

Mechanism of Eucalyptus Volatile Oil Application for Preventing and Treating Pseudomonas Aeruginosa Infection in Vitro

Lei Yang (✉ leiyang_Method@126.com)

Guangxi Traditional Chinese Medical University Affiliated First Hospital <https://orcid.org/0000-0001-8186-9338>

Qing Huang

Guangxi Traditional Chinese Medical University Affiliated First Hospital

Xiaodong Huang

GuangXi University of Chinese Medicine

Zhimin Lin

GuangXi University of Chinese Medicine

Xingxin Gao

Guangxi Medical University First Affiliated Hospital

Songlin Chen

Guangxi Traditional Chinese Medical University Affiliated First Hospital

Lili Zhao

The General Hospital of the Armed Police Force of Guangxi

Yinghui Lin

Guangxi Traditional Chinese Medical University Affiliated First Hospital

Guangzong Hua

Guangxi Traditional Chinese Medical University Affiliated First Hospital

Sheng Li

Research

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Abstract

Background The mechanism of eucalyptus volatile oil application on the prevention and treatment of burn infections caused by *Pseudomonas aeruginosa* (PA) was need to examined.

Methods The effect of eucalyptus volatile oil on bacterial biofilm was investigated using a PA biofilm model. The expression of *LasI* mRNA in PA was detected by RT-PCR.

Results The minimum inhibitory concentration test showed that the volatile oil of *Eucalyptus urophylla* in 20% concentration or more could exert an antibacterial effect. However, neither high nor low concentration of the eucalyptus volatile oil had a zone of inhibition. In scanning electron microscopy, the volatile oil group exhibited a significant delay compared with the control group. The volatile oil group also had a significantly lower *LasI* mRNA expression than the control group.

Conclusion As a Chinese medicine, the volatile oil of *E. urophylla* can affect PA proliferation and biofilm formation by interfering with the expression of *LasI*, thereby successfully preventing and treating burn infection caused by PA.

Background

Infection is one of the most common clinical complications of burn. It can cause septic shock and multiple organ dysfunction syndrome, which ultimately lead to death(1, 2). Sepsis is a complex process and a clinical manifestation that occurs in severe infection cases with systemic inflammatory response syndrome(3). As pathogens invade the blood circulation system, they multiply and produce toxins, causing acute systemic infections. Synthesized by pathogenic bacteria, endotoxin stimulates inflammatory cells to produce a multitude of inflammatory mediators, inducing immune dysfunction. Subsequently, immune cell function is inhibited, causing septic shock, multiple organ failure, and even death.

Pseudomonas aeruginosa (PA) is a common pathogen of burn(4), and biofilm formation is its most important survival strategy. In a mature PA biofilm, the extracellular polysaccharide fiber or the polysaccharide–protein complex is entangled and interlaced into a network. In addition, a negatively charged alginate, which constitutes the external environment of biofilm, fills the gap, creating a strong diffuse barrier. This barrier hinders the penetration of antibiotics, especially positively charged ones(5, 6), greatly enhancing bacterial viability(7). To regulate the formation of toxic factors, PA can use the Quorum Sensing (QS) system named Las system (*lasI/lasR*)(8). *LasI* encodes the signal molecule 3-oxo-dodecanoyl homoserine lactone (3-OXO-C12-homoserinelactone, 3-O-C12-HSL) and other signaling molecules that regulate transcriptional activators. *LasR* and *rhIR* gene defects can affect the ability of PA to form a biofilm in vitro, and the QS system plays an important role in the establishment and chronic development of PA lung infection(9, 10).

Traditional Chinese medicine possesses unique advantages in preventing infection caused by a bacterial biofilm; it can also inhibit bacterial biofilm formation. This type of medicine has become the focus of further anti-infective drugs following antibiotics because of its wide source of drugs, affordability, and minimal side effects(11). *Eucalyptus urophylla* is a kind of Myrtaceae plant cultivated in Guangxi Province. Eucalyptus leaves have been widely used by the public. They are commonly applied to the skin to prevent mosquito bites. As confirmed by modern medicine, eucalyptus leaves contain gallic acid, phenols, mellow alcohol, and *Eucalyptol*. The tests of gallic acid in vitro extracted from *E. urophylla* leaves inhibited *Staphylococcus aureus*, pneumococcus, typhoid, paratyphoid bacillus, and PA; thus, eucalyptus leaves have good antibacterial and anti-inflammatory effects(12, 13). Therefore, this study aimed to investigate the effect of *E. urophylla* volatile oil on patients with burn for preventing and treating concurrent PA infections.

Materials And Methods

1.1 Experimental materials and reagents

In this research, PA was obtained from clinical patients. RT-PCR-related extraction, reverse transcription, and amplification reagents were purchased from TAKARA (Japan). Eucalyptus leaves were collected from Hengxian, Guangxi, and the First Affiliated Hospital of Guangxi University of Chinese Medicine extracted and completed the volatile oil. Scanning electron microscope (SEM, EDAX-AMETEK) was provided by Guangxi Medical University. Moreover, RT-PCR detection, bacterial biofilm model construction, and MIC tests were completed by the laboratory of the abovementioned hospital.

1.2 GC-MS analysis of eucalyptus leaf oil

1.2.1 Material 6890A Gas Chromatograph-5973N Mass Spectrometer (GC-MS; Agilent, USA).

1.2.2 GC-MS analysis conditions

1.2.2.1 Gas chromatographic condition

Column: 1) Agilent HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). 2) Heating program: Initial temperature was 60 °C, which was then increased to 70 °C at 2 °C/min and held for 10 min. The temperature was raised to 140 °C at 3 °C/min, further elevated to 250 °C at 5 °C/min, and then maintained for 10 min. 3) Carrier gas: He. 4) Flow rate: 1 mL/min. 5) Injection volume: 1.0 μL. 6) Split ratio: 50:1.

1.2.2.2 Mass spectrometry conditions

1) Ionization mode: Electron ionization. 2) Ionization energy was 70 eV, inlet temperature was 250 °C, and ion source temperature was 230 °C. 3) Column flow rate: 1.0 mL/min.

1.2.2.3 Analysis methods

After extraction, the eucalyptus leaf oil was analyzed by GC-MS. To confirm each chromatographic peak, we searched the mass spectrum in NIST98 standard mass spectrum database (HPMSD ChemStation).

1.3 MIC test

Considering that the volatile oil of eucalyptus leaves is insoluble in water, cosolubilization was performed using the cosolvent Tween-80. The ratio of volatile oil and cosolvent is 5:1000. We extracted 10 μ L of 0.5 MCF of PA01 bacterial suspension and inoculated it into 1 mL of LB medium. Then, we added soluble volatile oil to configure a gradient-mixed suspension with a drug concentration of 10% to 50% in volume. Next, we incubated it in a CO₂ incubator for 24 h and transferred to the blood plate to examine bacterial growth.

1.4 Protein fingerprint analysis

The protein fingerprint of PA in each group was tested and analyzed by mass spectrometry (Micyoflex LT/SH, BD, US) and FlexAnalysis (Bruck, US), respectively. For validation and specification issues, we used the standard MBT method, which is commonly employed for typical laboratory standard samples. MS/Parent mode: On. Initial laser power: 30%, and maximal laser power: 40%. Allow only: 80 satisfactory shots per raster spot. Matrix blaster: Initially, 10 shots were fired, with a laser power of 40%.

1.5 Biofilm construction and SEM observation[10-12]

1.5.1 Biofilm model construction: We poured 20 mL of LB medium into a 50 mL centrifuge tube, inoculated 1 mL of 0.5 McFarley bacterial suspension, placed a sterile gastric tube (1 cm) into the bacterial suspension, and positioned it in a shaker at 45° angle. After shaking for 24 h, a bacterial film was formed on the surface of the sterile gastric tube, indicating the biofilm model.

1.5.2 Preparation before SEM observation: 1) Biological specimens were fixed with 3% glutaraldehyde for 2 h. 2) We immersed 0.1 mol/L PBS buffer solution and washed it thrice for 10 min each. 3) Osmium tetroxide was fixed for 1 h. 4) We again immersed 0.1 mol/L PBS buffer solution and washed thrice for 10 min each. 5) We soaked 50%, 70%, 80%, 90%, and 100% of ethanol thrice and dehydrated for 10 min each, 6) We immersed 100% pure hexamethyldisilazane thrice for 10 min each and then vacuum-dried. 7) The sample was pasted to the sample holder, placed on the IB3 ion sputtering instrument, and subsequently observed under an electron microscope.

1.6 RT-PCR detection

After constructing the biofilm model, we gently washed the gastric tube with sterile PBS and sonicated the bacteria on the inner and outer surfaces of the gastric tube to obtain the biofilm. After enrichment, the sample RNA was extracted using the TAKARA genome extraction kit and then reversed. After 15 min of recording, the LasI expression (6 cases per group) was tested using an RT-PCR detector (Redstone SLAN-96P, Shanghai, China).

1.7 Statistical methods

For statistical analysis and picture processing, we used the SPSS 19.0 and GraphPad, respectively. Measurement data were analyzed by *t* test. Sample means were expressed as mean \pm standard deviation. The test level was $\alpha = 0.05$. Furthermore, $P < 0.05$ indicated a statistically significant difference.

Results

2.1 Major chemical components of eucalyptus volatile oil analyzed by GC-MS

We conducted the GC-MS test and obtained the total ion current map of eucalyptus volatile oil (Figure 1). The main chemical component of eucalyptus volatile oil was eucalyptol ($C_{10}H_{18}O$), and the six other chemical ingredients were α -pinene ($C_{10}H_{16}$), camphene ($C_{10}H_{16}$), *o*-cumene ($C_{10}H_{16}$), limonene ($C_{10}H_{16}$), α -terpineol ($C_{10}H_{18}O$), Cyclohexanol, 1-methyl-4-(1-methylethyl)-, 1-acetate ($C_{12}H_{20}O_2$).

2.2 Antibacterial effect of eucalyptus volatile oil

Eucalyptus volatile oil at 20% concentration or more can yield an ideal antibacterial effect (Figure 2a). Drug diffusion experiments showed that various concentrations of eucalyptus volatile oil have no bacteriostatic ring (Figure 2b); thus, the evaporative capacity of eucalyptus is poor, and its antibacterial effect can only be achieved by direct contact.

2.3 Observation of PA after intervention with eucalyptus volatile oil

Figure 3 illustrates the PA (blue curve) in the control group and PA (red curve) in the experimental group after intervention with medication (drug concentration: 10%). The mass-to-nucleus (*m/z*) was significantly different at 7594 and some other areas. Thus, eucalyptus volatile oil has an effect on various proteins of the bacteria, and at the same time, it interferes with the PA QS system. In future studies, we will further explore and specify these proteins. Most of these proteins are related to bacterial resistance.

Figure 4 shows the surface biofilm morphology observed by SEM after the successful construction of the bacterial biofilm model. The control group formed a thick early biofilm. In the group treated with eucalyptus volatile oil, the biofilm morphology was relatively thin, indicating that the eucalyptus volatile oil has an inhibitory effect on bacteria and that biofilm formation was delayed.

As depicted in Figure 5, the RT-PCR detection results showed that the *LasI* mRNA expression in the experimental group was (30.68 ± 0.087) after being converted by standard RT-PCR curve [$Y = -3.346\text{LOG}(X) + 34.52$, $R = 0.998$]. Meanwhile, the average value of the control group was (31.12 ± 0.063). Compared with that in the control group, the *LasI* mRNA expression in the experimental group was significantly reduced after the eucalyptus volatile oil intervention, and the difference was statistically significant ($P < 0.05$).

Discussion

Guangxi Province is one of the regions in China where *Zhuang* people live, with rich medical resources and culture. Thus, the research on Chinese medicine is the advantage and characteristic of this article. Considering the numerous ethnic minorities in Guangxi, its diverse ethnic culture and unique local customs and activities are rare intangible cultural heritages in China; however, it also causes burden to the society. For instance, numerous people suffer from injuries and burns yearly caused by firework and firecracker exposure, consuming huge medical resources for the treatment. Therefore, while protecting China's intangible cultural heritage, burn treatment is particularly important and necessary as well.

The biofilm-forming system can lead to high resistance of PA, suggesting as one of the important factors causing clinical intractable infection(14). Thus, people explore other means to treat PA infection, such as the development of new antibacterial drugs, alginate monoclonal antibody, gene regulation, and biomedical material improvement; however, these methods are expensive and have a narrow application range, resulting in limited clinical application(15). The QS system plays an important role in PA biofilm formation(16), and it is a mechanism of information transmission between bacteria. Cells cannot sense the presence of bacteria themselves but rather the concentration of signal molecules. Signal molecule concentration depends on the density of the cells. The QS system participates in regulating the expression of virulence factors, and interference with the QS signaling system may affect the regulation of the pathogenic factors of PA.

Our study results showed that the *E. urophylla* volatile oil has a lower MIC and better bactericidal effect and the growth of PA can be fully inhibited by at least 20% concentration. Furthermore, SEM results revealed that bacterial biofilm formation can be delayed after exposing the PA to the eucalyptus volatile oil. Biofilm formation is closely related to bacterial resistance(17-20). Hence, destroying drug resistance is one of the mechanisms of the eucalyptus volatile oil that can be used in clinical bacterial treatment. In addition, the RT-PCR detection results demonstrated that the expression of *LasI* mRNA, which is a QS system-related gene, can be reduced by the eucalyptus volatile oil. The QS system regulates the secretion, virulence factors (e.g., elastin, exotoxin A, and pyocyanin), and biofilm formation of PA(21). Therefore, the eucalyptus volatile oil has a significant effect not only on bacteriostatic but also on the virulence-related system.

In summary, the volatile oil of *E. urophylla* has a high application value in the treatment of clinical infections caused by PA. Its main mechanism of action includes the following: directly inhibiting bacterial growth and reproduction, delaying bacterial biofilm formation to destroy its drug resistance, and reducing the expression of the *LasI* gene to reduce its virulence. However, the *E. urophylla* volatile oil also has its shortcomings. For example, its preparation process emits a pungent odor, and professional distillation and condensation recovery equipment is required for its extraction. Thus, this oil cannot be readily used.

In the current worldwide battle against the new coronavirus (2019-nCoV), Chinese medicine has become essential in patient treatment, rehabilitation, and daily infection prevention(22, 23). The use of integrative medicine for 2019-nCoV treatment has exhibited a significant clinical efficacy in Wuhan, Hubei Province,

and even the entire China(24). Therefore, we hope that the theory of traditional Chinese medicine be extended to the worldwide medical field. In the future research, we will further work on its therapeutic mechanism but also improve the shortcomings in exploring and promoting its use value.

Conclusion

As a Chinese medicine, the volatile oil of *E. urophylla* can affect PA proliferation and biofilm formation by interfering with the expression of LasI, thereby successfully preventing and treating burn infection caused by PA.

Declarations

Ethical Approval and Consent to participate

All experiments were performed in accordance with the Federation of European Laboratory Animal Science Association guidelines and WMA(2008). And the protocols were approved by the Animal Ethics Committee of The First Affiliated Hospital of Guangxi University of Chinese Medicine (Nanning, China).

Consent for publication

Not applicable.

Availability of supporting data

The datasets generated and/ or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

We declare no competing interests.

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Authors' contributions

Lei Yang conceived and designed the study; Leiyang, Xingxin Gao and Qing Huang wrote the manuscript; Zhimin Lin and Songlin Chen collected the data; Xiaodong Huang and Jinpeng Feng analyzed and

interpreted the data; Lili Zhao, Yinghui Lin and Guangzong Hua provided critical revisions; Sheng Li approved the final version of the manuscript.

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Authors' information

The First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning, Guangxi, China

Lei Yang, Qing Huang, Songlin Chen, Yinghui Lin, Guangzong Hua, Sheng Li

The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China

Xingxin Gao

Guangxi University of Chinese Medicine, Nanning, Guangxi, China China

Xiaodong Huang, Zhimin Lin,

The General Hospital of the Armed Police Force of Guangxi, Nanning, Guangxi, China

Lili Zhao

Corresponding author

Yinghui Lin, Guangzong Hua, Sheng Li

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Figures

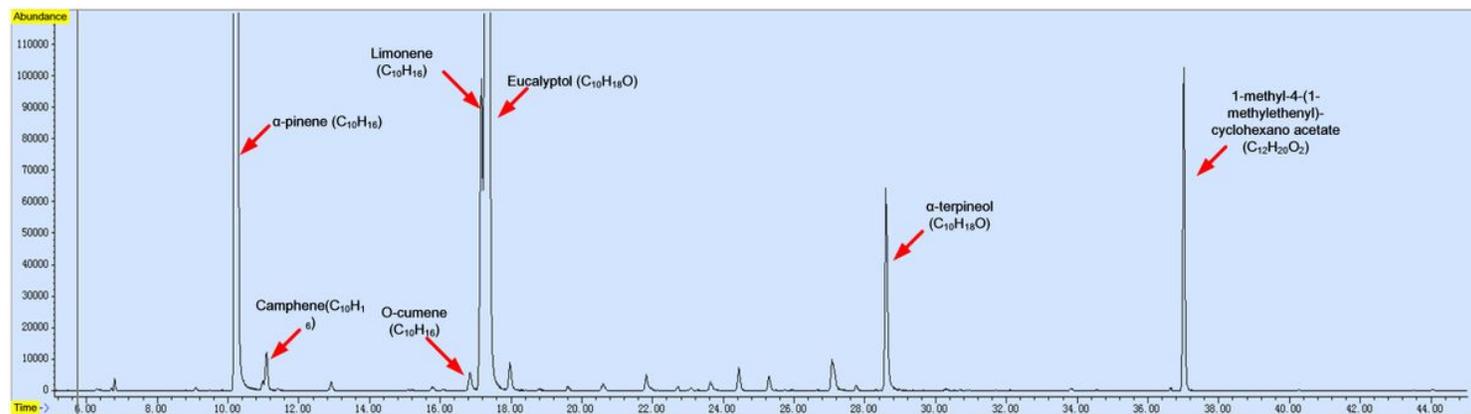


Figure 1

Main chemical components of eucalyptus volatile oil analyzed by GC-MS The total ion current map of eucalyptus volatile oil was obtained by GC-MS. Eucalyptus volatile oil is composed of eucalyptol (C₁₀H₁₈O), which is the main component, and 6 other chemical ingredients (α -pinene, camphene, o-cumene, limonene, α -terpineol, and 1-methyl-4-(1-methylethenyl)-cyclohexano acetate.), as pointed by the red arrow.

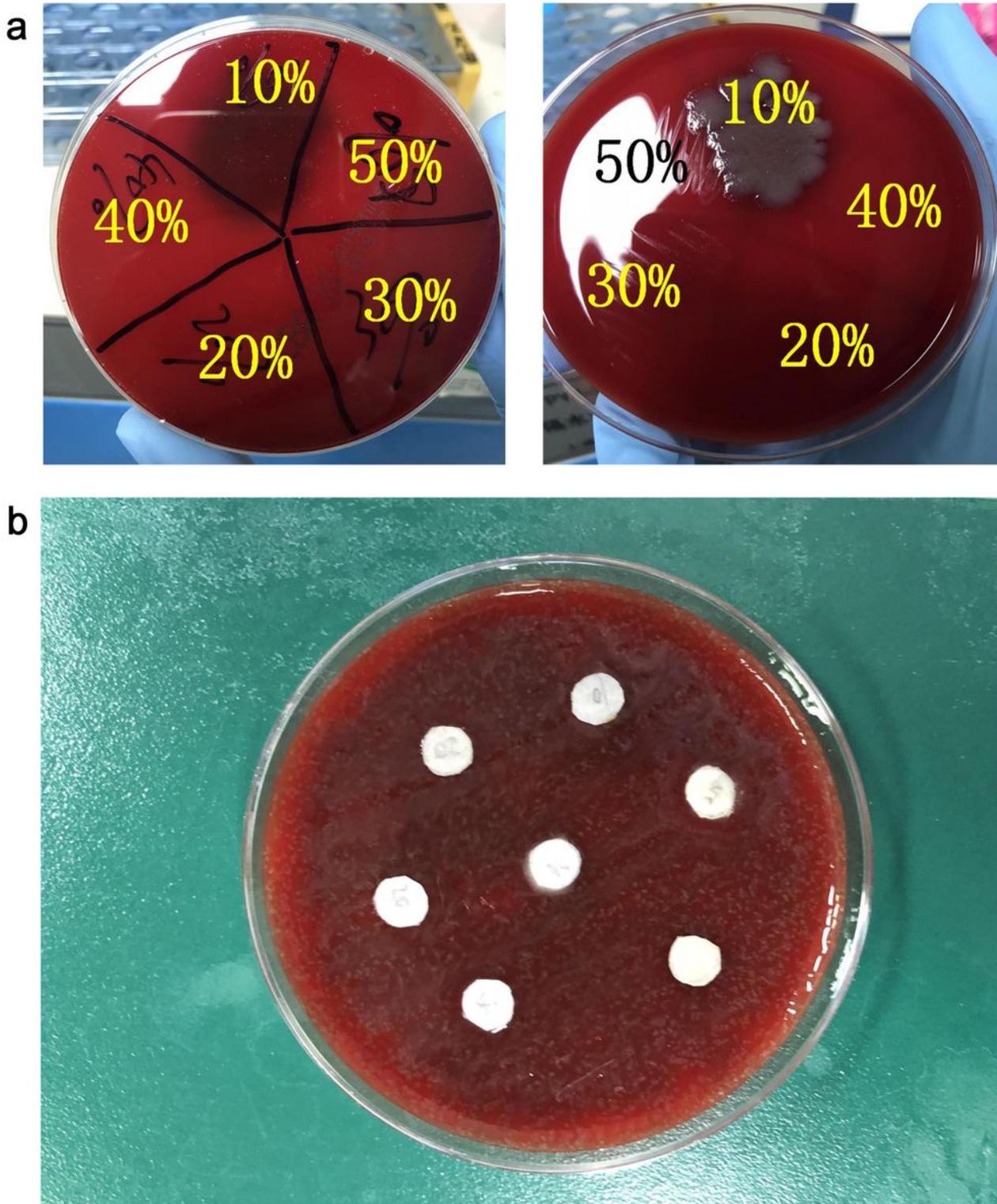


Figure 2

Antibacterial assay of eucalyptus volatile oil Figure 2a An ideal antibacterial effect can be achieved by using more than 20% concentration of eucalyptus volatile oil. Figure 2b Subsequent drug diffusion experiments showed that regardless of the concentration of eucalyptus volatile oil, no bacteriostatic ring could be observed (Figure 2b), indicating that the evaporative capacity of eucalyptus is poor and its antibacterial effect can only be attained by direct contact.

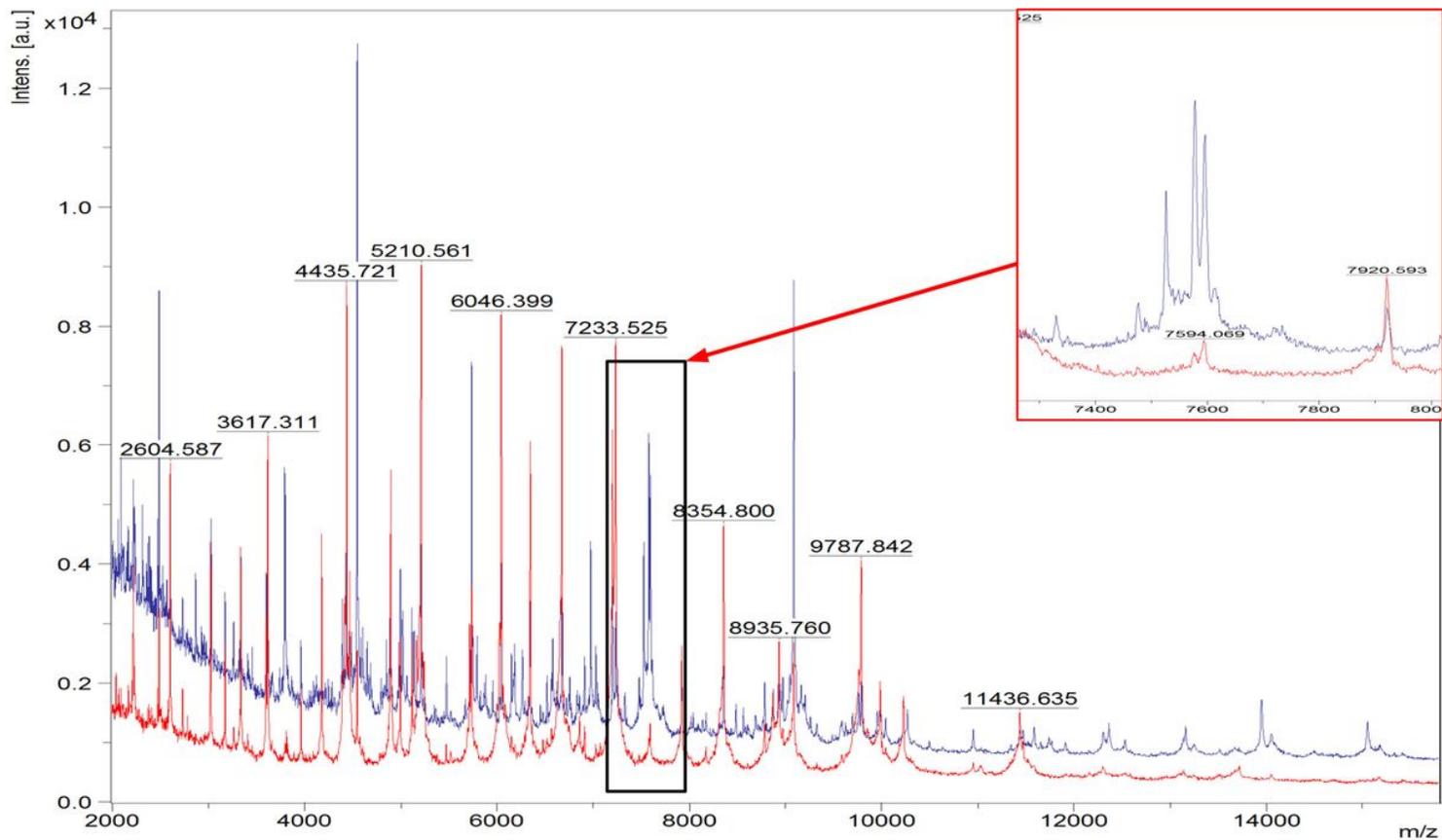
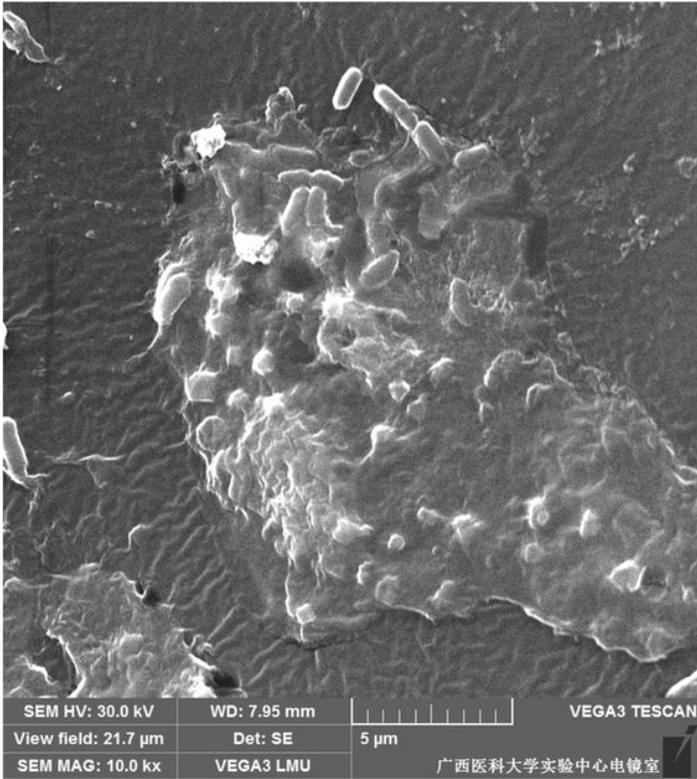
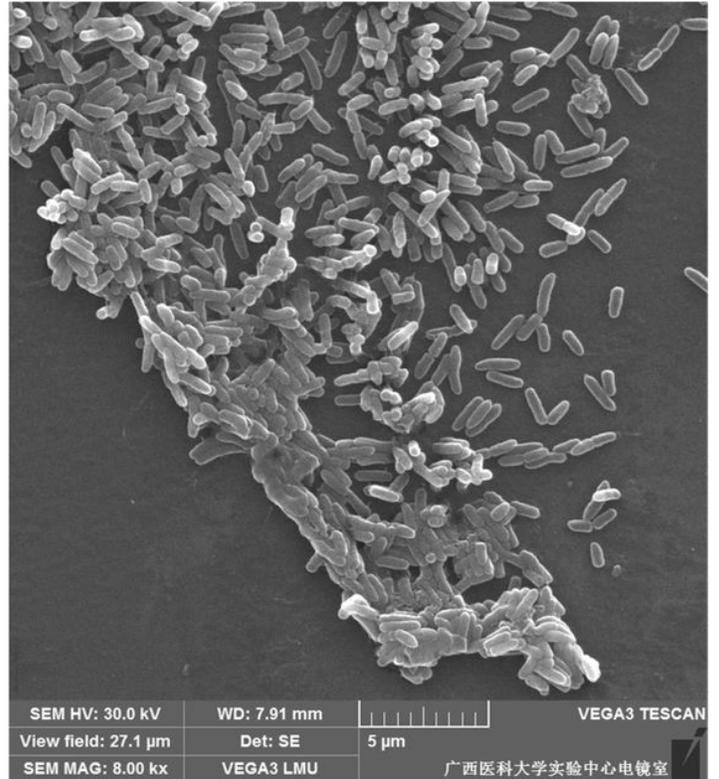


Figure 3

Protein fingerprint analysis by MS. PA (blue curve) in the control group and PA (red curve) in the experimental group after intervention with medication (drug concentration: 10%). The mass-to-nucleus (m/z) was significantly different at 7594 and some other areas.



Control Group



Observer Group

Figure 4

Scanning electron microscopy (SEM) observation. The control group had formed a thick early biofilm. In the group treated with eucalyptus volatile oil, the biofilm morphology is relatively thin, implying that the eucalyptus volatile oil inhibits biofilm formation.

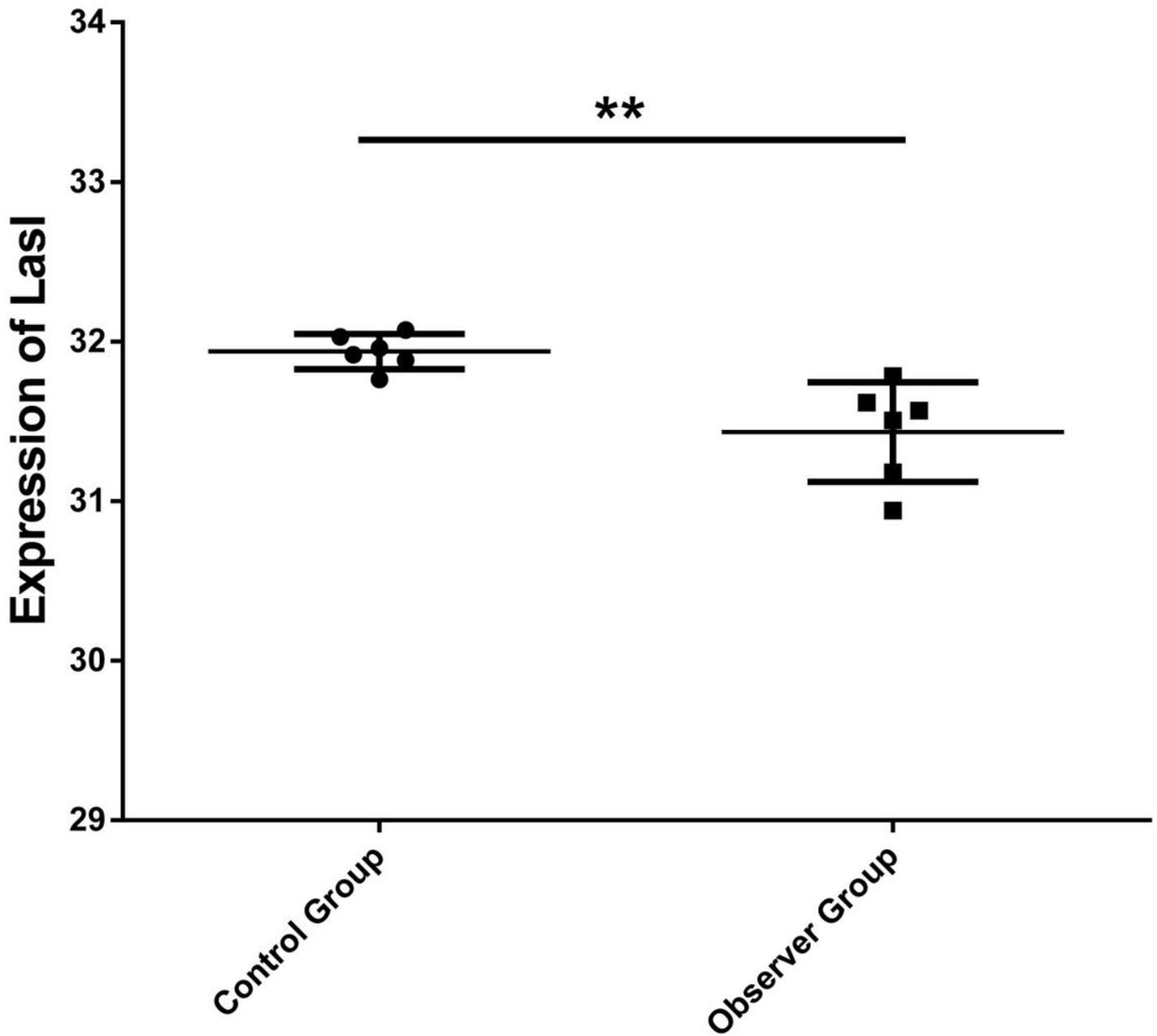


Figure 5

Detection of LasI mRNA expression in bacterial biofilm by RT-PCR. The RT-PCR detection results revealed that the LasI mRNA expression in the experimental group was (30.68 ± 0.087) after being converted by the standard RT-PCR curve $[Y = -3.346\text{LOG}(X) + 34.52, R = 0.998]$, whereas the average value of the control group was (31.12 ± 0.063) . The LasI mRNA expression in the experimental group was significantly reduced after eucalyptus volatile oil intervention, and the difference was statistically significant.