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Article

Enhancing activity of antibiotics against *Staphylococcus aureus* with Shuang-Huang-Lian

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SUMMARY

***Staphylococcus aureus* is a major pathogen in both hospitals and the community and can cause systemic infections such as pneumonia. Multi-drug resistant strains, such as Methicillin-resistant *S. aureus* (MRSA) are particularly worrisome. Bacterial antibiotic resistance can be attributed to various mechanisms including antibiotic inactivation. In order to reduce the development of bacterial resistance, we hypothesized that two selected traditional Chinese medicines, Shuang-Huang-Lian (SHL) and Lan-Qin, would be effective against *S. aureus*. In this study, we carried out the zone of inhibition determination of five antibiotics including amoxicillin, gentamicin, penicillin, clarithromycin, cefazolin and the traditional Chinese medicines SHL and Lan-Qin. The preliminary screening results showed that all the drugs except Lan-Qin exhibited antibacterial effects. Then, we conducted quantitative determination experiments to further explore the bacteriostasis of different drugs. The results of minimum inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) determination indicated that penicillin had the best inhibitory effect on bacterial growth, and clarithromycin killed all the bacteria at the lowest concentration. Also, we tested the combined effect of SHL with different antibiotics against *S. aureus* and calculated the fractional inhibitory concentration (FIC) of the antibiotics. The results showed that SHL had a synergistic effect with gentamicin as well as additive effects with penicillin and cefazolin against *S. aureus* by decreasing MICs of antibiotics compared with using antibiotics alone. Our study provides a reference for the clinical treatment of *S. aureus* infection in combination with traditional Chinese medicines.**

INTRODUCTION

Staphylococcus aureus is a gram-positive, round, thick-walled bacterium and is a common member of the human microbiota. It is frequently found in the upper respiratory tract and on the skin, usually tests positive for peroxidase and nitrate reduction, and is a facultative anaerobic bacterium that does not require oxygen to grow (1). Although *S. aureus* usually acts as a commensal organism of the human microbiota, it can also be an opportunistic pathogen. Pathogenic strains promote infection through the production of virulence factors, such as potent protein toxins and the expression of cell surface proteins that bind to active sites and inactivate antibodies (1). *S. aureus* can cause a range of diseases from minor skin infections such as acne, pustules, boils, cellulitis, folliculitis, carbuncles, scalded skin syndromes, and abscesses to life-threatening conditions like pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and septicemia. It remains one of the five most common causes of hospital-acquired infections and is often the cause of post-surgical wound infections. Each year, approximately 500,000 patients in US hospitals are infected with staphylococci, primarily caused by *S. aureus* (2). In the United States, up to 50,000 people die from *S. aureus* infections each year (3).

Antibiotics are the most important type of antibacterial agent against bacterial infections and are widely used to treat and prevent infections by killing or inhibiting the growth of bacteria (4). Amoxicillin, gentamicin, penicillin, clarithromycin, and cefazolin are all commonly prescribed antibiotics against *S. aureus* based on literature survey. Penicillin, amoxicillin, and cefazolin kill bacteria by inhibiting the completion of peptidoglycan synthesis, a structural component of the bacterial cell wall (5). Gentamicin is a bactericidal antibiotic that negatively affects protein synthesis by binding to the 30S subunit of the bacterial ribosome (6). Clarithromycin prevents bacterial multiplication by acting as a protein synthesis inhibitor, binding to 23S rRNA and thereby inhibiting peptide translation (7). The traditional Chinese medicines Shuang-Huang-Lian (SHL) and Lan-Qin are famous modern formulae prepared from medicinal herbs, which can be used for the treatment of upper respiratory tract infections and have heat-clearing and detoxification effects (8). Because *S. aureus* infection can also cause these symptoms, it is important to understand these two traditional medicines and their interplay with the standard antibiotics mentioned above.

As a special drug to control *S. aureus* infection, antibiotics have been widely used. Along with the serious drug resistance of pathogenic bacteria, especially the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA), new challenges arise for clinical anti-infection treatment. MRSA is a group of bacteria that are genetically distinct from other *S. aureus* strains and have acquired multiple resistance to beta-lactam antibiotics by natural selection or by horizontal gene transfer (9). MRSA is common in hospitals, prisons, and nursing homes, and people with open wounds, invasive

devices such as catheters, and weakened immune systems are at greater risk of hospital-acquired infections. Antibiotic-resistant strains of *S. aureus* are a global problem in clinical medicine. Despite extensive research and development, no approved *S. aureus* vaccine is currently available yet (2).

The rise of bacterial resistance highlights the urgency of finding new drugs with new modes of action to treat bacterial infections. Bacterial resistance to antibiotics can be attributed to a variety of mechanisms, including antibiotic inactivation, target gene mutations that impair drug binding, reduced membrane protein permeability, and efflux mechanisms that pump out drugs (10). Scientists have developed several approaches to overcome bacterial resistance such as combination therapies that include association of two or more antibiotics and the discovery of innovative antibiotics and antibiotic classes with novel targets. Moreover, referring to nature as a source of antimicrobials yielded some promising bactericidal candidates, like antimicrobial peptides (11).

With the misuse of antibiotics, increasing numbers of *S. aureus* have been reported to develop resistance, making it particularly important to explore some effective traditional Chinese medicine. We hypothesized that the two selected herbal medicines, SHL and Lan-Qin, would be effective against *S. aureus*. Herein, we measured the zone of inhibition of five antibiotics including amoxicillin, gentamicin, penicillin, clarithromycin, cefazolin and two kinds of traditional Chinese medicine to screen for drugs that might inhibit S. aureus. Furthermore, we evaluated the drugs that exhibited effectiveness for their antibacterial activity by minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) determination. In the end, we tested the combined effect of SHL with different antibiotics against *S. aureus* and calculated the fractional inhibitory concentration (FIC) of the antibiotics. The results showed that SHL could improve the antibacterial activity of some antibiotics compared with antibiotics alone, while Lan-Qin was not effective against *S. aureus*.

RESULTS

Through the inhibition zone experiment, we found that amoxicillin, gentamicin, penicillin, clarithromycin, cefazolin, and SHL were effective against *S. aureus*. Then we determined the MICs and MBCs of these drugs to screen out the optimal antibiotic. Finally, we studied the antibacterial effect of SHL combined with different antibiotics on *S. aureus* further.

Zone of inhibition determination

We carried out Kirby-Bauer tests to determine the zone of inhibition, in which the drug on the filter paper disk penetrated into the culture medium, thus inhibiting the growth of bacteria (12). After 18 hours of incubation, we observed and measured the diameters of inhibition zones of different drugs. The experiment was repeated three times and the average diameter of the inhibition measurements was

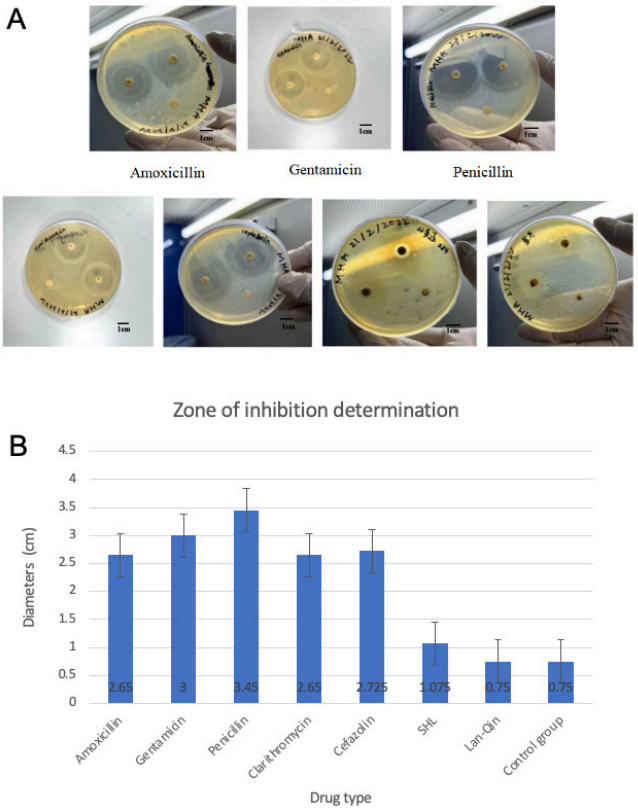

Figure 1: Zone of inhibition determination. A) Petri dishes with the three filter paper disks showing the inhibition zones of the seven different drugs; two disks were exposed to the drug as the experimental group and one disk was the drug-free control group. B) Graph representing the difference in diameter of zones of inhibition. The experimental group exhibited a larger average zone of inhibition than the control group.

calculated. **Figure 1A** demonstrated that all drugs except Lan-Qin had an obvious transparent zone around the tested disks, and no antibacterial zones were generated around the control disks on each plate. The diameters of the antibacterial zones of different antibiotics varied little, ranging from 2.65 cm to 3.45 cm (**Figure 1B**). Among these circles, the largest diameter was from penicillin (3.45 cm) and the smallest diameter was from amoxicillin and clarithromycin (2.65 cm). For the traditional Chinese medicines, the bacteriostatic zone of SHL was 1.05 cm, while Lan-Qin had the same diameter as the control disk. One-way ANOVA (analysis of variance) indicated all antibiotics and SHL produced statistically significant inhibition when compared to the control ($p < 0.0001$), while the diameter of the inhibition zone treated with Lan-Qin was not significantly different than that of bacteria without treatment ($p > 0.9999$). Through the inhibition zone experiment, we selected amoxicillin, gentamicin, penicillin, clarithromycin, cefazolin, and SHL for further testing.

MICs and MBCs determination

In order to quantitatively determine the bacteriostatic effect of each drug, we conducted MIC tests against *S.*

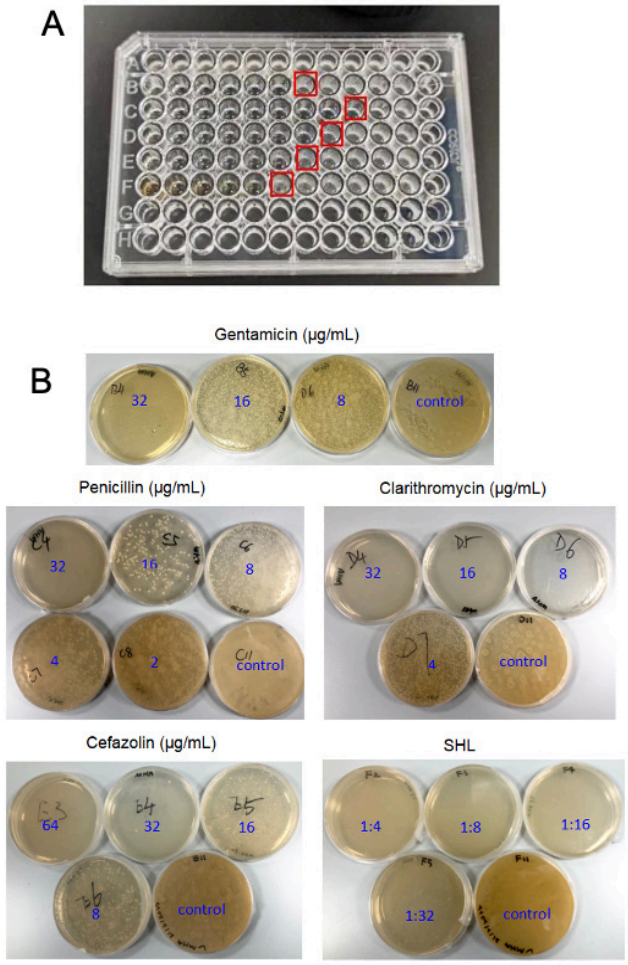

Figure 2: MICs and MBCs of different drugs determination. A) A 96-well plate was used to demonstrate the growth of bacteria. One drug was placed in each horizontal row and the concentration was gradually reduced. Column 11 without drugs served as a positive control and column 12 containing MHB medium only was the negative control. Inside the red box were the bacteria in wells that started to get muddy. B) Plates were inoculated with dilutions of MIC, 2×MIC, 3×MIC, 4×MIC, and 5×MIC that showed no visible growth of bacteria. Plates B to F contained different antibiotics at different concentrations labeled on the plates respectively. The last plate shown with lots of colonies acted as the positive control for each group.

aureus of amoxicillin, gentamicin, penicillin, clarithromycin, cefazolin, and SHL. All the liquid of column 11 in 96-well plate as positive controls was turbid, indicating bacteria grew well and all the liquid of column 12 in 96-well plate as negative controls was clear, which showed there were no other bacterial contamination. These control groups eliminated the interference of irrelevant factors in the experimental results. To eliminate human error by just eyeballing the plate, the optical density of bacteria in each column was recorded at 600 nm (OD 600), which represented the concentration of the bacteria in the fluid. For each drug, the liquid in the well gradually became cloudy and the absorption value increased gradually as the drug changed from higher concentration to lower concentration, indicating that lower concentrations of drug cannot inhibit bacterial growth (**Figure 2A, Table 1**). The MIC was regarded as the lowest concentration of drug that prevented visible turbidity, at which the absorption value was approximately equal to zero (13). The MIC of gentamicin, penicillin, clarithromycin, cefazolin, and SHL was 8 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, and 1:32, respectively (**Table 1**).

Unlike MIC, which determines the minimum concentration of the drug to inhibit bacterial growth, MBC is the minimum concentration of the drug to kill all bacteria. In order to determine whether the columns with greater than or equal to MIC values were really free of live bacteria, the following methods were adopted to measure the MBC values of drugs. After incubation of dilutions with MIC, 2×MIC, 3×MIC, 4×MIC, and 5×MIC that showed no visible growth of bacteria overnight in the in 96-well plate mentioned above, we inoculated the dilutions to the plates and conducted a colony count on the plates (14). For each drug, we could then find the value of MBC, the lowest concentration of antibiotics that killed all bacteria, which showed no bacterium on the plate and all the positive control plates had numerous colonies (**Figure 2B**). In addition, we found that, with the decrease of drug concentration, the number of bacterial colonies on the plate gradually increased, indicating that the bactericidal effects of the drug depend on the concentration of drugs (**Figure 2B**). The MBC of gentamicin, penicillin, clarithromycin, cefazolin and SHL was 32 μg/mL, 32 μg/mL, 8 μg/mL, 64 μg/mL, and 1:16, respectively (**Table 1**).

Combined effect of SHL with antibiotics

In order to search for antibiotic modulatory activity, we performed a combination assay between different drugs and SHL. An FIC index ≤0.5 indicated that the combination of the two drugs was synergistic. On the other hand, 0.5 < FIC≤1 indicated that the combination of the two drugs was additive, 1 < FIC < 2 indicated that the combination of two drugs was unrelated, and FIC > 2 indicated that the combination of two drugs was antagonistic (15). Significant ($p < 0.0001$) interactions between the combined effect of SHL with the antibiotics compared to the single-drug effect are indicated.

A

Antibiotic concentration (μg/mL) / Antibiotic type	Value of OD 600 in each well (W)											
	W 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 10	W 11	W 12
	256	128	64	32	16	8	4	2	1	0.5	Positive	Negative
Amoxicillin	0.60	0.62	0.65	0.73	0.87	0.90	0.92	1.14	1.21	1.23	1.49	0
Gentamicin	0.01	0.02	0.01	0.01	0.01	0.01	0.31	0.42	0.48	0.58	1.48	0
Penicillin	0.01	0.02	0.01	0.01	0.02	0.02	0.01	0.01	0.13	0.21	1.50	0
Clarithromycin	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.21	0.32	0.39	1.49	0
Cefazolin	0.01	0.02	0.01	0.01	0.01	0.01	0.28	0.45	0.51	0.62	1.51	0

B

SHL concentration / Drug	Value of OD 600 in each well (W)											
	W 1	W 2	W 3	W 4	W 5	W 6	W 7	W 8	W 9	W 10	W 11	W 12
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Positive	Negative
SHL	0.01	0.01	0.01	0.01	0.01	0.26	0.52	0.56	0.64	0.68	1.50	0

Table 1: Optical density of bacteria. Table 1A and 1B show the bacteriostatic effects of five type of antibiotics and SHL, respectively. As the drug concentration decreased, the bacterial concentration increased, thus, the light absorption value increased. The MIC is labeled in blue and MBC in red for each drug.

Drug Combination	MIC of different drugs （μg/mL）				FIC Index
	Antibiotic		SHL		
	Alone	Mixture form	Alone	Mixture form	
Gentamicin+SHL	8	1	1:32	1:256	0.25
Penicillin+SHL	2	1	1:32	1:4096	0.51
Clarithromycin+SHL	4	0.5	1:32	1:32	1.13
Cefazolin+SHL	8	2	1:32	1:64	0.75

Table 2: MIC and FIC values of antibiotics alone and in combination with SHL for S. aureus.

The MICs of gentamicin for *S. aureus* decreased eight-fold (MF= 8; FIC = 0.25) after adding SHL, revealing SHL exhibited the best modulation effect on the gentamicin (**Table 2, Figure 3**). The other two antibiotics penicillin and cefazolin also showed an additive effect in combination with SHL, by decreasing the MIC value for *S. aureus* two-fold and four-fold, respectively, compared with using the antibiotics alone.

DISCUSSION

In the present study, we tested various types and concentrations of antibiotics and compared their activity when used alone or in association with the Chinese traditional medicine SHL. The results obtained showed that all the tested drugs excepted for Lan-Qin had a statistically significant inhibitory effect on S. aureus, and this inhibitory effect was concentration dependent, which increased with the increase of drug concentration. Interestingly, SHL also had antibacterial effect, and the combination with other antibiotics reduced the MIC value of the antibiotics examined here, indicating that the combination of Chinese and Western drugs may have synergistic or additive effects against S. aureus. Our study provides a reference for the clinical treatment of *S. aureus* infection. Especially when SHL is used in combination with antibiotics, drugs with additive and synergistic effects should be selected, while avoiding ineffective antagonistic effects between drugs.

In recent years, with the widespread use of antibiotics, the number of drug-resistant strains increased year after year.

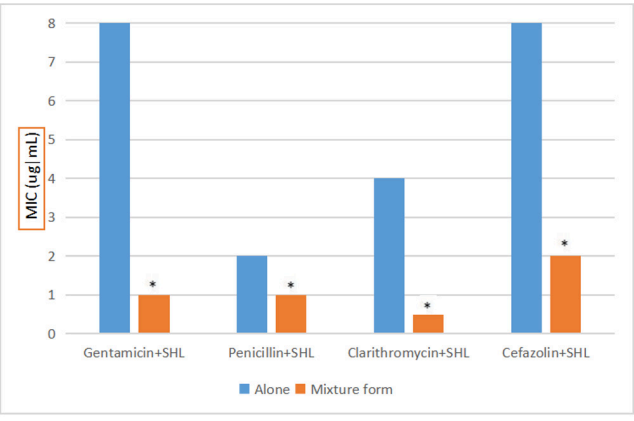

Figure 3: MIC values of antibiotics alone and in combination with SHL for *S. aureus*. Bacteria were cultured and added to a 96-well plate with different antibiotics either alone (blue bars) or in combination with SHL (orange bars) to treat the bacteria. $p < 0.001$ is shown as *.

In view of this medical problem, the combined application of Chinese and Western medicine as one of the solutions has become a research hotspot. SHL is one of the famous modern formulae refined from three medicinal herbs including Flos Lonicerae, Radix Scutellariae, and Fructus Forsythiae. With antibacterial, antiviral, and anti-inflammatory activities, SHL is used for clinical treatment of acute respiratory tract infection, bacterial infection, pneumonia, and other diseases (8). Despite previous work on SHL, there was no literature available on the combination of antibiotics and SHL for in vitro inhibition of *S. aureus* growth. Therefore, our study provides a new insight for the treatment of *S. aureus* infection.

During our experiment, controlling the concentration of bacteria in the appropriate range was the key to success. At the beginning, we did not pay much attention to controlling the growth state of the bacteria in the logarithmic growth phase, so we did not obtain bacteriostatic results. Through literature review, we improved the experiment by measuring the optical density value of the bacterial liquid with a microspectrophotometer so as to ensure that the cell density of the tested strain was controlled at 5×10^5 colony-forming units (CFUs)/mL. The McIntosh turbidimetric method is also a commonly used method to determine the concentration of bacteria, but it would cause great error to judge the concentration of bacteria by visual observation compared with spectrophotometer measurement (16).

Moreover, we acknowledge some limitations of our study. We only tested the activity of drugs against one reference *S. aureus* strain. Further work should be performed including more strains like MRSA to assess if medicine activity could be influenced by the resistant strain. And we should also explore the use of electron microscopy to characterize the morphology of the bacteria treated with the various antibiotics and combined drugs.

In conclusion, the combination of the antibiotics with SHL tested in the present study was highly active against *S. aureus* in vitro. A synergistic effect was highlighted when associating SHL with antibiotics, suggesting that they could be a promising option as a therapy for the treatment of *S. aureus* infection. Further studies using animal models and well-conducted clinical trials are needed to further evaluate combination therapy and its positioning in the management of these infections.

MATERIALS AND METHODS

Bacterial strain

S. aureus ATCC 25923 was bought from Beijing Zhongke Quality Inspection Biotechnology Co. LTD and stored in -80°C refrigerator.

Medium

Mueller Hinton Agar (MHA) plates and Mueller Hinton Broth (MHB) medium were prepared as following, 25 g Luria-Bertani (LB) powder was weighed and dissolved in 1 L distilled water. After 20 min of autoclaving in a 121°C autoclaving

steam sterilization pot, they were stored in refrigerator 4°C for bacteria culturing.

Tested reagent

Drug susceptibility filter paper disks of amoxicillin, gentamicin, penicillin, clarithromycin, and cefazolin were bought from Beijing Zhongnuo Taian Technology Co., LTD. Amoxicillin (Macklin, 26787-78-0), penicillin (Solarbio, 69-52-3), and cefazolin (Solarbio, 23325-78-2) were dissolved in pH 6.0 phosphate buffered solution (PBS) buffer, and gentamicin (Solarbio, 1405-41-0) and clarithromycin (Solarbio, 81103-11-9) were dissolved in pH 7.8 PBS buffer to obtain the suitable concentration.

Zone of inhibition determination

S. aureus strains were incubated in 50 mL MHB medium overnight at 37°C, then 100 µL volume of cell suspension was plated onto MHA plates to create a uniform bacterial layer. Each of the seven plates was divided into three sections. Two filter paper disks containing one of the seven drugs including amoxicillin, gentamicin, penicillin, clarithromycin, cefazolin, SHL, and Lan-Qin were placed in two sections of each petri dish as the experimental group and one filter paper disk without the drug was placed in the third section as the control group. The plates were incubated for 18 hours at 37°C and the diameter of the zones of inhibition was measured after incubation. The statistical significance of differences between groups were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test using GraphPad Prism software. Relationships with $p < 0.05$ were considered statistically significant.

MICs and MBCs determination

Bacteria were incubated in MHB medium overnight at 37°C with shaking and diluted into fresh MHB at a cell density of 5 $\times 10^5$ CFUs/mL. A 90 µL volume of cell suspension was dispensed in each well of a sterile 96-well plate from column 1 to column 11, and 100 µL of MHB was dispensed in column 12 for negative control in order to check the sterility of the medium used. A 10 µL sample of one type of antibiotic was added to the horizontal wells (columns 1-10) to a final concentration of 256, 128, 64, 32,16, 8, 4, 2, 1, and 0.5 µg/mL, respectively from list A to E, which was labelled longitudinally in the 96-well plate. SHL was diluted to a final concentration of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1: 512, and 1:1024 with sterile water and added to each well of columns 1-10 in list F. Column 11 without drugs served as positive controls (untreated bacteria) in order to check the bacterial viability. The plate was incubated at 37°C for 18–24 hours in an incubator. Then the turbidity degree of the liquid in each well was observed, and the light absorption value at 600 nm was further determined by a spectrophotometer (Nano-300, SHENG AO). Three replicates were generated for each test sample. In the present study, the MIC was regarded as the lowest concentration of drug that prevented visible turbidity. Then a 50 µL10-1 dilution of the first five wells that showed no visible growth of above overnight cultures and the positive control cultures were plated onto MHA plates and incubated at 37°C for 18 hours. The lowest concentration of peptides that prevent any residual colony formation is the MBC (14).

Combination effect of SHL with antibiotics

In order to test whether antibiotics and SHL have synergistic effects on bacteriostasis, the MIC values of the antibiotics in the presence of SHL were measured. Bacteria were cultured and added to 96-well plate as described above with sterility control in column 12.

A nine×ten checkerboard test was carried out in which different concentrations of SHL was added in horizontal mode and different concentrations of one type of antibiotic in vertical mode (14). Thus, the concentration of SHL in each horizontal well was 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, 0.0078125, and 0.0039625 times the MIC, respectively, and the concentration of antibiotics in each horizontal well was constant. The concentration of antibiotics in each vertical well was 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.0078125 times the MIC, at the same time the concentration of SHL in each vertical well was constant. The plate was incubated at 37 °C for 18–24 hours in an incubator. Three replicates were generated for each test sample. Statistical analyses were performed to test the combination effect compared to the single-drug effect.

The modulation factor (MF) was used to evaluate the modulating effects of compounds on MICs of antibiotics according to the following formula: MF = MIC antibiotics/(MIC antibiotics + modulator). In this experiment, SHL was the modulator. The FIC of the antibiotics was also calculated according to the following formula: FIC = (MIC of antibiotic A in combination/MIC of antibiotic A alone) + (MIC of antibiotic B in combination/MIC of antibiotic B alone) (15).

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