Study on the Mechanism of Application of Eucalyptus Volatile oil in Prevention and Treatment of Burn Wound Infections in Vitro

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Abstract

Objective To investigate working mechanism of Eucalyptus volatile oil on the prevention and treatment of burn wound infections.

Methods Pa biofilm model was used to investigate the effect of Eucalyptus volatile oil on bacterial biofilm. The expression of LasI mRNA in Pa was detected by RT-PCR.

Results MIC test showed that the volatile oil of Eucalyptus Urophylla in a concentration of 20% or more could exert anti-bacterial effect. However, no zone of inhibition could be observed in the neither high nor low concentration of the Eucalyptus volatile oil. Scanning electron microscopy results showed a significant delay in volatile oil groups when compared with the control group. The expression of LasI mRNA in the volatile oil group was significantly lower than that in the control group.

Conclusion As Chinese medicine, the volatile oil of Eucalyptus Urophylla can affect the proliferation of Pa and biofilm formation by interfering with the expression of LasI, thus achieving the purpose of preventing and treating infection of burn patients.

Introduction

Infection is one of the most common clinical complications of burn patients. It can cause septic shock and multiple organ dysfunction syndrome (MODS), which lead to death[1, 2]. Sepsis is a complex process and clinical manifestation which happened in a severely infected person with systemic inflammatory response syndrome (SIRS)[3]. When the blood circulation system is invaded by pathogens, acute systemic infections were caused following pathogens multiplying and toxin production. Endotoxin produced by pathogenic bacteria activates inflammatory cells to produce a large number of inflammatory mediators, which can induce immune dysfunction. It will inhibit the function of immune cells, and then cause septic shock, multiple organ failure, and even death.

Pseudomonas aeruginosa (Pa), is a common pathogen of burn wounds[4], and the most important survival strategy of Pa is the formation of biofilm. In the mature Pa. biofilm, the extracellular polysaccharide fiber or the polysaccharide-protein complex is entangled and interlaced into a network, and the gap is filled with a negatively charged alginate, which constitutes the external environment of BF. And this strong diffuse barrier hinders the penetration of antibiotics, especially positively charged ones[5, 6], greatly improving the viability of bacteria[7]. The current studies found that Pa can use Quorum Sensing (QS) systems to regulate toxic factor formation named Las system (lasI/lasR)[8]. LasI encode the signal molecule 3-oxo-dodecanoyl homoserine lactone (3-OXO-C12-homoserinelactone, 3-O-C12-HSL) and transcriptional activator regulatory signaling molecules. Studies have shown that LasR and rhlR gene defects can affect the ability of P.a. to form biofilm in vitro, and that QS system plays an important role in the establishment and chronic development of Pa. lung infection[9, 10].
Traditional Chinese medicine has unique advantages in preventing infection caused by bacterial biofilm, but also can inhibit the formation of bacterial biofilm. It has become the focus of further anti-infective drugs following antibiotics base on its wide source of drugs, low price and small side effects[11]. Eucalyptus Urophylla is a kind of Myrtaceae plant cultivated in Guangxi provinces, The use of Eucalyptus leaves has been circulating in the public. They are applied to the skin to prevent mosquito bites. Modern medicine has confirmed that the eucalyptus leaves contain gallic acid, phenols, mellow alcohol, and eucalyptus. The tests of gallic acid in vitro extracted from Eucalyptus Urophylla leaves inhibited Staphylococcus aureus, Pseudomonas aeruginosa, pneumococcus, typhoid and paratyphoid bacillus, which proved that eucalyptus leaves have good antibacterial and anti-inflammatory effects[12, 13]. Therefore, this study aimed to investigate the effect of Eucalyptus urophylla volatile oil on burn patients for prevention and treatment of concurrent infections.

1 Materials And Methods

1.1 Experimental materials and reagents

PAO1 used in this research was purchased from ACTT. RT-PCT related extraction, reverse transcription and amplification reagents were purchased from TAKARA (Japan). Eucalyptus leaves were collected from Hengxian, Guangxi, and the volatile oil was extracted and completed by the First Affiliated Hospital of Guangxi University of Chinese Medicine. Scanning electron microscope (SEM, EDAX-AMETEX) was provided by Guangxi Medical University. RT-PCR detection, bacterial biofilm Model construction and MIC tests were completed by the Laboratory of the the First Affiliated Hospital of Guangxi University of Chinese Medicine.

1.2 GC-MS Analysis of Eucalyptus Leaf Oil

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

1.2.2 GC-MS analysis conditions

1.2.2.1 Gas chromatographic condition Column: 1) Agilent HP-5MS capillary column (30m×0.25nm×0.25μm). 2) Heating program: Initial temperature was 60°C, warm to 70°C at 2°C/min and hold for 10min. Raised the temperature to 140°C at 3°C/min and raised to 250 °C at 5 °C/min, and then kept for 10 minutes. 3) Carrier gas: He. 4) Flow rate: 1ml/min. 5) Injection volume: 1.0 μL. 6) Split ratio: 50: 1.

1.2.2.2 Mass spectrometry conditions: 1) Ionization mode: EI. 2) Ionization energy was 70eV, inlet temperature was 250 °C and ion source temperature was 230 °C. 3) Column flow rate: 1.0ml/min.

1.2.2.3 Analysis methods

The Eucalyptus leaf oil was taken and analyzed by GC-MS. The mass spectrum was searched in NIST98 standard mass spectrum database (HPMSD Chem-Station) to confirm each chromatographic peak.
1.3 MIC test

Because the volatile oil of Eucalyptus leaves is insoluble in water, the co-solvent Tween-80 is used for co-solubilization, and the ratio of volatile oil and co-solvent is 5: 1000. Take 10 μL 0.5 MCF of PAO1 bacterial suspension and inoculate it into 1 mL of LB medium, and then add soluble volatile oil to configure a gradient mixed suspension with drug concentration of 10% to 50%, and incubate in a CO2 incubator for 24 hours, and then transfer to the blood plate to observe the growth of bacteria.

1.4 protein fingerprint analysis

Mass spectrometer (Micyoflex LT/SH, BD, US) was used for testing and FlexAnalysis (Bruck, US) for analysis. Standard MBT method, usual for typical laboratory standard samples, used for validation and specification issues. MS/Parent Mode: On. Initial Laser Power: 30%, and maximal laser power: 40%. Allow Only: 80 satisfactory shots per raster spot. Matrix Blaster: Fire initially 10 shots with a laser power of 40%.

1.5 BF Construction and SEM observation [10-12]

1.5.1 Biofilm model construction: Take 20mL of LB medium into a 50mL centrifuge tube, inoculate 1mL of 0.5 McFarley bacterial suspension, put a sterile gastric tube(1cm) into the bacterial suspension, and place it in a shaker at an angle of 45 degrees. After shaking for 24 hours, after that a bacterial film was formed on the surface of the sterile gastric tube, which is a biofilm model.

1.5.2 Preparation before SEM observation: 1) Biological specimens were fixed with 3% glutaraldehyde for 2 hours, 2) 0.1 mol/L PBS buffer solution was immersed and washed 3 times for 10 min each, 3) Osmium tetroxide was fixed for 1 hour, 4) 0.1mol/L PBS buffer solution for 3 times and 10min each time, 5) Ethanol from 50%, 70%, 80%, 90%, 100% (three times) soak and dehydrate for 10min each time, 6) 100% pure six Methylidisilazane was immersed three times for ten minutes each time, put into a vacuum dryer and vacuum dried, 7) Paste the sample to the sample holder, put it on the IB3 ion sputtering instrument and observe it under an electron microscope.

1.6 RT-PCR detection

After constructing the biofilm model according to the above steps, the gastric tube was gently washed with sterile PBS and the bacteria on the inner and outer surfaces of the gastric tube were collected with a spatula. After enrichment, the sample RNA was extracted using the TAKARA genome extraction kit and reversed. After 15 minutes of recording, use RT-PCR detector (Redstone SLAN-96P, Shanghai, China) to test the LasI expression.

1.7 Statistical methods

SPSS 19.0 and GraphPad were used for statistical analysis and picture processing. Measurement data were analyzed by t test. Sample means were expressed in the form of mean ± standard deviation. The
test level $\alpha = 0.05$. When the $P$ value is less than 0.05, it means the difference was statistically significant.

2 Results

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

The GC-MS test was performed and a total ion current map of Eucalyptus volatile oil was obtained (Fig 1). The main chemical component of eucalyptus volatile oil was Eucalyptol ($C_{10}H_{18}O$), and there are 6 other chemical ingredients, including $\alpha$-pinene ($C_{10}H_{16}$), Camphene ($C_{10}H_{16}$), O-cumene ($C_{10}H_{16}$), Limonene ($C_{10}H_{16}$), $\alpha$-terpineol ($C_{10}H_{18}O$), 1-methyl-4-(1-methylethenyl)-cyclohexano acetate ($C_{12}H_{20}O_2$).

2.2 Anti-bacterial effect of Eucalyptus volatile oil

It was found that eucalyptus volatile oil (20% or more) can make an ideal antibacterial effect (Figure 2a). Drug diffusion experiments showed that no bacteriostatic ring could be observed in different concentrations of Eucalyptus volatile oil (Figure 2b), which could indicate that the evaporative capacity of eucalyptus is poor, and its anti-bacterial effect can only be achieved by direct contact.

2.3 Observation of $P.a.$ after intervention with Eucalyptus volatile oil

$P.a.$ (Blue curve) in the control group and $P.a.$ (Red curve) in the experimental group after intervention with medication (drug concentration: 10%) was shown in the Fig. 3a. It was found that the mass-to-nucleus (m/z) was significantly different at 7594 and some other places. The differences lead us to consider that Eucalyptus volatile oil has an effect on various proteins of the bacteria and at the same time it interferes the $P.a.$ QS system as well. And we will continue to explore which kinds of these protein are in the future study. But what can be confirmed is most of these proteins are related to bacterial resistance.

As shown in Figure 3b, after the successful construction of the bacterial biofilm model, the surface biofilm morphology was observed by SEM. We found that the control group had formed an early biofilm with a thick state. In the observer group treated with Eucalyptus volatile oil, the biofilm morphology is relatively thin, which can be indicated that the Eucalyptus volatile oil of has inhibitory effect on bacteria, and the formation of biofilm was also delayed.

As shown in Figure 3c, the results of RT-PCR detection of LasI mRNA expression of LasI mRNA in the experimental group was $(30.68 \pm 0.087)$ after conversion by standard RT-PCR curve [$Y = -3.346 \log(X) + 34.52, R = 0.998$], and the average value of the control group was $(31.12 \pm 0.063)$. LasI mRNA expression in the experimental group was significantly reduced after intervention of Eucalyptus volatile oil, and the difference was statistically significant ($P < 0.05$).

3 Discussion
Guangxi Provence is an area lived Zhuang people, with abundant medical resources and culture. Therefore, the research on Chinese medicine is the advantage and characteristic of this article. At the same time, due to the large number of ethnic minorities in Guangxi, its diverse ethnic culture and unique local customs and activities are rare intangible cultural heritages in China, but it also brings problems: fireworks and firecrackers every year cause a large number of people suffering from injuries, burns, etc., which consume huge medical resources in the treatment of such patients. Therefore, while protecting China's intangible cultural heritage, it is particularly important and necessary to treat the burns as well.

The biofilm-forming system can cause high-resistance of Pa., which is one of the important factors causing clinical intractable infection[14]. Therefore, people are beginning to explore other ways to treat Pa. infection, such as the development of new antibacterial drugs, alginate monoclonal antibody, gene regulation and improvement of biomedical materials, but these methods still cost too much and have a narrow application range, causing clinical application limited[15]. The Quorum Sensing (QS) system plays an important role in Pa. biofilm formation[16], and it is a mechanism of information transmission between bacteria. Cells do not sense the presence of bacteria themselves, but rather the concentration of signal molecules, and the concentration of signal molecules depends on the density of the cells. In the case of the QS system involved in the regulation of the expression of virulence factors, interference with the QS signaling system may affect the regulation of the pathogenic factors of Pa.

The results in this study showed that the Eucalyptus urophylla volatile oil has a lower minimum inhibitory concentration(MIC) and better bactericidal effect, 20% concentration can fully inhibit the growth of Pa, and at the same time, results under scanning electron microscope(SEM) show that after the intervention of Pa. in the Eucalyptus urophylla volatile oil, the formation of bacterial biofilm can be delayed. Many reports have put that the formation of biofilm is closely related to bacterial resistance. This indicates that one of the mechanisms of the Eucalyptus urophylla volatile oil can be used in clinical bacterial treatment is to destroy its drug resistance. In addition, the results of RT-PCR detection show that the QS system related gene LasI mRNA can be reduced by the Eucalyptus urophylla volatile oil, and some studies have found that the secretion, virulence factors (elastin, exotoxin A, pyocyanin, etc.) and biofilm formation of Pa. are regulated by the QS system[17], so we can consider that the Eucalyptus urophylla volatile oil has a significant effect not only on bacteriostatic, but also on the virulence-related system.

In summary, the Eucalyptus urophylla volatile oil has high application value in the treatment of clinical infections. Its mechanism of action is mainly manifested in the following aspects: directly inhibiting bacterial growth and reproduction, delaying the formation of bacterial biofilm to destroy its drug resistance, and reducing LasI gene expression so as to reduce its virulence. However, the Eucalyptus urophylla volatile oil also has its shortcomings. For example, the pungent odor is emitted during the preparation process, and its extraction requires professional distillation and condensation recovery equipment. In daily life and clinical departments can not be used on demand.

In the recent worldwide battle against the new coronavirus (2019-nCoV), Chinese medicine has played an important role in patient treatment, rehabilitation, and daily infection prevention[18, 19]. Treatment of
integrative medicine has made significant clinical efficacy in Wuhan, Hubei Province and even the whole China[20]. Therefore, we hope to extend the theory of traditional Chinese medicine to the worldwide medical field, and in the future research, we will further work on its therapeutic mechanism, but also improve the shortcomings in exploring and promoting its use value.

**Declarations**

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

Competing interests

There is no competing interests and all authors agree with the author list.

Author contribution

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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**Figures**

**Figure 1**

The main chemical components of eucalyptus volatile oil analyzed by GC-MS. The GC-MS test was performed and a total ion current map of Eucalyptus volatile oil was obtained as shown in Fig 1. The main chemical component of eucalyptus volatile oil was Eucalyptol (C10H18O), and there still exist 6 other chemical ingredients (α-pinene, Camphene, O-cumene, Limonene, α-terpineol, 1-methyl-4-(1-methylethenyl)-cyclohexano acetate.) as red arrow pointed.
Figure 2
Antibacterial assay of Eucalyptus volatile oil Fig2a More than 20% concentration of eucalyptus volatile oil can make an ideal antibacterial effect. Fig 2b Subsequent drug diffusion experiments showed that no bacteriostatic ring could be observed regardless of the concentration of Eucalyptus volatile oil (Figure 2b), which could indicate that the evaporative capacity of eucalyptus is poor, and its antibacterial effect can only be achieved by direct contact.
Figure 3

Observation of P.a. after intervention with Eucalyptus volatile oil Figure 3a Protein fingerprint analysis by MS. P.a. (Blue curve) in the control group and P.a. (Red curve) in the experimental group after intervention with medication (drug concentration: 10%) was shown in the Figure 3a. It was found that the mass-to-nucleus (m/z) was significantly different at 7594 and some other places. Figure 3b Scanning electron microscopy (SEM) observation. We found that the control group had formed an early biofilm with a thick state. In the observer group treated with Eucalyptus volatile oil, the biofilm morphology is relatively thin, which can be indicated that the Eucalyptus volatile oil has inhibitory effect on the formation of biofilm. Figure 3c Detection of LasI mRNA expression in bacterial biofilm by RT-PCR. The results of RT-PCR detection of LasI mRNA expression of LasI mRNA in the experimental group was \((30.68 \pm 0.087)\) after conversion by standard RT-PCR curve \([Y = -3.346\text{LOG}(X) + 34.52, R = 0.998]\), and the average value of the control group was \((31.12 \pm 0.063)\). LasI mRNA expression in the experimental group was significantly reduced after intervention with Eucalyptus volatile oil, and the difference was statistically significant \((P < 0.05)\).