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ORIGINAL ARTICLE

Inhibitory Activity of Lupinifolin Isolated from *Derris reticulata* Stem against Biofilm Formation of *Streptococcus mutans* and *Staphylococcus aureus*

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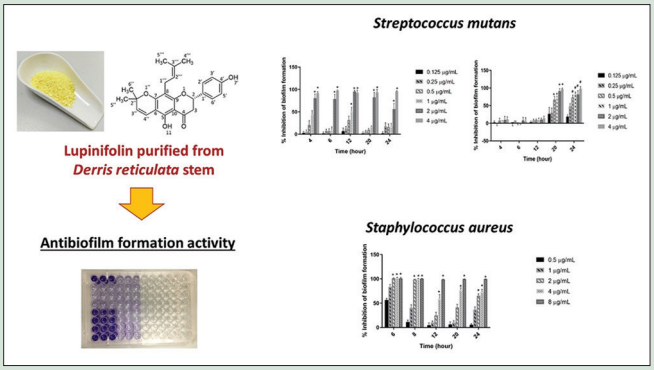
ABSTRACT

Background: Biofilm formation activity of pathogenic bacteria plays an important role in the pathogenesis and progression of various diseases caused by bacterial infections. It has been reported that lupinifolin, a major phytochemical isolated from *Derris reticulata* stem, possesses an antibacterial activity against *Streptococcus mutans* and *Staphylococcus aureus*. Nonetheless, its actions on biofilm formation properties of *S. mutans* and *S. aureus* have not been clearly established. **Objectives:** This study aimed to investigate the antibacterial and antibiofilm formation activities of lupinifolin derived from *D. reticulata* stem against *S. mutans* and *S. aureus*. **Subjects and Methods:** The minimum inhibitory concentration (MIC) was evaluated using the microbroth dilution method. The antibiofilm formation activity of lupinifolin was conducted at various incubation periods using the crystal violet biofilm formation assay. **Results:** The MICs of lupinifolin against *S. mutans* and *S. aureus* were 4 and 8 μg/mL, respectively. Lupinifolin at the concentrations of sub-MICs had significant inhibitory actions against both sucrose-dependent and sucrose-independent biofilm formations of *S. mutans*. The lowest median inhibitory concentrations (IC_{50s}) were found at the incubation periods of 12 h (0.57 ± 0.08 μg/mL) and 20 h (0.21 ± 0.04 μg/mL) against sucrose-dependent and sucrose-independent *S. mutans* biofilm formations, respectively. In addition, at its sub-MICs, lupinifolin also produced a significant inhibition against *S. aureus* biofilm formation with the lowest IC_{50} of 0.22 ± 0.03 μg/mL observed at 6-h incubation. **Conclusion:** These results evidently indicated that lupinifolin can potentially be developed further as a natural product-derived antibiofilm-forming agent for the prevention and/or treatment of biofilm-associated bacterial infections.

Keywords: Biofilm, *Derris reticulata*, lupinifolin, *Staphylococcus aureus*, *Streptococcus mutans*

SUMMARY

- This research work is the first report of the inhibitory action of lupinifolin, isolated from *Derris reticulate* stem, against biofilm formation of *Streptococcus mutans* and *Staphylococcus aureus*. The antibiofilm formation activities of lupinifolin in various incubation periods, which represent distinct stages of bacterial biofilm formation, were also demonstrated for the first time in this study. The antibiofilm formation activity of lupinifolin was exhibited even at the concentrations of its sub-MICs against both *S. mutans* and *S. aureus*. These results suggest a potential role of lupinifolin as an antibiofilm-forming agent used for some biofilm-associated infections caused by *S. mutans* and *S. aureus*.



Abbreviations Used: MIC: Minimum inhibitory concentration, IC_{50}: Median inhibitory concentration.

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INTRODUCTION

Several kinds of bacteria can form surface-associated communities called biofilm which plays a significant role in the pathogenesis of various chronic bacterial infections.[1] Biofilm produced by diverse oral microbes, especially Gram-positive *Streptococcus mutans*, contributes to localized acid-induced destruction of tooth surface, a pivotal etiology of dental caries. Biofilm-forming capability is one of the crucial virulence factors of *S. mutans*, together with its ability to produce and tolerate dietary carbohydrate-derived acids.[2] Biofilm originating from *Staphylococcus aureus*, another Gram-positive coccus, involves in many serious bacterial infections including osteomyelitis, indwelling medical device infections, endocarditis, and chronic wound infection.[3] The bacterial-derived extracellular polymeric matrix in biofilm holds the bacterial cells together and provides the secure and appropriate environment for the living of the sessile bacteria. Bacteria residing in the biofilm are exceptionally resistant to both antibacterial agents and host defenses. It was reported that when compared to the planktonic bacteria, the sessile bacteria in biofilm were almost 1000 times less

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sensitive to the antibacterial drugs.[4] The protective nature of the biofilm impedes drug penetration by acting as a diffusion barrier and/or an antimicrobial chelator. In addition, the extracellular polymeric matrix of biofilm concentrates crucial determinants required for bacterial survival, including extracellular DNA encoding drug resistance genes, antibacterial-destroying enzymes, and also essential nutrients. Biofilm likewise serves as a bacterial reservoir since bacteria which detach from the original site of biofilm can further colonize in the new location.[5] Thus, the biofilm-associated bacterial infections are genuinely difficult to eradicate. Currently, drug directly targeting against bacterial biofilm formation has not been clinically available yet. In general, plants naturally produce various kinds of biologically active metabolites in order to fight against microbial invasion. Therefore, plant-derived phytochemicals, especially polyphenols and flavonoids, are invaluable sources of drug candidates potentially used as antimicrobial and antibiofilm-forming agents.[6]

Derris reticulata Craib. (Leguminosae-Papilonoideae) is a medicinal plant used for the treatment of productive cough and throat diseases in Thai traditional medicines.[7] It has been documented that the crude extracts of *D. reticulata* stem exhibit various pharmacological actions including antioxidant, antidiabetic, and antimicrobial activities.[8-11] The ethanolic extract of *D. reticulata* stem was found to have antibacterial and anticariogenic activities against *S. mutans*.[12] The extract at the sub-MICs inhibited against the cariogenic properties of *S. mutans*, including surface adherence, biofilm formation, and glycolytic acid production.[12] A major pharmacologically active phytochemical derived from the stems of *D. reticulata* is a prenylated flavanone known as lupinifolin.[13] Lupinifolin can also be isolated from other medicinal plants such as *Albizia myriophylla*, *Eriosema chinense,* and *Myriopteron extensum*.[14-16] Lupinifolin has been found to possess diverse pharmacological activities including antidiabetics, antimycobacterials, antivirals as well as antibacterials.[16-20] Antibacterial activity of lupinifolin against Gram-positive cocci, specifically *S. mutans* and *S. aureus,* was previously documented with the MICs of 2 and 8 μg/mL, respectively.[18,19] Lupinifolin exhibited its bactericidal activity via disrupting cell membrane integrity and inducing leakage of cytoplasmic content.[18,19] It was recently demonstrated that lupinifolin at the sub-MICs significantly inhibited biofilm formation activity in certain strains of enterococci, *Enterococcus faecalis,* and *Enterococcus faecium*.[20] Nonetheless, the effects of lupinifolin against biofilm formation capability of other pathogenic bacteria, particularly *S. mutans* and *S. aureus*, have not been clearly established yet. This study thus aimed to investigate the antibacterial and antibiofilm formation activities of lupinifolin isolated from *D. reticulata* stem against *S. mutans* and *S. aureus*.

SUBJECTS AND METHODS

Isolation of lupinifolin from *Derris reticulata* stem

The stems of *D. reticulata* were purchased from the local herb store in Bangkok, Thailand. The authentication of the sample was conducted as described previously.[12] The purified lupinifolin from *D. reticulata* stem was obtained from our previous study.[21] The isolated lupinifolin was kept at − 20°C before using in the experiment.

Determination of the minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined using a modified microbroth dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[22] Lupinifolin was dissolved in 0.1 M NaOH and prepared in serial two-fold dilutions. A bacterial suspension of *S. mutans* (DMST 18777) in BHI broth or *Staphylococcus aureus* (DMST 8013) in TSB broth was prepared from the broth culture and adjusted to approximately 1.5×10^8 CFU/mL (McFarland no. 0.5). In each well of 96-well microplate, the bacterial suspension (180 μL) was mixed with lupinifolin at various concentrations (20 μL). The final concentrations of lupinifolin ranged from 0.125 to 32 μg/mL. The final bacterial cell concentration was approximately 5×10^5 CFU/mL. The microplates were incubated in the incubator at 37°C. For *S. mutans,* the microplate was kept in the incubator with 5% CO_2. The MIC was recorded as the lowest concentration totally inhibiting visible bacterial growth in the well after 24 h of incubation. 0.1M NaOH (vehicle) was used as a negative control. Three independent experiments were performed to obtain the mean MICs of lupinifolin against *S. mutans* and *S. aureus*.

Biofilm formation assay

The crystal violet biofilm formation assay was performed following the method of Hasan *et al*., 2015.[23] with slight modifications. *S. mutans* (1.5×10^6 CFU/mL, 50 μL) were grown in 96-well microplate containing BHI (130 μL) with or without 5% (w/v) sucrose. Meanwhile, *S. aureus* (1.5×10^6 CFU/mL, 50 μL) were grown in 96-well microplate containing TSB (130 μL). The bacteria-containing microplates were incubated with various concentrations of lupinifolin (0.125–8 μg/mL) or vehicle (0.1 M NaOH) at the volume of 20 μL in the incubator at 37°C (with 5% CO_2 for *S. mutans*). The incubation periods for *S. mutans* were 4, 6, 12, 20, or 24 h, whereas *S. aureus* were incubated for 6, 8, 12, 20, or 24 h. The blank wells were conducted by replacing the bacterial suspension with the broth media after incubation at the specific period, and the media were decanted from the microplate to remove the planktonic cells. The microplate wells were washed gently with sterile deionized water to remove the remaining unattached cells. The adhered biofilm was fixed by adding 200 μL of formalin (37%, diluted 1:10) with 2% sodium acetate. The fixed biofilm was stained with 0.1% crystal violet (100 μL) and left for 15 min. The microplate wells were washed three times with sterile deionized water (300 μL), and then, the biofilm-bound dye was removed by adding 120 μL of 95% ethanol. The microplate was shaken for 10 min, and the volume of 80 μL was taken out to measure its optical density at 600 nm. Inhibition of biofilm formation was calculated as a percentage from the following equation:

% Inhibition of biofilm formation = ([OD_{600} vehicle − OD_{600} lupinifolin]/[OD_{600} vehicle]) × 100.

The median inhibitory concentration (IC_{50}) of lupinifolin against biofilm formation was obtained from the concentration inhibitory curve plotted using GraphPad Prism software version 8.0.

Statistical analysis

The data were expressed as mean ± standard deviation (MIC and IC_{50}) or mean ± standard error of the mean (% inhibition of biofilm formation). The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test or Kruskal–Wallis test followed by the Dunn-Bonferroni test. The data were considered as a significant difference when $P < 0.05$.

RESULTS

Antibacterial activity of lupinifolin against *Streptococcus mutans* and *Staphylococcus aureus*

Lupinifolin inhibited the growth of *S. mutans* and *S. aureus* with the MICs of 4 ± 0 and 8 ± 0 μg/mL, respectively ($n = 3$).

Antibiofilm formation activity of lupinifolin against *Streptococcus mutans*

Lupinifolin produced a concentration-dependent inhibitory action against both sucrose-dependent and sucrose-independent biofilm formations of *S. mutans*. Lupinifolin at the sub-MIC of 2 μg/mL significantly inhibited sucrose-dependent biofilm formation at every incubation period tested (4, 6, 12, 20, and 24 h), with the maximal inhibition of 95.26 ± 3.32% (n = 4, P < 0.05) observed at the incubation time of 12 h [Figure 1]. At the 12-h incubation, lupinifolin at the lower concentration of 1 μg/mL also had a significant inhibitory action against sucrose-dependent biofilm formation with the % inhibition of 51.72 ± 8.31 (n = 5, P < 0.05) [Figure 1]. Lupinifolin at its sub-MICs (0.5, 1, and 2 μg/mL) also showed a statistically significant inhibition against sucrose-independent *S. mutans* biofilm formation at the incubation times of 20 and 24 h. However, at the incubation periods of 4, 6, and 12 h, lupinifolin at any concentration tested did not inhibit sucrose-independent biofilm formation [Figure 2].

The IC_{50s} of lupinifolin against *S. mutans* biofilm formation are shown in Table 1. The lowest IC_{50s} were found at the incubation periods of 12 h (0.57 ± 0.08 μg/mL) and 20 h (0.21 ± 0.04 μg/mL) against sucrose-dependent and sucrose-independent biofilm formations, respectively.

Antibiofilm formation activity of lupinifolin against *Staphylococcus aureus*

Lupinifolin significantly inhibited the biofilm formation of *S. aureus* in a concentration-dependent manner. At its sub-MIC of 4 μg/mL, lupinifolin produced a significant inhibition against *S. aureus* biofilm formation at every incubation period tested (6, 8, 12, 20, and 24 h). The maximal antibiofilm activity of 101.90 ± 0.98% was found at 6-h incubation (n = 5, P < 0.05) [Figure 3]. Lupinifolin at the lower concentration of 2 μg/mL also had a significant inhibition against *S. aureus* biofilm formation at the incubation times of 6, 8, and 24 h [Figure 3].

The IC_{50s} of lupinifolin against *S. aureus* biofilm formation are shown in Table 2. Lupinifolin produced the highest potency against *S. aureus* biofilm at 6-h incubation with the IC_{50} of 0.22 ± 0.03 μg/mL.

Table 1: The median inhibitory concentration of lupinifolin against *Streptococcus mutans* biofilm formation (mean±standard deviation, n=3-5)

Incubation time (hours)	Conditions	IC_{50} (μg/mL)
24	With sucrose	1.25±0.24
	Without sucrose	0.27±0.07
20	With sucrose	1.33±0.31
	Without sucrose	0.21±0.04
12	With sucrose	0.57±0.08
	Without sucrose	N/A
6	With sucrose	1.58±0.53
	Without sucrose	N/A
4	With sucrose	1.27±0.45
	Without sucrose	N/A

N/A: Data is not available; IC_{50}: Median inhibitory concentration

Table 2: The median inhibitory concentration of lupinifolin against *Staphylococcus aureus* biofilm formation (mean±standard deviation, n=4-6)

Incubation time (hours)	IC_{50} (μg/mL)
24	1.31±0.35
20	2.31±0.15
12	3.18±0.61
8	0.84±0.18
6	0.22±0.03

IC_{50}: Median inhibitory concentration

DISCUSSION

The results showed that lupinifolin, isolated from *D. reticulata* stem,

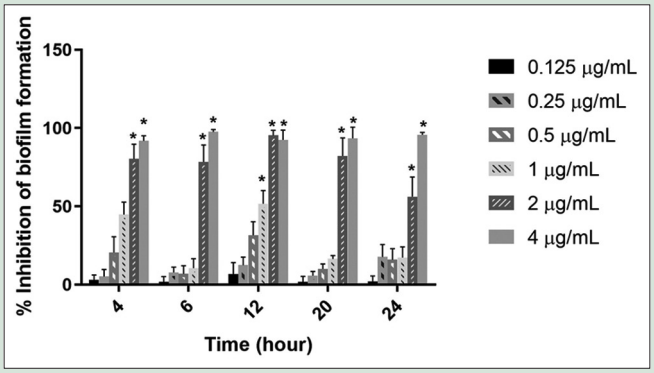

Figure 1: The effects of lupinifolin on sucrose-dependent biofilm formation of *Streptococcus mutans*. *P < 0.05 when compared with the negative control (mean ± standard error of the mean, n = 4–7) (Kruskal–Wallis test, followed by Dunn-Bonferroni test)

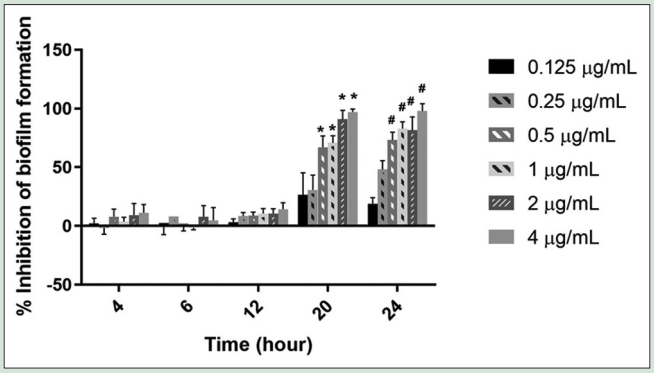

Figure 2: The effects of lupinifolin on sucrose-independent biofilm formation of *Streptococcus mutans*. *P < 0.05 when compared with the negative control (mean ± standard error of the mean, n = 3–6) (one-way ANOVA followed by Bonferroni *post hoc* test, n = 3); #P < 0.05 when compared with the negative control (mean ± standard error of the mean, n = 3–6) (Kruskal–Wallis test, followed by Dunn-Bonferroni test)

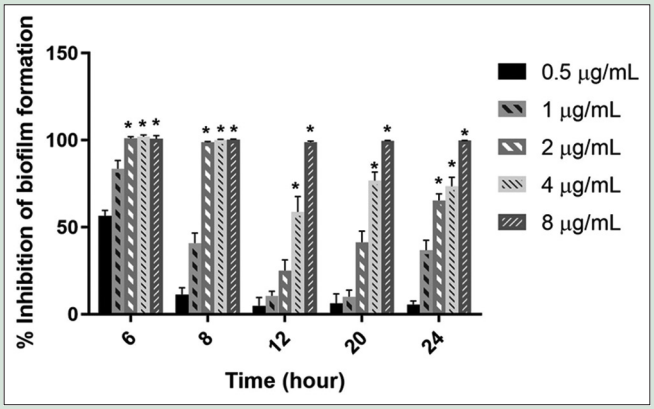

Figure 3: The effects of lupinifolin on biofilm formation of *Staphylococcus aureus*. *P < 0.05 when compared with the negative control (mean ± standard error of the mean, n = 4–9) (Kruskal–Wallis test, followed by Dunn-Bonferroni test)

possessed the antibacterial activity against both *S. mutans* DMST 18777 and *S. aureus* DMST 8013 with the MICs of 4 and 8 μg/mL, respectively. The antibacterial activity of lupinifolin, derived from *A. myriophylla*, against *S. mutans* was reported previously with slightly different values of MICs.[14,19,24] From the studies of Joycharat *et al.* and Joycharat *et al.,* the reported MIC of lupinifolin against *S. mutans* ATCC 25175 was relatively low at 1 μg/mL.[14,24] Meanwhile, from the study of Limsuwan *et al*., the antibacterial activity of lupinifolin against *S. mutans* ATCC 25175 and clinical isolated *S. mutans* was quite comparable to our study with the MICs of 2 and 2–4 μg/mL, respectively.[19] The difference in *S. mutans* strains tested is likely to be the main reason for the discrepancy of the MICs reported between studies. The antibacterial activity of lupinifolin against *S. aureus* TISTR 1466 was previously reported by Yusook *et al.* with the MIC of 8 μg/mL.[18] This is in agreement with our results in which the lupinifolin's MIC of 8 μg/mL was demonstrated against *S. aureus* DMST 8013. It should be noted that a similar vehicle, 0.1 M NaOH, was used in the current study and the study of Yusook *et al.*[18] A water solubility of lupinifolin is remarkably low (0.009 mg/mL), and thus, it is essential to choose the appropriate vehicle for its dilution before adding into the aqueous media. A basic solution of 0.1 M NaOH was used in our study to enhance the aqueous solubility of lupinifolin according to the suggestion of Yusook *et al.*[18] On the other hand, DMSO was used as a vehicle in the studies of Joycharat *et al.,* Joycharat *et al.,* and Limsuwan *et al.*[14,19,24] Therefore, in addition to the bacterial strains tested, the vehicle used for lupinifolin dissolution as well as other factors, such as the conditions for bacterial culture, can also influence the bacterial sensitivity to antibacterial agents. Rios and Recio suggested that the MIC of the isolated phytochemical compound should not exceed 100 μg/mL.[25] The MIC of 10 μg/mL or lower even further signified the potential use of the compound as the antibacterial agent. The apparently low MICs of lupinifolin against *S. mutans* (4 μg/mL) and *S. aureus* (8 μg/mL) thus potentially assert its role as a candidate antibacterial agent.

The antibacterial mechanism of lupinifolin against *S. mutans* and *S. aureus* has been recently explored by Yusook *et al.* and Limsuwan *et al.*[18,19] Lupinifolin exhibited its antibacterial activity via interfering with *S. aureus* cell membrane structure and functions.[18] The growth of *S. aureus* was inhibited by lupinifolin within the 1st h of incubation. Its bactericidal action was found to occur faster than that of ampicillin, a cell wall synthesis inhibitor. Lupinifolin also rendered a loss of cell membrane integrity and leakage of cytoplasmic materials in *S. mutans* without causing any cell lysis.[19] However, the target binding site of lupinifolin has not been established yet. Most antibacterial agents currently used for the treatment of *S. aureus* infection, both anti-methicillin-sensitive *S. aureus* antibiotics (such as cloxacillin and dicloxacillin) and anti-methicillin-resistant *S. aureus* drugs (such as vancomycin, teicoplanin, telavancin, and fosfomycin), act via inhibition against bacterial cell wall synthesis. Therefore, a combination of lupinifolin and these cell wall synthesis inhibitors may potentially produce a synergistic antibacterial activity. The checkerboard assay should be further conducted to determine this speculation.

Lupinifolin had no cytotoxic effect when tested at the concentration of up to 40 μg/mL in rabbit red blood cells.[18] The cytotoxicity tests (MTT and trypan blue exclusion assays) of lupinifolin were also investigated in HepG2 cells with the IC_{50} of 78 and 67 μg/mL.[18] The *in vivo* study indicated that lupinifolin had no acute toxicity when given at the maximal dose of 5 g/kg body weight in mice, and there was no toxicity when the mice were observed for further 14 days.[26] Thus, lupinifolin at its relatively low concentration of MICs against *S. mutans* and *S. aureus* (4 and 8 μg/mL, respectively) is likely to be acceptably safe. Nonetheless, an *in vivo* chronic toxicity test and clinical study are still required to assure its practical application in humans.

Lupinifolin produced significant concentration-dependent inhibitory actions against both sucrose-dependent and sucrose-independent biofilm formations of *S. mutans*. Lupinifolin at its MIC against *S. mutans* (4 μg/mL) substantially inhibited biofilm formation as expected due to its antibacterial activity. Interestingly, lupinifolin at the concentrations of sub-MIC also possessed a significant inhibitory action against *S. mutans* biofilm formation. The IC_{50s} of lupinifolin against *S. mutans* biofilm formation at 24-h incubation were 1.25 ± 0.24 and 0.27 ± 0.07 μg/mL in the presence and absence of sucrose, respectively. This indicated that lupinifolin exhibited a half-maximal inhibition against *S. mutans* biofilm formation when used at only approximately 25% (1 μg/mL) and 5% (0.2 μg/mL) of its MIC (4 μg/mL) in the presence and absence of sucrose, respectively. Our previous study reported that the ethanolic extract of *D. reticulata* stem at its sub-MICs had a significant antibiofilm formation activity against *S. mutans*.[12] The results from this study, therefore, steadily support that lupinifolin is an active phytochemical in *D. reticulata* stem acting against *S. mutans* biofilm formation. Lupinifolin at the concentration of 2 μg/mL significantly inhibited sucrose-dependent *S. mutans* biofilm formation at every incubation period. *S. mutans* biofilm formation can be divided into several stages including (1) initial attachment phase (4 h), (2) attachment phase (6 h), (3) active accumulated phase (12 h), (4) initial plateau accumulated phase (20 h), and (5) plateau accumulated phase (24 h).[27,28] Thus, lupinifolin at the low concentration of 2 μg/mL can substantially interfere with every stage of *S. mutans* biofilm formation. The lowest IC_{50} of lupinifolin (0.57 ± 0.08 μg/mL) against sucrose-dependent *S. mutans* biofilm formation was observed at 12-h incubation, in which bacterial production of extracellular polysaccharides arises. The bacterial glucosyltransferase (GTF) enzymes play a prominent role in a production of extracellular glucan, an essential extracellular matrix component in *S. mutans* biofilm. The enzymes primarily generate extracellular glucan from a sucrose substrate. Some natural products were reported to inhibit function and/or gene expression of GTFs, such as the crude extract and the methanolic fraction of *Zingiber officinale* rhizomes, the methanolic extract of *Dryopteris crassirhizoma* roots, and epigallocatechin gallate.[23,29,30] Since lupinifolin produced a substantial antibiofilm activity during the active accumulated phase of sucrose-dependent *S. mutans* biofilm formation, it may also cause an inhibition against GTFs in the same manner. However, the exact role of lupinifolin against GTFs is currently unknown and deserves to be explored further.

S. mutans can form biofilm even in the absence of sucrose. However, due to a lack of extracellular glucan production, the biofilm formed in this condition is relatively unstable and can be destroyed or washed off more easily.[31] From this study, the levels of sucrose-independent biofilm formation of *S. mutans* were very low at the incubation periods of 4, 6, and 12 h. To this extent, lupinifolin did not produce any inhibitory action against sucrose-independent *S. mutans* biofilm formation at these incubation points. Nonetheless, at the 20 and 24 incubations, lupinifolin produced a significant concentration-dependent inhibition against sucrose-independent *S. mutans* biofilm formation. The inhibitory action of lupinifolin at its MIC (4 μg/mL) against sucrose-independent *S. mutans* biofilm formation again was caused by its antibacterial activity. At the sub-MICs of 0.5, 1, and 2 μg/mL, lupinifolin significantly inhibited sucrose-independent *S. mutans* biofilm formation at 20- and 24-h incubations. At these incubation periods, the IC_{50s} of lupinifolin against sucrose-independent *S. mutans* biofilm formation were approximately 5 times lower than those in the presence of sucrose [Table 1]. The inhibitory potency of lupinifolin against *S. mutans* biofilm formation was thus greater when sucrose was omitted from the incubation. This is an agreement with the unsteady nature of sucrose-independent *S. mutans* biofilm formation as a result of a lack of extracellular glucan. Several surface proteins, especially antigen I/II, involve in sucrose-independent

S. mutans biofilm formation.[32] Some herbal extracts, such as the *Morus alba* leaf extract and the crude and methanolic fraction of *Z. officinale* rhizomes, were described to inhibit expression of antigen I/II on *S. mutans* cell surface.[23,27] The compelling antibiofilm formation activity of lupinifolin found in the absence of sucrose thus suggested that lupinifolin also possibly acts via the inhibition against antigen I/II expression. Further study is still required to justify its antibiofilm mechanisms of action in *S. mutans*.

Lupinifolin at the sub-MICs also significantly inhibited against *S. aureus* biofilm formation. The lowest IC_{50} of lupinifolin (0.22 ± 0.03 μg/mL) was achieved at the incubation period of 6 h, in which the attachment phase of *S. aureus* occurred. At the incubation times of 6, 8, and 24 h, the antibiofilm formation activity of lupinifolin was present significantly even at the considerably low concentrations of 1/4MIC (2 μg/mL) and 1/2MIC (4 μg/mL). Sianglum *et al.* previously reported the antibiofilm formation activity of lupinifolin against *S. aureus* ATCC25923.[20] However, the inhibitory action of lupinifolin against *S. aureus* biofilm formation shown in their study was substantially lower than that found in this study, with the % inhibition of only approximately 20% at 24-h incubation. Moreover, no inhibitory action against *S. aureus* biofilm formation was detected at the lupinifolin concentration of 1/4 MIC in their study. A difference in *S. aureus* strains is likely to be a major reason for this discrepancy. It was demonstrated that diverse strains of *S. aureus* possess different biofilm-forming capabilities.[33] In addition, bacterial culture conditions, nutrient availability, and polysaccharide intercellular adhesin (PIA)-producing ability can also influence on the biofilm formation capacity of *S. aureus*.[34,35] Several surface proteins, such as autolysin, biofilm-associated protein (Bap), fibronectin-binding protein (FnBPs), are likewise essential for biofilm formation capability of *S. aureus*.[36] Furthermore, the existence of efflux pumps was also documented to be linked with *S. aureus* biofilm formation.[37] It is currently unknown whether lupinifolin performed its antibiofilm formation activity via interrupting the expressions and/or functions of these crucial determinants in *S. aureus*. Several flavonoids were described to inhibit *S. aureus* biofilm formation activity.[38-40] It was reported that the antibiofilm formation activity of aglycone flavonoids was more potent than that of its glycone counterparts.[38] The presence of O-glycosidic bond in glycone flavonoids was proposed to hinder the binding of the flavonoids to its site of action on bacterial cell surface. An aglycone structure of lupinifolin thus possibly contributed to its potent inhibitory action against *S. aureus* biofilm formation.

From our study, lupinifolin exhibited its inhibitions against *S. mutans* and *S. aureus* biofilm formations even at the low concentration of sub-MICs, in which no antibacterial activity arrived. This evidence strongly suggested that lupinifolin can potentially be developed further as an antibiofilm agent for the prevention and/or treatment of *S. mutans* and *S. aureus* biofilm-related diseases, especially dental caries (*S. mutans*) and indwelling medical device infections (*S. aureus*). However, further experiments exploring the mechanisms of its antibiofilm formation activity and an *in vivo* biofilm formation study are still required to justify its promising clinical use.

CONCLUSION

Lupinifolin derived from *D. reticulata* stem inhibited the growth of both *S. mutans* and *S. aureus* with the MICs of 4 and 8 μg/mL, respectively. Lupinifolin at the sub-MICs produced a significant inhibitory action against both *S. mutans* (sucrose-dependent and sucrose-independent fashions) and *S. aureus* biofilm formations. These results convincingly indicated that lupinifolin has a promising role as a natural product-derived antibiofilm-forming agent for the prevention and/or treatment of biofilm-associated bacterial infection in the future.

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Conflicts of interest

There are no conflicts of interest.

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