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Short Communication

Synergistic Antibacterial Activity of Lupinifolin and Protein Synthesis Inhibitors Against *Enterococcus faecalis* and *Enterococcus faecium*

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ABSTRACT

Antibacterial enhancers can increase the activity of currently-available antibacterial drugs, and their development is one of the feasible strategies to counteract antibacterial resistance. This study aims to investigate the effects of lupinifolin (isolated from *Derris reticulata* stems) in combination with antibacterial drugs acting as protein synthesis inhibitors—including streptomycin, chloramphenicol, and tetracycline—against *Enterococcus faecalis* and *Enterococcus faecium*. The Fractional Inhibitory Concentration Index (FICI) was obtained from a checkerboard assay to indicate the antibacterial activity of a lupinifolin–drug combination. The combinations of lupinifolin and streptomycin or chloramphenicol showed synergistic antibacterial activity against *E. faecalis* (TISTR 379 and DMST 4736) and *E. faecium* (TISTR 2058), with median FICIs of ≤ 0.5 (n=4–5); additionally, lupinifolin combined with streptomycin showed a potential synergism against *E. faecium* DMST 4743 (FICI < 0.5156, n=3). However, the combination of lupinifolin and tetracycline resulted in no interaction against any enterococcal strain examined (FICIs ≥ 1). In conclusion, lupinifolin potentially plays a role as an antibacterial intensifier against *E. faecalis* and *E. faecium* when used in combination with certain protein synthesis inhibitors, specifically streptomycin and chloramphenicol; nonetheless, more research is needed to examine the synergistic antibacterial mechanism of the combinations.

Keywords: *Enterococcus faecalis, Enterococcus faecium*, Lupinifolin, Protein synthesis inhibitors, Synergism, Antibacterial activity.

Introduction

Enterococci are gram-positive, facultative anaerobes that are remarkably resilient to a range of severe conditions, such as high salt concentrations and extreme pH and temperature.[1] Although they are commensal gut bacteria in healthy individuals, enterococci—primarily *Enterococcus faecalis* and *Enterococcus faecium*—can cause life-threatening nosocomial infections including endocarditis, surgical site infection, urinary tract infection, and bacteremia in vulnerable hosts.[2] Enterococci are intrinsically resistant to a number of currently available antimicrobials, such as β-lactam antibiotics and aminoglycosides.[1,3,4] Additionally, enterococci can readily acquire external antimicrobial resistance genes, through horizontal exchange of genetic material encoding resistance determinants or through mutation of intrinsic genes.[4] Moreover, the versatile nature of *Enterococcus* spp. renders them a reservoir of drug-resistant genes, which can be transmitted to other bacteria. Several approaches to fight enterococcal infections have been proposed, including the development of novel antibacterial drugs and the application of antibacterial drug combinations.[5,6] Nonetheless, antimicrobial resistance acquired by enterococci has been increasing globally.[7] In addition to genetic factors, antibiotic overuse and the persistence of resistant bacteria in hospital settings are some of the reasons contributing to the rise of antimicrobial resistance.

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Given the above, novel therapeutic strategies are needed to combat this critical clinical challenge. Apart from the development of novel antibacterial drugs with distinct mechanisms of action, the discovery of antibacterial enhancers—which can potentiate the antibacterial activity of currently-available antibacterials—is another feasible option. Plants naturally create a range of physiologically active substances to combat microbial invasion. With their antibacterial and anti-virulence properties, the phytochemicals derived from plants—particularly flavonoids—are a significant source of potential therapeutic candidates.[8] The combination of certain flavonoids with specific antibacterial drugs has been shown in numerous studies to have notable additive and/or synergistic effects.[8,9] Lupinifolin is classified according to its chemical structure as a prenylated flavanone; it can be extracted from a variety of medicinal herbs, including *Derris reticulata* Craib. stems.[10,11] Lupinifolin has been documented as possessing potent antibacterial activities against several gram-positive pathogenic bacteria, such as *Streptococcus mutans*, *Staphylococcus aureus* (both methicillin-sensitive and methicillin-resistant *S. aureus*), and *Staphylococcus epidermidis*, as well as *Enterococcus* spp. including *E. faecalis* and *E. faecium*, at reported minimum inhibitory concentrations (MICs) of 0.5–16 µg/mL.[10,12–15] It has been reported that lupinifolin exerts its antibacterial mechanism by targeting the bacterial cell membrane, which subsequently results in cell membrane disruption and leakage of cytoplasmic content.[13,15,16]

Theoretically, when antimicrobial agents are used in combination, they can have synergistic effects if each active agent has a distinct mode of action and takes aim at a different target or signaling pathway.[17] It is thus possible that lupinifolin and certain therapeutically available antibacterial drugs, such as cell wall active agents or protein synthesis inhibitors (which target different sites of action from lupinifolin) could have synergistic antibacterial effects. In our earlier studies, the combination of lupinifolin and ampicillin or cloxacillin demonstrated synergistic antibacterial action against methicillin-sensitive *S. aureus* (MSSA), with fractional inhibitory concentration index values of 0.5.[18] In addition, the bactericidal synergy of a combination of lupinifolin and streptomycin against methicillin-resistant *S. aureus* (MRSA) was

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demonstrated in a time-kill assay.[19] These previous findings suggest that lupinifolin is a potential antibacterial enhancer when used in combination with antibacterial drugs against certain gram-positive pathogens. However, the effects of lupinifolin combined with antimicrobial drugs acting as protein synthesis inhibitors on the growth of *Enterococcus* spp. have not been established. In the current study, lupinifolin was combined with antibacterial drugs acting as protein synthesis inhibitors—specifically streptomycin, tetracycline, and chloramphenicol—to examine the antibacterial activity of these combinations.

Materials and Methods

Isolation of lupinifolin from D. reticulata Craib. stems

This experiment employed lupinifolin, which was extracted from *D. reticulata* Craib. stems and obtained from a previous investigation.[19] The isolation and identification process of lupinifolin has been described in detail in our previous work. Lupinifolin was primarily authenticated by thin-layer chromatography (TLC) and elucidated by nuclear magnetic resonance (1H-NMR and 13C-NMR) and mass spectrometry.[20] The purified lupinifolin was kept at -20°C before use in the experiment.

Determination of the MIC

The minimum inhibitory concentration (MIC) was obtained using a modified microbroth dilution technique in accordance with Clinical and Laboratory Standards Institute (CLSI) procedures.[21] Stock solutions of lupinifolin, streptomycin (Sigma-Aldrich®, S6501), chloramphenicol (Sigma-Aldrich®, C0378), and tetracycline (Sigma-Aldrich®, T7660) were prepared in two-fold serial dilutions using the appropriate vehicles (0.1 M NaOH for lupinifolin, and sterile deionized water for the antibacterial drugs). *E. faecalis* (TISTR 379) and *E. faecium* (TISTR 2058) were obtained from the Thailand Institute of Scientific and Technological Research's culture collection center in Thailand, and *E. faecalis* (DMST 4736) and *E. faecium* (DMST 4743) were acquired from the Department of Medical Sciences, Ministry of Public Health Thailand. Suspensions of *E. faecalis* (TISTR 379 and DMST 4736) and *E. faecium* (TISTR 2058 and DMST 4743) were prepared in Tryptic Soy Broth (TSB, Himedia®, India) at a concentration of $1.5x10^6$ CFU/mL. The test agent or its vehicle (20 µL), bacterial suspension (50 µL), and TSB (130 µL) were applied to each well of a 96-well microplate and incubated for 24 hours at 37°C. At that point, the MIC was determined, defined as the lowest concentration of the test agent that prevented any visible bacterial growth. The median MIC was obtained from at least three independent experiments.

Checkerboard assay

Checkerboard assay, as described by Orhan *et al.*, was used to evaluate the antibacterial activity of lupinifolin in combination with the tested antibacterial drugs (streptomycin, chloramphenicol, and tetracycline).[22] Briefly, a mixture of TSB (130 µL) containing different concentrations of lupinifolin (10 µL) and the tested antibacterial drug (10 µL) was mixed with 50 µL of bacterial suspension (1.5×10^6 CFU/mL). The lupinifolin concentrations were 2-fold serially diluted along the abscissa, and the antibacterial drug concentrations used in testing were similarly serially diluted along the ordinate. Each drug was utilized at a maximum concentration of at least 4xMIC. The MICs for each combination of lupinifolin and the tested antibacterial drug were established following a 24-hour incubation period at 37°C. Then, the following equation was used to determine the fractional inhibitory concentration (FIC) index (FICI): FICI = FIC of lupinifolin + FIC of antibacterial drug. The FIC of the antibacterial drug was determined by dividing the MIC of the antibacterial drug in combination by the MIC of the drug alone, and the FIC of lupinifolin was determined by dividing the MIC of lupinifolin in combination by the MIC of lupinifolin alone. A combination is categorized as "antagonism," "no interaction," or "synergy" if its FIC index is >4.0, >0.5–4.0, or ≤0.5, respectively.[23] The results were represented by the median of at least three independent experiments.

Statistical analysis

The MIC and FICI data were analyzed using IBM SPSS Statistics Version 29 (IBM, USA) and expressed as a median.

Results and Discussion

The MICs of lupinifolin and the antibacterial drugs used in this experiment (streptomycin, chloramphenicol, and tetracycline), both alone and in combination, against the four tested strains of *E. faecalis* and *E. faecium* are shown in **Table 1**.

Since the bacterial uptake of aminoglycosides is an oxygen-dependent transport, enterococci are intrinsically resistant (low-level) to aminoglycosides, due to their facultative nature;[24] accordingly, the CLSI has no MIC breakpoint criteria for streptomycin against *Enterococcus* spp. Both strains of *E. faecalis* (TISTR 379 and DMST 4736) and one strain of *E. faecium* (TISTR 2058) can be considered as having low-level aminoglycoside resistance, with streptomycin MICs of ≤ 128 µg/mL. In contrast, *E. faecium* DMST 4743 had a lower streptomycin sensitivity (MIC > 128 µg/mL) and might have had high-level aminoglycoside resistance. In the checkerboard assay, the combination of lupinifolin and streptomycin showed synergistic antibacterial activity against *E. faecalis* TISTR 379, *E. faecalis* DMST 4736, and *E. faecium* TISTR 2058, with FICI values of 0.3750 (n=4), 0.3125 (n=5), and 0.500 (n=4), respectively (**Table 1**). The synergistic effect of lupinifolin and streptomycin against these enterococcal species aligns with their previously-described synergistic action against MRSA (DMST 20645).[19] However, the combination of lupinifolin and streptomycin resulted in a borderline yet inconclusive action against *E. faecium* DMST 4743, with a FICI value of < 0.5156 (**Table 1**). As in our earlier research, the time-kill experiment demonstrated the synergistic bactericidal activity of streptomycin and lupinifolin against MRSA;[19] at the same time, that checkerboard assay yielded a FICI value of < 0.625, which is higher than the combination's FICI against *E. faecium* DMST 4743 found in the current study. Therefore, a time-kill assay should be carried out in the future to verify if synergistic activity against *E. faecium* DMST 4743 was present. In general, *E. faecium* has a higher propensity to adapt and acquire antibiotic resistance, due to its higher level of genetic variation than *E. faecalis*.[25] This could lead to the lower sensitivity to the combination effects of lupinifolin and streptomycin found for *E. faecium* DMST 4743; it is also consistent with the other results, which showed that while lupinifolin and streptomycin exerted a synergistic effect against another strain of *E. faecium* (TISTR 2058), this FICI value (0.5) was higher than the FICI values for both tested strains of *E. faecalis* (0.3750 and 0.3125).

The membrane permeability of aminoglycosides, the rate-limiting step for aminoglycoside antibacterial action, occurs slowly.[24] Since lupinifolin targets and enhances bacterial cell membrane permeability[10,13,15], it potentially causes a synergistic antibacterial effect with aminoglycosides—as demonstrated in this study—by accelerating the rate-limiting step of aminoglycoside action. There are currently few reports on the synergistic effect of aminoglycosides and flavonoids against enterococci; nonetheless, some flavonoids, including baicalein and 7-*O*-isopropylnaringenin oxime, have been proposed to produce synergistic antibacterial activity against enterococcal species by increasing the penetration of antibacterial drugs into bacterial cells.[26,27]

The virulence factors of *Enterococcus* spp.—particularly their capacity to withstand severe environments, such as high concentrations of salt and bile and wide ranges of pH values (4.5–10.0) and temperatures (5–65°C)—depend on the integrity and function of their cell membranes.[1] To this end, it has been documented that lupinifolin caused a reduction in the salt tolerance of *Enterococcus* spp.[14] Therefore, in addition to its antibacterial action, lupinifolin may have anti-virulence properties that could enhance the antibacterial effects of other antimicrobials.

Enterococcus spp. can also possess high-level aminoglycoside resistance, which is externally acquired and allows them to withstand gentamicin and streptomycin even at the very high concentrations of >500 and >2,000 µg/mL, respectively.[28] The mechanism underlying high-level aminoglycoside resistance is the synthesis of aminoglycoside-modifying enzymes (AMEs), which add functional groups to the -OH or -NH2 sites of the 2-deoxystreptamine nucleus and prevent its action.[29]

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Table 1: MICs and FIC index of lupinifolin, streptomycin, chloramphenicol, and tetracycline against *E. faecalis* and *E. faecium*

Bacteria	MIC alone (µg/mL)				MIC in combination (µg/mL)			FIC index (n)		
	Lupinifolin	Streptomycin	Chloramphenicol	Tetracycline	Lupi + Strep	Lupi + Chloram	Lupi + Tetra	Lupi + Strep	Lupi + Chloram	Lupi + Tetra
E. faecalis (TISTR 379)	128	128	8	2	32^a+16^b	$32^a + 0.25^b$	$0.125^a + 2^b$	0.3750 (4)	0.2813 (3)	1.0010 (5)
E. faecium (TISTR 2058)	128	64	8	1	$32^a + 16^b$	$32^a + 0.25^b$	$128^a + 0.5^b$	0.5000 (4)	0.2813 (3)	1.5000 (5)
E. faecalis (DMST 4736)	128	128	8	4	$32^a + 8^b$	$0.125^a + 4^b$	$128^a + 2^b$	0.3125 (5)	0.5010 (3)	1.5000 (3)
E. faecium (DMST 4743)	128	>128	8	2	$64^a + 2^b$	$64^a + 0.25^b$	$64^a + 2^b$	<0.5156 (3)	0.5313 (3)	1.5000 (3)

Data are expressed as median values.
a = MIC of lupinifolin in combination
b = MIC of antibacterial drug in combination
Lupi = lupinifolin; Strep = streptomycin; Chloram = chloramphenicol; Tetra = tetracycline
The combination is classified as "synergy", "no interaction", or "antagonism" when the FIC index is ≤0.5, >0.5–4.0, or >4.0, respectively.

Thus, AME inhibition is another strategy to tackle aminoglycoside resistance.[30] However, few cases document natural compounds (such as quercetin and aranorosin) that have an inhibitory effect against AMEs.[31,32] According to reports, *E. faecium* exhibits greater levels of high-level aminoglycoside resistance than *E. faecalis*.[33] Since *E. faecium* DMST 4743 was found in this study to be less sensitive to streptomycin (MIC > 128 µg/mL), it may possess high-level aminoglycoside resistance involving AME production. Further investigation is necessary to ascertain whether lupinifolin has an AME-inhibiting function even though the checkerboard assay did not clearly demonstrate the synergistic activity of lupinifolin with streptomycin in *E. faecium* DMST 4743 (FICI < 0.5156).

When lupinifolin was used in combination with chloramphenicol, a FICI value of 0.2813 was found against both *E. faecalis* TISTR 379 and *E. faecium* TISTR 2058, indicating synergistic antibacterial activity (Table 1). In addition, a FICI value of 0.5010 was produced when lupinifolin was used in combination with chloramphenicol against *E. faecalis* DMST 4736, again indicating a synergistic effect. According to the CLSI's criteria, every strain of *E. faecalis* and *E. faecium* employed in this study is chloramphenicol-susceptible (MIC ≤ 8 µg/mL);[21] thus, it is possible that the synergistic action between lupinifolin and chloramphenicol against *E. faecalis* (TISTR 379 and DMST 4736) and *E. faecium* (TISTR 2058) found in this study was not linked with a reversal of a chloramphenicol resistance mechanism (which involves the production of enzymes, including chloramphenicol acetyltransferase and rRNA methyltransferase).[34,35] Rather, the mechanism of synergistic action between lupinifolin and chloramphenicol found here may be due to lupinifolin's enhancement of bacterial membrane permeability, which in turn increases the bacterial uptake and the antibacterial activity of chloramphenicol.

At the same time, the current study showed that the combination of lupinifolin and chloramphenicol had no interaction on *E. faecium* DMST 4743 (FICI = 0.5313) (**Table 1**). In our previous study, this combination also showed indifferent action against MSSA and MRSA, which were both susceptible to chloramphenicol at FICI values of 0.5078 and 1.0078, respectively.[36] It has also been reported that baicalein, a flavone, does not produce a synergistic effect with chloramphenicol against MRSA.[37] This disparity could be caused by variations in the structure of bacterial cell membranes: the membrane permeability for chloramphenicol in MSSA, MRSA, and *E. faecium* DMST 4743 may already be adequate for its action, and thus its antibacterial activity is not substantially improved by the addition of lupinifolin (which targets the permeability of bacterial cell membranes). The synergistic effect of chloramphenicol in combination with other flavonoids against *Enterococcus* spp. has not been documented. The combination of rutin and florphenicol, another phenicol, produced a synergistic action against *Aeromonas hydrophila*, a gram-negative bacillus, with a FICI value of 0.5;[38] however, the mechanism of their synergistic antibacterial activity is still unclear. Accordingly, more research is needed to prove the synergistic mechanism of lupinifolin and chloramphenicol.

All strains of enterococcal species used in this experiment are sensitive to tetracycline, according to the CLSI criteria (MIC ≤ 4 µg/mL).[21] As a result, it is unlikely that they have a tetracycline resistance mechanism, which mainly involves the drug efflux pump.[6] Given that the enterococcal species used in the study are tetracycline-susceptible, tetracycline most likely experienced sufficient penetration of *Enterococcus* spp. cell membranes to bind to its target site. This is supported by the finding that when lupinifolin was present, the tetracycline MICs against every tested enterococcal strain were either unchanged or only decreased by twofold. In other words, the combination of lupinifolin and tetracycline exhibited an indifferent antibacterial action against all four tested strains of *E. faecalis* and *E. faecium*, with FICI values of ≥ 1, as shown in **Table 1**. In previous work, our earlier findings showed a synergistic effect of lupinifolin and tetracycline against MSSA, with a FICI value of 0.0937;[36] since MSSA primarily uses efflux pumps to wield its tetracycline resistance, lupinifolin possibly sensitized the antibacterial activity of tetracycline against MSSA through suppression of the efflux pump.[39] Nevertheless, more research is needed to determine whether lupinifolin has distinct

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effects on the efflux pump transporter that is expressed in different bacterial species, including enterococci.

Conclusion

This study was the first to demonstrate the synergistic action of lupinifolin when combined with antibacterial drugs acting as protein synthesis inhibitors—specifically streptomycin and chloramphenicol—against *E. faecalis* and *E. faecium*. As observed in this study, the synergistic actions of lupinifolin and protein synthesis inhibitors are specific to a certain drug class. Consequently, lupinifolin could potentially be employed as an antibacterial intensifier to treat antibiotic-resistant enterococcal infections in the future. Nonetheless, more research is needed to determine the precise mechanism of the synergistic activity between lupinifolin and protein synthesis inhibitors.

Conflict of interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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