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Antibacterial Activity and Mechanism of Aureusidin Against Staphylococcus Aureus

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Abstract: *Aureusidin is a novel compound with antibacterial activity against Staphylococcus aureus (S. aureus), yet the mechanism of aureusidin against S. aureus has not been completely described. The objective of this study aimed at evaluating the inhibition of the growth of S. aureus, investigating the antibacterial activity and mechanism of action of aureusidin against S. aureus. The mechanism of action of aureusidin was investigated by analysing its effects on the cell morphology, the membrane permeability, the electrical conductivity, the nucleic acid leakage and the protein release at MIC. The respiratory rate of S. aureus decreased when the concentration of aureusidin was increased to MIC, and was the same as malonic acid. This inferred that the TCA cycle was restrained, reducing the energy transfer of S. aureus and inhibiting the growth of S. aureus. Thus, these results showed that aureusidin had a strong antibacterial effect on inhibiting the growth of S. aureus and provided application potential in the field on food preservatives.*

Keywords: *Aureusidin; Staphylococcus aureus; MIC; Bacteriostatic activity; Bacteriostatic mechanism*

1. Introduction

In recent years, with the rapid development of human medicine and a large environment of deeply reflecting on the relationship between human and nature, natural medicines have been increasingly recognized due to their advantages of high efficacy and low toxicity [1]. Flavonoids are a kind of naturally occurring natural products with low molecular weight, which are widely found in vegetables, fruits and other plants [2]. As of today, there are more than a thousand flavonoids that have been discovered [3]. *Aureusidin* is an aurora compound, which is not very widely distributed in nature and is mainly found in fruits and flowers, where it plays an important role in plant pigmentation [4]. It is one of the less known representatives of flavonoids and possesses various biological activities, including antiviral, antibacterial, anti-inflammatory, antitumor, antimalarial, antioxidant, and other pharmacological activities [5].

S. aureus is ubiquitous in nature and often parasitizes the skin surface and mucosa of warm-blooded animals and human beings [6]. The cells are spherical with a diameter of 0.5-1.0 μm and *S. aureus* is arranged in a string or a single arrangement. Enterotoxin secreted by *S. aureus* can cause human gastroenteritis bacterial poisoning, and some strains can produce epidermal shedding toxin and toxic shock syndrome toxin which cause scalded skin syndrome [7].

In this work, the paper reports that *aureusidin* has excellent antibacterial effect, and the objective of this study were determined to the minimum inhibitory concentrations (MIC) and the antibacterial activity of *aureusidin*. The authors proposed the antibacterial mechanism of *aureusidin* against *S. aureus* by experimental study of cell membrane permeability, DAPI staining experiments and results of respiratory metabolism experiments. The scanning electron microscope also proved the correctness of the antibacterial mechanism of *aureusidin*.

2. Materials and methods

2.1. Materials

The *S. aureus* was kept by medicinal chemistry laboratory, Changzhou University and preserved in refrigerator at -80℃. Synthesis and purification of *aureusidin* was carried out in medicinal chemistry laboratory (purity > 99%).

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2.2. Test of Minimum Inhibitory Concentration

The lowest concentration of *aureusidin* without visible *S. aureus* growth is the minimum inhibitory concentration. Logarithmic phase *S. aureus* were diluted to 1.0×10^7 CFU/mL with nutrient broth and 100 mL of nutrient broth was mixed with 0.4 uL (200 mM). Then 100uL *S. aureus* solution was injected to 96 - well microtiter plates and the concentration of *aureusidin* was 50, 100, 200 uM. The plates were incubated at 37℃ for 24 h. Test of minimum inhibitory concentration was repeated in duplicate.

2.3. Determination of the inhibition of aureusidin growth curve

The inhibition of *aureusidin* on the growth of *S. aureus* was measured by Ultraviolet-Visible Spectrophotometer. Logarithmic phase *S. aureus* was adjusted to 1.0×10^9 CFU/mL with broth medium and 300 uL of the bacterial solution was injected into a conical flask consisted of 30 mL nutrient broth. *Aureusidin* was injected into the nutrient broth to keep a final concentration of 1 ×MIC. The *S. aureus* culture without *aureusidin* was measured as control. All cultures were on an orbital shaker (120 rpm at 37.0℃). The bacterial concentration was monitored every 4 h by measuring the OD_{600} values with an UV spectrophotometer.

2.4. Effect of aureusidin in integrity of cell membrane

The integrity of cell membrane is detected by detecting intracellular molecules overflowing absorbance at 260 nm reported by Zhao and Lin. Diluted *S. aureus* in logarithmic growth phase was diluted to 1.0×10^7 CFU/mL and injected into a medium containing *aureusidin* (1 ×MIC) at 37.0℃. The *S. aureus* suspension (2 mL) was taken out at five times intervals in 0, 2, 4, 6, 8 h. Centrifuging *S. aureus* suspension to obtain supernatant and detecting with UV spectrophotometer at 260 nm.

2.5. Cell electroconductibility assay

Activated and incubated cultured *S. aureus* to logarithmic phase and added *S. aureus* to 30 mL of broth medium to a final concentration of 200 uL. Inoculated in 30 mL broth medium to 2% inoculation amount and shook culture at 120 rpm and 37℃. After culturing for 1, 2, 4, 8 h, 1mL of culture solution was taken respectively, the *S. aureus* suspension centrifuged at 8000 rpm for 10 min, and the supernatant was diluted 20 times, then the conductivity was measured by DD-307A conductivity meter.

The experiment was repeated 3 times with blank medium as control, and the average value was taken. The formula for conductivity change rate is as follows:

$$R\ (\%) = (Rs-Rc)/Rc \times 100\% \quad (1)$$

R (%): Change of Relative Conductivity

Rs: Electric Conductivity treated with *aureusidin*

Rc: Electric Conductivity without *aureusidin*

2.6. Cell metabolism assay

The activated *S. aureus* suspension was prepared by liquid culture medium. The suspension was centrifuged at 4000 rpm for 10 minutes, then the supernatant was poured out, washed three times with normal saline, and then diluted into a bacterial suspension with OD_{600} to 0.6. Typical inhibitors and determination of respiratory rate of *aureusidin*: 1.8 mL of 1% bacterial suspension at pH 7.2 and 0.1 mol/L of phosphate buffer solution, 0.5 mL of 1% bacterial suspension, stirred in the open air for 5 minutes, the dissolved oxygen content in suspension of bacteria was measured, and the whole system should be sealed. Three typical inhibitors, iodoacetic acid, malonic acid and sodium phosphate, were added into syringes to make the final concentration of 500 mg/L and 200 mM respectively. The control group was not added with inhibitors. Three parallel experiments were conducted in each group, and the mean values (respiratory rate is the oxygen consumption per unit time and mass of microorganisms) were taken. According to the change of dissolved oxygen in the suspension, the respiratory rate of bacteria can be calculated.

The formula for respiratory inhibition rate of bacteria is as follows:

$$I_R = (R_0 - R_1)/R_0 \times 100\% \quad (2)$$

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I_R: Inhibitory rate of inhibitors on bacterial respiration

R_0: Respiratory rate in control group

R_1: Respiratory rate of bacteria in inhibitory group

The formula for superposition rate of bacteria is as follows:

$$R_R=(R_1-R_1')/R_1 \times 100\% \quad (3)$$

R_R: Superposition Rate of Typical Inhibitors to *aureusidin*

R_1: Respiratory rate of bacteria after adding *aureusidin*

R_1': Respiratory rate of bacteria after adding *aureusidin* and typical inhibitors

2.7. Measure of variance ratio in DNA and RNA contents

The cell staining of *S. aureus* was determined by DAPI exclusion with slight modification. *S. aureus* was activated and cultured to log phase by adding aureomycin to 30 ml of broth medium to a final concentration of 200 uL. Inoculated in 30 mL broth medium to 2% inoculation amount, and shook culture at 120 rpm and 37℃. The *S. aureus* suspension was injected with *aureusidin* at concentration of 1 ×MIC at 37 ℃ for 24 h. Centrifuge *S. aureus*, discard supernatant, and wash thallus with PBS for 3 times. *S. aureus* being stained with 10 ug/mL DAPI for 10min in the dark, the cell suspension was measured by fluorescent inverted microscope.

0.05 mL of *aureusidin*-treated bacterial samples (OD_{600} value of 0.6) for 6 h, 12 h, 18 h and 24 h were mixed with 0.150 mL of DAPI staining solution, shaken in the dark for 10 min, then centrifuged at 8000 rpm, discarded supernatant, and added 0.2 mL sterile water. Then the fluorescence intensity of bacterial DNA and RNA was determined by fluorescence spectrophotometer at 364 nm and 400 nm excitation wavelengths of DNA and RNA. Experiment was repeated three times, and the control group was bacterium-like solution without *aureusidin*.

2.8. Test of scanning electron microscope

Scanning electron microscope (SEM) assay could observe the morphological changes of *S. aureus* added with *aureusidin*. The logarithmic phase *S. aureus* solution (1.0×10^7 CFU/mL) in nutrient broth was incubated with *aureusidin* (1 × MIC) at 37℃ and 120 rpm for 24 h. After that, the *S. aureus* suspension was centrifuged at 8000 rpm for 10 min and washed with PBS. After the solution was centrifuged, the supernatant was discarded, and the *S. aureus* was fixed with an aqueous solution of osmium tetroxide for 2 hours, after which the supernatant was centrifuged, fixed with 5% glutaraldehyde for 3 hours, and then dehydrated with absolute ethanol. The bacteria cells were measured by SEM.

2.9. SDS-PAGE analysis of bacterial protein

The changes of bacterial protein content before and after *aureusidin* treatment were proved by SDS-PAGE analysis. *Aureusidin* was added to the suspension of activated bacterial cell to obtain a final concentration of 200Mm. A control sample without *aureusidin* was prepared. The sample of *S. aureus* treated with *aureusidin* for 6, 12, 18, 24 h (OD_{600} value of 0.6) was taken as 1 mL. The *S. aureus* cells were collected by centrifugation at 4000 rpm for 10 min at 4℃, then were washed three times with PBS, resuspended in 100 uL sterile water. The suspension of *S. aureus* was mixed with 25 uL of the sample buffer (1 M Tris-HCl, pH 6.8, 10% SDS, 5% bromophenol blue). Then, sample were heated at 100℃ for 10 min and centrifuged at 8000 rpm for 10 min. The supernatants were soluble protein. The marker and soluble protein samples were run at a constant voltage of 80V through the stacking gel. When they were passed though the separating gel at 120 V for 60 min until the dye reached the bottom of the plate. The gel was removed from the apparatus and dyed with Coomassie Brilliant Blue R250 for 60 min. Then, the gel was decolorized with a decoloring agent. After 10 h, protein bands were visualized on the gel imager.

3. Results

3.1. MIC of aureusidin against S. aureus

The minimum inhibitory concentration of *aureusidin* against *S. aureus* was subject to broth

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microdilution method. It was observed from Table1 that MIC of *aureusidin* against *S. aureus* was 200 uM.

Table 1: MIC of aureusidin against S. aureus

Bacteria	The concentration of *aureusidin* against *S. aureus*(mmol/L)			
	0.00	512.00	1024.00	2048.00
S. aureus	--	-	+	++

--, No effect to bacteria; -, No antibacterial activity; +, Have effect to antibacterial activity and observed growth of bacteria; ++, No visible growth of bacteria; MIC, minimum inhibitory concentration

3.2. Determination of the inhibition of aureusidin growth curve

According to the antibacterial activity of *aureusidin* against S. aureus, the growth curve of *aureusidin* against *S. aureus* in the presence of 1 ×MIC and 2 ×MIC *aureusidin* were plotted. As shown in Fig.1, The control group *S. aureus* entered logarithmic growth phase after a short delay period, and the number of bacteria increased exponentially. *S. aureus* growth was completely inhibition at the concentration of 1 ×MIC *aureusidin* and 2 ×MIC. The inhibition growth curve results showed that *aureusidin* can effectively inhibit the growth of *S. aureus* within 24 hours.

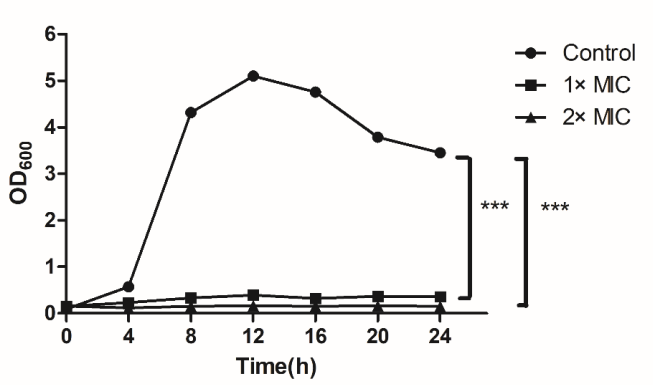

Figure 1: Effect of aureusidin on growth curve of S. aureus

3.3. Effect of aureusidin on cell membrane permeability

Detection of intracellular components can reveal the integrity of the cell membrane. When the integrity of the cell membrane is destroyed, small molecules and ions overflow the cell first, and then biological macromolecules such as nucleic acids overflow the cell. Therefore, the cell integrity can be judged by detecting the dsDNA content in the supernatant after centrifugation at 260 nm absorbance. The absorbance of the *S. aureus* supernatant was shown in Fig.2. The absorbance of the solution increased significantly, and the absorbance of *S. aureus* solution added in *aureusidin* was significantly higher than that of the control. We believe that *aureusidin* destroyed the integrity of cell membrane, resulting in the overflow of intracellular substances.

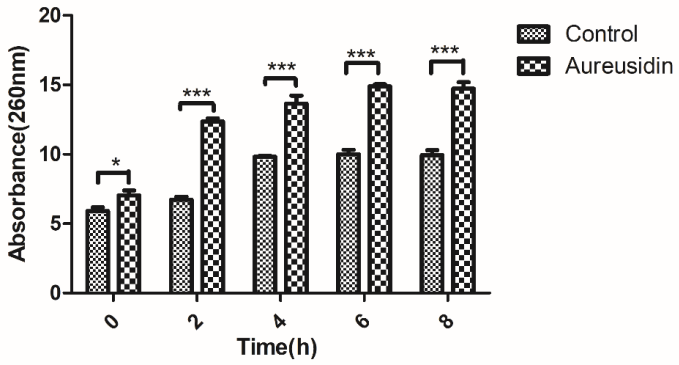

Figure 2: Effect of aureusidin on nucleotide release of S. aureus

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3.4. Effect of aureusidin on electrical conductivity of S. aureus

The results showed that when *aureusidin* was added to the bacterial suspension for 1 hour, the conductivity in the culture medium increased significantly, indicating that cytoplasmic leakage occurred in bacterial cells, thus increasing the conductivity of the experimental group (Cui, H., Zhao, C., & Lin, L. 2015). When cultured for 6 hours, the experimental group reached the maximum rate of change of 7.91%. The change of the conductivity of the culture solution can reflect the change of the cell membrane permeability. After adding *aureusidin*, the conductivity of the bacterial solution increased, which indicated that *aureusidin* can increase the permeability of the cell membrane and cause the cell cytoplasm to leak out. *Aureusidin* may destroys the integrity of cell membrane of *S. aureus*.

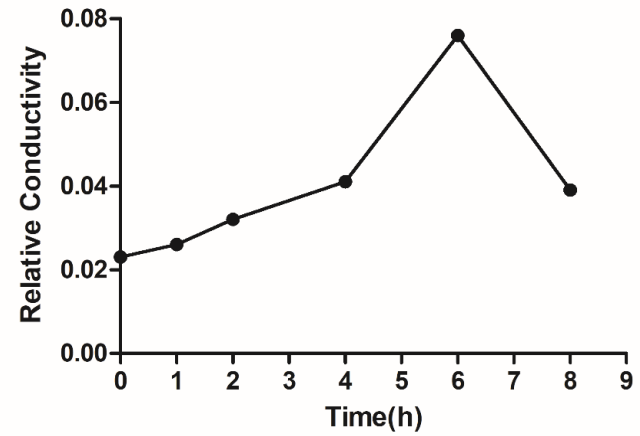

Figure 3: The change of electrical conductivity

3.5. Effect of aureusidin respiratory metabolism of S. aureus

Sodium phosphate, iodoacetic acid and malonic acid are typical inhibitors. of pentose phosphate pathway, glycolytic pathway and tricarboxylic acid circulation pathways in organisms respectively. As shown in the figure, *aureusidin*, like the three typical inhibitors, has inhibitory effect on respiratory metabolism of *S. aureus*.

Table 2: Respiratory inhibition rate of typical inhibitors and aureusidin against S. aureus

Inhibitor	Concentration	Respiration rate R_0	Respiration rate R_1	Respiratory inhibition rate I_R
Malonic acid	0.5 g/L	0.1213	0.1213	18.34%
Iodoacetic acid	0.5 g/L	0.1425	0.1052	35.35%
Sodium phosphate	0.5 g/L	0.1201	0.1032	16.37%
Aureusidin	200 mM	0.1200	0.1008	16%

The specific pathway of *aureusidin* inhibiting microbial respiratory metabolism can be judged by comparing the superposition rate of typical inhibitors to *aureusidin*. The smaller the superposition rate is, the weaker the synergistic effect between *aureusidin* and typical inhibitors is, and the greater the possibility that *aureusidin* and typical inhibitors have the same metabolic pathway. As can be seen from fig. 3-2, the smaller the superposition rate of *aureusidin* and malonic acid, therefore *aureusidin* mainly acts by inhibiting the tricarboxylic acid circulation pathway.

Table 3: Composition inhibition rate of typical inhibitors and aureusidin against S. aureus

Inhibitor	Respiration rate		Superposition rate
Aureusidin	0.1201		/
Aureusidin+ Malonic acid	0.1095		8.83%
Aureusidin+Iodoacetic acid	0.0976		18.73%
Aureusidin+Sodium phosphate	0.1032		14.82%

3.6. Effect of aureusidin fluorescence intensity of S. aureus

DAPI is a fluorescent dye that can bind to DNA and RNA. It is widely used in the dyeing of nucleic acids. The effect is that the larger the nucleic acid content, the stronger the fluorescent brightness of the dye after binding to the nucleic acid. Therefore, DAPI staining experiments can demonstrate whether the

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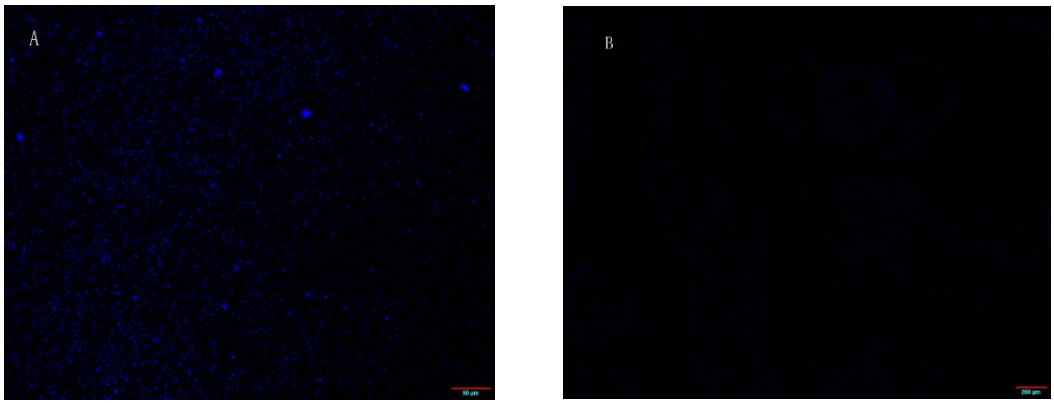
drug inhibits the *S. aureus* nucleic acid. The DAPI staining results of this experiment showed that the fluorescence intensity of *S. aureus* treated with *aureusidin* was significantly lower than the control group (Fig. 4). Therefore, the results suggest that the *aureusidin* can inhibit the synthesis of DNA and RNA in S. aureus.



(A) Control; *(B) S. aureus cells treated with aureusidin at MIC level*

Figure 4: Cell viability of S. aureus were observed with Fluorescent Inverted microscope.

3.7. Effect of aureusidin on DNA and RNA content of S. aureus

As can be seen from the figure, the nucleic acid content of *S. aureus* is obviously reduced after the action of *aureusidin*. *Aureusidin* may inhibit the growth of *S. aureus* by inhibiting the synthesis of DNA and RNA.

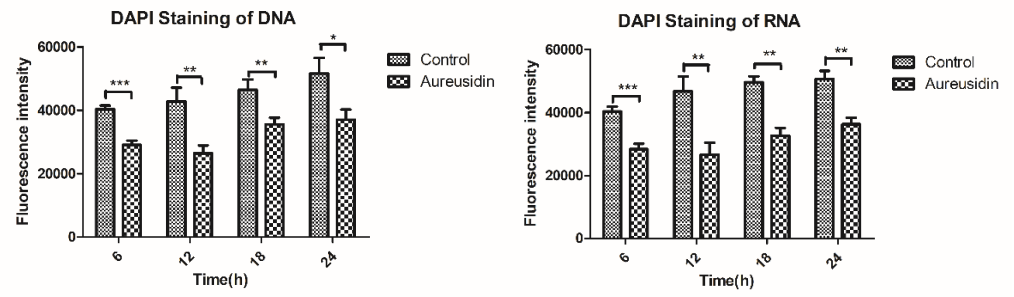
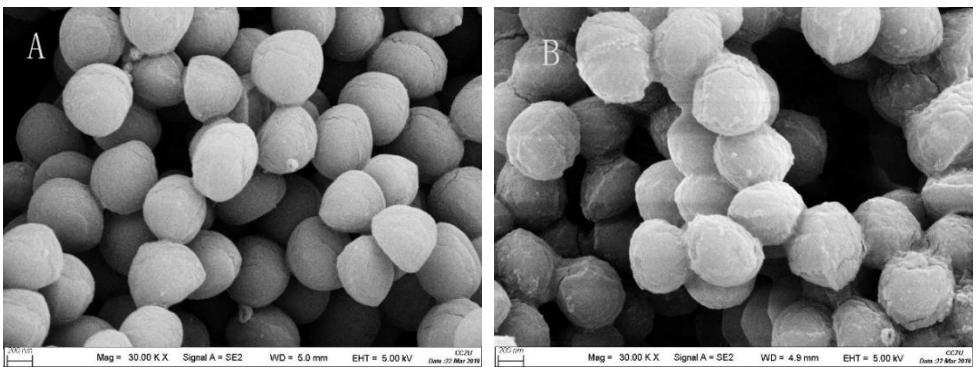


Figure 5: DAPI Staining of DNA and RNA

3.8. Effects of aureusidin on morphology of S. aureus

The morphological changes of *S. aureus* and the integrity of cell membrane can be observed through scanning electron microscope experiments. As shown in Fig. 6, the surface of *S. aureus* bacteria in the control group was smooth and without depression, but the group treated with *aureusidin* at concentration of MIC showed morphological changes.



(A) Control; *(B) S. aureus treated with aureusidin at MIC*

Figure 6: Morphology of S. aureus cells were showed with SEM.

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4. Discussion

As we all know, *S. aureus* is a Gram+ bacteria with a diameter of about 1μm. At the same time, *S. aureus* is an extremely common pathogen. *S. aureus* is permanently parasitic in 20% of the population, and is commonly found on the skin surface and upper respiratory tract mucosa [8]. *S. aureus* is a common pathogen causing food poisoning. Usually *S. aureus* infections in humans or animals arise from food poisoning [9]. For example, food itself carries bacteria before processing or is contaminated during processing, resulting in enterotoxins and food poisoning. People who eat food contaminated by *S. aureus* and its toxins will cause acute gastroenteritis with nausea, vomiting, abdominal pain, diarrhea and other symptoms [10]. Severe cases lead to shock or delay. *S. aureus* can pass through skin wounds, causing local suppuration infection [11]. Under the condition of decreased resistance, it will multiply in large numbers in human body, thus causing multiple abscesses and inflammation. *Aureusidin* effectively inhibits the reproduction of *S. aureus* and has high bactericidal activity. Meanwhile, the total synthesis of *aureusidin* was completed by Hu Kun's research group at Changzhou University.

Cell membrane is the boundary membrane surrounding cytoplasm and organelles, also known as plasma membrane. Their main function is to separate the living substances in cells from the external environment and maintain the stability of cell specific internal environment [12]. In addition, the cell membrane also performs many other functions, including material transport, signal transmission, cell recognition and so on [13]. The results from the 96-well microtiter plate method, followed by measurement of MIC indicated that the *aureusidin* had strong inhibitory effects against S. aureus. The cell membrane permeability test found that dsDNA leaked out of the cell membrane and the absorbance increased with time. The experimental results directly showed that the cell membrane permeability changed under the action of drugs. At the same time, the conductivity experiment also proved that the bacteria leaked under the action of drugs and electrolyte overflowed the cells, thus increasing the conductivity of the bacterial suspension. The above experiment showed that *aureusidin* changes the permeability of cell membrane. Respiratory metabolism experiment measured the respiratory rate decline of *S. aureus* under the action of drugs. Through the comparison of *aureusidin* and three typical inhibitors, it was found that *aureusidin* has the function of respiratory function of S. aureus. Through further calculation of the superposition rate of *aureusidin* with malonic acid, sodium phosphate and iodoacetic acid, it was found that the superposition rate of *aureusidin* and malonic acid is the smallest. Finally, it was proved that the pathway of *aureusidin* inhibiting respiratory metabolism of *S. aureus* may be the tricarboxylic acid circulation pathway. DAPI staining solution can stain DNA and RNA, and the intensity after staining is 20 times of its own fluorescence intensity. DAPI staining test directly proved the effective inhibitory effect of *aureusidin* on *S. aureus* DNA and RNA. Following the scanning electron microscope experiment, the morphology of *S. aureus* treated with *aureusidin* was observed. it was found that the cell membrane of *S. aureus* in the experimental group was partially dissolved and changed in morphology, while the morphology of *S. aureus* in the control group was intact and showed no signs of rupture. The scanning electron microscope experiment also verified the correctness of the results of the cell membrane permeability experiment.

This paper reports for the first time that *aureusidin* has very high antibacterial activity, and the antibacterial growth curve *aureusidin* can effectively inhibit the growth of S. aureus. The cell membrane integrity test and conductivity test revealed that *aureusidin* can damage the integrity of the cell membrane of S. aureus. The DAPI staining assay proved that the antibacterial mechanism not only destroyed the cell membrane structure, but also inhibited the synthesis of DNA and RNA. The morphological changes of *S. aureus* were directly proved by scanning electron microscope experiments, thus verifying the correctness of the above experiments.

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