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Research Article
Antibiofilm Formation Activity of Lupinifolin Against Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Background and Objective: Biofilm formation activity of Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the crucial factors rendering this pathogenic bacterium difficult to be eradicated. It has been reported that lupinifolin, is a major phytochemical agent isolated from *Derris reticulata* Craib. stem possesses antibacterial activity against MRSA. This study aimed to investigate the effects of lupinifolin and its combinations with some antibacterial drugs, including ampicillin, cloxacillin or vancomycin, on the biofilm formation activity of MRSA. **Materials and Methods:** The crystal violet biofilm formation assay was performed to evaluate the biofilm formation activity. **Results:** Lupinifolin produced a significant inhibitory activity against MRSA biofilm formation with the median inhibitory concentration (IC_{50}) of 7.96±3.05 μg mL^{-1} (n = 6) at 24 hrs incubation. Lupinifolin at the concentrations of sub-MICs (1, 2, 4 and 8 μg mL^{-1}) combined with the antibacterial drugs at their sub-MICs also exhibited substantial antibiofilm formation activities. The maximal antibiofilm activity was found with the combination of lupinifolin (8 μg mL^{-1}) and vancomycin (1 μg mL^{-1}) by the percentage inhibition of 102.39±0.89 (n = 8). The antibiofilm formation activities of the combinations between lupinifolin and the antibacterial drugs at various concentrations tested were also significantly higher than those of lupinifolin alone. **Conclusion:** These results indicated that lupinifolin can potentially be developed as an antibacterial enhancer for the management of biofilm-associated bacterial infections caused by MRSA, in which the current pharmacological treatment is still limited.

Key words: Lupinifolin, *Derris reticulata* Craib., biofilm, methicillin-resistant *Staphylococcus aureus*, MRSA

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an evolved strain of *S. aureus* which possesses unique penicillin-binding protein 2a (PBP2a), encoded by *mecA* gene. PBP2a expressed in MRSA cannot be targeted by β-lactamase-resistant penicillins (e.g., methicillin, cloxacillin, dicloxacillin) and other β-lactam antibiotics, except 5th generation cephalosporins, i.e., ceftaroline fosamil and ceftobiprole[1]. The MRSA, similar to its methicillin-sensitive counterpart (MSSA) can cause several notorious infectious diseases, including skin and soft tissue infections, endocarditis, pericarditis, pneumonia as well as bone and joint infections[2]. One of the crucial virulence factors of *S. aureus*, both MSSA and MRSA, is their ability to produce biofilm[3]. Distinct mechanisms of biofilm formation are exploited by MSSA and MRSA. The MSSA produces polysaccharide intercellular adhesin (PIA)-dependent biofilm, whilst MRSA generates PIA-independent biofilm[1]. In other words, the biofilm of MRSA and MSSA can be classified as proteinaceous-type and polysaccharide-type biofilms, respectively. The MRSA biofilm necessarily involves expressions of sortase-anchored surface proteins, major autolysin (Alt) and release of extracellular DNA (eDNA)[3]. Methicillin-resistance trait has been found to suppress polysaccharide-type biofilm production and induce proteinaceous-type biofilm[4]. Biofilm produced by *S. aureus* is the most common cause of medical device-related infections, which can substantially increase morbidity and mortality of the infectious diseases[1-3]. The treatment of biofilm-associated infections is challenging since biofilm behaves as a protective barrier to secure the enclosed bacteria inside. The bacteria inhabited inside biofilm are protected from host immune response and antibacterial drugs. The sessile bacteria were found to be approximately 1,000-fold less sensitive to antibacterial drugs when compared to their planktonic state[5]. Bacteria residing deep inside the biofilm can become dormant or persisted cells, which exist in a slow-growing state. These persisted cells are genuinely not sensitive to several antibacterial drugs, such as β-lactam antibiotics and other cell wall synthesis inhibitors, which exclusively act against actively growing cells. The survival of dormant cells extensively contributes to difficulty in the management of biofilm-related infections. Biofilm also creates a suitable niche for transferring drug-resistance genes across the embedded bacteria[2]. Biofilm-associated infections are thus authentically difficult to be eradicated. The development of anti-biofilm strategies is therefore considered essential for the successful management of MRSA biofilm-related infections. Plants naturally produce bioactive metabolites as an effective measure to combat invading microorganisms. Therefore, plant-derived phytochemicals, especially flavonoids, are potential sources of antibacterial and antibiofilm forming agents[6].

Lupinifolin is a prenylated flavanone, which can be primarily isolated from *Derris reticulata* Craib. (Leguminosae-Papilionoideae). It has been demonstrated that lupinifolin possesses antibacterial activity against certain gram-positive cocci including *Streptococcus mutans*, *Enterococcus faecalis*, *Enterococcus faecium*, MSSA as well as MRSA[7-14]. Lupinifolin was found to exert its antibacterial activity via disruption of bacterial cell membrane structure and functions[9-10]. Therefore, lupinifolin operates a distinct antibacterial mechanism of action from that of cell wall synthesis inhibitors, including β-lactam antibiotics and vancomycin, which are primarily used for the treatment of MSSA and MRSA infections. The previous study recently reported that the combinations of lupinifolin with ampicillin or cloxacillin produced synergistic effects against MSSA and MRSA with a FICI of $\leq$0.5[14]. Lupinifolin at its sub-MICs significantly exhibited inhibitory actions against biofilm formation of *S. mutans*, *E. faecalis*, *E. faecium* and MSSA[11,13]. However, the inhibitory action of Lupinifolin against MRSA biofilm formation has not been established. This study thus aimed to investigate the effects of lupinifolin either alone or in combinations with antibacterial drugs against MRSA biofilm formation.

MATERIALS AND METHODS

Study area: The experiments were carried out at the Faculty of Pharmacy, Maha Sarakham University, Thailand from November, 2021 to February, 2022.

Isolation of lupinifolin from *D. reticulata* stem: The purified lupinifolin used in the experiment was obtained from our previous study[13]. The authentication of *D. reticulata* samples and the isolation of lupinifolin were conducted as previously described in our earlier publication[15]. The isolated lupinifolin was kept at -20°C before using in the experiment.

Determination of the MIC: A modified micro broth dilution method was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines[14]. Stock solutions of lupinifolin, ampicillin (Sigma-Aldrich®, A8351), cloxacillin (Sigma-Aldrich®, C9393) and vancomycin (Sigma-Aldrich®, V2002) were prepared in 2-fold serial dilutions in their respective vehicles (0.1 M NaOH for lupinifolin and sterile deionized water for ampicillin, cloxacillin and vancomycin). A bacterial suspension of methicillin-resistant *Staphylococcus aureus* (MRSA, DMST 20645) was prepared in Tryptic Soy Broth (TSB) and adjusted to the concentration of

1.5×10^6 CFU mL^{-1}. The bacterial suspension (50 µL) was mixed with TSB broth (130 µL) and the test agent (20 µL) in each well of the 96-well microplate. After 24 hrs incubation at 37°C, the lowest concentration of the test agent which cause no visible bacterial growth was determined as the MIC. Three independent experiments were performed to obtain the median MICs of the test agents against MRSA.

Biofilm formation assay: The crystal violet biofilm formation assay was performed following the method of Hasan *et al.*[16] with slight modifications. The 50 µL of MRSA suspension (1.5×10^6 CFU μL^{-1}) were grown in TSB with 1% glucose (130 µL) containing various concentrations of lupinifolin either alone (20 µL) or lupinifolin (10 µL) in combinations with ampicillin, cloxacillin or vancomycin (10 µL). The bacterial suspension was omitted in the blank well. Two hundred milliliters of formalin (37%, diluted 1:10) with 2% sodium acetate was added into each well after 24 hrs incubation. Subsequently, 100 µL of 0.1% crystal violet was applied to stain the fixed biofilm. The microplate wells were washed thrice with sterile deionized water (300 µL) and then 95% ethanol (120 µL) was added to solubilize the biofilm-bound dye. After 10 min shaking, 80 µL of the mixture was pipetted into a new 96-well microplate to measure its optical density at the wavelength of 600 nm. Antibiofilm formation activity was indicated by inhibition (%) of biofilm formation, calculated by the following equation:

$$\frac{OD_{600}\ \text{vehicle-}OD_{600}\ \text{test}}{OD_{600}\ \text{vehicle}}\times100$$

The concentration-inhibitory curve was later plotted to obtain the median inhibitory concentration (IC_{50}).

Statistical analysis: The data were expressed as median (MIC), Mean±SEM (inhibition (%) of biofilm formation) or Mean±SD (IC_{50}). The data of inhibition (%) of biofilm formation was statistically analyzed by One-way ANOVA or Kruskal-Wallis Test. A significant difference was indicated if the $p<0.05$.

RESULTS

Antibacterial activity of lupinifolin, ampicillin and vancomycin against MRSA: Lupinifolin inhibited the growth of MRSA with a median MIC of 16 µg mL^{-1} (n =10). The median MICs of vancomycin and ampicillin against MRSA were 2 and 128 µg mL^{-1}, respectively (n = 7). Cloxacillin had no antibacterial activity against MRSA when tested at the maximal concentration of 128 µg mL^{-1} (n = 8).

Effects of lupinifolin, ampicillin, cloxacillin and vancomycin on MRSA biofilm formation: Lupinifolin at the concentration of 8 µg mL^{-1} (0.5 MIC), 16 µg mL^{-1} (MIC) and 32 µg mL^{-1} (2 MIC) significantly inhibited MRSA biofilm formation with the inhibitions (%) of biofilm formation of 39.53±9.92, 94.36±3.31 and 100.63±1.52, respectively (n = 6-9, $p<0.05$) in Fig. 1a. Ampicillin at the concentrations of 64 µg mL^{-1} (0.5MIC) and 128 µg mL^{-1} (MIC) and 4 g mL^{-1} (2MIC) significantly inhibited MRSA biofilm formation with the inhibitions (%) of biofilm formation of 74.88±12.30 and 94.10±5.21, respectively (n = 5, $p<0.05$) in Fig. 1b. Vancomycin at the concentrations of 1 µg mL^{-1} (0.5 MIC) and 2 µg mL^{-1} (MIC) also significantly inhibited MRSA biofilm formation with the inhibitions (%) of biofilm formation of 54.84±12.15, 98.96±0.80 and 98.93±1.45, respectively (n = 6, $p<0.05$) in Fig. 1c. The median inhibitory concentration (IC_{50}) of lupinifolin, ampicillin and vancomycin against biofilm formation was subsequently obtained from the concentration-inhibitory curve. The IC_{50s} of lupinifolin, ampicillin and vancomycin against MRSA biofilm formation were 7.96±3.05, 55.10±21.20 and 1.04±0.27 µg mL^{-1}, respectively (n = 6). On the contrary, cloxacillin at every concentration tested did not inhibit MRSA biofilm formation in Fig. 1d.

Inhibitory activities of lupinifolin in combinations with ampicillin, cloxacillin or vancomycin against MRSA biofilm formation: Lupinifolin at the sub-MIC of 8 µg mL^{-1} (0.5 MIC) in combinations with ampicillin at every concentration tested (at the sub-MICs) of 0.125, 0.25, 0.5, 8, 16, 32 and 64 µg mL^{-1} significantly inhibited MRSA biofilm formation with the inhibitions (%) of biofilm formation of 87.75±8.09, 98.28±1.28, 101.18±0.66, 99.93±0.40, 99.39±1.35, 99.87±1.39 and 99.68±1.11, respectively (n = 5, $p<0.05$) in Fig. 2a. The combinations of lupinifolin (8 µg mL^{-1}) and cloxacillin at every concentration tested (0.125, 0.25, 0.5, 8, 16, 32 and 64 µg mL^{-1}) significantly inhibited MRSA biofilm formation with the inhibitions (%) of 97.04±1.87, 98.79±1.54, 97.88±1.50, 99.39±1.83, 98.99±0.68, 98.88±0.92 and 99.20±0.70 (n = 5-8, $p<0.05$) in Fig. 2b. The combinations of lupinifolin (8 µg mL^{-1}) and vancomycin at every concentration tested (0.125, 0.25, 0.5 and 1 µg mL^{-1}) also caused a significant inhibition against MRSA biofilm formation with the inhibitions (%) of 83.11±5.50, 92.50±4.81, 94.85±2.49 and 102.39±0.89 (n = 8, $p<0.05$) in Fig. 2c. The antibiofilm formation activities of the combinations between lupinifolin (8 µg mL^{-1}) and the antibacterial drugs at various concentrations tested were also significantly higher than those of lupinifolin (8 µg mL^{-1}) alone (n = 5-8, $p<0.05$).

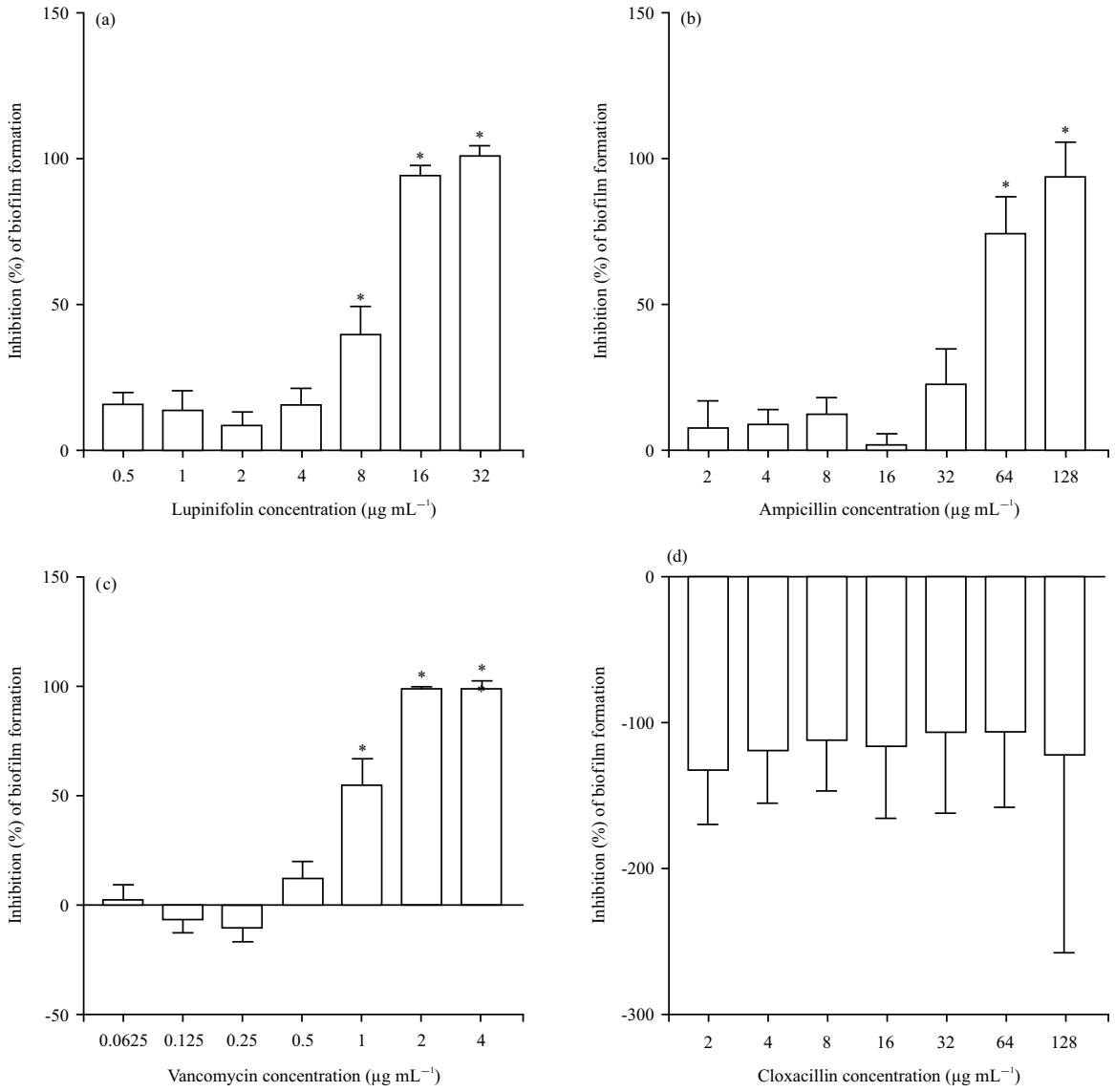

Fig. 1(a-d): Effects of biofilm formation on MRSA, (a) Lupinifolin, (b) Ampicillin, (c) Vancomycin and (d) Cloxacillin
*p<0.05 when compared with the negative control (Mean±SEM, n = 5-9) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test)

The combinations of lupinifolin at the concentration of 4 µg mL^{-1} (0.25 MIC) and ampicillin at the concentrations of 0.5, 8, 16, 32 and 64 µg mL^{-1} had a significant inhibition against MRSA biofilm formation with the inhibitions (%) of biofilm formation of 38.42±9.57, 37.96±7.81, 65.83±6.22, 98.36±1.59 and 100.70±1.27, respectively (n = 4-5, p<0.05) in Fig 3a. Lupinifolin (4 µg mL^{-1}) in combinations with cloxacillin at the concentrations of 8, 16, 32 and 64 µg mL^{-1} significantly inhibited MRSA biofilm formation with the inhibitions (%) of 56.71±8.59, 60.31±3.90, 81.39±7.30 and 73.93±7.65, respectively (n = 6-8, p<0.05) in Fig. 3b. The combinations of lupinifolin (4 µg mL^{-1}) and vancomycin at every concentration tested (0.125, 0.25, 0.5 and 1 µg mL^{-1}) also produced a significant inhibition against biofilm formation with the inhibitions (%) of 33.48±4.27, 46.26±4.64, 63.49±7.73 and 99.46±0.80 (n = 6-8, p<0.05) in Fig. 3c.

The combination of lupinifolin at the concentration of 2 µg mL^{-1} (0.125 MIC) and ampicillin only at the highest concentration tested (64 µg mL^{-1}) had a significant inhibition against MRSA biofilm formation with the inhibition (%) of 100.41±1.60 (n = 5, p<0.05) in Fig. 4a. On the other hand, the combinations of 2 µg mL^{-1} of lupinifolin with any tested concentration of cloxacillin (0.125-64 µg mL^{-1}) did not inhibit MRSA biofilm formation in Fig. 4b. The combinations of lupinifolin (2 µg mL^{-1}) and cloxacillin at the concentrations of 0.25 and 0.5 µg mL^{-1} significantly decreased the

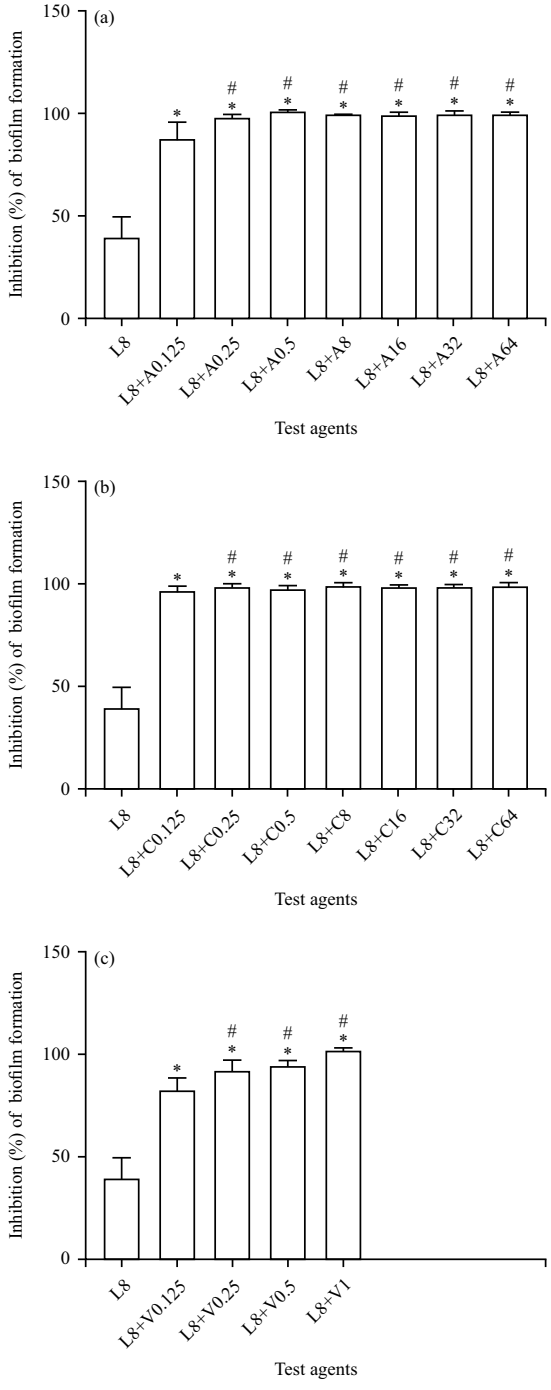

Fig. 2(a-c): Effects of lupinifolin at the concentration of 8 μg mL^{-1} (0.5 MIC) in combinations on biofilm formation of MRSA, (a) Ampicillin, (b) Cloxacillin and (c) Vancomycin

L: Lupinifolin, A: Ampicillin, C: Cloxacillin, V: Vancomycin, followed by the concentration tested in μg mL^{-1}, *p<0.05 when compared with the negative control (Mean±SEM, n = 5-8) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test), #p<0.05 when compared with lupinifolin (8 μg mL^{-1}) (Mean±SEM, n = 5-8) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test)

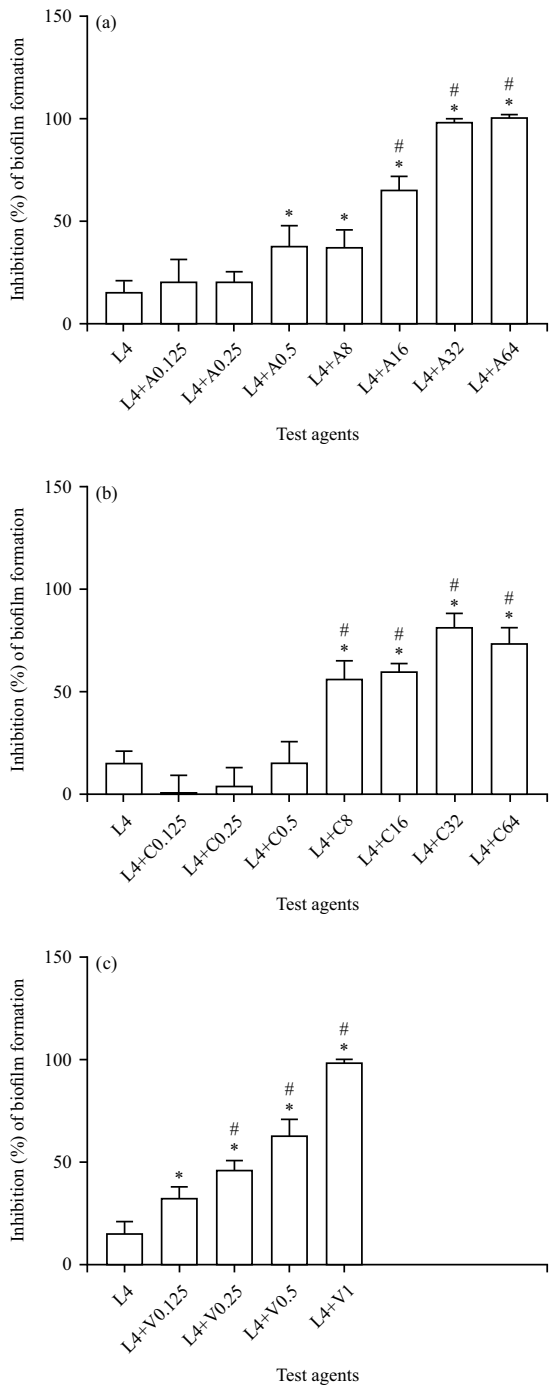

Fig. 3(a-c): Effects of lupinifolin at the concentration of 4 μg mL^{-1} (0.25MIC) in combinations on biofilm formation of MRSA, (a) Ampicillin, (b) Cloxacillin and (c) Vancomycin

L: Lupinifolin, A: Ampicillin, C: Cloxacillin, V: Vancomycin, followed by the concentration tested in μg mL^{-1}, *p<0.05 when compared with the negative control (Mean±SEM, n = 5-8) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test), #p<0.05 when compared with lupinifolin (4 μg mL^{-1}) (Mean±SEM, n = 5-8) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test)

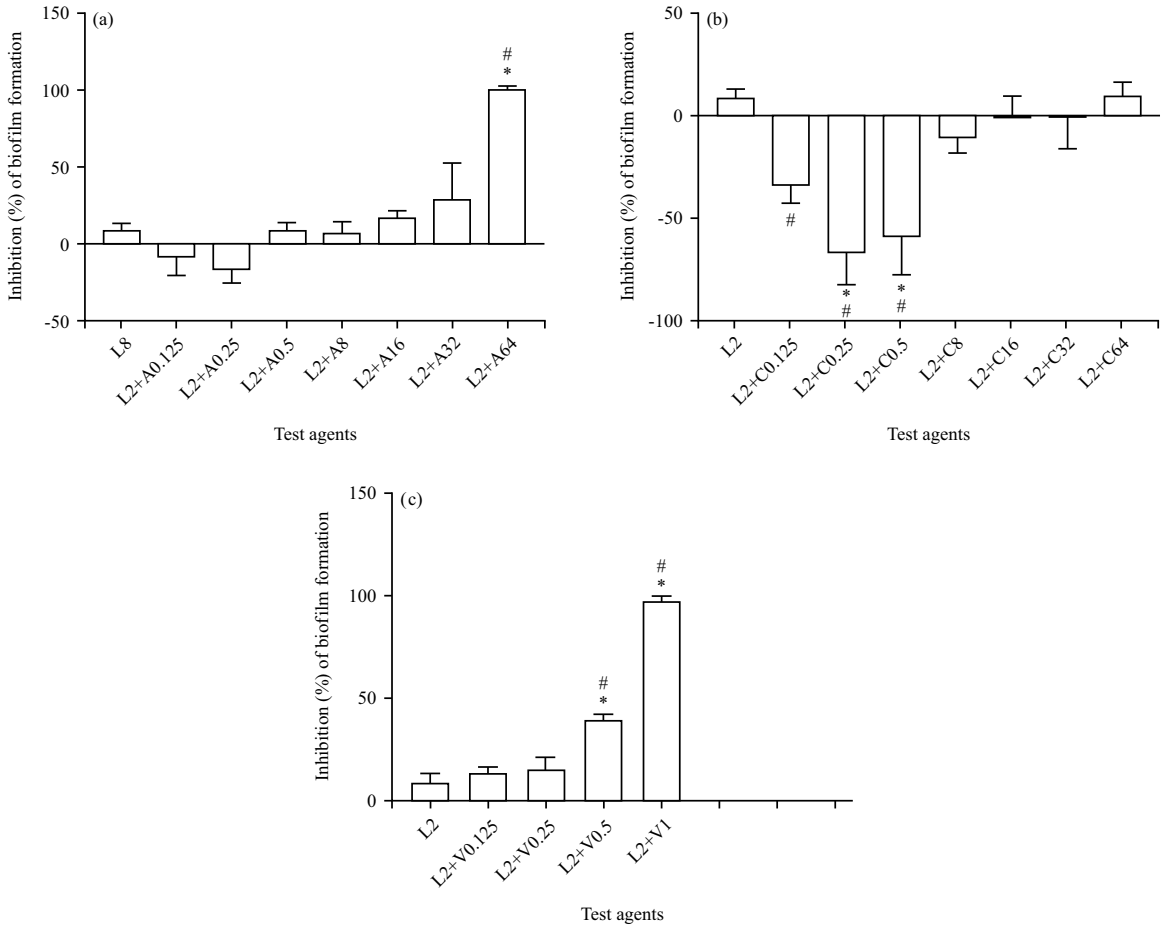

Fig. 4(a-c): Effects of lupinifolin at the concentration of 2 μg mL^{-1} (0.125 MIC) in combinations on biofilm formation of MRSA, (a) Ampicillin, (b) Cloxacillin and (c) Vancomycin

L: Lupinifolin, A: Ampicillin, C: Cloxacillin, V: Vancomycin, followed by the concentration tested in μg mL^{-1}, *p<0.05 when compared with the negative control (Mean±SEM, n = 5-8) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test), #p<0.05 when compared with lupinifolin (2 μg mL^{-1}) (Mean±SEM, n = 5-8) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test)

inhibition (%) of biofilm formation (i.e., there is a significant increase in MRSA biofilm formation) with the inhibitions (%) of biofilm formation of -66.73±15.90 and -59.05±18.34 (n = 4, p<0.05) in Fig. 4b. The inhibition (%) of biofilm formation in the presence of lupinifolin (2 μg mL^{-1}) and cloxacillin (0.125 μg mL^{-1}) also significantly lowered than that of lupinifolin (2 μg mL^{-1}) alone (inhibition (%) of biofilm formation of -34.17±8.58, n = 4, p<0.05) in Fig. 4b. The combinations of lupinifolin (2 μg mL^{-1}) and vancomycin at the concentrations of 0.5 and 1 μg mL^{-1} significantly inhibited MRSA biofilm formation with the inhibitions (%) of 39.34±2.76 and 97.18±2.32 (n = 6-8, p<0.05) in Fig. 4c).

Lupinifolin at the concentration of 1 μg mL^{-1} (0.0625 MIC) significantly inhibited MRSA biofilm formation only in the presence of 32 or 64 μg mL^{-1} of ampicillin with the inhibitions (%) of biofilm formation of 70.37±13.05 and 99.31±0.55, respectively (n = 6, p<0.05) in Fig. 5a. There was no inhibition against MRSA biofilm formation observed when cloxacillin at every concentration tested (0.125-64 μg mL^{-1}) was combined with 1 μg mL^{-1} of lupinifolin in Fig. 5b. The combinations of 1 μg mL^{-1} of lupinifolin and cloxacillin at the certain concentrations (0.125, 0.25 and 0.5 μg mL^{-1}) significantly decreased the inhibition (%) of biofilm formation (i.e., there is a significant increase in MRSA biofilm formation) with the inhibitions (%) of biofilm formation of -60.50±11.81, -117.37±18.48 and -97.36±17.84, respectively (n = 6, p<0.05) (Fig. 5b). The combinations of lupinifolin (1 μg mL^{-1}) and 0.5 or 1 μg mL^{-1} of vancomycin exhibited significant inhibition against MRSA biofilm formation with the inhibitions (%) of biofilm formation of 40.00±3.52 and 77.61±9.89, respectively (n = 6, p<0.05) in Fig. 5c.

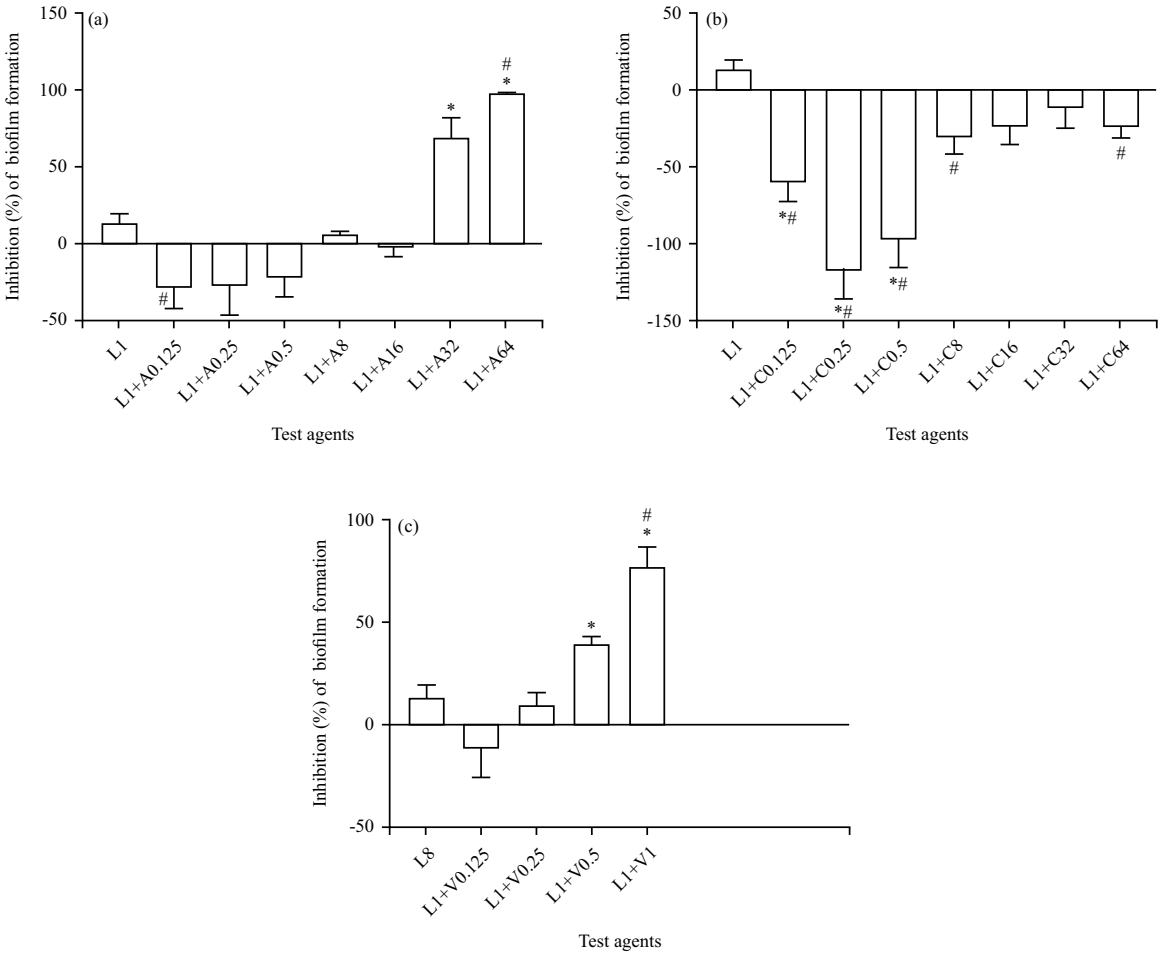

Fig. 5(a-c): Effects of lupinifolin at the concentration of 1 μg mL^{-1} (0.0625MIC) in combinations on biofilm formation of MRSA, (a) Ampicillin, (b) Cloxacillin and (c) Vancomycin

L: Lupinifolin, A: Ampicillin, C: Cloxacillin, V: Vancomycin, followed by the concentration tested in μg mL^{-1}, *p<0.05 when compared with the negative control (Mean±SEM, n = 5-8) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test), #p<0.05 when compared with lupinifolin (1 μg mL^{-1}) (Mean±SEM, n = 5-8) (Kruskal-Wallis Test, followed by Dunn-Bonferroni Test)

DISCUSSION

Lupinifolin exhibited a concentration-dependent inhibition against MRSA biofilm formation with the IC_{50} of 7.96±3.05 μg mL^{-1}. Thus, the antibiofilm formation activity of lupinifolin can be attained despite being at its sub-MICs. Ampicillin and vancomycin also produced antibiofilm formation activities against MRSA with the IC_{50s} of 55.10±21.20 and 1.04±0.27 μg mL^{-1}, respectively. The potency of lupinifolin against MRSA biofilm formation was thus substantially higher than that of ampicillin. The antibiofilm formation activity of both lupinifolin and vancomycin against MRSA was achieved at similar concentration ranges. The IC_{50s} of lupinifolin and vancomycin against MRSA biofilm formation were both at approximately half of their MICs (0.5 MIC). On the contrary, cloxacillin typically favoured MRSA biofilm formation. This agrees with the previous study which demonstrated that the exposure of sub-MICs of cloxacillin induced MRSA biofilm formation via induction of eDNA release[17]. From our previous study, the antibiofilm formation activity of lupinifolin against Methicillin-sensitive *Staphylococcus aureus* (MSSA) was documented with the IC_{50} of 1.31±0.35 μg mL^{-1} at 24 hrs incubation[13]. The potency of lupinifolin against MRSA biofilm formation is thus slightly lower than its potency against MSSA biofilm formation. Nonetheless, the IC_{50s} of lupinifolin against both MRSA and MSSA biofilm formation are still at relatively low concentrations of less than 10 μg mL^{-1}. Additionally, lupinifolin substantially executed the antibiofilm formation activity against both MRSA and MSSA even at the

concentrations of sub-MICs. This suggested that lupinifolin at the sub-MICs can disrupt MRSA and MSSA biofilm formations without causing selective pressure, which usually occurs when a high concentration of antibacterial agents at ≥MICs is introduced. However, since MRSA and MSSA form distinct types of biofilm known as polysaccharide-type and proteinaceous-type biofilms, respectively[3]. Thus, lupinifolin is likely to employ different mechanisms of antibiofilm formation in these two varieties of *S. aureus*.

From our previous study, it was demonstrated that lupinifolin can potentiate the antibacterial activity of ampicillin and cloxacillin against MRSA with the FIC indices of <0.5625 and <0.5156 respectively[14]. This indicated the synergistic effects of lupinifolin when used in combinations with these two penicillins. However, lupinifolin caused an indifferent action when combined with vancomycin with an FIC index of 0.75[14]. The combinations of lupinifolin and ampicillin, cloxacillin or vancomycin at their sub-MICs produced significant inhibitory actions against MRSA biofilm formation in this study. Interestingly, the antibiofilm formation activities of the combinations between lupinifolin and the antibacterial drugs at various concentrations tested were also significantly higher than those of lupinifolin alone. The antibiofilm formation activities of lupinifolin in combinations with these antibacterial drugs can conceivably arise from their antibacterial activity. The inhibition against bacterial growth would consequently impede their biofilm formation capacity. Nonetheless, turbidity of the media, which indicates bacterial growth, can still be observed in the presence of lupinifolin in combinations with the antibacterial drugs, particularly at their relatively low concentrations (e.g., 1 μg mL^{-1} of lupinifolin plus 0.5 μg mL^{-1} of vancomycin) in this study. Therefore, the antibiofilm formation activity found with these combinations would be potentially caused by direct inhibitory action against biofilm formation without affecting bacterial growth.

It has been shown that glabridin, an isoflavane, prevented biofilm formation of clinical isolate MRSA via down regulation of several surface-associated adhesins, including fibronectin-binding proteins (FnbA, FnbB), serine-aspartate repeat-containing protein D (SdrD) and immunoglobulin-binding protein G (Sbi)[18]. The LPXTG motif-containing proteins, which play a key role in MRSA biofilm formation, are anchored to bacterial cell wall peptidoglycan by sortase, an extracellular transpeptidase enzyme. Sortase is considered one of the virulence factors of *S. aureus* and its function is therefore necessary for MRSA biofilm formation[19]. It has been reported that various flavonoids, including flavones, flavonols, isoflavones as well as prenylated flavanones, can inhibit sortase enzyme[20]. Kurarinol, a 5-methoxyflavanone derivative isolated from *Sophora flavescens* roots, was found to inhibit the sortase enzyme of *S. aureus* ATCC 3538p with the IC_{50} of 107 μM[21]. Eriodictyol (3,4,5,7-tetrahydroxyflavanone), another flavanone derived from citrus fruits, was also reported to have sortase inhibitory action with the IC_{50} of 7.73 μM[22]. From the study by Wang *et al.*[22], eriodictyol also exhibited concentration-dependent inhibition against MRSA biofilm formation. Additionally, subcutaneous injection of eriodictyol at the doses of 100 μg kg^{-1} significantly reduced MRSA infection and increased animal survival in the mouse pneumonia model[22]. Therefore, it should be investigated further whether lupinifolin, a prenylated flavanone, can modulate sortase enzyme activity, which is essentially required for surface protein expression and MRSA biofilm formation.

Autolysin (Alt), which induces bacterial cell lysis and releases extracellular DNA (eDNA), is another essential component in MRSA biofilm formation[23]. It has been reported that the addition of DNAase I, which can destroy eDNA, inhibited MRSA biofilm formation without affecting mature preformed MRSA biofilm[24]. Therefore, eDNA is specifically involved in the initial attachment or early stage of MRSA biofilm formation[3]. Myrtenol, a bicyclic alcohol mono-terpene plant derivative commonly known for its pleasant aroma, at the concentrations of 75-300 μg mL^{-1} significantly inhibited MRSA biofilm formation via inhibition against autolysis and eDNA synthesis[25]. Previous experiments showed that lupinifolin at its sub-MICs significantly inhibited MSSA biofilm formation at the initial attachment stage of 6 hour-incubation with the IC_{50} of 0.22±0.03 μg mL^{-1}[13]. It is therefore, interesting to investigate whether lupinifolin disturbs the attachment phase of MRSA biofilm formation via inhibition against bacterial cell autolysis and/or release of eDNA.

It has been documented that some flavonoids can enhance the antibiofilm formation activity of certain antimicrobial agents. Myricetin (80 μg mL^{-1}) was reported to potentiate antibiofilm formation activity of miconazole (0.0625-8 μg mL^{-1}) against *Candida albicans*[26]. Epigallocatechin-3-gallate (EGCG, 0.3 μg mL^{-1}) significantly potentiated the antibacterial activity of cationic peptides (KR-12-a5, 0.6 μg mL^{-1}) against biofilms of bacteria associated with endodontic infections, including *S. mutans* and *A. israelii*, *E. faecalis* and *F. nucleatum*[27]. However, the evidence of antibiofilm activity of phytochemicals in combinations with antibacterial agents against MRSA biofilm is relatively scarce. It was reported that thymol enhanced the antibacterial and the biofilm eradication efficiency of rifampicin against MRSA and also reduced the formation of persisters[28]. The present study indicated that the combinations of lupinifolin and ampicillin, cloxacillin or vancomycin, can enhance their

antibiofilm formation activities. The results from this study revealed that lupinifolin either alone or in combinations with antibacterial drugs possessed the inhibitory action against MRSA biofilm formation even at their sub-MICs. Therefore, lupinifolin, specifically at its sub-MICs, can be an effective antibiofilm agent for the management of MRSA infection since it would not provoke selective pressure due to a lack of antibacterial activity. Nonetheless, lupinifolin produced its activity against MRSA biofilm formation in a concentration-dependent manner. Thus, the concentrations of lupinifolin obtained at the site of infection must reach the appropriate levels in which the antibiofilm formation activity ensues. Therefore, further *in vivo* studies are required to examine the pharmacokinetics of lupinifolin to estimate its concentration in a specific niche of biofilm-associated bacterial infections.

CONCLUSION

Lupinifolin produced a significant inhibition against MRSA biofilm formation with the IC_{50} of 7.96±3.05 μg mL^{-1}. The antibiofilm formation activity of lupinifolin was substantially exhibited even at the concentrations of sub-MICs. This suggested that lupinifolin can primarily disturb MRSA biofilm formation, independent of its antibacterial activity. The combinations of lupinifolin with ampicillin, cloxacillin or vancomycin at their sub-MICs also possessed significant inhibitory actions against MRSA biofilm formation. Interestingly, the antibiofilm formation activities of the combinations were significantly higher than those of lupinifolin alone. In summary, this study reports an inhibitory activity of lupinifolin either alone or in combinations with antibacterial drugs against MRSA biofilm formation for the first time. Lupinifolin is thus potentially a good candidate to be developed as an antibacterial enhancer against biofilm-associated MRSA infection.

SIGNIFICANCE STATEMENT

This research work is the first report of the inhibitory action of lupinifolin, isolated from *D. reticulata* stem, against biofilm formation of Methicillin-resistant *Staphylococcus aureus* (MRSA). The antibiofilm formation activity of lupinifolin was exhibited even at the concentrations of its sub-MICs against MRSA. Additionally, the combinations of lupinifolin and some antibacterial drugs tested at their sub-MICs also exhibited significant antibiofilm formation activities with higher inhibition (%) of biofilm formation than those of lupinifolin alone. These results suggest a potential role of lupinifolin as an antibiofilm forming agent used for some biofilm-associated infections caused by MRSA.

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