In Vitro Antibacterial Activity of Panduratin A against Enterococci Clinical Isolates

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Panduratin A, a natural chalcone compound isolated from the rhizome of fingerroot (*Boesenbergia rotunda* (L.) Mansf. A). The antibacterial activity of panduratin A against clinical enterococci isolates was compared in terms of minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) to those of commonly used antimicrobials, according to the CLSI guidelines. Time–kill curves were constructed to assess the concentration between MIC and bactericidal activity of panduratin A at concentrations ranging from 0× MIC to 4× MIC. The activity of panduratin A against biofilm-producing enterococcal strains was also evaluated. The growth of all clinical enterococci isolates ($n=23$) were inhibited by panduratin A at a concentration of 2 μg/ml. Panduratin A was able to kill all clinical enterococci isolates with a MBC of 8 μg/ml. The time–kill curves demonstrated that the bactericidal endpoint for clinical enterococci was reached after 30 min of incubation at a panduratin A concentration of 4× MIC. The growth of biofilm-producing enterococcal strains can be inhibited and eradicated by panduratin A at concentrations of $\leq$4 μg/ml and $\leq$16 μg/ml, respectively. The antibacterial activity of panduratin A against all clinical enterococci isolates was generally more potent than commonly used antimicrobials. Panduratin A has stronger activity against biofilm-producing enterococcal strains than daptomycin and linezolid. Panduratin A is an antimicrobial agent with high *in vitro* activity against clinical enterococci, including organisms resistant to other antimicrobials.

Key words antibacterial activity; enterococci; panduratin A

Enterococci, *Enterococcus faecalis*, and *E. faecium*, are the most common Gram-positive cocci in the intestinal tract and have been documented to cause infection of the urinary tract and other sites of humans.[1—5] Increased antimicrobial resistance in enterococci has become a problem in recent years.[6,7] Vancomycin is often used as a drug of last resort in treatment of antibiotic-resistant, Gram-positive bacterial infections caused by organisms such as multiresistant enterococci, but the treatment is frequently unsuccessful.[8] Thus, the identification of novel agents effective in inhibiting the growth of these strains has gained renewed urgency.[9] Further, an interest in plants with antimicrobial properties has been revived as a consequence of the current problems associated with the use of antibiotics.[10,11]

Panduratin A, a natural chalcone compound isolated from the rhizomes of fingerroot (*Boesenbergia rotunda* (L.) Mansf, A.), has been reported to posses antibacterial activity against *Prevotella intermedia*, *P. loescheii*, *Porphyromonas gingivalis*, *Propionibacterium acnes*, and *Streptococcus mutans*, as well as antibiofilm activity against multispecies oral biofilms, *in vitro*.[12—15] Panduratin A has also been reported to have strong antimicrobial activity against clinical *Staphylococcus* strains.[16] However, the antimicrobial activity of panduratin A against other pathogenic bacteria, such as enterococci, has not been investigated. In this study, we compared the *in vitro* activity of panduratin A against clinical enterococci isolates with those of common antimicrobial agents, including ampicillin, daptomycin, gentamycin, erythromycin, levofloxacin, linezolid, tetracycline, and vancomycin.

MATERIALS AND METHODS

Microorganisms A reference strain of *E. faecalis* (ATCC 29212), was obtained from American Type Culture Collection (Rockville, MD, U.S.A.). Twenty-three isolates of clinical enterococci consisting 10 and 13 isolates of *E. faecalis* and *E. faecium*, respectively, were obtained from The Research Institute of Bacterial Resistance, College of Medicine, Yonsei University, Korea. The strains were isolated from blood, genital secretions, pus, or sputum of patients in 2008. The species were identified by conventional methods or by using the Vitek system (bioMerieux SA, Marcy l'Etoile, France), according to the manufacturer's instructions.

Panduratin A and Commercial Antibacterial Agent Preparation Panduratin A (Fig. 1) was isolated in a pure form from an ethanol extract of fingerroot (*B. rotunda*) rhizome using the method of Park *et al.* and dissolved in 10% dimethylsulfoxide (DMSO) to generate a stock solution of 1024 μg/ml.[13] Ampicillin, erythromycin, gentamicin, tetracycline, and vancomycin were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Daptomycin was obtained from Cubist Pharmaceuticals Inc. (Lexington, MA, U.S.A.). Levofloxacin was purchased from Sigma-Fluka Co. (Steinheim, Germany) and linezolid was provided by Dong-A Pharmaceutical Co. (Seoul, Korea). Stock solutions of commercial antimicrobial agents were prepared according to the manufacturers' instructions.

Minimum Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs) Determination *In vitro* susceptibility tests were performed in 96-well microtiter plates to determine MICs of panduratin A and other

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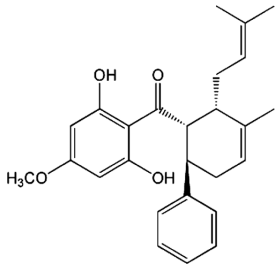

Fig. 1. Chemical Structure of Panduratin A

antimicrobial agents against *E. faecalis* strain ATCC 29212 and 23 isolates of clinical enterococci using the method described in the CLSI M7-A6 guidelines.[17] A 2-fold dilution of panduratin A stock solution or other antimicrobial agent preparation was mixed with the test organisms (5×10^5 colony forming unit (CFU)/ml) in Mueller–Hinton broth (MHB) medium (Difco Becton Dickincon, Sparks, MD, U.S.A.). The dilutions were started from the wells in column 12 of the microtiter plate. Thus, column 12 of the microtiter plate contained the highest concentrations of panduratin A or other antimicrobial agents, and column 3 contained the lowest concentrations of panduratin A or other antimicrobials agents. Column 2 served as the positive control for all samples (only medium and inoculum or antimicrobial agent-free wells), and column 1 was the negative control (only medium, no inoculum, no antimicrobial agent). Microtiter plates were incubated aerobically at 37 °C for 24 h. The MIC was defined as the lowest concentration of antimicrobial agent that resulted in the complete inhibition of visible growth.

Panduratin A was diluted in 10% DMSO followed by 2-fold dilutions in the test wells; thus, the final concentration of DMSO would be serially decreased. We examined the effect of DMSO on the growth and viability of all clinical enterococci tested. DMSO at ≤10% was found not to affect growth or viability of the enterococci tested. These results suggest that DMSO had no effect on activity and that all measured antimicrobial activity was due to panduratin A. Specific MIC breakpoints (in μg/ml) were as follows (S, susceptible; I, intermediate; and R, resistant): ampicillin, ≤8, —, ≥16; daptomycin, ≤4, —, —; erythromycin, ≤0.5, 1—4, ≥8; levofloxacin, ≤2, 4, ≥8; linezolid, ≤2, 4, ≥8; tetracycline, ≤4, 8, ≥16; and vancomycin, ≤4, 8—16, ≥32.[17]

MBCs were determined for each agent for all clinical enterococci isolates, as described for determination of MICs, by removing the medium from each well that showed no visible growth and subculturing onto MHA (MHB supplemented with 1.5% bacterial agar) plates. Briefly, medium (approximately of 100 μl) from each well showing no visible growth was spread onto MHA plates. Wells in column 2, the positive controls (antimicrobial agent-free wells), and wells in column 1, growth-negative controls, were included in the MBC test. Plates were incubated at 37 °C for 24 h or until growth was seen in the growth positive control plates. MBC was defined as the lowest concentration of antimicrobial agent at which all bacteria in the culture are killed or the lowest concentration at which no growth occurs on MHA plates.[18,19]

Time–Kill Assay A time–kill assay was performed on each of all enterococci clinical isolates and *E. faecalis* reference strain ATCC 29212. Time–kill assays were performed in MBH medium, according to the method of Lorian[18] and Pankey and Ashcraft with modification.[20] Briefly, the adjusted inoculum suspension of 5×10^7 CFU/ml was diluted 1 : 10 in MHB medium to a final concentration of 5×10^6 CFU/ml. Each concentration of panduratin A was diluted 1 : 10 in MHB medium containing 5×10^6 CFU/ml. This yielded an initial inoculum of 4.5×10^6 CFU/ml. Final concentrations of panduratin A were 0× MIC, 0.5× MIC, 1× MIC, 2× MIC, and 4× MIC for each *Enterococcus* isolate. Cultures (5 ml final volume) were incubated at 37 °C with 200 rpm agitation. At pre-determined time points (0, 15, 30, and 45 min as well as 1, 2, and 4 h), 100-μl aliquots were removed and transferred to Eppendorf tubes, centrifuged (3900 rpm at 4 °C for 1 min) and rinsed twice with 900 μl of sterile distillated water to obtain panduratin A-free cells. Pellets were suspended in 100 μl of MHB medium and serially diluted. An appropriate volume (100, 50, or 25 μl, depending on the dilution and the concentration of panduratin A) was spread onto MHA plates and incubated at 37 °C for 48 h or more, until the colonies appeared on the plates, to determine the number of CFU/ml. Assays were carried out on three different occasions, in triplicate.

***In Vitro* Biofilm Formation** Enterococcal biofilms were allowed to form in the wells of commercially available, presterilized, polystyrene flat-bottomed 96-well microtiter plates, as previously described by Sandoe *et al.*[21] with modification. Briefly, the wells of microtiter plates were filled with 100 μl of brain heart infusion (BHI) medium. Biofilms were generated by pipetting 100 μl of the standard inoculum (5×10^6 CFU/ml) into selected wells of the microtiter plates, resulting in final inoculums of 2.5×10^6 CFU/ml. The plates were covered and sealed with parafilm. Plates were incubated at 37 °C without agitation for 24 h. After incubation, the medium was discarded, and nonadherent cells were removed by thoroughly washing the biofilm three times with sterile phosphate buffered saline (PBS). The plates were inverted and drained by blotting them with paper towels to remove any residual medium. Biofilms were then ready to be assessed for their biofilm-forming capacity and sensitivity to panduratin A and others antibacterial agents.

Quantification of Enterococcal Biofilm Quantification of staphylococci biofilms were carried out using 0.1% crystal violet as previously described.[22,23] Briefly, washed adherent cells, as described in the section: "*in vitro* biofilm formation," were stained with 0.1% crystal violet. The crystal violet was solubilized using 30% glacial acetic acid for 15 min. Relative biofilm formation was assayed by reading optical density at 550 nm using a microtiter plate reader (VERSA$_{MAX}$, Sunnyvale, CA, U.S.A.).

Sessile Minimal Inhibitory Concentrations (SMICs) and Minimal Biofilm Eradication Concentrations (MBECs) SMICs and MBECs of panduratin A and other antibacterial agents on established staphylococci biofilms were verified in commercially available, presterilized, polystyrene, flat-bottomed 96-well microtiter plates, as described above. Briefly, washed adherent cells were filled with 200 ml 2-fold dilutions of the panduratin A or other antibacterial agents in BHI, ranging from the 1—512 μg/ml. The dilutions were started from the wells in column 12 of the microtiter plate. Thus, column 12 of the microtiter plate contained 512 μg/ml of panduratin A or other antimicrobial agents, and column 3 contained 1 μg/ml of panduratin A or other antimi-

crobials agents. Column 2 served as the positive control for all samples (only medium and inoculum or antimicrobial agent-free wells), and column 1 was the negative control (only medium, no inoculum, no antimicrobial agent). The plates were incubated for 24 h at 37 °C. The biofilms where then washed and stained, as described above. The optical density (OD_{550}) was measured at time 0 h and after incubation for 24 h. The SMIC was defined as the lowest concentration where no growth occurred in the supernatant fluid, confirmed by no increase in OD_{550} compared to the initial reading. $SMIC_{50}$ was defined as the lowest concentration of antibacterial agent at which 50% of staphylococci biofilms OD_{550} were inhibited. $SMIC_{90}$ was defined as the lowest concentration of antibacterial agent at which 90% of staphylococci biofilms OD_{550} were inhibited.

MBECs were determined, as follows: washed biofilms from the bottom of treated wells were scarred with a metal loop and spread over the surface of BHA (BHI supplemented with 15% bacterial agar) plates then incubated for 24 h at 37 °C. The MBEC was determined to be the lowest concentration at which no bacterial growth occurred on the BHA plates. $MBEC_{50}$ was defined as the lowest concentration of antibacterial agent at which 50% of enterococcal biofilms showed no bacterial growth on the BHA plates. $MBEC_{90}$ was defined as the lowest concentration of antibacterial agent at which 90% of enterococcal biofilms showed no bacterial growth on the BHA plates. Data from at least four time points of experiment with four replicates each were evaluated and calculated.

RESULTS AND DISCUSSION

The MICs and MBCs of panduratin A against 23 isolates of clinical enterococci are shown with those of ampicillin, daptomycin, erythromycin, eugenol, gentamicin, levofloxacin, linezolid, tetracycline, and vancomycin in Table 1. Based on the CLSI break points, all clinical enterococci isolates were resistant to ampicillin and erythromycin.[17] Moreover, more than 90% of clinical enterococci isolates were resistant to levofloxacin and vancomycin, and 40% were resistant to tetracycline. All clinical enterococci isolates were susceptible to daptomycin and linezolid. The break point of gentamicin against enterococci is not available in CLSI.[17]

Our results show that all isolates were susceptible to panduratin A, with MICs of $\leq 2\,\mu$g/ml and MIC_{90} of 1 μg/ml. In our previous report, the MIC of panduratin A against *P. gingivalis*, *P. loescheii*, and *S. mutans* was 4 μg/ml, while that of panduratin A against *P. intermedia* and *P. acnes* was 2 μg/ml.[12—14] Moreover, panduratin A had a MIC_{90} of 1 μg/ml for clinical staphylococcal isolates ($n=108$).[16] These results show that panduratin A has stronger activity against clinical enterococci isolates than against *P. gingivalis*, *P. loescheii*, and *S. mutans*, and comparable or equal to that against *P. intermedia*, *P. acnes*, and clinical *Staphylococcus* strains. Panduratin A has been shown to prevent biofilm formation of primary multi-species oral bacteria (*Actinomyces viscosus*, *S. mutans*, and *S. sanguis*) *in vitro*.[15]

The MIC_{90} of panduratin A against all clinical enterococci isolates (2 μg/ml) was also much lower than that of ampi-

Table 1. Comparative *in Vitro* Activities of Panduratin A and Other Antimicrobial Agents against Clinical Enterococci Isolates

Enterococci (no. of isolates tested) and antimicrobial agents	MIC (μg/ml)			Susceptibility (%)[a]			MBC (μg/ml)		
	Range	MIC_{50}	MIC_{90}	S	I	R	Range	MBC_{50}	MBC_{90}
E. faecalis ($n=10$)									
Ampicillin	16—128	64	128	0	—	100	32—256	128	256
Daptomycin	0.5—4	2	4	100	—	—	1—8	4	8
Erythromycin	32—256	128	256	0	0	100	64—512	256	512
Gentamycin	64—256	128	256	—	—	—	256—>512	512	>512
Levofloxacin	1—64	32	32	10	0	90	16—256	64	128
Linezolid	0.25—1	1	1	100	0	0	0.5—8	4	8
Tetracycline	0.25—0.5	0.5	0.5	100	0	0	2—8	4	8
Vancomycin	8—64	64	64	0	10	100	32—266	128	256
Panduratin A	0.125—2	0.5	1	—	—	—	0.25—8	4	4
E. faecium ($n=13$)									
Ampicillin	16—128	64	128	0	—	100	64—256	128	256
Daptomycin	1—4	1	4	100	—	—	2—8	4	8
Erythromycin	32—256	128	256	0	0	100	64—>512	128	256
Gentamycin	128—512	256	512	—	—	—	256—>512	256	>512
Levofloxacin	4—64	16	32	0	7.5	92.5	16—256	64	128
Linezolid	1—2	1	2	100	0	0	4—16	4	8
Tetracycline	1—64	4	32	30	15	55	4—256	8	64
Vancomycin	8—128	64	128	0	7.5	92.5	16—256	256	512
Panduratin A	1—2	1	2	—	—	—	4—8	4	8
All isolates ($n=23$)									
Ampicillin	16—128	64	128	0	—	100	32—256	128	256
Daptomycin	0.5—4	2	4	100	—	—	1—16	4	>512
Erythromycin	32—256	64	256	0	0	100	128—512	512	8
Gentamicin	64—512	256	512	—	—	—	128—>512	512	>512
Levofloxacin	1—64	16	32	4.5	4.5	91	2—256	64	128
Linezolid	0.25—2	1	2	100	0	0	0.5—32	4	16
Tetracycline	0.5—64	1	64	60	0	40	1—256	64	256
Vancomycin	8—128	64	128	0	4.5	95.5	16—512	256	512
Panduratin A	0.125—2	1	2	—	—	—	0.25—8	2	4

a) S, susceptible; I, intermediate; R, resistant; —, CLSI breakpoint is not available.

cillin (256 μg/ml), erythromycin (256 μg/ml), gentamicin (512 μg/ml), levofloxacin (32 μg/ml), tetracycline (64 μg/ml), and vancomycin (128 μg/ml), indicating that panduratin A has stronger anti-enterococcal activity against clinical enterococci isolates than any of these commonly used antimicrobial agents. Our results show that the growth of all clinical enterococci isolates was inhibited by ≤2 μg/ml of panduratin A. The activity of panduratin A against all clinical enterococci isolates was comparable or equal to that of daptomycin (≤4 μg/ml) and linezolid (≤2 μg/ml).

The *in vitro* MBCs of panduratin A with an endpoint of 24 h demonstrated that panduratin A was able to kill all clinical enterococci isolates with MBCs of ≤8 μg/ml. Even though, the MICs of daptomycin (≤4 μg/ml) and linezolid (≤2 μg/ml) against all clinical enterococci isolates was equal with the MIC of panduratin (≤2 μg/ml), but the MBCs of daptomycin (≤16 μg/ml) and linezolid (≤32 μg/ml) were higher than that of panduratin A (≤8 μg/ml), indicating that panduratin A has a stronger antimicrobial activity against all clinical enterococci isolates. The MBC of panduratin A against all clinical enterococci isolates was also much lower than that of ampicillin (≤256 μg/ml), erythromycin (512 μg/ml), gentamicin (>512 μg/ml), levofloxacin (≤256 μg/ml), tetracycline (≤256 μg/ml), and vancomycin (512 μg/ml). These results suggest that panduratin A has very strong bactericidal activity against all clinical isolates. In addition, the MBC of panduratin A against *P. ginggivalis*, *P. loescheii*, and *S. mutans* was 8 μg/ml, the MBC of panduratin A against *P. intermedia* and *P. acnes* was 4 μg/ml, and the MBC of panduratin A against 108 isolates of clinical *Staphylococcus* was ≤8 μg/ml.[12—15)]

In the time–kill assays, panduratin A significantly inhibited bacterial growth compared to control cultures (Fig. 2). The bactericidal activity of panduratin A was fast-acting against *E. faecalis* ATCC 29212, *E. faecalis* clinical isolate #10, and *E. faecium* clinical isolate #6; the reduction in the CFU/ml was >3 log units (99.9%) at a panduratin A concentration of 2× MIC. The bactericidal endpoints for *E. faecalis* ATCC 29212, *E. faecalis* clinical isolate #10, and *E. faecium* clinical isolate #6 were reached after 45 min of incubation at a panduratin A concentration of 2× MIC or after 30 min of incubation at a panduratin A concentration of 4× MIC.

Panduratin A was evaluated for antibiofilm activity against 23 isolates of clinical enterococci isolates. The results are summarized in Table 2 and discussed in comparison to daptomycin and linezolid. All clinical enterococcal isolates were susceptible to daptomycin and linezolid. Even though daptomycin and linezolid were effective against the planktonic growth of enterococcal strains, these drugs did not demonstrate strong activity against biofilm-producing enterococcal strains. The SMICs and MBECs of daptomycin and linezolid

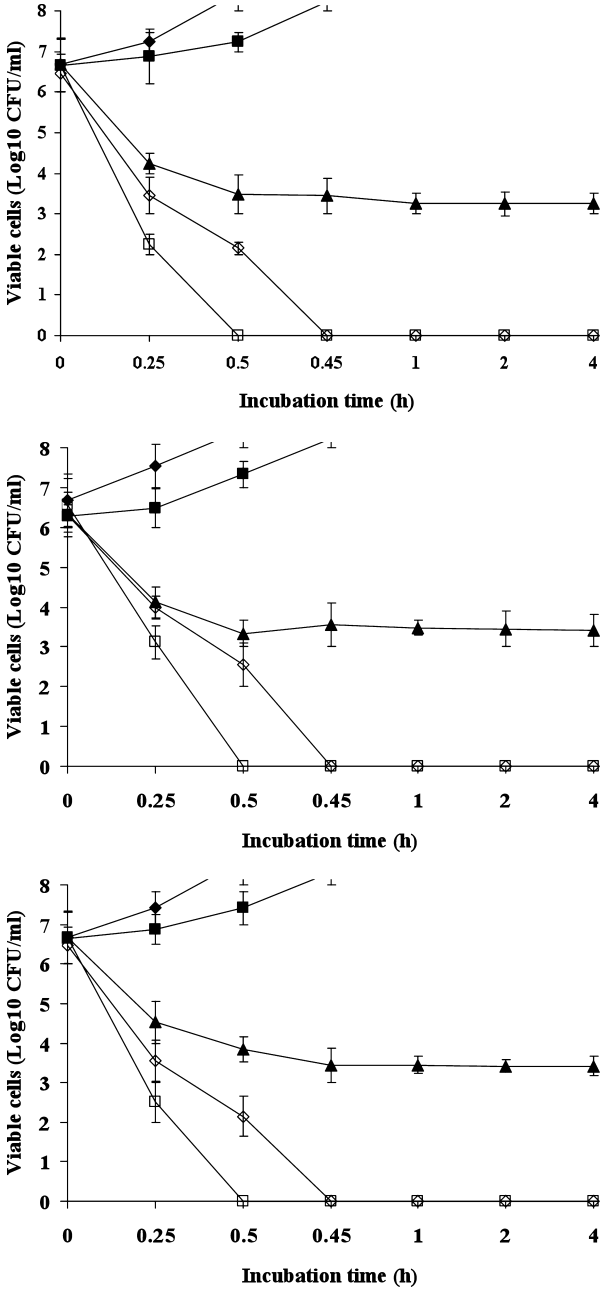

Fig. 2. Time–Kill Plots of *E. faecalis* ATCC 29212 (2A), and Representative Clinical Enterococci

E. faecalis clinical isolate #10 (2B) and *E. faecium* clinical isolate #6 (2C), following exposure to panduratin A at 0× MIC (filled diamonds, control), 0.5× MIC (filled squares), 1× MIC (filled triangles), 2× MIC (open diamonds), and 4× MIC (open squares) after endpoint (48 h), *E. faecalis* ATCC 29212 (0, 0.5, 1.0, 2.0, 4.0 μg/ml); *E. faecalis* clinical isolate #10 (0, 0.25, 0.5, 1.0, 2.0 μg/ml); *E. faecium* clinical isolate #6 (0, 0.5, 1.0, 2.0, 4.0 μg/ml). Values given in the brackets after species are 0× MIC (control), 0.5× MIC, 1× MIC, 2× MIC, and 4× MIC, respectively.

Table 2. Comparative *in Vitro* Activities of Panduratin A and Other Antimicrobial Agents against Enterococcal Biofilms

Enterococcal group (n^a) or antimicrobial agents	SMIC (μg/ml)			MBEC (μg/ml)		
	Range	50%	90%	Range	50%	90%
All isolates (n=23)						
Daptomycin	4—16	8	16	32—64	64	64
Linezolid	4—16	16	16	16—64	32	64
Panduratin A	1—4	2	4	8—16	16	16

against biofilm-producing enterococcal strains were ≤16 μg/ml and ≤64 μg/ml, respectively. Interestingly, the growth of biofilm-producing enterococcal strains can be inhibited by panduratin A at concentrations of ≤4 μg/ml. The biofilm-producing enterococcal strains can be eradicated by panduratin A at concentrations of ≤16 μg/ml (Table 1). These results indicate that panduratin A has stronger activity against biofilm-producing enterococcal strains than daptomycin and linezolid.

In conclusion, panduratin A is an antimicrobial agent with high *in vitro* activity against clinical isolates, including multidrug-resistant strains. Our results strongly suggest that panduratin A should undergo further testing to assess its potential for the treatment of diseases caused by enterococci. It is important to note that the activities of phytochemicals like panduratin A might be non-specific and affected in the presence of serum. In this research, all tests have been conducted *in vitro*, thus further studies are necessary to elucidate its antibacterial activities *in vivo*.

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