

# Antibacterial Effect and Mechanism of the Ethyl Acetate Extract of *Cichorium Intybus* L. Against *Staphylococcus Aureus*

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**Abstract:** Objective: *Cichorium intybus* L. (CILEAE) has many functions, such as antibacterial, anti-inflammatory, hypoglycemic, lipid-lowering, liver-protection and anti-oxidation. The subject aims to investigate the antibacterial activity and mechanism of the ethyl acetate extract of CILEAE against *Staphylococcus aureus* (*S. aureus*). Methods: Firstly, the antibacterial activity of CILEAE against several kinds of frequently pathogenic bacteria was determined by microbroth dilution method, then the antibacterial mechanism was investigated by Scanning Electron Microscope (SEM), permeability of cell membrane, respiratory metabolic pathway, protein band change in SDS-PAGE of soluble-protein, and DAPI fluorescence staining. Results: The results of the Minimum Inhibitory Concentration (MIC) showed that CILEAE could significantly inhibit *S. aureus* and *Escherichia coli* in the tested concentration range. SEM results showed that 24 h after the application of CILEAE to *S. aureus*, the surface areas of the phage showed signs of shrinkage, dryness, distortion and deformation, vesicular or irregularly shaped protrusions and contraction of other structures. Culture fluid conductivity results showed that the cell membrane permeability was changed by CILEAE; The results of respiratory metabolism inhibition experiments showed minimal superimposition rates of CILEAE; The results of protein band analysis showed that CILEAE could inhibit protein synthesis in *S. aureus*, and the results of DAPI fluorescence staining showed that CILEAE treatment significantly reduced the nucleic acid content in vivo. Conclusion: CILEAE has strong bacteriostatic activity against *S. aureus*. Its bacteriostatic mechanism may be through destroying the integrity of bacterial cell wall and cell membrane, inhibiting bacterial respiratory metabolism, and inhibiting the synthesis of bacterial protein.

**Keywords:** *Cichorium intybus* L.; *S. aureus*; MIC; Bacteriostatic activity; Bacteriostatic mechanism

## 1. Introduction

With the widespread application of antibiotics, bacterial resistance to antibiotics has become an increasingly intractable and challenging serious problem in the clinic <sup>[1]</sup>. Among them, methicillin-resistant *S. aureus* (MRSA), which is multi-drug resistant, has become a major global concern due to its increasing clinical isolation rate year after year, difficulty in treatment and high morbidity and mortality rate. <sup>[2]</sup> It is therefore imperative to find a novel and highly effective drug to replace the traditional antibiotics to reduce the spread and morbidity of drug-resistant strains. The effect of common antibacterial drugs on drug-resistant bacteria becomes weaker, while the development of new chemosynthetic drugs is difficult and has a long cycle <sup>[3]</sup>. A large number of studies have shown that Chinese herbal medicines with heat clearing and detoxification effects mostly have antibacterial effects, and are commonly used in the clinic to treat infectious diseases and achieve good results <sup>[4]</sup>. Because Chinese herbal medicines (TCM) have a wide antimicrobial spectrum, low toxicity, less occurrence of drug resistance, extensive drug sources and low cost, domestic and foreign scholars have paid more and more attention to the extraction of antimicrobial components from plants <sup>[5]</sup>. Therefore, it is of great significance to search for antibacterial drugs from natural product pathways to replace antibiotics.

CILEAE also known as bitter chicory, is a biennial or perennial herb of the genus chicory in the family Asteraceae, is a popular medicinal herb of Uyghur <sup>[6]</sup>. Love to grow on the sides of sunny fields, slopes and so on. Due to its special composition, chicory is of great value for food, medicine and forage. The main components of chicory include polysaccharides, flavonoids, terpenoids and phenolic acids <sup>[7]</sup>. In the present study, we found that chicory has many effects including antimicrobial, anti-inflammatory, glucose and lipid lowering, hepatoprotective and antioxidant properties <sup>[8]</sup>. It has the effects of diuresis and swelling, clearing heat and detoxifying, and invigorating stomach.

## 2. Materials and methods

### 2.1. Materials

The *S. aureus* was kept by the medicinal chemistry laboratory, Changzhou University and preserved in the refrigerator at -80 °C. The whole herb of CILEAE was obtained from Chinese Herbal Medicine Company in Bozhou City (Anhui, China), and was indentified by Prof. Guangtong Chen, Nantong University, Jiangsu, PR China.

### 2.2. Test of Minimum Inhibitory Concentration

Logarithmic phase *S. aureus* was diluted to  $1.0 \times 10^7$  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 into 96-well microtiter plates and the concentration of CILEAE was 50, 100, 200 uM. The plates were incubated at 37°C for 24 h. Test of minimum inhibitory concentration was repeated in duplicate.

### 2.3. Determination of the inhibition of CILEAE growth curve

The inhibition of CILEAE on the growth of *S. aureus* was measured by Ultraviolet-Visible Spectrophotometer. Logarithmic phase *S. aureus* was adjusted to  $1.0 \times 10^9$  CFU/mL with broth medium and 300 uL of the bacterial solution was injected into a conical flask consisted of 30 ml nutrient broth. CILEAE was injected into the nutrient broth to keep a final concentration of  $1 \times \text{MIC}$ . The *S. aureus* culture without CILEAE was measured as control. All culture was on an orbital shaker (120 rpm at 37.0 °C). The bacterial concentration was monitored every 4 h by using an UV spectrophotometer measuring the OD<sub>600</sub> values.

### 2.4. Effect of CILEAE 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 to  $1.0 \times 10^7$  CFU/mL and inject into culture medium containing CILEAE ( $1 \times \text{MIC}$ ) at 37.0 °C. The *S. aureus* suspension (2 mL) was taken out at five times intervals in 0, 2, 4, 6, 8 h. The supernatant was obtained by centrifugation of the *S. aureus* suspension and detected with a UV spectrophotometer at 260 nm.

### 2.5. Cell electroconductibility assay

Activate and culture *S. aureus* to logarithmic phase, and CILEAE was added to 30 mL of broth medium to make its final concentration to 200 uL. Inoculate in 30 mL of broth medium to 2% inoculation amount, and shake the culture at 120 rpm and 37 °C. After culturing for 1, 2, 4, 6, 8 h, 1 mL of the 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:

$$R (\%) = (R_s - R_c) / R_c \times 100\%$$

R (%): Change of Relative Conductivity

R<sub>s</sub>: Electric Conductivity treated with CILEAE

R<sub>c</sub>: Electric Conductivity without CILEAE

### 2.6. Measure of variance ratio in DNA and RNA contents

The cell staining of *S. aureus* was determined by DAPI exclusion with slight modifications. Activate and culture *S. aureus* to logarithmic phase, and CILEAE was added to 30 mL of broth medium to make its final concentration to 200 uL. Inoculate in 30 mL of broth medium to 2% inoculation amount, and shake culture at 120 rpm and 37 °C. The *S. aureus* suspension was injected with CILEAE at a concentration of  $1 \times \text{MIC}$  at 37 °C for 24 h. Centrifuge *S. aureus*, discard supernatant, and wash thallus with PBS for 3 times. *S. aureus* was stained with 10 µg/mL DAPI for 10 min in the dark, the cell suspension was measured by a fluorescent inverted microscope.

### 2.7. Test of scanning electron microscope

Scanning electron microscope (SEM) assay could observe the morphological changes of *S. aureus* added with CILEAE. The logarithmic phase *S. aureus* solution ( $1.0 \times 10^7$  CFU/mL) in nutrient broth was incubated with CILEAE ( $1 \times \text{MIC}$ ) at 37 °C 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.

### 2.8. SDS-PAGE analysis of bacterial protein

The changes of bacterial protein content before and after CILEAE treatment was proved by SDS-PAGE analysis. CILEAE was added to the suspension of activated bacterial cell to obtain a final concentration of 200 Mm. A control sample without CILEAE was prepared. The sample of *S. aureus* treated with CILEAE for 6, 12, 18, 24 h ( $\text{OD}_{600}$  value is 0.6) was taken as 1 mL. The *S. aureus* cell were collected by centrifugation at 4000 rpm for 10 min at 4 °C, then were washed three times with PBS, re-suspended in 100  $\mu\text{L}$  sterile water. The suspension of *S. aureus* was mixed with 25  $\mu\text{L}$  of the sample buffer (1M Tris-HCl, pH 6.8, 10% SDS, 5% bromophenol blue). Then, the sample were heated at 100 °C for 10 min and centrifuged at 8000 rpm for 10 min. The supernatant is soluble protein. The Marker and soluble protein samples were run at a constant voltage of 80 V through the stacking gel. When they were run 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 CILEAE against *S. aureus*

Microbroth dilution assay to determine the bacteriostatic activity of CILEAE against *S. aureus*, as shown in Figure 1, the results of the experiment showed that the bacteriostatic effect began to appear when the final concentration of CILEAE was 0.256 mg/mL, but completely inhibited the growth and reproduction of *S. aureus* at the concentration of 1.024 mg/mL, so the MIC of CILEAE against *S. aureus* for 24 h was 1.024 mg/mL.

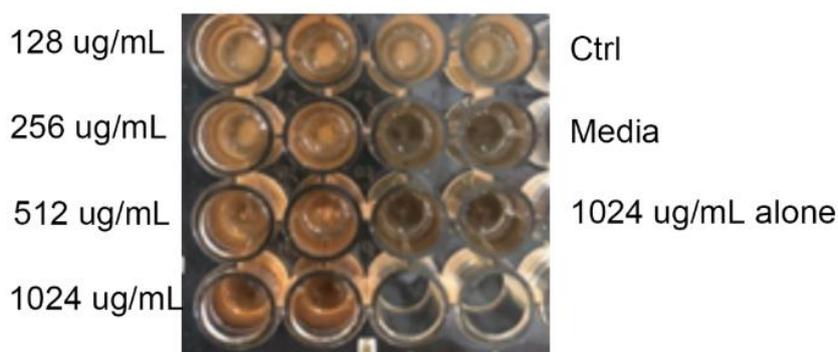


Figure 1: Determination of the bacteriostatic activity (MIC) of CILEAE against *S. aureus* by microbroth dilution

### 3.2. Inhibition of *S. aureus* growth curve by CILEAE

Figure 2 shows the *S. aureus* growth curve was significantly inhibited by CILEAE. As shown, the control group *S. aureus* not treated by CILEAE quickly entered the logarithmic phase of rapid growth after a short lag phase, and the culture around 10 h entered the highest period of cell growth, while in the CILEAE treated group, the logarithmic phase of rapid growth of bacteria did not appear, indicating that the growth of *S. aureus* in the logarithmic phase was inhibited by CILEAE.

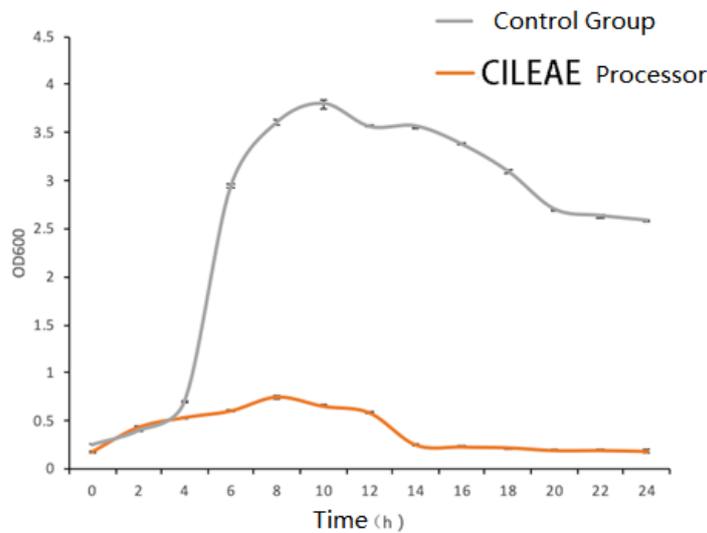


Figure 2: Effect of CILEAE on *S. aureus* growth curve

### 3.3. Effect of CILEAE on *S. aureus* bacteriophage morphology

The bacteria treated with CILEAE were analyzed by SEM to observe the morphological changes. As shown in Figure 3, the untreated *S. aureus* cells are full in appearance, refractile, and smooth in cell membrane characteristics. In contrast, after 24 h of CILEAE treatment, the morphology of *S. aureus* cells was significantly changed, and the surface showed a large number of wrinkles, shrivels, and distortions, and some of the phage cell surface showed obvious pits, vesicles, or irregular protrusion structures. These results suggest that CILEAE can induce morphological aberrations in *S. aureus* cells.

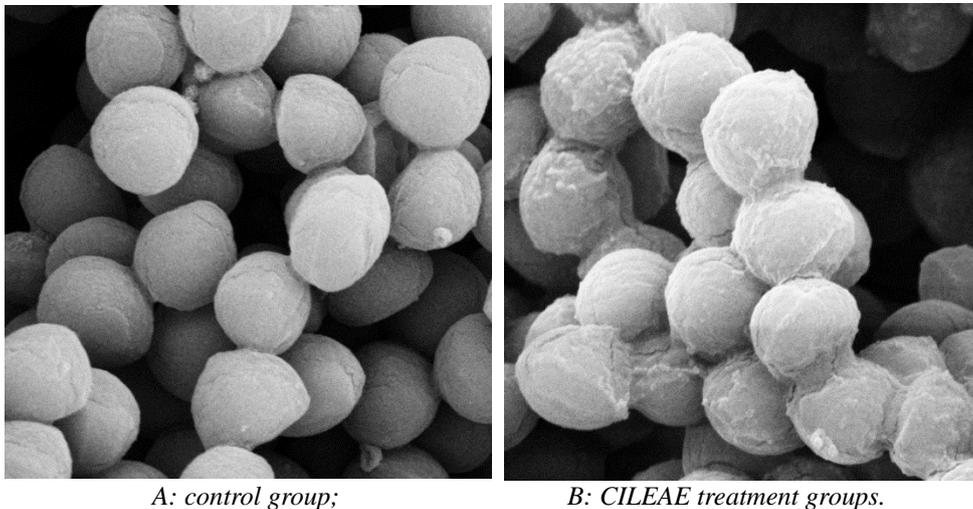
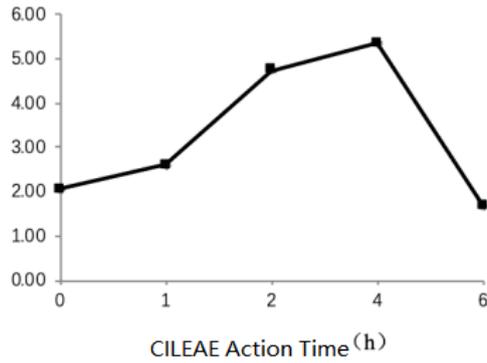


Figure 3: SEM observation of the effect of CILEAE on *S. aureus* morphology after 24h.

### 3.4. Effect of CILEAE on *S. aureus* electrical conductivity

Figure 4 showed the effect of CILEAE on the rate of change of electrical conductivity of bacteriophage cells. As shown, the conductivity of the bacterial medium after CILEAE treatment for 1 h was significantly increased, indicating that there was cytoplasmic leakage from the bacteriophage cells; The rate of change in conductivity reached a maximum of 5.35% when CILEAE was administered for 4 h. The changed conductivity of culture fluid due to CILEAE could reflect the increased permeability of the bacteriophage cell membrane, causing its cytoplasm to leak out of the cell, which might be caused by the induction of certain enzymes by CILEAE that could degrade the cell wall and membrane.

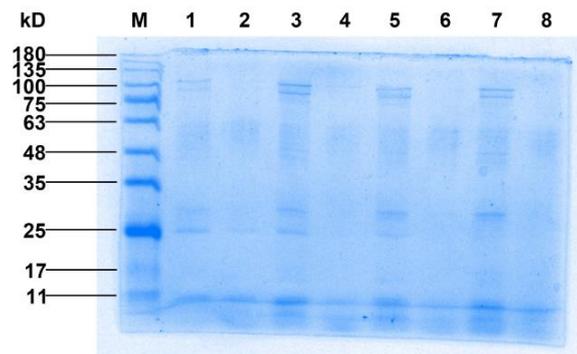


Bacterial medium conductivity after CILEAE treatment for 1, 2, 4, 6 h, respectively, was determined by a conductivity meter.

Figure 4: Effect of CILEAE on the rate of change of electrical conductivity of bacteriophage cells.

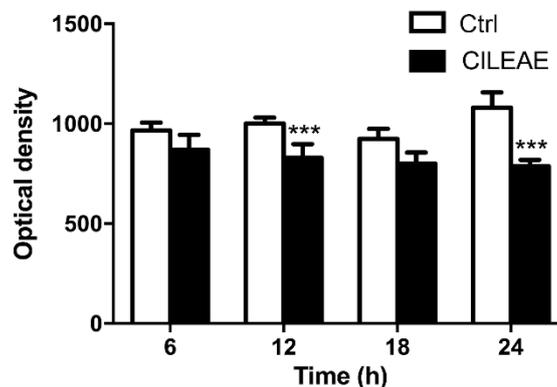
### 3.5. Effect of CILEAE on protein synthesis in *S. aureus*

Proteins are fundamental substances that are tightly associated with life and with various forms of life activities. To confirm whether CILEAE affected the synthesis of proteins in *S. aureus*, samples were taken after CILEAE (1.024 mg/mL) treatment for 6, 12, 18, 24 h, respectively, and SDS-PAGE electrophoresis was performed using the group without CILEAE as control, and the results are shown in figures 5.



M: marker; 1: 6 h versus control group; 2: The 6-h experimental group; 3: Versus the control group at 12 h; 4: The 12 h experimental group; 5: Control group at 18 h; 6: 18 h experimental group; 7: Control group at 24 h; 8: Experimental groups at 24 h.

Figure 5: Effects of CILEAE on in vivo soluble protein electrophoresis of *S. aureus* bacteria



*Aureus* treated with CILEAE for 6, 12, 18 and 24 h, respectively, and the OD value of soluble total protein in the phage changed. Error bars show the mean  $\pm$  SEM (standard error of the mean),  $n=3$ , \*\*\* $P<0.001$ .

Figure 6: CILEAE effects on soluble total protein OD values in vivo of *S. aureus* bacteria

The results of protein analysis showed that the expression of total protein in vivo of *S. aureus* was obviously reduced by CILEAE. *S. aureus* treated with CILEAE for 24 h resulted in an OD of 1080.1 for

total protein and a 27.2% reduction in total protein at 786.8 for untreated control *S. aureus*, and the protein expression was significantly reduced with the extension of the CILEAE treatment time with 135-25 KD of larger protein.

### 3.6. Effect of CILEAE on fluorescence intensity of *S. aureus* bacteriophage

The inhibition of total protein expression in vivo by CILEAE may be that it affects the expression of genes involved in DNA synthesis regulated by *S. aureus*. DAPI is a fluorescent dye that can penetrate the cell membrane into the interior of the cell, binding to DNA and RNA, the larger the amount of nucleic acid, the stronger the fluorescence brightness. The inhibitory effect of CILEAE on *S. aureus* nucleic acid was observed by inverted fluorescence phase contrast microscopy, and the results are shown in Figure 7. It is known from the figure that after 24 h of *S. aureus* treatment by CILEAE, its fluorescence intensity was obviously lower than that of the untreated control group. The reduced *S. aureus* nucleic acid content by CILEAE action may be due to its inhibition of *S. aureus* bacteriophage nucleic acid synthesis.

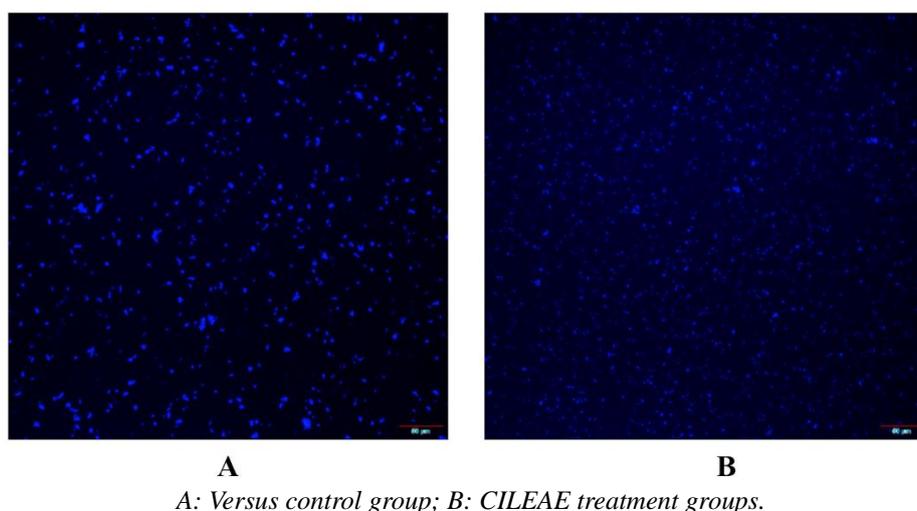


Figure 7: Inverted fluorescence phase contrast microscope was used to observe the effect of CILEAE on the fluorescence intensity of bacteriophage after 24 h of *S. aureus* action.

## 4. Discussion

Bacterial infection is an acute systemic infection caused by pathogenic bacteria or conditional pathogenic bacteria invading the blood circulation to grow and reproduce and produce toxins and other metabolites [9]. Clinically, it is characterized by shivering, high fever, rash, joint pain and hepatosplenomegaly, and some may have septic shock and migratory lesions [10].

In this paper, *S. aureus* was used as the experimental strain to evaluate the antibacterial activity of CILEAE. The results showed that CILEAE had obvious inhibitory effect on *S. aureus*, and the inhibitory effect increased with the increase of its concentration. The minimum inhibitory concentration of CILEAE against the bacterium was determined by micro broth dilution method. The results of minimum inhibitory concentration showed that the minimum inhibitory concentration of CILEAE against *S. aureus* was 1.024 mg/mL.

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. Maintain the stability of cell specific internal environment [11]. In addition, the cell membrane also performs many other functions, including material transport, signal transmission, cell recognition and so on [12]. The results of SEM and conductivity test showed that the morphology of *S. aureus* cells after CILEAE treatment for 24 hours changed significantly, with a large number of wrinkles, shrivels and distortions on the surface, and some cells showed obvious concave, vesicular or irregular protrusions on the surface; The results of conductivity experiment showed that the conductivity of bacterial culture medium cultured by CILEAE for a period of time increased significantly, indicating that there was cytoplasmic leakage in bacterial cells.

Respiration refers to the process in which organic substances undergo a series of oxidative

decomposition in cells to produce carbon dioxide or other products, release energy and produce ATP<sup>[13]</sup>. Cell respiration is the basic metabolism of living cells. Only cell respiration can provide energy for various life activities of cells. In order to evaluate the effect of CILEAE on the respiratory metabolism of *S. aureus*, the respiratory metabolism inhibition experiment was carried out. The results showed that CILEAE inhibited the pentose phosphate pathway in the glucose metabolism pathway.

Protein nucleic acid is a biological macromolecular compound polymerized by many nucleotides, which is one of the most basic substances of life<sup>[14]</sup>. Nucleic acids often combine with proteins to form nuclear proteins, which widely exist in all animal and plant cells and microorganisms. The changes of SDS-PAGE protein bands showed that the protein synthesis of *S. aureus* was significantly inhibited by CILEAE, and its total protein decreased with the extension of action time. After 24 hours of CILEAE, the protein content of higher molecular weight in bacteria decreased significantly, and its protein content decreased by 27.2%; DAPI fluorescence staining showed that the fluorescence intensity of *S. aureus* treated with CILEAE for different times was significantly lower than that of the control group. When CILEAE was treated for 12 hours, the fluorescence intensity of DNA was 27.16% lower than that of the control group, and the content of RNA was 42.29% lower than that of the control group after CILEAE was treated for 6 hours.

In conclusion, CILEAE has strong antibacterial activity against *S. aureus*. Its antibacterial mechanism may be achieved by destroying the integrity of bacterial cell wall and cell membrane, inhibiting bacterial respiratory metabolism and inhibiting the synthesis of bacterial DNA, RNA and protein. This provides clues for the further study of the antibacterial mechanism of CILEAE, and also provides a basic theoretical basis for the development of CILEAE into natural food additives or antibiotics.

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