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Antibiofilm formation activity of lupinifolin isolated from *Derris reticulata* stems against *Enterococcus faecalis*

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ABSTRACT

Introduction: The biofilm formation capability of *Enterococcus faecalis* is one of the key virulence factors contributing to its multidrug resistance and therapeutic challenges. Lupinifolin, a prenylated flavanone extracted from the stems of *Derris reticulata* Craib., possesses antibacterial properties against a range of gram-positive cocci. This study aimed to investigate the effects of lupinifolin alone or in combination with vancomycin against *E. faecalis* biofilm formation at different incubation durations, presumably representing different biofilm stages.

Methods: The crystal violet biofilm formation assay and the micro-broth dilution method were used to measure the antibiofilm and antibacterial activities, respectively.

Results: Lupinifolin did not inhibit *E. faecalis* growth at the highest concentration tested, i.e., minimum inhibitory concentration (MIC) > 128 µg/mL. However, lupinifolin at the sub-MICs (16-128 µg/mL) possessed a significant inhibition against *E. faecalis* biofilm formation at every incubation time of 6, 12, 24, and 36 hours. Lupinifolin had the lowest median inhibitory concentrations (IC_{50}) of 6.78 ± 3.04 µg/mL after incubation for 6 hours. Thus, the antibiofilm action of lupinifolin was most likely produced predominantly at the first stage of biofilm formation, known as initial surface attachment. The sub-MICs of lupinifolin also significantly enhanced the antibiofilm activity of vancomycin at relatively low concentrations of ¼ MIC (2 µg/mL) and $^1/_8$ MIC (1 µg/mL), specifically at 6- and 36-hour incubation ($P<0.05$).

Conclusion: These results point to lupinifolin's potential use against *E. faecalis* as an antibiofilm agent. Nonetheless, more research is needed to identify the exact antibiofilm mechanism of lupinifolin.

Implication for health policy/practice/research/medical education:
This work offers empirical support for the antibiofilm action of lupinifolin at its sub-MICs against *E. faecalis* during all incubation periods up to 36 hours. Lupinifolin and vancomycin also had synergistic antibiofilm effects at their respective sub-MICs. These results suggest that lupinifolin may be used as an anti-virulence agent to prevent the production of biofilms by *E. faecalis*.

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Introduction

The human gastrointestinal tract is a common habitat for commensal gram-positive bacteria like *Enterococcus faecalis* (1). *E. faecalis* is a non-pathogenic commensal gut species that is present in healthy persons and is even present in breastfed infants (2). In recent decades, *E. faecalis* has become a significant pathogen that is resistant to multiple drugs when it comes to susceptible hosts (3,4). Life-threatening nosocomial infections, including surgical site infections, endocarditis, urinary tract infections, bacteremia, and dental root canal infections, can be brought on by this type of enterococcus. When treating a susceptible *E. faecalis* infection, ampicillin is the preferred medication; in contrast, vancomycin should be used when treating infections caused by strains of bacteria that have significant levels of penicillin resistance (5). Nonetheless, the rising incidence of vancomycin-resistant enterococci (VRE) is concerning and presents a risk to public health. The incidence of antibiotic-resistant *E. faecalis* infections, associated with high mortality rates and limited therapeutic options, has been increasing globally (6). The capacity of *E. faecalis* to produce biofilms is one of the key mechanisms that contribute to its multidrug resistance (1,7,8). It was discovered that enterococci growing as biofilms were

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more tolerant to several antibacterial drugs, such as vancomycin, ampicillin, linezolid, and tigecycline, than their planktonic forms (9,10). The biofilm matrix severely limits the ability of antibacterial medications and host immune cells to penetrate. Persister cells inside the biofilm are another factor that leads to antibacterial tolerance and persistent infections. Additionally, enterococcal biofilms help spread antibiotic-resistant genes within and between different microbial species. Urinary tract infections linked to catheter use, endocarditis, and infections of the skin and soft tissues are among the biofilm-associated illnesses brought on by enterococci (1). Currently available antimicrobial medications used in clinical settings are often ineffective in eliminating enterococcal biofilms.

Bacterial cells in biofilms are not only resistant to antibacterial drugs, but they also can evade the host's immunological response. Furthermore, the antibiotics that are currently on the market are insufficient to treat infections linked to biofilms due to requirement of a higher dose, which may result in toxicity (8). Consequently, the sole available treatment option is to remove biofilm-forming medical equipment; unfortunately, this is not always feasible (7). Novel treatments are thus still required to address these notorious clinical issues. Drugs that target enterococcal biofilms, either by inhibiting biofilm development or inducing biofilm eradication, are promising options for combating biofilm-associated infection (8). Unfortunately, these antibiofilm agents are not yet clinically available. Phytochemicals obtained from medicinal plants are a great source of possible therapeutics that block important virulence factors of pathogenic microbes, like the production of biofilms.

Several medicinal plants, such as *Derris reticulata* Craib., *Myriopteron extensum*, *Eriosema chinense*, and *Albizia myriophylla* can yield lupinifolin, a prenylated flavanone (11-14). Methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA), *E. faecalis, E. faecium, Staphylococcus epidermidis, Bacillus subtilis, B. cereus*, and *Streptococcus mutans* are the gram-positive bacteria that lupinifolin has been shown to have antibacterial activity against (14-19). Lupinifolin's minimum inhibitory concentrations (MICs) against various gram-positive bacteria were recorded at comparatively modest levels of 1–16 μg/mL. It has also been documented that lupinifolin, at sub-MICs, inhibits the ability of MRSA, MSSA, *S. mutans*, and clinical isolates of *Enterococcus* species to form biofilms (18,20-22). Bacterial biofilm growth generally occurs in four stages: initial adhesion, microcolony production, biofilm maturation, and biofilm dispersal (1). In our previous study, lupinifolin showed concentration- and time-dependent antibiofilm activity against *S. mutans* and MSSA at its sub-MICs (21). Nevertheless, lupinifolin's antibiofilm activity against *E. faecalis* has not been investigated at varied incubation times, which likely correspond to distinct biofilm phases. The purpose of this work was to examine the effects of lupinifolin on the production of *E. faecalis* biofilms at varying incubation times, either in isolation or in combination with vancomycin.

Materials and methods

Study area

From August 2023 to January 2024, the experiments were conducted at Mahasarakham University's Faculty of Pharmacy in Thailand.

Isolation of lupinifolin from *D. reticulata* Craib. stems

Lupinifolin, isolated from *D. reticulata* Craib. stems, were acquired from our prior study and used in this experiment (20). The method used for lupinifolin isolation was described in detail in our previous publication (23). Concisely, hexane (400 mL) was used to conduct a Soxhlet extraction on the 60 g ground sample. After filtering the extract, it was heated to 65 °C until a clear yellow extract was obtained. Using a rotary evaporator, the hexane extract was evaporated until the turbid suspension was visible. The extract was stored at room temperature to facilitate crystallization. Before using the purified lupinifolin in the experiment, it was stored at -20 °C.

MIC determination

The MIC was ascertained using the modified microbroth dilution method in accordance with the Clinical and Laboratory Standards Institute's (CLSI's) standards (24). The 2-fold serial dilutions were made in sterile deionized water for vancomycin (Sigma-Aldrich®, V2002), and ampicillin (Sigma-Aldrich®, A8351) and in 0.1M NaOH for lupinifolin. The culture collection center of the Thailand Institute of Scientific and Technological Research provided *Enterococcus faecalis* (TISTR 379). In Tryptic Soy Broth (TSB), *E. faecalis* suspensions were made at a concentration of 1.5×10^6 CFU/mL. The sample or its solvent (20 μL), *E. faecalis* suspension (50 μL), and TSB (130 μL) were mixed in each well of the 96-well microplate. The lowest concentration of the test drug that results in no discernible bacterial growth during a 24-hour incubation period at 37 °C was identified as the MIC. The MIC was calculated as a median using data from at least five separate studies.

Biofilm formation assay

With some slight adjustments as mentioned in our previous works (20,22,25), the crystal violet biofilm assay was conducted in accordance with the report of Hasan et al. In a mixture of 50 μL of *E. faecalis* suspension (1.5×10^6 CFU/mL) and TSB supplemented with 0.5% glucose (130 μL), various concentrations of the tested agents, lupinifolin, ampicillin, or vancomycin, (10 or 20 μL) were applied. By removing the bacterial suspension, blank wells with identical concentrations of the test agents were performed. The media containing planktonic bacterial cells was carefully removed from the microplate by gently

decanting it after the designated incubation period of 6, 12, 24, or 36 hours at 37 °C. Each well was incubated for 15 minutes with 200 µL of formalin (37%, diluted 1:10) together with 2% sodium acetate to fix the adhering biofilm mass. Next, 100 µL of 0.1% crystal violet solution was added to each well to stain the fixed biofilm. The biofilm-bound dye was dissolved by pipetting 120 µL of 95% ethanol into the microplate wells following three 300 µL washes with sterile deionized water. Following the transfer of the mixture (80 µL) to a new 96-well microplate, the optical density (OD) of the mixture was determined at 600 nm. The following formula was used to calculate the antibiofilm formation activity, which was reported as %inhibition of biofilm formation:

$$[(OD_{600}\ vehicle - OD_{600}\ sample) / (OD600\ vehicle)] \times 100.$$

The appropriate blank's OD was deducted from the optical densities of the vehicle and sample, respectively, to get the OD_{600} vehicle and OD_{600} sample. To calculate the median inhibitory concentration (IC_{50}) and generate the concentration-inhibitory curve, GraphPad Prism version 8.0 was employed.

Statistical analysis

The data was presented as median (MIC), mean ± SEM (%inhibition of biofilm formation), or mean ± SD (IC_{50}). The results on the inhibition of biofilm formation were statistically analyzed using a two-way analysis of variance (ANOVA) and pairwise comparison. A difference was considered significant if the *P* value was less than 0.01. Simultaneously, one-way ANOVA and the Bonferroni post-hoc test were used to assess the IC_{50} results; a *P* value of less than 0.05 indicates a statistically significant difference.

Results

Antibacterial activity of lupinifolin, ampicillin, and vancomycin against *Enterococcus faecalis*

Enterococcus faecalis (TISTR 379) growth was inhibited by ampicillin and vancomycin, with MICs of 4 and 8 µg/mL, respectively. However, lupinifolin did not produce an antibacterial activity against *E. faecalis* (TISTR 379) when tested at the maximum concentration of 128 µg/mL (MIC > 128 µg/mL, n=10).

Antibiofilm formation activity of lupinifolin, ampicillin, and vancomycin against *Enterococcus faecalis*

The IC_{50s} of lupinifolin against *E. faecalis* biofilm formation at every incubation time were lower than that of its MIC (>128 µg/mL) (Table 1). With an IC_{50} of 6.78 ± 3.04 µg/mL (n = 8), the maximum inhibitory effectiveness against the development of biofilms was seen after 6 hours of incubation (Table 1). Lupinifolin at the sub-MICs of 16, 32, 64, and 128 µg/mL significantly inhibited *E. faecalis* biofilm formation at every incubation period (6, 12, 24 and 36 hours) ($P<0.01$; n=7-8) (Figure 1A). At a concentration of 32 µg/mL, lupinifolin exhibited the highest inhibitory activity at a 6-hour incubation period, with a percentage inhibition of biofilm formation of 116.60 ± 4.18 ($P<0.01$; n=8). Lupinifolin at the concentration of 8 µg/mL also significantly inhibited biofilm formation at 6 and 36-hour-incubation periods. Lupinifolin did not significantly suppress the production of biofilms at the lower tested concentrations (2 and 4 µg/mL). Additionally, when incubated for 12 and 24 hours, respectively, lupinifolin at these two concentrations greatly boosted biofilm development.

The IC_{50s} of ampicillin against *E. faecalis* biofilm formation at every incubation time were lower than that of its MIC (4 µg/mL) (Table 1). At 36-hour incubation, ampicillin produced the highest inhibitory potency against biofilm formation with the IC_{50} of 0.49 ± 0.05 µg/mL (n=8) (Table 1). However, the IC_{50s} of ampicillin were not significantly different between incubation periods according to the statistical analysis. Biofilm development was strongly suppressed at all incubation periods (6, 12, 24, and 36 hours) ($P<0.01$; n=5-8) by ampicillin at concentrations of 4 (MIC) and 8 (2MIC) µg/mL (Figure 1B). Ampicillin at the sub-MICs of 1 and 2 µg/mL also produced significant inhibition against biofilm formation at every incubation period ($P<0.01$; n=5-8) (Figure 1B). The maximal antibiofilm activity of 102.75 ± 0.63 ($P<0.01$; n=8) was found with 2 µg/mL ampicillin at 6-hour incubation. A significant increase in biofilm development was seen at a 6-hour incubation period with 0.5 µg/mL ampicillin, resulting in a percentage inhibition of -111.55 ± 40.21 ($P<0.01$; n=8). With a % reduction of biofilm

Table 1. Median inhibitory concentrations (IC_{50s}; µg/mL) of lupinifolin, ampicillin, and vancomycin against *Enterococcus faecalis* (TISTR379) biofilm formation at various incubation periods

Incubation time (hours)	Lupinifolin		Ampicillin		Vancomycin	
	IC_{50} (mean ± SD)	n	IC_{50} (mean ± SD)	n	IC_{50} (mean ± SD)	n
6	6.78 ± 3.04	8	1.27 ± 1.01	8	3.27 ± 2.21	8
12	14.03 ± 4.24	8	1.31 ± 0.80	8	3.22 ± 1.38	8
24	25.59 ± 7.60*	7	0.57 ± 0.10	5	7.16 ± 4.09#	5
36	14.85 ± 8.50	8	0.49 ± 0.05	8	4.30 ± 1.22	8

*$P<0.05$ when compared with the IC_{50s} of lupinifolin at 6, 12 and 36 hour-incubation; #$P<0.05$ when compared with the IC_{50s} of vancomycin at 6 and 12 hour-incubation (SD = standard deviation).

formation of -53.97 ± 9.77 ($P<0.01$; n = 5), ampicillin at a concentration of 0.25 µg/mL also significantly increased biofilm formation at 24-hour incubation (Figure 1B).

The IC_{50s} of vancomycin against *E. faecalis* biofilm formation were lower than its MIC (8 µg/mL) at all incubation times (Table 1). However, the IC_{50} of vancomycin at 24-hour incubation was significantly higher than those at 6- and 12-hour incubation periods. At all incubation times, biofilm formation was markedly reduced by vancomycin at doses of 8 (MIC) and 16 (2MIC) µg/mL (Figure 1C). The maximum inhibitory action of 101.27 ± 0.63 % (n = 8) was found with 16 µg/mL vancomycin at 6-hour incubation. Biofilm development was strongly suppressed by vancomycin at a sub-MIC of 4 µg/mL during the incubation periods of 6, 12, and 36 hours (Figure 1C). At all incubation times, vancomycin at the other sub-MICs examined did not significantly alter the production of biofilms.

Antibiofilm formation activity of lupinifolin in combination with vancomycin against *Enterococcus faecalis*

Vancomycin at 1 µg/mL did not prevent biofilm formation in any of the studied incubation times. Nevertheless, after 6-hour and 36-hour incubation, the combination of lupinifolin (4 and 8 µg/mL) and vancomycin (1 µg/mL) dramatically reduced the production of *E. faecalis* biofilm. The combined antibiofilm actions were significantly greater than those of vancomycin (1 µg/mL) used alone. The highest inhibition of 52.30 ± 7.26% (n=6, $P<0.01$)

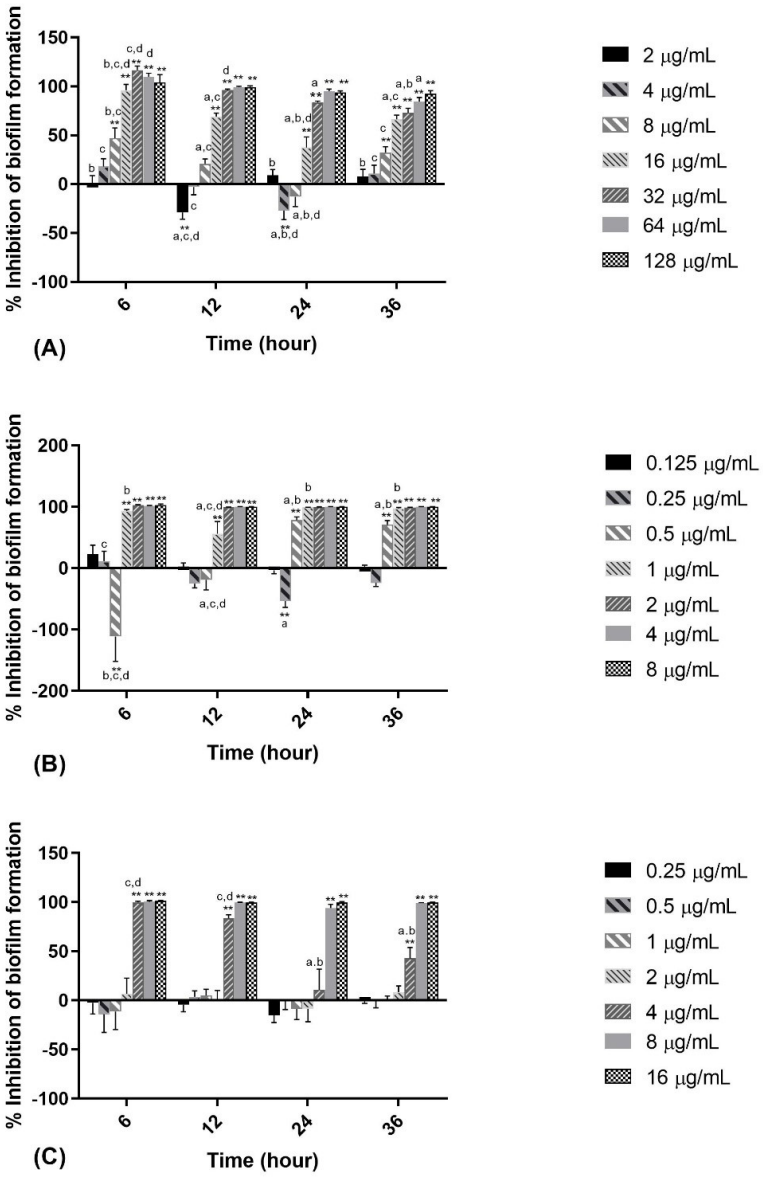

Figure 1. The effects of lupinifolin (A), ampicillin (B), and vancomycin (C) on biofilm formation of *Enterococcus faecalis* (TISTR379) at various incubation periods. ***P*<0.01 when compared with the negative control; [a,b,c,d] *P*<0.01 when compared with the same concentration tested at 6, 12, 24 and 36-hour incubation, respectively (mean ± SEM, n=7-8) (Pairwise comparison of two-way ANOVA).

was obtained when lupinifolin (8 µg/mL) and vancomycin (1 µg/mL) were combined at 6 hours (Figure 2A). The addition of lupinifolin did not significantly alter the effects of vancomycin (1 µg/mL) at the 12- or 24-hour incubation times.

At every incubation period, vancomycin at a dose of 2 µg/mL did not significantly alter the production of *E. faecalis* biofilms. The combination of vancomycin (2 µg/mL) with lupinifolin (2, 4, and 8 µg/mL) effectively suppressed the formation of *E. faecalis* biofilms at 6 hours of incubation (Figure 2B). The percentage inhibitions were 34.40 ± 3.14, 48.37 ± 3.55, and 73.87 ± 5.57, respectively (n=6, $P<0.01$). These levels of biofilm inhibition were significantly higher than those of vancomycin (2 µg/mL) alone. Significant inhibition of biofilm formation was also observed at 12- and 36-hour incubation when lupinifolin (8 µg/mL) and vancomycin (2 µg/mL) were combined; the percentage inhibitions were 39.28 ± 8.02 and 56.55 ± 7.37, respectively (n=6, $P<0.01$). On the other hand, the addition of lupinifolin did not alter the effects of vancomycin (2 µg/mL) over 24 hours.

Vancomycin at the concentration of 4 µg/mL alone significantly inhibited *E. faecalis* biofilm formation when incubated for 6 and 12 hours with the % inhibitions of 99.73 ± 1.26 and 83.39 ± 3.69, respectively (n=8, $P<0.01$) as shown in Figure 2C. These antibiofilm activities of vancomycin (4 µg/mL) at 6- and 12-hour incubation were significantly higher than their action at 24- and 36 hours. At 6 and 12 hour-incubation periods, the combination of vancomycin and lupinifolin did not change the biofilm inhibitory action when compared to those of vancomycin alone. At the concentration of 4 µg/mL, vancomycin did not significantly prevent the production of biofilms by *E. faecalis* after 24 hours. But after 24 hours, the combination of vancomycin (4 µg/mL) with lupinifolin (2, 4, and 8 µg/mL) greatly reduced the amount of biofilm that *E. faecalis* formed. At 24 hours, the combination of lupinifolin (8 µg/mL) and vancomycin (4 µg/mL) resulted in the maximum inhibition of 87.93 ±1.75% (Figure 2C). When lupinifolin (8 µg/mL) was added, the amount of biofilm inhibition was further raised significantly, with the % inhibition of 92.37 ± 1.05 (n=6, $P<0.01$), even though vancomycin (4 µg/mL) alone significantly prevented *E. faecalis* biofilm formation at 36 hour-incubation (42.90 ± 11.06 %, n=8).

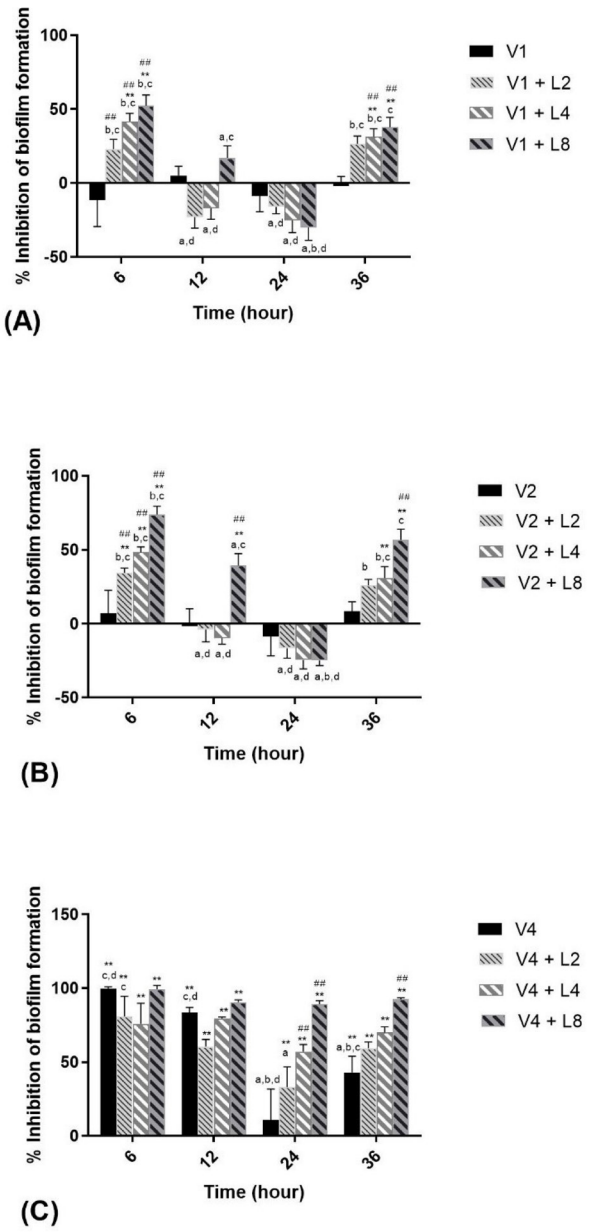

Figure 2. The effects of lupinifolin (2, 4, and 8 µg/mL) in combination with vancomycin at the concentration of 1 µg/mL (A), 2 µg/mL (B), and 4 µg/mL (C) on biofilm formation of *Enterococcus faecalis* (TISTR379). L=lupinifolin, V=vancomycin; followed by its concentration in µg/mL. $^{*}P<0.05$ and $^{**}P<0.01$ when compared with the negative control; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ when compared with vancomycin alone; $^{a,b,c,d}P<0.01$ when compared with the same combination tested at 6-, 12-, 24- and 36-hour incubation, respectively (mean ± SEM, n=6) (pairwise comparison of two-way ANOVA).

Discussion

Ampicillin and vancomycin had antibacterial activity against *E. faecalis* (TISTR 379) with MICs of 4 and 8 µg/mL, respectively. As per the MIC breakpoints established by the CLSI, enterococci are considered susceptible to ampicillin and vancomycin if the MICs are less than or equal to 8 and 4 µg/mL, respectively (24). Meanwhile, the intermediate breakpoint is indicated when the MIC of vancomycin is at 8-16 µg/mL (24). Therefore, the *E. faecalis* strain used in this study was susceptible to ampicillin but its sensitivity to vancomycin was intermediate. Lupinifolin had no antibacterial activity against *E. faecalis* (TISTR 379) when the maximum concentration of 128 µg/mL was tested. Sianglum et al reported that lupinifolin exhibited growth inhibition against the clinical strains of *E. faecalis* and *E. faecium*, with MICs of 0.5-2 µg/mL. The disparity in findings was probably caused by a variation in the bacterial strains that were employed. Additionally, the sensitivity of the bacteria may also be partially impacted by variations in the media that are employed (26,27).

The bacterial culture media used in this work was TSB, whereas Sianglum et al, used Muller-Hilton Broth (MHB). The MICs of essential oils against *S. aureus* cultured in TSB and MHB were found to differ by 122-138 μg/mL (27). The components and characteristics of the bacterial cell membrane have been revealed to be influenced by the cultured media used (28). Lupinifolin's antibacterial activity has been proven against a variety of gram-positive pathogenic bacteria, such as MRSA, MSSA, *S. mutans, E. faecium*, and *E. faecalis* by disrupting their cell membranes (14,16-18). Thus, a change in growth media, which alters the composition of bacterial cell membranes may partly affect lupinifolin's antibacterial activity. Future research should examine how culture media affect the antibacterial activity of lupinifolin.

Even at the maximum concentration of 128 μg/mL, lupinifolin did not exhibit any antibacterial action; nevertheless, it did significantly inhibit the production of *E. faecalis* biofilms during the whole incubation period. The IC_{50s} of lupinifolin against *E. faecalis* biofilm formation were essentially lower than its MIC. Lupinifolin exhibited the lowest IC_{50} of only 6.78 ± 3.04 g/mL at 6-hour incubation. The antibiofilm action of lupinifolin was observed throughout the incubation period examined for up to 36 hours. However, its inhibition against biofilm formation was less pronounced at 24 hour-incubation, with the IC_{50} of 25.59 ± 7.60 μg/mL. Biofilm maturation, defined by considerable extracellular polymeric material formation and bacterial growth, most likely occurs after a 24-hour incubation period (1). Lupinifolin may have a comparatively minor effect on the biofilm maturation stage of biofilm development. To ascertain whether lupinifolin impacts the exopolysaccharide and eDNA, two main components of the extracellular polymeric substance of the *E. faecalis* biofilm, more future investigation is necessary. It is most likely that lupinifolin's antibiofilm effect was mostly generated at the first surface attachment stage of *E. faecalis* biofilm development. This finding aligns with our earlier research, which demonstrated that lupinifolin's antibiofilm action against MSSA peaked at the 6-hour incubation period, when biofilm formation was only getting started (21). Both biotic and abiotic surfaces are susceptible to enterococcal surface attachment, particularly those found inside medical devices such as orthopedic implants, artificial heart valves, and catheters (1). The surface attachment stage involves a number of bacterial components, such as cell wall-anchoring enzymes and surface proteins. It was discovered that the endocarditis and biofilm-associated pilus (Ebp) and sortase C (SrtC) enzyme are necessary for *E. faecalis* surface attachment and biofilm formation (29). It has been shown that cell wall attachment and Ebp assembly depend on SrtC. Sortases are transamidase enzymes that attach to the proteins containing a C-terminal LPXTG-like motif to bacterial peptidoglycan by covalent bonding (30). Adhesin to collagen from *E. faecalis* (Ace), aggregation substance (Agg), biofilm-associated glycolipid synthesis A (BgsA), and enterococcal surface protein (Esp) are other critical surface adhesins needed for *E. faecalis* surface attachment (31-34). As a result, a number of surface proteins contribute to the formation of enterococcal biofilms.

Since sortase enzyme is readily available in the bacterial cell membrane and is not necessary for bacterial growth, sortase enzyme inhibition has been proposed as a potential target for antivirulence medicines against gram-positive bacteria that are resistant to several drugs (35). Sortase from a variety of gram-positive pathogenic bacteria is inhibited by a number of flavonoids, including prenylated flavanones (36,37). Kurarinol, a trihydroxyflavanone that was extracted from *Sophora flavescens* roots, showed a significant inhibition of *S. aureus* sortase, with an IC_{50} of 107 μM (38). It has also been observed that eriodictyol (3,4,5,7-tetrahydroxyflavanone) inhibits *S. aureus* sortase more effectively (IC_{50} = 7.73 M) (39). Remarkably, eriodictyol greatly reduced the formation of *S. aureus* biofilms but did not affect bacterial viability (39). The results of this investigation, which showed that lupinifolin exhibited strong antibiofilm activity without preventing *E. faecalis* growth, are thus consistent with those of Wang and colleagues' results. Naturally occurring substances that inhibit the production of biofilms without compromising bacterial viability, like lupinifolin, may be superior to traditional antibiotics because they exert less selective pressure on evolution and lessen the chance that resistant genes would arise (36).

There are currently few investigations on how phytochemicals affect the sortases and surface proteins unique to *E. faecalis*. According to *in vitro* binding tests and *in silico* docking, certain phytochemicals, such as curcumin, berberine, and myricetin, have a high binding affinity for *E. faecalis* sortase A (EfSrtA) (40). To find out if the significant antibiofilm effect of lupinifolin at the 6-hour incubation was also caused by an inhibition of *E. faecalis* sortase and consequently resulted in the suppression of several bacterial surface protein expressions and functions, more tests should be conducted. *E. faecalis* sortase A is occupied by benzylpenicillin and cefotaxime at the same binding region where the natural substrate's LPXTG motif binds from the *in silico* docking experiment (41). Sortase A inhibitory effects of these medications may stop *E. faecalis* from developing biofilms. Ampicillin at various sub-MICs exerted a significant inhibition against *E. faecalis* biofilm formation in this study. The IC_{50s} of ampicillin against *E. faecalis* biofilm inhibition were consistent across the incubation times examined and substantially lower than its MIC. Benzylpenicillin and ampicillin are in the same β-lactam antibiotic class of β-lactamase-labile penicillins. Since they have nearly identical chemical structures, ampicillin may also bind and inhibit *E. faecalis* sortase similar to that of benzylpenicillin.

Enterococcus faecalis biofilm production was significantly increased by ampicillin at doses of 0.25

and 0.5 μg/mL, particularly during 24- and 6-hour incubation, respectively. Similarly, at low concentrations of 2 and 4 μg/mL, lupinifolin induced *E. faecalis* biofilm formation after 12 and 24 hours, respectively. This agrees with the earlier findings which reported that the effects of some β-lactam antibiotics, including ampicillin, methicillin, and cloxacillin, on MRSA biofilm formation were biphasic response with a biofilm stimulation at the low concentration but an antibiofilm action at the high concentration (22,42). Sub-MIC doses of methicillin have been shown to promote the formation of MRSA biofilms by inducing the release of extracellular DNA (eDNA) in an autolysin-dependent manner (42). The potential for biofilm formation brought on by bacterial stress to persist in the presence of antibacterial drugs at sub-MICs (43). Therefore, it is necessary to employ the appropriate concentrations of ampicillin and lupinifolin to prevent the generation of biofilm development. The mechanism behind the *E. faecalis* biofilm production of ampicillin and lupinifolin found in this work requires more investigation.

Following their initial attachment to surfaces, bacteria proliferate and produce minute quantities of biofilm matrix to create aggregates referred to as microcolonies. It is uncertain, nevertheless, which particular enterococcal elements regulate the establishment of microcolonies (1). The next stage of biofilm development is called biofilm maturation, and it calls for the active synthesis and expansion of extracellular matrix constituents such as lipoteichoic acid, polysaccharides, and eDNA. eDNA is the matrix component of enterococcal biofilm that has been studied the most (1). It was shown that the autolysis and release of eDNA are regulated by both serine protease (SprE) and gelatinase (GelE), and that this helps *E. faecalis* form biofilms (44). However, early biofilm formation in *E. faecalis* was observed to produce eDNA by live cells without the need for cell lysis (45). Numerous phytochemicals have been shown to prevent *E. faecalis* from producing the components of the biofilm matrix (46-49). Plant-derived quercetin, a flavonol, has shown antibiofilm action against *E. faecalis* by interfering with multiple biofilm formation pathways, such as the glycolytic, protein translation-elongation, and folding pathways (48). Within biofilms generated by *E. faecalis* and/or *Candida albicans*, luteolin (3′,4′,5,7-tetrahydroxyflavone) at doses of ¼ MIC and ½ MIC dramatically reduced the biofilm matrix components, including proteins, polysaccharides, and eDNA (49). Trans-cinnamaldehyde, the main phytochemical found in cinnamon essential oil, inhibited the growth of *E. faecalis* biofilms and decreased their exopolysaccharide content at sub-MIC levels. Additionally, it suppressed the *fsr* locus and the downstream gene, *gelE*, of the biofilm-associated quorum sensing pathway, which is implicated in eDNA release and the development of biofilms (47). Two terpenoid derivatives, rhodrin and rubrivivaxin, notably reduced the development of *E. faecalis* biofilms and exopolysaccharide synthesis (46). Significant antibiofilm activity was developed by lupinifolin at sub-MICs after 12 and 24 hours of incubation. Consequently, it might have prevented the synthesis of extracellular matrix during the later phases of biofilm formation, referred to as microcolony formation and biofilm maturation. Further investigation is required to ascertain whether lupinifolin also impacts the extracellular components of the *E. faecalis* biofilm, namely exopolysaccharide as well as eDNA. The antibiofilm activity of lupinifolin at the sub-MICs was demonstrated across all incubation durations up to 36 hours, where the biofilm dispersal was anticipated to begin. The final stage of the biofilm development process, known as biofilm dispersal, is when the biofilm structure disintegrates and individual bacterial cells separate from the biofilm to resume their planktonic existence (50). In order to colonize a new area, the dislodged planktonic cells have the ability to move and attach. Another potential target for biofilm control is an activation of biofilm dispersal. *E. faecalis* biofilm dispersal has been linked to certain phytochemicals (49,51). By downregulating sortase A and Esp, berberine was reported to disperse the biofilms of *E. faecalis* UTI isolates (51). It was noted that luteolin considerably dispersed *E. faecalis* biofilm that had already been produced (49). In the preformed biofilm of *E. faecalis*, it was shown that chitosan-propolis nanoparticle formulation physically disrupted the biofilm structure and reduced the quantity of bacteria (52). To determine whether lupinifolin also facilitates the spread of *E. faecalis* biofilms, more research is necessary.

Ampicillin and vancomycin at their concentrations of MIC and 2 MIC significantly inhibited *E. faecalis* biofilm formation at every incubation time. It was anticipated that ampicillin and vancomycin's ability to limit bacterial growth would give rise to their antibiofilm activities. At every stage of the incubation process, ampicillin at ½ MIC (2 μg/mL) and ¼ MIC (1 μg/mL) could greatly inhibit the formation of biofilms. At 1/8 MIC (0.5 μg/mL), ampicillin also significantly produced antibiofilm activity at 24 and 36 hours. Since the bacteria's viability was unaffected at these sub-MICs of ampicillin, it is plausible that the antibiofilm activity of ampicillin at these sub-MICs resulted from its direct effect against the biofilm-forming process. This result was consistent with a previous study, which found that after a 24-hour incubation period, sub-MICs of ampicillin reduced the creation of biofilm by *E. faecalis* and downregulated the expression of biofilm-linked genes (53). At 6, 12, and 36 hours, vancomycin at ½ MIC (4 μg/mL) dramatically reduced the production of *E. faecalis* biofilms. Nevertheless, at any incubation period, vancomycin at ¼ MIC (2 μg/mL) and 1/8 MIC (1 μg/mL) could not prevent the formation of biofilms. This is consistent with the research by de Moura et al, which demonstrated that the formation of *E. faecalis* biofilms was unaffected by vancomycin at its sub-MICs (54).

Since vancomycin at the concentrations of ¼ MIC and 1/8 MIC did not have an antibiofilm activity against

E. faecalis, the subsequent experiment was conducted to determine whether lupinifolin enhances the biofilm inhibition of vancomycin. Vancomycin's antibiofilm action at ¼ MIC (2 µg/mL) and 1/8 MIC (1 µg/mL) was greatly enhanced by lupinifolin at its sub-MICs (2, 4, or 8 µg/mL), particularly during the 6-hour incubation period. The antibiofilm activity of lupinifolin was most potent at 6 hours with the lowest IC_{50} of 6.78 ± 3.04 µg/mL. Therefore, these sub-MICs of lupinifolin may have enhanced the antibiofilm effects of vancomycin mainly by disrupting the bacterial surface attachment, which is the early stage in the development of biofilms. The synergistic effect of lupinifolin and vancomycin (1 or 2 µg/mL) was also observed at 36-hour incubation. Therefore, these combinations may influence the late stage of biofilm formation by inducing biofilm dispersal at a 36-hour period. However, it should be noted that the synergistic antibiofilm effect of the combination of lupinifolin and ¼ MIC or 1/8 MIC of vancomycin was not observed at 24-hour incubation, where the biofilm maturation apparently arises. When the higher concentration of vancomycin at ½ MIC (4 µg/mL) was employed in combination with lupinifolin, the synergistic antibiofilm activity was found only at 24- and 36-hour incubation which presumably represents biofilm maturation and subsequent biofilm dispersal. Nonetheless, 4 µg/mL of vancomycin by itself already demonstrated significant antibiofilm activity at 6- and 12-hour incubation. It was therefore probable that the synergistic antibiofilm activity of vancomycin and lupinifolin would depend on both concentration and time. To precisely understand the mechanism underlying the synergistic antibiofilm activity of vancomycin and lupinifolin, more research needs to be done.

Specific flavonoids can augment the efficacy of select antimicrobial medications in inhibiting the formation of gram-positive bacteria's biofilms (55-57). Nonetheless, there is relatively little data to support the antibiofilm effectiveness of phytochemicals when used in conjunction with antibacterial drugs to reduce the growth of *E. faecalis* biofilm. Thymol was found to increase rifampicin's antibacterial and biofilm-eradication capabilities against MRSA and decrease the formation of persister cells (55). Our earlier research demonstrated that ampicillin, cloxacillin, and vancomycin's antibiofilm action against MRSA was considerably enhanced by sub-MICs of lupinifolin (4 and 8 µg/mL) (22). Since vancomycin and lupinifolin did not show a synergistic effect on MRSA growth (FIC index of 0.75), the antibiofilm action of the combination was most likely caused by its direct impact on the MRSA biofilm formation process (19). Epigallocatechin-3-gallate (EGCG, 0.3 µg/mL) greatly improved the antibacterial activity of cationic peptides (KR-12-a5, 0.6 µg/mL) against biofilms of bacteria associated with endodontic infections, including *E. faecalis* (56). It was observed that apigenin by itself did not significantly reduce the biomass of the *E. faecalis* biofilm; however, when apigenin was combined with reduced graphene oxide, a considerable reduction in biofilm biomass was found (57). According to the current findings, lupinifolin has joined EGCG and apigenin as potential natural product-derived antibiofilm enhancers against *E. faecalis*.

According to this study, there was a considerable antibiofilm activity against *E. faecalis* by the sub-MICs of lupinifolin, either by itself or in combination with the sub-MICs of vancomycin. This work reported the first evidence of lupinifolin and vancomycin's synergistic antibiofilm activity. Whether administered alone or in combination with vancomycin, lupinifolin's antibiofilm action was concentration- and time-dependent. Consequently, for the antibiofilm activity to occur, the concentration of lupinifolin acquired at the infection site needs to reach the proper values. First-stage biofilm development, or initial surface attachment, was largely interfered with lupinifolin. As a result, it ought to be used as soon as possible, before the biofilm formation has become well established. Since it is unlikely that lupinifolin at a relatively low concentration of sub-MICs will induce antibacterial drug resistance, using it as an antibiofilm agent has potential benefits. These findings suggest that lupinifolin may be used as an anti-virulence drug to increase the antibacterial effects against biofilm-associated *E. faecalis* infections. However, the *in vitro* experiment results were unable to adequately capture the intricate conditions that exist within the body during an *E. faecalis* infection. Thus, more *in vitro* and *in vivo* research needs to be done in order to validate our findings.

Conclusion

At the highest concentration of 128 µg/mL, lupinifolin exhibited no antibacterial action against *E. faecalis* (TISTR 379) (MIC > 128 µg/mL). But across all incubation periods, lupinifolin alone at sub-MICs of 16–128 µg/mL strongly reduced the development of *E. faecalis* biofilms. The lowest IC_{50} of 6.78 ± 3.04 µg/mL of lupinifolin was observed at 6-hour incubation. As a result, lupinifolin might have acted as an antibiofilm agent mainly by preventing bacterial surface attachment. The antibiofilm activity of vancomycin at ¼ MIC (2 µg/mL) and 1/8 MIC (1 µg/mL) was also markedly increased by the sub-MICs of lupinifolin, specifically at 6- and 36-hour incubation. Therefore, it was predicted that the synergistic antibiofilm action of vancomycin and lupinifolin would be influenced by both concentration and incubation duration. To fully understand the mode of action of lupinifolin's antibiofilm activity, more research is necessary.

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Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

The authors have diligently addressed ethical considerations, including plagiarism, data fabrication, and double publication.

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