



Antibacterial effect of sophoraflavanone G by destroying the cell wall of *Enterococcus faecium*

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ABSTRACT

Enterococcus faecium has appeared as an important opportunistic pathogen that can cause urinary tract infections, surgical site infections, bacteremia, and endocarditis. Therefore, it is imperative to develop alternative therapeutic methods to treat enterococcal infections. Sophoraflavanone G (5,7,2',4'-tetrahydroxy-8-lavandulylflavanone, SPF-G) exhibited the strongest antibacterial activity based on the minimum inhibitory concentration values on two *E. faecium* strains (6.25 and 12.5 µg/ml at 24 and 48 hours of treatment, resp.) in the broth microdilution assay among the tested compounds and a remarkable bactericidal effect with a 12.5 µg/ml minimum bactericidal concentration value. Sophoraflavanone G (12.0 ± 2.3 mm inhibition zone for Korean Agricultural Culture Collection, Korea (KACC) 11954 and 11.0 ± 3.0mm for Culture Collection of Antimicrobial Resistant Microbes, Korea (CCARM) 5506) also exhibited the highest susceptibility based on the agar diffusion assay. Membrane-permeabilizing agents with a low dose of sophoraflavanone G synergistically activated anti-*E. faecium* activity through a 67% reduction of *E. faecium* growth, and *E. faecium*-derived peptidoglycans (PGN) blocked the antibacterial activity. These results indicate that sophoraflavanone G could bind to the bacterial cell wall and induce *E. faecium* cell wall damage. Transmission electron microscopy (TEM) images of *E. faecium* treated with sophoraflavanone G also exhibited cell lysis, followed by leakage of intracellular components, confirming that sophoraflavanone G has anti-*E. faecium* activity by binding to the PGN and disrupting the cell wall. This study showed the possible usage of sophoraflavanone G as an effective natural anti-*E. faecium* compound.

INTRODUCTION

Enterococci commonly reside within the human gastrointestinal and genitourinary tracts and can cause enterococcal bacteremia, infective endocarditis, and urinary tract infection (Graham and Gould, 2002; Morrison *et al.*, 1997; Murdoch *et al.*, 2002; Murray, 1990). Over the last decades, *Enterococci* isolated by hospital-acquired infections have showed increased intrinsic or acquired antibiotics resistance to the most currently approved agents. Particularly, enterococcal or nosocomial infection caused by resistant strain is life-threatening in humans (Vignaroli *et al.*, 2010).

Among the *Enterococci* species, *Enterococcus faecium* has appeared as a serious opportunistic pathogen with major causation of multidrug-resistant enterococcal infection especially in immunocompromised patients. Even though *Enterococcus faecalis* is isolated more frequently, *E. faecium* isolates often showed resistance to vancomycin, ampicillin, penicillin, and oxacillin. The drug resistance of *E. faecium* made that species difficult to be treated, and therefore the incidence of the problem is increased. Approximately 40% of recent medical device-associated infections were due to multidrug-resistant *E. faecium*. Therefore, it is crucial to the development of new antimicrobial agents and alternative therapy to treat multidrug-resistant *Enterococci* infections (Miller *et al.*, 2015; Vignaroli *et al.*, 2010).

Natural compounds have been referred to as those produced by plants or animals and are investigated because they possess potential bioactive materials that have antimicrobial activity, anticancer activity, and so on (Kim *et al.*, 2004).

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Eleven plant-originated compounds were used to screen the antibacterial activity against *E. faecium* and found sophoraflavanone G as a compound that has the strongest antibacterial activity among the tested ones.

Sophoraflavanone G, also known as a 5,7,2'4'-tetrahydroxy-8-lavabdulyflavanone, is a crenelated flavonoid that can be isolated from the root of *Sophora flavescens* (Figure 1). Sophoraflavanone G has a variety of biological activities including antibacterial activity against opportunistic bacteria including *Streptococcus sobrinus*, *Streptococcus mutans*, and *Staphylococcus aureus* (Gerber *et al.*, 2009; Hsiang *et al.*, 2001; Orsi *et al.*, 2011; Tsai *et al.*, 2004).

This study presented the antibacterial mechanisms of sophoraflavanone G to treat *E. faecium* infections. The antimicrobial activity of sophoraflavanone G was obtained by determining the minimum inhibitory concentration (MIC) using the broth microdilution method, and then the antibacterial mechanism of sophoraflavanone G by disrupting the *E. faecium* cell wall was investigated by growing in combination with an *E. faecium*-derived peptidoglycan (PGN), detecting the release of UV-absorbing material, and images captured using transmission electron microscopy.

MATERIALS AND METHODS

Bacterial strains

Enterococcus faecium (KACC 11954 and CCARM 5506) were purchased from KACC (Korean Agricultural Culture Collection, Korea) or CCARM (Culture Collection of Antimicrobial Resistant Microbes, Korea). The bacterial strains were maintained on Muller-Hinton (MB cell, USA) agar plates and incubated at 37°C.

Antibacterial activity of sophoraflavanone G against *E. faecium*

The broth microdilution method was applied to determine the MICs using microplates (Clinical and Laboratory Standards Institute, 2006; Tsai *et al.*, 2004). *Enterococcus faecium* strains were cultured in Muller-Hinton broth for 24 hours at 37°C and then transferred to a 96-well plate of 0.5 McFarland standard. Serial twofold dilutions of sophoraflavanone G (Sigma, St. Louis, MO) were prepared in Muller-Hinton broth. The MIC value was determined as the minimum compound concentration that inhibited bacterial growth after the indicated time. Ampicillin, vancomycin, and streptomycin were used as positive controls.

The minimum bactericidal concentration (MBC) was obtained using a microdilution assay according to the standard with slight modification (Clinical and Laboratory Standards Institute, 2006). After bacteria were cultured in the same manner as the MIC test, *E. faecium* strains were inoculated onto the agar plates and incubated for 24 hours at 37°C. MBCs were determined by the lowest concentration of compound which exhibit no viable bacteria colony. The experimental and control groups then were assigned to the wells in triplicate.

Susceptibility test

Broth inoculums with 0.5 McFarland standard were spread onto sterile Muller-Hinton broth by cotton swabs. The 8 mm sterile paper discs were impregnated with 100 µg/ml compounds, and the inhibition zones were measured after 18 hours (Climo *et al.*, 1999; Jorgensen and Ferraro, 2009).

Growth curve assay

The growth curve was obtained using the previously described 96-well microplate. After this, sophoraflavanone G was added to the cells and incubated at 37°C, for an indicated time (Lee YS, 2015). To assess the anti-*E. faecium* synergistic effect of sophoraflavanone G with the membrane-permeabilizing chemical, 3.125 µg/ml of sophoraflavanone G was added in combination with 0.01% Triton X-100. Neither compound inhibited bacterial growth (Lim and Strynadka, 2002; Mun *et al.*, 2014, Sasaki *et al.*, 2012).

To analyze the effect of the PGN on anti-*E. faecium* activity of sophoraflavanone G, bacterial inoculum (OD₆₀₀ = 0.1) was added to the Mueller-Hinton broth containing different concentrations of sophoraflavanone G and/or PGN and then incubated for 24 hours. The growth of *E. faecium* was evaluated by measuring OD₆₀₀ using microplate reader (Mun *et al.*, 2014; Viktoriya and Vitaly, 2014).

Isolation of PGN from cell walls of gram-positive bacteria

Overnight bacteria cultures were washed twice with saline, and then prewarmed 20 ml of 30% aqueous phenol was added to the bacteria for 20 minutes at 65°C–68°C.

After cooling to 20°C, the mixture was centrifuged 3 times for 10 minutes at 1700 Revolutions per minute (RPM), with the removal of the supernatant after each cycle. At this stage, the noncovalently associated contaminant was removed. The product obtained in the preceding step was stirred at 100°C for 3 hours with 300 ml of distilled water and 3 ml of 100% acetic acid to eliminate trace amounts of lipopolysaccharide (LPS). After the precipitate was washed 3 times with water, dialysis with 0.05M sodium acetate solution was carried out at 21°C ± 2°C (Desmarais *et al.*, 2014; Furukawa *et al.*, 1993; Kuhner *et al.*, 2014; Viktoriya and Vitaly, 2014).

Detection of release of UV-absorbing materials

The release of UV-absorbing materials was measured using a UV spectrophotometer with slight modifications. Sophoraflavanone G was applied to *E. faecium* strains at a final concentration of 0–50 µg/ml. Absorbance measurements of the supernatant at 260 nm and 280 nm (A₂₆₀ and A₂₈₀) were recorded (Septama *et al.*, 2017).

Transmission electron microscopy analysis

Enterococcus faecium were treated with 6.25 µg/ml of sophoraflavanone G for 30 minutes. After removal of supernatant, the pellets were fixed with a microscopy grid (Ted Pella). The specimens were analyzed using a transmission electron microscope (JEM-1010, JEOL, Seoul, Korea, 80 kV) (Mun *et al.*, 2014).

Statistical analysis

All experiments were performed at least three times, and data were expressed as the median with individual data including standard deviation.

RESULTS

Sophoraflavanone G possesses anti-*E. faecium* activity

Antibacterial susceptibility tests of sophoraflavanone G against *E. faecium* strains were performed based on the standard

broth microdilution method. The MIC values of sophoraflavanone G against *E. faecium* were 12.5 µg/ml (KACC 11954 and CCARM 5506), but the MIC values of ampicillin and streptomycin used as positive controls were 6.25 µg/ml (KACC 11954 and CCARM 5506), >100 µg/ml (KACC 11954), and 0.78 µg/ml (CCARM 5506), respectively. Both strains exhibited resistance against vancomycin (MIC value >100 µg/ml) (Table 1).

The MBC value was obtained by subculturing the samples from the wells in the MIC assays. MBC values were not altered for *E. faecium* strain (12.5 µg/ml) after 24 hours at 37°C following further incubation for 18–24 hours (Table 2).

A bacterial susceptibility test was also performed to determine the antibacterial effect of sophoraflavanone G on the solid agar plate. Antibacterial activity of sophoraflavanone G was confirmed by measuring the diameters of the zone of growth inhibition, and the highest susceptibility was shown to *E. faecium* (12 ± 2.3 mm for CCARM 5506; 11 ± 3 KACC 11954 (Table 3)).

To confirm the antibacterial effect of sophoraflavanone G, the growth curve was used, and sophoraflavanone G exhibited concentration-dependent inhibitory activity against both *E. faecium* strains. Interestingly, 6.25 µg/ml of sophoraflavanone G completely blocked bacterial growth (Fig. 2).

Sophoraflavanone G synergistically inhibited *E. faecium* growth with membrane-permeabilizing agents

Enterococcus faecium strain was treated with a combination of sophoraflavanone G and the membrane-

permeabilizing agent Triton X-100 (0.01%) to determine whether the sophoraflavanone G altered membrane permeability. The nonionic detergents of Triton X-100 were used to increase the permeability of the outer membrane. At the tested concentration, the treatment of single compound did not significantly affect *E. faecium* growth, but the combination of the two did decrease *E. faecium* growth (Fig. 3).

Sophoraflavanone G increased release of UV-absorbing materials

Release of UV-absorbing materials was measured using a UV spectrophotometer to confirm cell wall disturbance by sophoraflavanone G. The absorption change indicated material leakage from the cell including proteins and nucleic acids (Septama *et al.*, 2017). The higher absorption value suggested greater cell disruption. Significant increases in absorption at 260 and 280 nm after treatment with sophoraflavanone G suggest that sophoraflavanone G binds to and disrupts the cell wall (Fig. 4).

Sophoraflavanone G inhibited *E. faecium* growth by binding to PGNs

To determine whether sophoraflavanone G blocks *E. faecium* growth by binding to PGNs that induce cell wall perturbation, PGNs were added to the culture with sophoraflavanone G.

Table 1. MIC values against *E. faecium*.

Compounds	MIC value (µg/ml)			
	KACC 11954		CCARM 5506	
	24 hours	48 hours	24 hours	48 hours
Ampicillin	1.56	6.25	3.125	6.25
Vancomycin	>100	>100	0.78	0.78
Streptomycin	>100	>100	>100	>100
Acetylshikonin	25	50	12.5	12.5
6,8-Diprenylorobol	25	50	25	50
Kurarinone	25	50	25	50
Sophoraflavanone G	6.25	12.5	6.25	12.5
Pimaric acid	100	100	100	100
Corosolic acid	12.5	25	12.5	25
Oleanolic acid	>100	>100	>100	>100
Ursolic acid	>100	>100	>100	>100

Table 2. MBC values of sophoraflavanone G and ampicillin against *E. faecium* CCARM 5506.

MIC (µg/ml)		MBC (µg/ml)	
Sophoraflavanone G	Ampicillin	Sophoraflavanone G	Ampicillin
12.5	6.25	25	50

Table 3. Inhibition diameters of sophoraflavanone G against *E. faecium*.

Compounds	Inhibition zone (mm)	
	KACC 11954	CCARM 5506
Ampicillin	18 ± 1.5	24 ± 2
Sophoraflavanone G	12 ± 2.3	11 ± 3

PGNs dose-dependently blocked the antibacterial activity of sophoraflavanone G even at low concentrations of 6.25 and 12.5 $\mu\text{g/ml}$. This result suggests that sophoraflavanone G binds to bacterial PGNs to block bacteria growth (Fig. 5a and b).

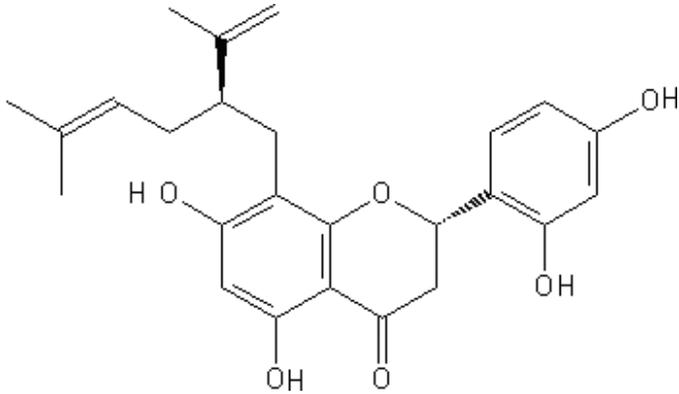


Figure 1. Chemical structure of sophoraflavanone G.

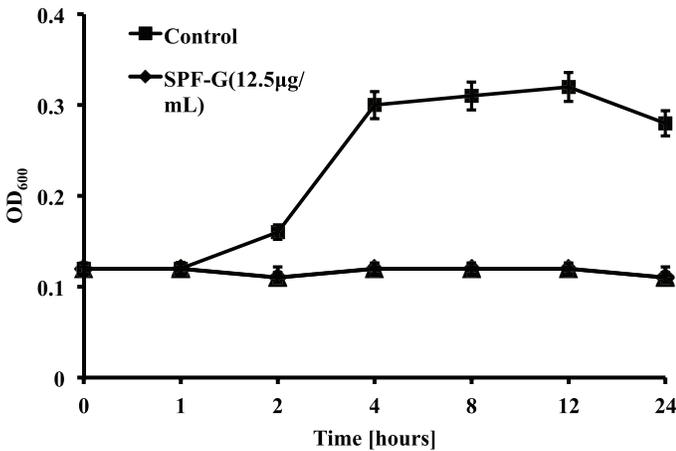


Figure 2. Sophoraflavanone G inhibited the growth of *E. faecium*. Control: untreated control *E. faecium*; SPF-G: sophoraflavanone G (12.5 $\mu\text{g/ml}$) and sophoraflavanone G (6.25 $\mu\text{g/ml}$).

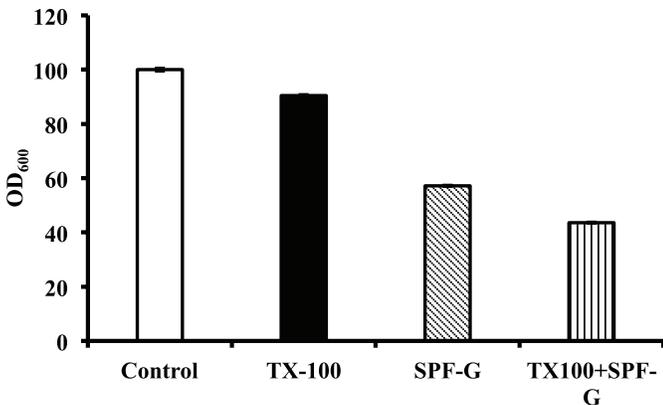


Figure 3. Sophoraflavanone G (3.125 $\mu\text{g/ml}$) synergistically inhibited growth of *E. faecium* with membrane-permeabilizing agents. Bacterial viability was determined by absorbance at 600 nm after incubation for 24 hours with sophoraflavanone G (3.125 $\mu\text{g/ml}$) and/or 0.01% Triton X-100. Control: untreated control *E. faecium* cells, TX-100: 0.01% Triton X-100, SPF-G: sophoraflavanone G (3.125 $\mu\text{g/ml}$), and TX-100+SPF-G: 0.01% Triton X-100 + sophoraflavanone G (3.125 $\mu\text{g/ml}$).

Transmission electron microscopy

To confirm whether sophoraflavanone G disrupted *E. faecium* cells, sophoraflavanone G-treated bacterial cells were examined by TEM. A 24-hours exposure of 6.25 $\mu\text{g/ml}$ of

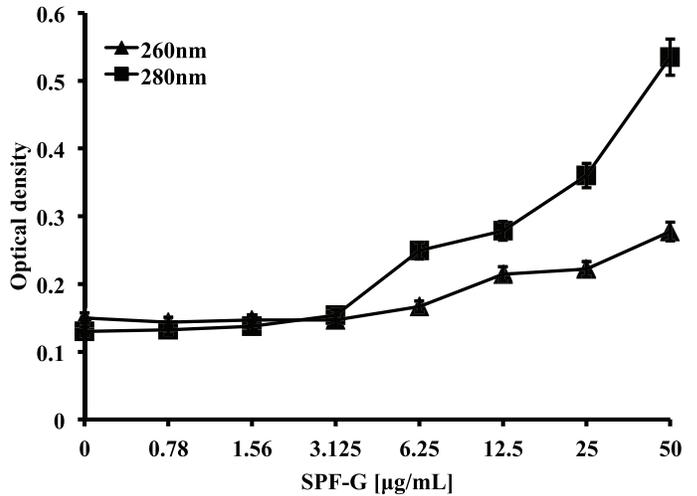


Figure 4. Sophoraflavanone G increased the leakage of cell materials.

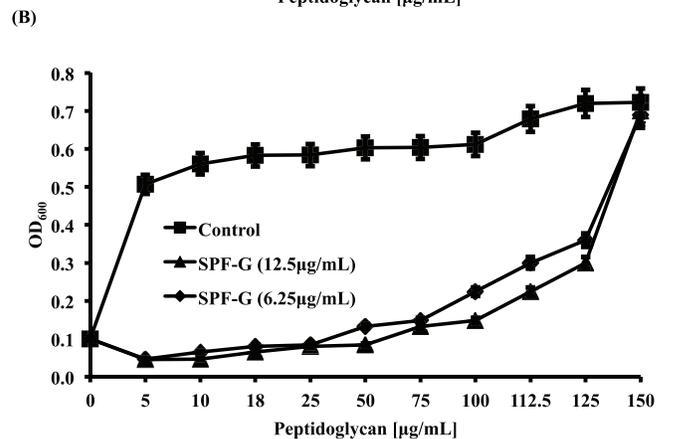
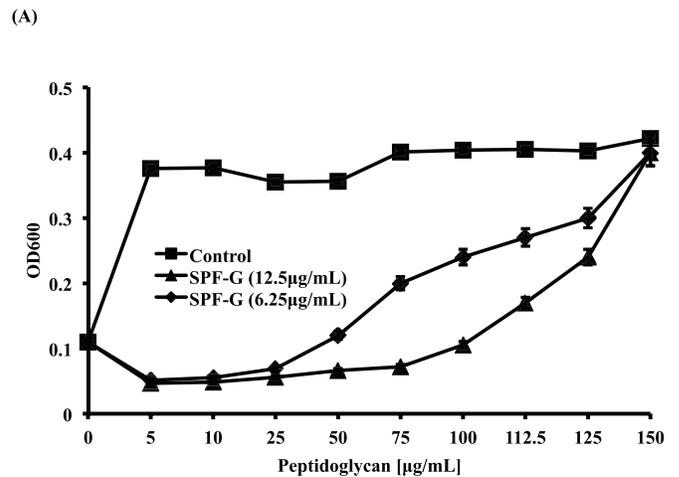


Figure 5. *E. faecium* cell wall PGN inhibited the antibacterial activity of sophoraflavanone G (6.25 $\mu\text{g/ml}$) against *E. faecium*. (A) Control: *E. faecium*; SPF-G: sophoraflavanone G (12.5 $\mu\text{g/ml}$) and sophoraflavanone G (6.25 $\mu\text{g/ml}$). (B) Control: *S. aureus*; SPF-G: sophoraflavanone G (12.5 $\mu\text{g/ml}$) and sophoraflavanone G (6.25 $\mu\text{g/ml}$).

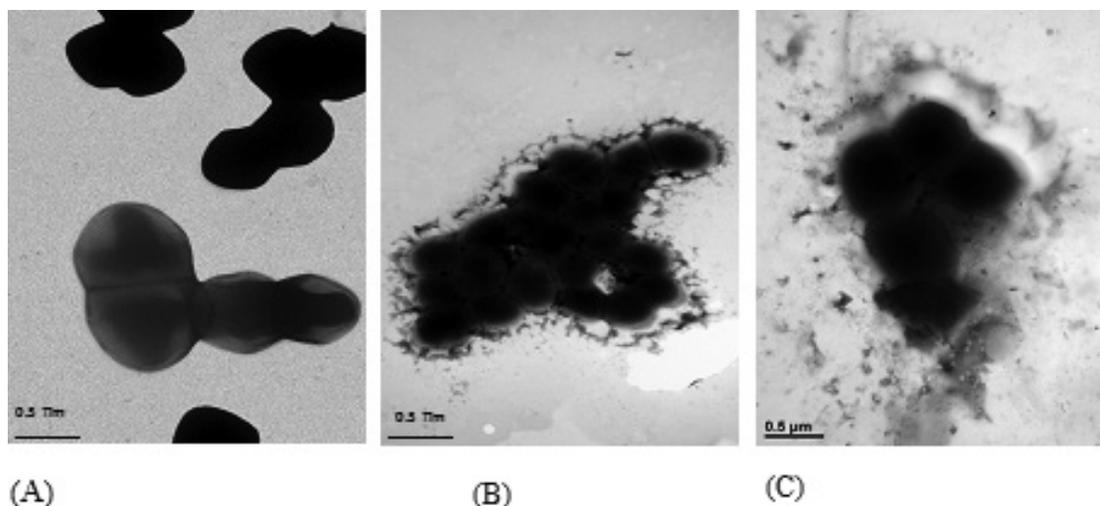


Figure 6. Transmission electron microscopy images of sophoraflavanone G-treated *E. faecium* cells. (A) Untreated control *E. faecium* cells. (B, C) *E. faecium* cells treated with sophoraflavanone G (6.25 µg/ml 1/2 MIC).

sophoraflavanone G induced cell lysis and release of cytoplasmic contents (Fig. 6b and c) compared with untreated normal bacterial cells (Fig. 6a).

DISCUSSION

The occurrence of resistant strains to antibiotics has become a problem of the world because of the extended duration of treatment and cost. *Enterococci* commonly showed the intrinsic or acquired resistance to most of the antibiotics used in humans (Climo *et al.*, 1999).

Sophoraflavanone G is a component extensively found in medicinal plants and has been used to treat the atopic dermatitis and more serious medical issues like malaria and myeloid leukemia. In particular, *Sophora* species containing sophoraflavanone G showed an antibacterial activity against *S. aureus*, *S. mutans*, *Porphyromonas gingivalis*, and *S. sobrinus* (Gerber *et al.*, 2009; Hsiang *et al.*, 2001; Kuroyanagi *et al.*, 1999; Orsi *et al.*, 2011; Tsai *et al.*, 2004).

This study showed that sophoraflavanone G inhibited *E. faecium* growth by disturbing bacterial cell walls. Sophoraflavanone G showed the highest stable antibacterial activity compared with acetylshikonin, 6,8-diprenylorobol, kurarinone, pimaric acid, and corosolic acid, which were used to treat *E. faecium* (Table 1).

The membrane perturbing effect of some of the antimicrobial flavanones was studied in association with a reduction of membrane fluidity. Accordingly, sophoraflavanone G is regarded to decrease the integrity of the cell wall, and this is confirmed by treatment of Triton X-100 that accelerates the membrane perturbing the activity of sophoraflavanone G (Lee *et al.*, 2015; Mun *et al.*, 2014). PGN is the major component of the cell wall of gram-positive bacteria including *E. faecium* and a substantial part of osmotic conservation. As shown in Figure 5, sophoraflavanone G (12.5 µg/ml) showed anti-*E. faecium* activity by binding to PGN, and this suggests that direct binding of sophoraflavanone G to the bacterial PGN induced damage of the bacterial cell wall. TEM images of sophoraflavanone G-treated *E. faecium* also confirmed the disruption of the cell wall of *E. faecium* by sophoraflavanone G (Lee *et al.*, 2015; Mun *et al.*, 2014).

This study provided evidence that sophoraflavanone G possesses anti-*E. faecium* activity by disrupting the cell wall through binding to PGNs. Therefore, sophoraflavanone G should be a good candidate to treat multidrug-resistant *E. faecium* infection. However, more studies including toxicity, bioavailability, and combination tests with existing antibiotics are required to confirm the availability as an antibacterial agent.

CONFLICT OF INTEREST

Authors declared that there is no conflicts of interest.

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