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Research Article
Synergistic Activity of Lupinifolin in Combinations with Antibiotics Against *Staphylococcus aureus*

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Abstract

Background and Objective: Antibacterial resistance is one of the top global public health problems. The use of natural substances, which can enhance the antibacterial activity of currently used medications, is a promising alternative to oppose antibacterial resistance. The pharmacological activities of lupinifolin, a prenylated flavanone isolated from stems of *Derris reticulata* Craib., against growth and biofilm formation of *Streptococcus mutans* and *Staphylococcus aureus* have been previously documented. Nonetheless, interactions between lupinifolin and other antibacterial agents have not been determined. This study aimed to investigate the effects of lupinifolin in combinations with some antibacterial agents, specifically ampicillin, cloxacillin or vancomycin, against *S. mutans*, Methicillin-Sensitive *S. aureus* (MSSA) and Methicillin-Resistant *S. aureus* (MRSA). **Materials and Methods:** The checkerboard assay was performed to determine the antibacterial activity of lupinifolin plus the testing antibacterial agents. The Fractional Inhibitory Concentration Index (FICI) was calculated to indicate the interaction between lupinifolin and the antibacterial agent tested. **Results:** Lupinifolin exerted the synergistic activity when using in combination with ampicillin or cloxacillin against MSSA with the FICIs of $\leq$0.5. The potential synergistic effect was also observed with lupinifolin plus ampicillin or cloxacillin against MRSA. However, the combination of lupinifolin plus vancomycin resulted in no interaction against MRSA. The combined effects of lupinifolin and ampicillin or cloxacillin against *S. mutans* were somewhat ambiguous with the borderline values of FICI of 0.5156 and 0.5625, respectively. **Conclusion:** Lupinifolin potentially plays a role as an antibacterial intensifier against some pathogenic gram-positive bacteria, particularly MSSA and MRSA. Nonetheless, further experiments are required to explain the precise mechanism of synergy.

Key words: Lupinifolin, *Streptococcus mutans*, methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, synergistic effect, antibacterial intensifier

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

INTRODUCTION

Bacteria use several modes of action to combat against antibacterial agents including (1) Alteration of the drug target binding site, (2) Production of enzymes that destroy or modify the chemical structure of antibacterial agents, (3) Expression of efflux pumps which extrude the drug out of the microorganism and (4) Changing in the cell membrane or cell wall permeability[1]. These approaches lead to antibacterial resistance and treatment failure which are one of the major public health problems worldwide[2]. Drug-resistant pathogenic bacteria aggressively emerge as a result of misuse and overuse of antimicrobial agents. Various problems derived from antimicrobial resistance include higher medical costs, prolonged hospital stays and increased disability as well as mortality. *Staphylococcus aureus* is one of the prevailing gram-positive pathogenic bacteria which rapidly acquire antibiotic resistance[3]. Initially, *S. aureus* produces β-lactamase enzyme to survive in the presence of β-lactamase-labile penicillin's, for example, penicillin, ampicillin and amoxicillin. When β-lactamase-resistant penicillin's, such as methicillin, oxacillin and cloxacillin, have been developed, *S. aureus* resists this penicillin group by harbouring another penicillin-binding protein, named PBP2a, which cannot be inhibited by the drugs. This *S. aureus* type is so-called, Methicillin-Resistant *S. aureus* (MRSA). Furthermore, *S. aureus* can also produce biofilm which substantially enhances antibacterial resistance[4]. The generated biofilm creates a suitable environment for bacterial survival by impeding the penetration of host immune cells as well as antibacterial agents. Additionally, the biofilm can concentrate crucial determinants required for bacterial durability, including extracellular DNA encoding drug resistance genes, antibacterial-destroying enzymes and also essential nutrients. The sessile bacteria in biofilm are almost 1,000 times less sensitive than their planktonic counterpart to the antibacterial drugs[5]. *Streptococcus mutans,* another gram-positive coccus, significantly contributes to the pathogenesis of dental caries by its ability to produce biofilm or dental plaque along with its acidogenicity and aciduricity competences[6]. It was reported that *S. mutans* exhibited the highest frequency of antibiotic resistance among oral streptococci isolated from active dental infections in adults[7]. The antibiotic resistance found in *S. mutans* was presumably linked with its genetic diversity.

A search for novel effective treatment strategies for antibacterial-resistance infections is still crucially needed. It is compelling to develop new drugs with unique mechanisms of antibacterial action. However, the development of a novel antibacterial drug is a time-consuming and very expensive procedure. Moreover, pathogenic bacteria can still evolve their resistant tools to escape from the drug swiftly[8]. Another promising alternative is the use of natural substances which can enhance the antibacterial activity of currently used antibacterial agents. Several reports have evidenced that various flavonoids produce substantial synergistic and/or additive effects when using in combinations with certain antibacterial agents[1,9]. Several flavonoids, such as kaempferol, quercetin, rutin, morin, luteolin, apigenin, myricetin, catechin and epicatechin gallate, have been reported to sensitize the bacteria to some antibacterial agents to which they used to be previously resistant[10-18]. In addition to their direct antibacterial activity, numerous flavonoids significantly impede the bacterial virulence factors by employing various means, including (1) Inhibition against biosynthesis of essential enzymes and proteins such as β-lactamase , sortase, α-toxin, coagulase[17,19-21], (2) Stimulation of bacterial cell aggregation[22] as well as (3) Inhibition of biofilm formation[23,24].

Lupinifolin is a prenylated flavanone that can be isolated from certain medicinal plants including stems of *Derris reticulata* Craib. (Leguminosae-Papilionoideae)[25]. Lupinifolin exhibits diverse pharmacological activities such as antidiabetic, antimycobacterial, antiviral as well as antibacterial actions[24-32]. The antibacterial activities of lupinifolin against *S. mutans* and *S. aureus*, including MSSA and MRSA, have been documented with the MIC ranges of lower than 10 μg mL^{-1} [24,25,28-30,32]. Lupinifolin also disrupted the biofilm formation activity in certain strains of enterococci, *Enterococcus faecalis* and *Enterococcus faecium*[31]. Additionally, it was recently demonstrated that lupinifolin at the sub-MICs also significantly inhibited the biofilm formation activity of *S. mutans* and *S. aureus*[24]. Lupinifolin exhibited its antibacterial activity via interfering with bacterial cell membrane structure and functions[25,30]. The antibacterial action of lupinifolin against *S. aureus* was observed earlier than that of ampicillin which acts as a cell wall synthesis inhibitor[25]. The antibacterial mechanism of lupinifolin is likely to be different from that of cell walls synthesis inhibitors such as penicillin's, which are generally used for the treatment of *S. mutans* and *S. aureus* infections. Therefore, a combination of lupinifolin and drugs targeting bacterial cell wall synthesis may potentially produce a synergistic antibacterial activity. Nonetheless, the interaction between lupinifolin and an antibacterial agent has not been determined.

This study thus aimed to investigate the effects of lupinifolin in combinations with some antibacterial agents, specifically ampicillin, cloxacillin or vancomycin, against *S. mutans*, MSSA and MRSA.

MATERIALS AND METHODS

Study area: The study was carried out at the Faculty of Pharmacy, Mahasarakham University, Thailand from November, 2020-January, 2021.

Isolation of lupinifolin from *D. reticulata* stem: The sample of dry *D. reticulata* stems was obtained from the local herb store in Bangkok, Thailand. The herbs were authenticated and lupinifolin was subsequently isolated as described earlier[27,33]. The purified lupinifolin prepared from *D. reticulata* stems was obtained from our previous study[27].

Determination of the MIC: The Minimum Inhibitory Concentration (MIC) was determined by using a modified micro broth dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines[34]. The stock solution of lupinifolin was prepared in 0.1 M NaOH and diluted in serial two-fold dilutions. A bacterial suspension of *Streptococcus mutans* (DMST 18777) in BHI broth or methicillin-sensitive *Staphylococcus aureus* (MSSA, DMST 8013) and methicillin-resistant *Staphylococcus aureus* (MRSA, DMST 20645) in TSB broth was prepared from the broth culture and adjusted to approximately 1.5×10^6 CFU mL^{-1}. In each well of the 96-well microplate, the bacterial suspension (50 µL) was mixed with lupinifolin at various concentrations (20 µL) and their respective broth (130 µL). The final concentrations of lupinifolin ranged from 0.125-32 µg mL^{-1}. 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 defined as the lowest concentration of lupinifolin producing a complete inhibition of bacterial growth as detected with the unaided eye after 24 hrs incubation. 0.1M NaOH (vehicle for lupinifolin) was used as a negative control. At least three independent experiments were performed to obtain the median MICs of lupinifolin against *S. mutans,* MSSA and MRSA.

Checkerboard determination: Checkerboard assay was conducted according to the method of Orhan *et al.*[35] to determine the antibacterial activity of lupinifolin in combinations with testing antibacterial agent (ampicillin, cloxacillin or vancomycin). A bacterial suspension of *Streptococcus mutans* (DMST 18777), MSSA (DMST 8013) or MRSA (DMST 20645) was prepared in BHI or TSB broth and adjusted to approximately 1.5×10^6 CFU mL^{-1}. The bacterial suspension (50 µL) was mixed with testing antibacterial agent (10 µL) and lupinifolin (10 µL) at various concentrations. The testing antibacterial agent was serially diluted along the ordinate, while the purified lupinifolin was serially diluted along the abscissa. The serial twofold dilutions of each drug were prepared to start from the maximal concentration of at least 4x MIC. Total 130 µL of Brain Heart Infusion (BHI) or Tryptic Soy Broth (TSB) broth was subsequently distributed into each well of the 96-well microplates. The microplates were incubated at 37°C in the incubator (with 5% CO_2 for *S. mutans*). The MICs were determined for each combination of testing antibacterial agent and lupinifolin after 24 hrs incubation. Fractional Inhibitory Concentration (FIC) of lupinifolin is MIC of lupinifolin in combination/MIC of lupinifolin alone, whereas FIC of antibacterial agent is MIC of antibacterial agent in combination/MIC of the antibacterial agent alone. The FIC index of the combination was calculated by using the following Eq.[36]:

$$\text{FIC index} = \text{FIC of lupinifolin} + \text{FIC of antibacterial agent}$$

The combination is identified as "synergy" when the FIC index is equal to or less than 0.5. The FIC index of >0.5-4.0 indicates "no interaction" of the combination, while the values above 4.0 represent "antagonism"[36]. The MICs and FIC indices are presented as the median values obtained from at least three independent experiments.

RESULTS

The MICs of lupinifolin and testing antibacterial agents (ampicillin, cloxacillin or vancomycin) against *S. mutans*, MSSA and MRSA are shown in Table 1. The FIC indices of 0.5156 and 0.5625, which indicated the borderline between synergy and no interaction, was observed when lupinifolin was used together with ampicillin or cloxacillin against *S. mutans*. When lupinifolin was used in combination with ampicillin or cloxacillin, the MICs of lupinifolin and these penicillins against both MSSA and MRSA were substantially decreased. The synergistic activity of lupinifolin and ampicillin or cloxacillin against MSSA was indicated with the FIC indices of $\leq$0.5. Since MRSA was resistant to both ampicillin and cloxacillin (their MICs were >32 µg mL^{-1}), the exact FIC indices of lupinifolin and these penicillin in combinations could not be specified. The FIC indices of <0.5625 and <0.5156 were found with lupinifolin plus ampicillin or cloxacillin, respectively against MRSA. Therefore, the potential synergistic action of lupinifolin and the testing of penicillin against MRSA was also suggested. On the other hand, the combination of lupinifolin and vancomycin produced no interaction against MRSA with the FIC index of 0.75.

Table 1: MIC and FIC index of lupinifolin, ampicillin, cloxacillin and vancomycin against *S. mutans*, MSSA and MRSA

Bacteria	Test agent	MIC alone (μg mL^{-1})	MIC in combination (μg mL^{-1})	FIC index	n
S. mutans	Lupinifolin	8	0.125	0.5156	5
	Ampicillin	0.125	0.0625		
S. mutans	Lupinifolin	8	4	0.5625	5
	Cloxacillin	0.25	0.0156		
MSSA	Lupinifolin	16	4	0.5000	5
	Ampicillin	0.5	0.125		
MSSA	Lupinifolin	16	0.125	0.5078	3
	Cloxacillin	0.5	0.25		
MRSA	Lupinifolin	16	8	<0.5625	3
	Ampicillin	>32	2		
MRSA	Lupinifolin	16	8	<0.5156	3
	Cloxacillin	>32	0.5		
MRSA	Lupinifolin	8	2	0.7500	4
	Vancomycin	2	1		

Data are expressed as median values, MSSA: Methicillin-sensitive *S. aureus*, MRSA: Methicillin-resistant *S. aureus*, MIC: Minimum inhibitory concentration, FICI: Fractional inhibitory concentration index

DISCUSSION

Lupinifolin produced an antibacterial activity against *S. mutans* (DMST 18777), MSSA (DMST 8013) as well as MRSA (DMST 20645) with the MICs of 8, 16 and 16 μg mL^{-1}, respectively. The antibacterial activity of lupinifolin against *S. mutans*, MSSA and MRSA was previously documented with the MICs of 2-4, 8 and 8 μg mL^{-1}, respectively[1 24,25,28-30,32]. The differences in the bacterial strains tested and solvent used for the preparation of lupinifolin stock solution possibly caused a modest variation of the MICs reported between studies. Additionally, there is a widely accepted norm in MIC testing that MICs can vary by a factor of 2 during testing[9,36]. The antibacterial activity of lupinifolin shown in this study is thus in agreement with those reported earlier.

The effects of lupinifolin on antibacterial activity of ampicillin or cloxacillin against *S. mutans* were at the borderline level with the FICIs of 0.5156 and 0.5625, respectively. These FICI values may be roughly interpreted as no interaction. However, a time-kill assay should be performed to undoubtedly identify its action. The checkerboard assay revealed the synergistic effects of lupinifolin plus ampicillin or cloxacillin against MSSA with the FIC indices of 0.5. The current results indicated that MSSA was not susceptible to ampicillin alone (MIC $\geq$0.25 μg mL^{-1})[34]. Interestingly, lupinifolin plus ampicillin was able to sensitize MSSA to be ampicillin-sensitive with the MIC of 0.125 μg mL^{-1}. Additionally, the potential synergy was identified with lupinifolin plus ampicillin or cloxacillin against MRSA. MRSA was not susceptible to both ampicillin and cloxacillin with MICs of >32 μg mL^{-1}. The combinations of lupinifolin plus ampicillin or cloxacillin substantially reduced the MICs of these penicillins against MRSA to 2 and 0.5 μg mL^{-1}, respectively. The FIC indices of <0.5625 and <0.5156 thus essentially suggests the promising synergistic effect between lupinifolin and ampicillin or cloxacillin. A synergy between lupinifolin and other antibacterial agents acting via other modes of action, specifically cell wall synthesis inhibitors, is primarily expected. The synergy between lupinifolin and ampicillin or cloxacillin against MSSA found in this study thus supports this speculation. Nonetheless, the combination of lupinifolin and vancomycin resulted in no interaction against MRSA (FICI = 0.75). Although penicillin and vancomycin share a similar antibacterial mechanism of action as cell wall synthesis inhibitors, their specific sites of action are different[37]. In addition to its action on the bacterial cell membrane, lupinifolin was also reported to cause bacterial cell wall damage[25]. Accordingly, modification of bacterial cell wall by targeting distinct site of action is possibly another mechanism for the synergy observed with lupinifolin plus penicillin tested. It is not known whether the no interaction observed between lupinifolin and vancomycin against MRSA was due to their common target sites in the bacterial cell wall.

The precise mechanisms of synergy between lupinifolin and penicillin have not been established yet. Flavonoids have been documented to exert their synergistic activity via multiple mechanisms of action[9]. It was evidenced that some flavonoids, such as luteolin and apigenin, partly expressed their synergy with b-lactam antibiotics (amoxicillin and ceftazidime) via augmenting cell membrane permeability against amoxicillin-resistant *Escherichia coli* and ceftazidime-resistant *Enterobacter cloacae*[12,13]. Since lupinifolin also executes its antibacterial activity via disruption of cell membrane integrity, it possibly exerts the synergy when using in combination with ampicillin or cloxacillin against MSSA and also MRSA through this mode of action.

Many strains of Staphylococci, especially *Staphylococcus aureus* and *Staphylococcus epidermidis* produce

β-lactamase enzyme which inactivates β-lactamase labile penicillin, such as ampicillin, penicillin and amoxicillin, by hydrolyzing the peptide bond of the b-lactam ring[38]. It has been reported that some flavonoids, such as quercetin and galangin, can substantially inhibit β-lactamase enzymes produced by certain types of bacteria[12,17]. The inhibitory action of these flavonoids towards β-lactamase has been proposed to be associated with their synergistic activity with β-lactamase-labile penicillin against amoxicillin-resistant *S. epidermidis* and *S. aureus*. Therefore, inhibition against bacterial β-lactamase enzyme may also be involved in the synergistic action of lupinifolin plus ampicillin against MSSA demonstrated in this study.

An alteration of PBP into PBP2a, encoded by *mecA* gene, renders *S. aureus* to be not susceptible to β-lactamase-resistant penicillins[34]. The problematic microorganism is the so-called Methicillin-Resistant *S. aureus* (MRSA). PBP2a has a very low affinity for β-lactam antibiotics because of the closed conformation of its active site[39]. Lupinifolin was able to potentiate the antibacterial activity of both ampicillin and cloxacillin but not vancomycin, against MRSA. Therefore, lupinifolin might cause some changes in the structure and/or function of PBP2a of MRSA, which sensitize the bacteria towards the binding of these penicillins. Epicatechin gallate (Ecg), a flavanol, could sensitize MRSA to oxacillin and other β-lactam antibiotics via delocalization of PBP2 from the site of cell division[40]. It should be noted that both PBP2 and PBP2a mutually function in the presence of β-lactamase-resistant penicillin, possibly as a multienzyme complex[8]. The PBP2 transglycosylase activity is required for peptidoglycan synthesis since the PBP2a moiety is non-functional. It is still not known whether lupinifolin exerts its ampicillin/cloxacillin-sensitizing action against MRSA similarly to that of Ecg. The potential synergy of lupinifolin plus ampicillin or cloxacillin against MRSA is rather intriguingly since the prevalence of MRSA-related serious infections is still rising[2]. Vancomycin is a current drug of choice for fighting against MRSA infections[41]. However, the high use of vancomycin can lead to an occurrence of Vancomycin-Intermediate resistant *S. aureus* (VISA) and Vancomycin-Resistant *S. aureus* (VRSA) due to selection pressure. The combination of lupinifolin plus ampicillin or cloxacillin may probably provide an alternative for the treatment of MRSA infections.

It has been documented that a suppression of the efflux pump is another possible mechanism for the synergistic or additive effect between flavonoids and antibacterial agents. The efflux pumps, acting as a first-line defence mechanism are membrane proteins that operate to detoxify antibacterial agents out of bacterial cells[42]. The expression of *abcA* efflux pump was reported to be responsible for the resistance against b-lactam antibiotics in *S. aureus*[43]. The combination of catechin and Ecg could downregulate mRNA expressions of the MRSA efflux pumps, *norA*, *nor C* and *abcA*, which conceivably linked to the synergistic effect between these flavanols and β-lactam antibiotics against MRSA *in vitro* and *in vivo*[14]. A further experiment is required to investigate whether lupinifolin can also modulate the efflux pump of *S. aureus*.

From our previous study, lupinifolin at the sub-MICs possessed inhibitory activity against biofilm formations of both MSSA and *S. mutans*[24]. Biofilm forming capability of the bacteria also plays an essential role in the development of antibacterial resistance[4]. The biofilm provides an ecological niche that allows the bacteria to endure even in a harsh environment. Therefore, the inhibitory activity of lupinifolin against biofilm formation of *S. aureus* may conceivably be linked with its synergistic activity revealed in this study.

CONCLUSION

In conclusion, lupinifolin, isolated from *D. reticulata* stems, substantially exerted the synergistic activity against MSSA when using together with ampicillin or cloxacillin with the FICIs of $\leq$0.5. The potential synergistic effect was also observed with lupinifolin plus ampicillin or cloxacillin against MRSA. However, the combination of lupinifolin plus vancomycin resulted in no interaction against MRSA. This prenylated flavanone produced ambiguous effects in combinations with ampicillin or cloxacillin against *S. mutans* with the borderline values of the FICI index. Lupinifolin thus potentially play a role as an antibacterial intensifier against some pathogenic gram-positive bacteria, particularly MSSA and MRSA. Nonetheless, further experiments are required to explain the precise mechanism of synergy.

SIGNIFICANCE STATEMENT

The synergistic activity of lupinifolin in combination with ampicillin or cloxacillin against MSSA was firstly revealed in this study. The potential synergy between lupinifolin and ampicillin or cloxacillin against MRSA was also essentially discovered. Thus, the use of lupinifolin as an antibacterial intensifier to overcome antibacterial-resistant *S. aureus* infections may promisingly arrive in the future.

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