grow easily in the hospital environment and have a high potential to develop resistance are easily transmitted between patients and hospital staff. This leads to multidrug-resistant bacteremia and sepsis, where antibiotherapy options are extremely limited. In order to manage the rise in the incidence of multidrug-resistant bacteria in emergency situations and determine treatment protocols, it is important to test different combinations of antibiotics and publish the results. Future studies should explore baicalin's synergy with other antibiotics, test its effects on polymicrobial biofilms, and validate its efficacy in animal models.

Conclusions

Using the biofilm microplate method, biofilm inhibition was observed in 7 isolates that produced strong (3+) biofilms with baicalin MIC > 1024 μg/mL and MIC/2 of 512 μg/mL, based on the sub-MIC value. The combination of ceftazidime and colistin was evaluated using the checkerboard method for these 7 isolates with strong (3+) biofilm production, and no synergy was observed.

These results suggest that baicalin may serve as an adjunct therapy to prevent biofilm formation, particularly in multidrug-resistant *P. aeruginosa*. However, further studies are needed to confirm its in vivo efficacy and explore its role in combination therapies.

Given the rising prevalence of multidrug-resistant *P. aeruginosa*, developing anti-biofilm strategies is critical. This study contributes to understanding the limitations of antibiotic synergy and the potential of natural compounds like baicalin in combating biofilm-associated infections.

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Conflict of interests

No conflict of interests is declared.

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