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Antibiofilm and antibacterial activities of lupinifolin in combination with protein synthesis inhibitors against methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA)-derived biofilm formation is a crucial virulence factor, which essentially contributes to therapeutic challenges. This study aims to evaluate the antibiofilm and antibacterial formation activities of lupinifolin, a prenylated flavanone derived from *Derris reticulata* Craib. stem, in combination with protein synthesis inhibitors.

Methods: The crystal violet biofilm formation assay was performed to determine the biofilm formation activity. The synergistic antibacterial activities were evaluated using the checkerboard and time-kill assays.

Results: Lupinifolin and tetracycline significantly reduced MRSA biofilm formation with IC_{50} values of 15.32 ± 5.98 and 13.42 ± 5.90 µg/mL, respectively. On the contrary, the individual treatment of streptomycin and clindamycin tended to enhance biofilm formation. Lupinifolin at the sub-MIC of 8 µg/mL in combination with certain sub-MICs of tetracycline (8 and 16 µg/mL), streptomycin (16, 32, and 64 µg/mL), or clindamycin (4, 8, and 16 µg/mL) caused significant inhibitions against MRSA biofilm formation (P<0.05). The combination of lupinifolin and streptomycin exhibited a synergy (FIC index <0.625), confirmed in the time-kill assay. Conversely, the combination of lupinifolin and tetracycline or clindamycin resulted in no interaction (FIC indices of 1.0078 and <1.0156, respectively).

Conclusion: The antibacterial synergy of lupinifolin and streptomycin possibly contributed to their antibiofilm-forming activity. However, the combinations of lupinifolin and tetracycline or clindamycin conceivably executed their antibiofilm activity directly against the MRSA biofilm formation process. These findings indicate a potential role for lupinifolin as an antibiofilm enhancer to diminish MRSA biofilm formation.

Implication for health policy/practice/research/medical education:
This study provides scientific evidence that the combination of lupinifolin (8 µg/mL) and antibacterial drugs acting as protein synthesis inhibitors, specifically tetracycline, streptomycin, and clindamycin, at their sub-MICs, can significantly inhibit MRSA biofilm formations. These findings suggest the potential use of lupinifolin as an enhancer against MRSA biofilm formation.

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Introduction

Antimicrobial resistance has been declared by World Health Organization (WHO) as one of the top global public health threats requiring urgent coordinated actions from multiple sectors (1). *Staphylococcus aureus* is a well-adaptive gram-positive pathogenic bacterium evolving to be resistant to an array of antibacterial drugs. The mechanisms of antimicrobial resistance in *S. aureus* primarily involve enzymatic inactivation of antibiotics, drug efflux, and drug target modification. Penicillin-resistant *S. aureus,* which produces beta-lactamase enzyme hydrolyzing the beta-lactam ring of penicillin's chemical structure, was first discovered in 1942 (2). Most *Staphylococcal* isolates (more than 90%) are capable of producing β-lactamase and are resistant to penicillin. Methicillin-resistant *Staphylococcus aureus* (MRSA)

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was emerged for the first time in 1961, soon after the development of various β-lactamase-resistant penicillins such as oxacillin, cloxacillin, and methicillin (3). MRSA expresses modified penicillin binding proteins (PBPs) known as PBP2a (encoded by the *mecA* gene), which have low affinity for most of β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems. In addition to its tolerance to β-lactam antibiotics, MRSA has also been reported to be resistant to various antibacterial drugs, including tetracyclines, aminoglycosides, and lincosamides (4,5). MRSA can cause a variety of serious bacterial infections such as skin and soft tissue infections, bacteremia, infective endocarditis, osteomyelitis, pneumonia, as well as medical-device related infections. MRSA infections have been recognized as a leading cause of morbidity and mortality among infectious diseases worldwide. With estimations ranging from 28% (in Hong Kong SAR) to 73% (in Korea), the prevalence of MRSA infections in numerous Asian nations has been shown to be among the highest in the globe in the 2010s (6). In Thailand, 46% of *S. aureus* clinical isolates from the tertiary-care academic hospital were MRSA (7). The prognosis of MRSA-caused bacteremia has been reported to be relatively poor with 90-day mortality rate of more than 50% (8). According to a meta-analysis, the mortality of MRSA bacteremia was also significantly higher than that of methicillin-sensitive *Staphylococcus aureus* (MSSA) bacteremia (9). Accordingly, the development of antibacterial drugs effectively acting against multidrug-resistant microbes, including MRSA, has been listed as high priority by the WHO.

The treatment of MRSA infections is further challenged by the ability of this hazardous pathogen to develop biofilm. Biofilm-associated infections are particularly difficult to treat since the biofilm-embedded bacteria are shielded from both antimicrobial drugs as well as host immune defense mechanisms. It has been documented that sessile bacteria are approximately 1000-fold more tolerant to antibiotics than their planktonic counterparts (10). In addition to its role as a barrier, biofilm establishes an ecological niche in which bacteria can transfer survival factors such as necessary nutrients, and antimicrobial drug resistance genes. The bacteria in the biofilm also operate as a pathogen reservoir, allowing pathogens to detach and colonize a new surface area within the host (3). Altogether, biofilm-associated infections are basically difficult to eradicate. *S. aureus* is the most common pathogen causing biofilm-associated infections, especially in medical device-related infections (11). Multidrug resistant strains of MRSA, isolated from various types of infections, including bacteremia, diabetic foot and osteomyelitis, were reported to possess substantial biofilm-forming capacity (12). Inhibition against biofilm formation proposes another feasible approach for the management of biofilm-associated MRSA infections (13). Unfortunately, there is currently no therapeutically accessible drug that can specifically target the production of bacterial biofilms. Although much effort has been put into the research and development of antibacterial drugs in the past decades, these novel agents still face challenges such as antimicrobial resistance and adverse drug reactions. Plants usually defend themselves against invasive microbes by producing biologically active compounds. As a result, phytochemicals derived from medicinal plants are important sources of therapeutic candidates that act against pathogenic microorganisms (14). Additionally, the combination of antibacterial drugs and plant-derived phytochemicals may also provide an additional option for combating multidrug-resistant bacteria, such as MRSA.

Lupinifolin is a prenylated flavanone found in a variety of medicinal plants, including *Albizia myriophylla*, *Eriosema chinense*, *Myriopteron extensum*, and *Derris reticulata* Craib. (15-18). Lupinifolin has been shown to possess antimicrobial activities against various microorganisms, such as *Herpes simplex* virus (HSV-1), *Mycobacterium tuberculosis,* and some gram-positive pathogenic bacteria, including *Streptococcus mutans*, *Enterococcus faecalis*, *Enterococcus faecium*, MSSA, and MRSA (18-22). In addition to its antibacterial action, lupinifolin was reported to inhibit biofilm formation in clinical isolates of *E. faecalis* and *E. faecium* (22). The anti-biofilm formation activity of lupinifolin against the biofilm formation of *S. mutans* and *S. aureus,* both MSSA and MRSA, has been described in our previous studies (23,24). The combinations of lupinifolin and drugs acting as cell wall synthesis inhibitors, specifically ampicillin and cloxacillin, resulted in synergistic antibacterial activity against MSSA with the fractional inhibitory concentration (FIC) indices of 0.5000 and 0.5078, respectively (25). The potential antibacterial synergy against MRSA was also observed with the combinations of lupinifolin and ampicillin or cloxacillin with the FIC indices of <0.5625 and <0.5156, respectively (25). Our recent findings also demonstrated that when used at their sub-MICs, lupinifolin in combination with ampicillin, cloxacillin, or vancomycin significantly inhibited MRSA biofilm formation (24). Therefore, lupinifolin has a potential to be employed as an enhancer to boost the antibacterial and antibiofilm actions of antimicrobial drugs, which are used to treat MRSA infections. Nonetheless, the combined effects of lupinifolin and antimicrobial drugs acting as protein synthesis inhibitors on bacterial growth and biofilm formation of MRSA has not been established. In the current study, lupinifolin was combined with the antibacterial drugs, tetracycline, streptomycin, or clindamycin, which serve as protein synthesis inhibitors, to examine the antibacterial and antibiofilm formation activities of these combinations.

Materials and Methods

This work was done at the Faculty of Pharmacy, Mahasarakham University, Thailand from December 2022 to March 2023.

Isolation of lupinifolin from *Derris reticulata* stem

Derris reticulata stems were purchased from the local herb store in Bangkok, Thailand. The sample was authenticated according to a method previously described (26). A voucher specimen was deposited at the Herbarium unit of Pharmaceutical Chemistry and Natural Product Research, Faculty of Pharmacy, Mahasarakham University (code: MSU.PH-LEG-DR-01). The methods used for isolation and identification of lupinifolin from *D. reticulata* were performed according to the method explained in our previous study (27). The percentage yield of the isolated lupinifolin was 0.7251%. The obtained lupinifolin crystals were stored at -20°C until use.

Minimum inhibitory concentration determination

The microbroth dilution method was used to determine the minimum inhibitory concentration (MIC) (28). Lupinifolin, clindamycin (Sigma-Aldrich®, C5269), streptomycin (Sigma-Aldrich®, S6501), and tetracycline (Sigma-Aldrich®, T7660) were prepared as their stock solutions in two-fold serial dilutions by using their respective vehicles (0.1 M NaOH for lupinifolin and sterile deionized water for the other anti-bacterial drugs). MRSA (DMST 20645) suspensions with a concentration of 1.5×10^6 CFU/mL were prepared in Tryptic Soy Broth (TSB). In each well of the 96-well microplate, TSB (130 µL), MRSA suspension (50 µL) and the test agent or its vehicle (20 µL) were added. The MIC was the lowest concentration of the test agent, which resulted in no visible growth of the bacteria after 24-hour incubation at 37°C. The median MIC was determined from at least five independent experiments.

Biofilm formation assay

The biofilm formation assay was conducted according to the method of Hasan et al with slight modifications as described in our previous experiments (24,29). Lupinifolin with different concentrations, alone or in combination with the testing antibacterial drug (tetracycline, streptomycin, or clindamycin) (20 µL), was added to a mixture of 50 µL of MRSA suspension (1.5×10^6 CFU/mL) and TSB supplemented with 1% glucose (130 µL). The blank wells with similar concentrations of the test agents were carried out by excluding the bacterial suspension. After 24-hour incubation at 37 °C, the microplate was gently decanted to remove the media containing planktonic bacterial cells. The attached biofilm mass was fixed by adding 200 µL of formalin (37%, diluted 1:10) with 2% sodium acetate into each well and incubating for 15 minutes. The crystal violet solution (100 µL, 0.1%) was used to stain the fixed biofilm. After three sterile deionized water washes (300 µL), 120 µL of 95% ethanol was applied to the microplate wells to solubilize the biofilm-bound dye. The mixture (80 µL) was subsequently transferred to a 96-well microplate and its optical density was measured at a wavelength of 600 nm. The antibiofilm formation activity was expressed as %inhibition of biofilm formation, calculated by the following equation:

$$[(OD_{600}\text{ vehicle} - OD_{600}\text{ test}) / (OD_{600}\text{ vehicle})] \times 100.$$

By which, the optical density of the appropriate blank was subtracted from the optical densities of the vehicle and test agent to produce the OD_{600} vehicle and OD_{600} test, respectively. The median inhibitory concentration (IC_{50}) was obtained from the concentration-inhibitory curve generated using GraphPad Prism software version 8.0.

Checkerboard assay

Antibacterial drugs (tetracycline, streptomycin, or clindamycin), the checkerboard assay described by Orhan et al was used (30). Concisely, 50 µL of MRSA bacterial suspension (1.5×10^6 CFU/mL) was added to a mixture of TSB (130 µL) containing various concentrations of lupinifolin (10 µL) and the testing antibacterial drug (10 µL). The concentrations of lupinifolin used were 2-fold serially diluted along the abscissa; likewise, the concentrations of the testing antibacterial drug were serially diluted in the similar way along the ordinate. The maximum concentration used for each drug was at least 4xMIC. After 24-hour incubation at 37 °C, the MICs for each combination of lupinifolin and the testing antibacterial drug were determined. Subsequently, the FIC index was calculated by using the following equation:

FIC index = FIC of lupinifolin + FIC of the antibacterial drug.

FIC of lupinifolin was calculated by dividing the MIC of lupinifolin in combination with the MIC of lupinifolin alone, whereas FIC of the antibacterial drug was calculated by dividing the MIC of the antibacterial drug in combination by the MIC of the drug alone. The combination was classified as "synergy", "no interaction", or "antagonism", when the FIC index was ≤0.5, >0.5-4.0, or >4.0, respectively (31). The results obtained from at least three independent experiments were expressed as the median.

Time-kill assay

The time-kill assay was performed to evaluate the bactericidal synergism in order to confirm the antibacterial synergy determined in the checkerboard assay. The combination of lupinifolin and an antibacterial drug that showed the potential synergistic effect was selected

to be investigated, in the time-kill assay. Therefore, the combination of lupinifolin and streptomycin with the FIC index of <0.6250 was chosen. The experiment was conducted according to the method of Siriwong et al (32). The viability of MRSA over a 24-hour incubation period, presented as CFU/mL, was determined in the presence of the individual test agent (at its half-MIC) or the combination (at the concentration producing the potential synergistic FIC index). Accordingly, lupinifolin (8 µg/mL), streptomycin (128 µg/mL), or the combination of lupinifolin (8 µg/mL) and streptomycin (32 µg/mL) were tested. Briefly, the test agent at the specified concentrations (0.5 mL) or vehicle control was mixed with TSB (3.25 mL) and MRSA suspension (1.25 mL, 1.5×10^6 CFU/mL). The samples were collected at 0, 6, 10, and 24-hour incubation to determine the viable counts of MRSA. The colonies were counted after subsequent dilution plating on tryptic soy agar (TSA) and 24 hours of incubation at 37 °C. The experiments were done in triplicate. The synergistic effect was indicated when the numbers of bacterial growth (log10 CFU/mL) in the combinations at 24 hours was reduced by ≥ 2 log10 CFU/mL comparing to those in the most active single agent (33).

Statistical analysis

In this study, one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test or Kruskal–Wallis test followed by the Dunn-Bonferroni test was used to statistically analyze the data of the %inhibition of biofilm formation. A significant difference was indicated if the *P* value was less than 0.05. The data were expressed as mean ± SEM (%inhibition of biofilm formation and colony count), median (MIC and FIC index), or mean ± SD (IC_{50}).

Results

Antibacterial and antibiofilm activities of lupinifolin, tetracycline, streptomycin, and clindamycin when given as a single agent

The MICs of lupinifolin and tetracycline against MRSA were 16 and 32 µg/mL, respectively (Table 1). Lupinifolin at the concentrations of 16, 32, and 64 µg/mL had significant inhibitory actions against MRSA biofilm formation with the %inhibitions of 87.78 ± 5.88, 98.75 ± 0.65 and 95.86 ± 1.09, respectively ($P < 0.05$; n=10) (Figure 1a). Tetracycline at the concentrations of 8, 16, 32, and 64 µg/mL also produced significant antibiofilm formatting actions with the %inhibitions of 31.85 ± 4.84, 58.78 ± 2.73, 91.27 ± 8.24, and 96.50 ± 1.22%, respectively ($P < 0.05$; n=7) (Figure 1b). The median inhibitory concentrations (IC_{50s}) against MRSA biofilm formation of lupinifolin and tetracycline were 15.32 ± 5.98 µg/mL (n=10) and 13.42 ± 5.90 µg/mL (n=7), respectively.

The MICs of streptomycin and clindamycin against MRSA were found to be higher than the highest concentrations tested in this study (>256 and >128 µg/mL, respectively) (Table 1). Correspondingly, these two antibacterial drugs did not have antibiofilm activity against MRSA (Figures 1c and 1d). Despite not being statistically significant, the presence of streptomycin and clindamycin tended to promote the production of MRSA biofilms.

Antibiofilm activity of lupinifolin in combination with tetracycline, streptomycin, or clindamycin

Lupinifolin at the concentration of 8 µg/mL (1/2 MIC) in combination with certain sub-MIC concentrations of tetracycline (8 and 16 µg/mL), streptomycin (16, 32, and 64 µg/mL), or clindamycin (4, 8, and 16 µg/mL) resulted in a significant reduction in MRSA biofilm formation ($P < 0.05$; n=8-9) (Figures 2a, 2b, and 2c). The highest antibiofilm was observed in the combination of lupinifolin (8 µg/mL) and streptomycin (16, 32, and 64 µg/mL) with the %inhibitions of 87.79 ± 7.31, 97.98 ± 0.99, and 98.98 ± 1.11, respectively ($P < 0.05$; n=8) (Figure 2b). The antibiofilm formation of the combination of lupinifolin (8 µg/mL) and streptomycin was also significantly higher than that of lupinifolin at 8 µg/mL alone (22.58 ± 12.59%, n=10).

Lupinifolin at a concentration of 4 µg/mL (1/4 MIC) caused a significant increase in MRSA biofilm formation with the %inhibition of biofilm formation of -32.28 ± 4.41 ($P < 0.05$; n=10). However, the combinations of lupinifolin at the sub-MIC of 4 µg/mL and the sub-MICs of

Table 1. Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) index of lupinifolin, clindamycin, streptomycin and tetracycline against methicillin-resistant *Staphylococcus aureus*

Test agent	MIC alone (µg/mL)	MIC in combination (µg/mL)	FIC index	N
Lupinifolin	16	0.125	1.0078	4
Tetracycline	32	32		
Lupinifolin	16	8	<0.6250	5
Streptomycin	>256	32		
Lupinifolin	16	16	<1.0156	3
Clindamycin	>128	2		

Data are expressed as median values.
The combination is classified as "synergy", "no interaction", or "antagonism", when the FIC index is ≤0.5, >0.5-4.0 or >4.0, respectively.

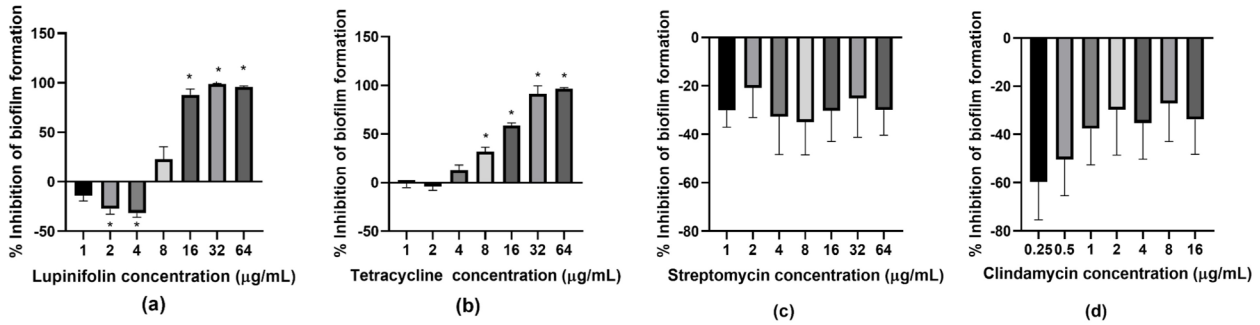

Figure 1. The effects of lupinifolin (a), tetracycline (b), streptomycin (c), and clindamycin (d) on biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA). **P*<0.05 when compared with the negative control group (mean ± SEM, n=7-10) (Kruskal-Wallis test, followed by Dunn-Bonferroni test).

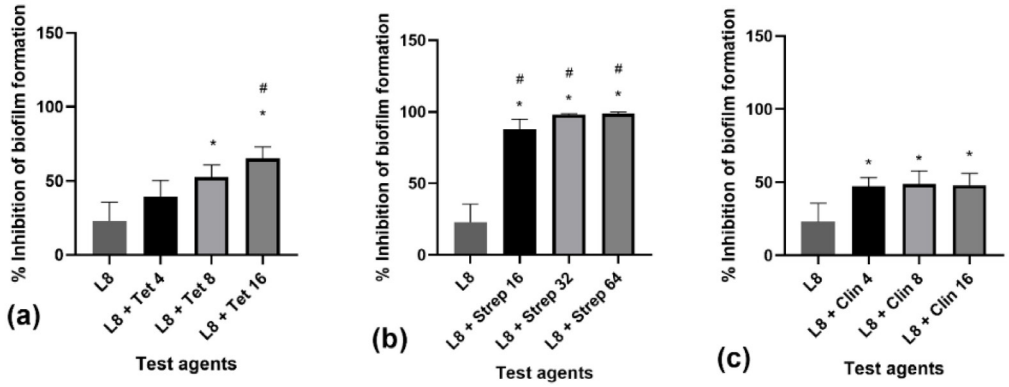

Figure 2. The effects of lupinifolin at the concentration of 8 µg/mL (1/2MIC) in combinations with tetracycline (a), streptomycin (b), or clindamycin (c) on biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA). (L=lupinifolin, Tet=tetracycline, Strep=streptomycin, Clin=clindamycin; followed by the concentration tested in µg/mL). **P*<0.05 when compared with the negative control (mean ± SEM, n=8-9) (Kruskal-Wallis test, followed by Dunn-Bonferroni test); #*P*<0.05 when compared with lupinifolin (8 µg/mL) (mean ± SEM, n=8-9) (Kruskal-Wallis test, followed by Dunn-Bonferroni test).

tetracycline (8 and 16 µg/mL) and streptomycin (64 µg/mL) significantly inhibited MRSA biofilm formation (Figures 3a and 3b). The highest antibiofilm activity of 49.50 ± 6.85% inhibition ($P < 0.05$, n = 8) was found with the combination of lupinifolin (4 µg/mL) and streptomycin (64 µg/mL). On the contrary, the mixtures of lupinifolin (4 µg/mL) and clindamycin (4, 8, and, 16 µg/mL) did not cause significant inhibition against MRSA biofilm formation (Figure 3c). However, these mixtures significantly counteracted lupinifolin's (4 µg/mL) promoting effect on MRSA biofilm development.

At 2 µg/mL (1/8 MIC), lupinifolin significantly increased MRSA biofilm formation with the %inhibition of -27.48 ± 5.16% ($P < 0.05$; n=10). A significant antibiofilm formation was observed with the combination of lupinifolin (2 µg/mL) and tetracycline (16 µg/mL), with the %inhibition of 48.76 ± 7.85 ($P < 0.05$; n=9) (Figure 4a). However, the combination of lupinifolin (2 µg/mL) with the sub-MICs of either streptomycin or clindamycin did not reduce MRSA biofilm formation (Figures 4b and 4c). These mixtures were likely to cause a modest but insignificant biofilm formation.

Antibacterial activity of lupinifolin in combination with tetracycline, streptomycin, or clindamycin

The MICs of lupinifolin and testing protein synthesis inhibitors (tetracycline, streptomycin, and clindamycin), either alone or in combination against MRSA, are shown in Table 1. The FIC index of the combination between lupinifolin and tetracycline (1.0078) was determined as indifference. Since the definite MICs of streptomycin and clindamycin against MRSA could not be detected in this study, the calculated FIC indices of the combination between lupinifolin and these two drugs were found at <0.6250 and <1.0156, respectively. Therefore, there was no interaction between the testing agents when lupinifolin and clindamycin were mixed. However, when lupinifolin and streptomycin were combined, a possible synergistic effect may have resulted. The time-kill experiment was subsequently performed, to justify this speculation.

From the time-kill assay, the combination of lupinifolin (8 µg/mL) and streptomycin (32 µg/mL) caused a substantial decrease in colony count at 6 hours after incubation (Figure 5). However, the viable count increased slightly after 10 hours of incubation. At 24-hour

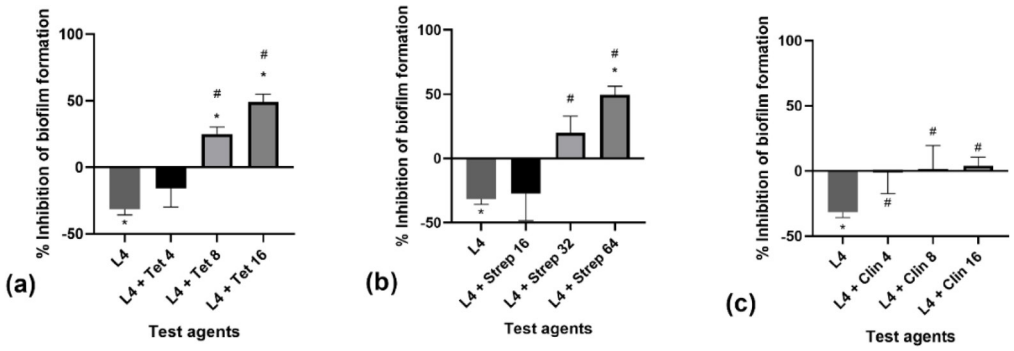

Figure 3. The effects of lupinifolin at the concentration of 4 µg/mL (1/4MIC) in combinations with tetracycline (a), streptomycin (b), or clindamycin (c) on biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA). (L=lupinifolin, Tet=tetracycline, Strep=streptomycin, Clin=clindamycin; followed by the concentration tested in µg/mL). **P* < 0.05 when compared with the negative control group (mean ± SEM, n=8-9) (Kruskal-Wallis test, followed by Dunn-Bonferroni test); #*P* < 0.05 when compared with lupinifolin (4 µg/mL) (mean ± SEM, n=8-9) (Kruskal-Wallis test, followed by Dunn-Bonferroni test).

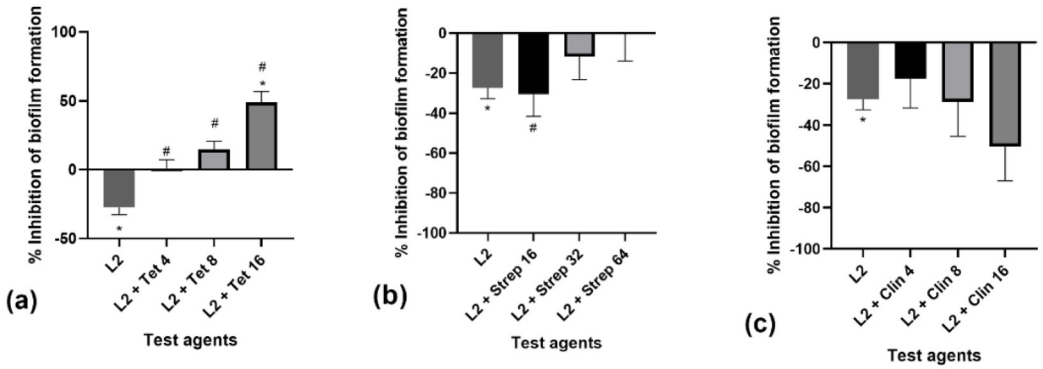

Figure 4. The effects of lupinifolin at the concentration of 2 µg/mL (1/8MIC) in combinations with tetracycline (a), streptomycin (b), or clindamycin (c) on biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA). (L=lupinifolin, Tet=tetracycline, Strep=streptomycin, Clin=clindamycin; followed by the concentration tested in µg/mL). **P* < 0.05 when compared with the negative control (mean ± SEM, n=8-9) (Kruskal-Wallis test, followed by Dunn-Bonferroni test); #*P* < 0.05 when compared with lupinifolin (2 µg/mL) (mean ± SEM, n=8-9) (Kruskal-Wallis test, followed by Dunn-Bonferroni test).

incubation period, this combination resulted in a mean reduction of 2.74±0.44 log10 CFU/mL of the colony count compared to lupinifolin (8 µg/mL) alone, which was the most active single agent (n=3). Thus, the bactericidal synergistic activity of this combination was confirmed.

Discussion

The MIC of lupinifolin (16 µg/mL) against MRSA found in this study was similar to those documented earlier in our previous studies (24,25). The MICs of tetracycline, streptomycin, and clindamycin against MRSA observed

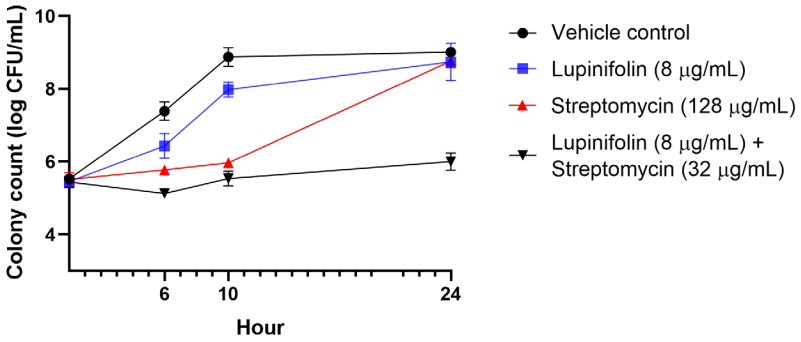

Figure 5 The effects of lupinifolin (8 µg/mL), streptomycin (128 µg/mL), and lupinifolin (8 µg/mL) plus streptomycin (32 µg/mL) on the viable count of methicillin-resistant *Staphylococcus aureus* (MRSA). The values are plotted as mean ± SEM (n=3).

here were 32, >256 and >128 μg/mL, respectively. According to the Clinical & Laboratory Standards Institute's (CLSI's) MIC breakpoints, *S. aureus* is considered sensitive to tetracycline and clindamycin, when the MICs are at ≤4 and ≤0.5 μg/mL, respectively (28). Therefore, the MRSA strain used in this study was classified as resistant to both tetracycline and clindamycin. Although the MIC breakpoint for streptomycin against *S. aureus* is not shown in CLSI 2018, the sensitivity of gentamicin, another aminoglycoside antibiotic, is indicated when its MIC is ≤4 μg/mL (28). Thus, the MRSA strain used in this study is apparently supposed to be resistant to streptomycin.

In addition to its resistance against β-lactam antibiotics, MRSA is well known to be resistant against multiple antibacterial drugs, including tetracyclines, aminoglycosides, and lincosamides (5,34). Two major mechanisms have been described as being associated with tetracycline resistance in *Staphylococcus spp.*, including active drug efflux and ribosomal (target site) protection. The active drug efflux is mediated via membrane proteins primarily encoded by *tetK* and *tetL* genes, whereas ribosomal protection, essentially encoded by *tetM* and *tetO* genes, involves a dissociation of the drug molecule from its target site (30S subunit of the ribosome) (5,34). It was reported that most MRSA isolates were typically *tetM, tetK,* or *tetKM* genotypes (34,35). Concurrently, *S. aureus* employs various mechanisms of resistance against macrolides, lincosamide, and streptogramins B (MLS-B) antibiotics. The most common mechanism of *S. aureus* resistance to MLS-B antibiotics involves enzyme modification of the drug target site by adenyl-N-methyltransferase erythromycin resistance methylase (Erm) enzymes, encoded by *erm* genes (5,36). It has been documented that more than 80% of MRSA strains exhibit concurrent resistance to MLSB antibiotics (36). Macrolide-resistant methicillin-resistant *S. aureus* (MAC-MRSA) infections generally have a poor clinical prognosis due to the limitations of therapeutic options. Meanwhile, the most common mechanism associated with aminoglycoside resistance in *S. aureus* is the enzymatic modification of aminoglycoside molecules via transferases (acetyltransferases, phosphotransferases, and nucleotidyltransferases) (5,37). The rates of aminoglycoside resistance in MRSA were reported to be approximately 75% in hospital settings (37). The most prevalent genes encoding aminoglycoside-modifying enzymes (AMEs) in clinical isolates of MRSA reported to be *aac (6')/aph (2')* genes, which generate aminoglycoside acetyl transferases (AACs) and aminoglycoside phosphotransferases enzymes (38,39). It has been documented that most MRSA strains contain both *tet* and AME encoding genes (40). Additionally, *S. aureus* residing within biofilm can also exhibit aminoglycoside resistance due to the lack of enzymes responsible for active transport of aminoglycosides into the bacterial cell (5).

Several flavonoids have been documented to possess synergistic antibacterial activity against MRSA when used in combination with aminoglycosides (33,41-43). The mixture of galangin, a major flavonol derived from *Alipinia officinarum,* and gentamicin was found to produce a synergistic antibacterial effect against 15 clinical isolates of MRSA with FIC indices of 0.19-0.25 (41). The combination of sophoraflavanone B, a prenylated flavanone isolated from *Desmodium caudatum* roots, with gentamicin resulted in FIC indices of 0.25-0.31 against both standard and clinical isolates of MRSA (44). A synergy between luteolin, a flavone, and gentamicin, an aminoglycoside, was observed against both reference and clinical strains of MRSA with FIC indices of 0.125-0.562 (42). Zuo et al demonstrated that morusinol, a prenylflavonoid isolated from *Morus alba* roots, had a synergistic effect against MRSA when using in combinations with aminoglycosides, either amikacin or streptomycin, with the FIC indices of 0.09-0.5. However, the synergy was not exhibited in the time-kill assay (33). Zuo et al also showed that multicaulisin, sanggenon G, and albanin G, which are flavonoids derived from *Morus alba* root barks, had a synergistic action with aminoglycoside antibiotics, including amikacin, etimicin, and gentamicin, against MRSA, with the FIC indices of the combinations ranging from 0.19 to 0.5 (43). The synergistic bactericidal activity of lupinifolin and streptomycin against MRSA found in this study is in consistent with the previous findings of other flavonoids.

Aminoglycosides have been known to possess a synergistic effect with a variety of antibacterial drugs (45). The transport of aminoglycosides across the cell membrane requires an energy-dependent transport pathway, which is more efficient when the energy is produced aerobically via active electron transport (46). Thus, oxygen-dependent processes are necessary for the antibacterial action of aminoglycosides. Anaerobic bacteria, as well as biofilm-residing bacteria, are therefore typically insensitive to aminoglycosides. Additionally, it was reported that adaptive resistance to aminoglycosides (amikacin) was linked with cell wall thickening in MRSA clinical isolates (47). Aminoglycoside transport can be enhanced by using them in combination with cell wall synthesis inhibitors, such as beta-lactam antibiotics or vancomycin. This combination generally results in a potent antibacterial synergy and is commonly used in the treatment of several serious infections, such as ventilator-associated pneumonia and sepsis, caused by multidrug resistant species (48). Lupinifolin, a prenylated flavanone, executes its antibacterial action in both MSSA and MRSA via disruption of the bacterial cell membrane (18,20). It has been documented that prenylated flavonoids are more hydrophobic than other flavonoids. Therefore, they may easily penetrate the bacterial cell barrier and facilitate the compounds targeting the active site (49,50).

The bactericidal synergy observed in the combination of lupinifolin and streptomycin was potentially caused by the enhancement of streptomycin transport via lupinifolin-induced bacterial cell membrane damage. Additionally, since the production of AMEs primarily contributes to aminoglycoside resistance in *S. aureus,* lupinifolin may possibly exert its antibacterial synergy with aminoglycosides by inhibiting AMEs. Further study is thus required to prove this speculation.

When used as a single agent at their ≥MICs, lupinifolin (16, 32, and 64 μg/mL) and tetracycline (32 and 64 μg/mL) caused significant inhibitions against MRSA biofilm formation. This antibiofilm formation activity was inevitably expected since no bacterial growth appeared at these concentrations of ≥MICs. Meanwhile, the sub-MICs of tetracycline at 1/4 MIC and 1/2 MIC (8 and 16 μg/mL) caused a significant inhibition against MRSA biofilm formation. Lupinifolin at the sub-MIC of 8 g/mL also caused a modest but statistically insignificant inhibition against MRSA biofilm formation. Thus, tetracycline and lupinifolin at these sub-MICs may possibly have direct and antibacterial-independent actions against the MRSA biofilm formation process. However, the antibiofilm mechanism of these agents has not been clearly studied. It has been shown that some antibacterial agents at the sub-MICs can inhibit biofilm formation without killing bacteria (51,52). Azithromycin at the sub-MICs was found to inhibit *Pseudomonas aeruginosa* biofilm formation via inhibitions against quorum-sensing and mucoid biofilm matrix polysaccharide alginate production (53,54). It was reported that certain antibacterial drugs at sub-MIC levels prevented *S. aureus* from forming biofilms by suppressing the expression of genes related to biofilms, such as *sarA, fnbA*, and *lrgA*. (52). More research is needed to determine whether the antibiofilm formation activity of sole tetracycline and lupinifolin at their sub-MICs involve the modulation of biofilm formation-related genes.

On the contrary, the lower concentrations tested of lupinifolin at 2 and 4 μg/mL significantly enhanced the MRSA biofilm formation. Streptomycin and clindamycin at every concentration tested also induced statistically insignificant, biofilm formation. It was evidenced that some antibacterial drugs, including streptomycin and clindamycin, significantly enhanced *S. aureus* biofilm formation when applied at the sub-MICs (51,55,56). Streptomycin at its sub-MICs was reported to cause bacterial stress by producing several changes in *S. aureus,* including an increase in hydrophobicity and a concomitant decrease in the surface charge, which favor surface attachment of the bacteria (55). Additionally, various biofilm formation-regulating factors, such as extracellular matrix binding proteins, were also upregulated on the surface of *S. aureus* due to a stress response to streptomycin (55). Clindamycin, at its sub-MICs, was found to upregulate the expression of numerous important MRSA biofilm-associated genes, including *atlA, lrgA, agrA,* the *psm* genes, *fnbA,* and *fnbB,* as well as the amount of extracellular DNA (56). MRSA treated with the sub-MICs of clindamycin also had bacterial morphological changes, including a loss of the spherical shape, inflation, and modification in cell wall thickness (56,57). Taken together, certain antibacterial agents, including streptomycin and clindamycin, at their sub-MICs, can trigger the stress response, which enhances the development of biofilm formation in MRSA, whereas the sub-MICs of tetracycline suppress biofilm formation. It is still unclear what factors influence a sub-MIC antibacterial agent to modulate the production of biofilms.

The significant antibiofilm formations were observed when lupinifolin at the sub-MIC of 8 μg/mL was combined with tetracycline, streptomycin, or clindamycin at every sub-MICs tested; the only exception was found with tetracycline at the lowest concentration used (4 μg/mL). Tetracycline at 8 and 16 μg/mL also caused a significant inhibition against biofilm formation when given solely; thus, the antibiofilm activity observed in these combinations was probably due to the action of tetracycline. Interestingly, significant antibiofilm formations were also prominently found with the combinations of lupinifolin (8 μg/mL) and every sub-MICs tested for streptomycin and clindamycin. When given as a single agent, lupinifolin, streptomycin, and clindamycin at these sub-MICs did not cause significant inhibition against biofilm formation. Additionally, a sole treatment of streptomycin or clindamycin substantially potentiated biofilm formation, as mentioned above. Therefore, the significant antibiofilm formation activity of the combinations between lupinifolin (8 μg/mL) and streptomycin or clindamycin arises only when these agents are used together. From the checkerboard assay, the combination of lupinifolin and clindamycin only produced indifferent antibacterial activity with the FIC index of <1.0516. Moreover, it should be noted that bacterial growth was still visibly observed in the combinations of lupinifolin (8 μg/mL) and clindamycin (4, 8 or 16 μg/mL). These combinations, hence possibly executed their antibiofilm formation activity by directly affecting the biofilm formation process, without having a compelling effect on bacterial growth. On the other hand, a promising synergistic antibacterial action was observed in the presence of lupinifolin and streptomycin with a FIC index of <0.6250. The bactericidal synergy was also evidently demonstrated in the time-kill assay. The combination of lupinifolin (8 μg/mL) and streptomycin (32 μg/mL) had remarkably diminished numbers of MRSA colonies of ≥100 fold (2 log10 CFU/mL) lower than those of the most active single agent. This suggests that the antibiofilm formation activity of lupinifolin and streptomycin combination was potentially derived from the synergic antibacterial action.

Lupinifolin at the sub-MICs of 2 and 4 μg/mL caused a significant decrease in antibiofilm formation activity as described earlier. The combinations of lupinifolin at 4 μg/mL and tetracycline (8 and 16 μg/mL) or streptomycin (64 μg/mL) reversed the biofilm forming induction of 4 μg/mL lupinifolin and produced a significant inhibition against biofilm formation. A similar effect was found with lupinifolin at a lower concentration of 2 μg/mL only in combination with tetracycline (16 μg/mL). Collectively, the antibiofilm formation activity was substantially decreased when lupinifolin at the lower concentrations of sub-MIC (4 and 2 μg/mL) were tested in combinations with the protein synthesis inhibitors. Therefore, the antibiofilm formation activity of the combination between lupinifolin and protein synthesis inhibitor is essentially dependent on the presence of appropriate concentrations of both agents.

Conclusion

The antibiofilm formation activity of the sub-MICs of lupinifolin (8 μg/mL) and streptomycin (16, 32 and 64 μg/mL) was potentially caused by their antibacterial synergy, as shown in the checkerboard and time-kill assays. On the other hand, the combinations of lupinifolin and tetracycline or clindamycin could possibly execute their antibiofilm formation activity directly toward the MRSA biofilm formation process since no interaction was observed in the checkerboard assay. These findings suggest the potential use of lupinifolin as an enhancer against MRSA biofilm formation when used in combination with tetracycline, streptomycin, or clindamycin. Nonetheless, further experiments are required to investigate the antibiofilm mechanism of lupinifolin when used in combination with antibacterial drugs acting as protein synthesis inhibitors.

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Authors' contributions

PP designed the study, conducted the experiment, performed the statistical analysis, wrote the manuscript, and revised the manuscript. PK, KC, TW, and SR conducted the experiments and analyzed the data. All authors reviewed and approved the final version of the manuscript.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The possible duplication was considered and the manuscript was checked for plagiarism.

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