Sclareol Protects *Staphylococcus aureus*-Induced Lung Cell Injury via Inhibiting Alpha-Hemolysin Expression

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Abstract

*Staphylococcus aureus* (*S. aureus*) is a common gram-positive bacterium that causes serious infections in humans and animals. With the continuous emergence of methicillin-resistant *S. aureus* (MRSA) strains, antibiotics have limited efficacy in treating MRSA infections. Accordingly, novel agents that act on new targets are desperately needed to combat these infections. *S. aureus* alpha-hemolysin plays an indispensable role in its pathogenicity. In this study, we demonstrate that sclareol, a fragrant chemical compound found in clary sage, can prominently decrease alpha-hemolysin secretion in *S. aureus* strain USA300 at sub-inhibitory concentrations. Hemolysis assays, western-blotting, and RT-PCR were used to detect the production of alpha-hemolysin in the culture supernatant. When USA300 was co-cultured with A549 epithelial cells, sclareol could protect the A549 cells at a final concentration of 8 µg/ml. The protective capability of sclareol against the USA300-mediated injury of A549 cells was further shown by cytotoxicity assays and live/dead analysis. In conclusion, sclareol was shown to inhibit the production of *S. aureus* alpha-hemolysin. Sclareol has potential for development as a new agent to treat *S. aureus* infections.

Keywords: Methicillin-resistant *Staphylococcus aureus*, virulence factor, sclareol, alpha-hemolysin

Introduction

*Staphylococcus aureus* is a gram-positive pathogen that causes a wide range of infections, ranging from minor skin infections to serious diseases such as endocarditis, pneumonia, and toxic shock syndrome in humans and animals [1]. Since methicillin-resistant *S. aureus* (MRSA) was first reported in 1961 [2], the prevalence of MRSA has been increasing worldwide. USA300, the first described strain of community-associated MRSA (CA-MRSA), has spread throughout the USA. Over the last two decades, infections with USA300 have been reported in an increasing number of countries [3–5]. USA300 is the most widespread MRSA strain and USA300 infections have threatened global public health. Infections caused by MRSA often fail to respond to antibiotic treatment, thus making them harder to cure. Consequently, new agents are urgently required for the treatment of *S. aureus* infections, especially MRSA infections.

Antibiotics are the commonly prescribed drugs for *S. aureus* infections. However, the development of bacterial resistance has been linked to antibiotic use. Because traditional antibiotics are used to control bacterial infections by killing or restraining the multiplication of pathogens, this increases the selective pressure on these pathogenic bacteria, leading to bacterial resistance [6]. So far, some studies have found that the pathogenicity of *S. aureus* is closely related to the secretion of virulence factors [1]. Alpha-hemolysin (Hla) is a major cytotoxin secreted by *S. aureus*. USA300 has been shown to highly express Hla and is highly virulent [7, 8]. Hla binds to target host cell membranes, altering cellular permeability and causing leakage of the cytoplasm, activation of stress-signaling pathways, and cell death [9]. Hla has been shown to play a
significant role in the pathogenesis of S. aureus infections [10] and the indispensable role of Hla makes it an ideal target for the development of antivirulence drugs against S. aureus infection.

According to previous studies, some natural products have been shown to inhibit the production and activity of S. aureus Hla, and to relieve symptoms of S. aureus pneumonia in a mouse model [11–14]. Sclareol ((1R,2R,4aS,8aS)-1-{[3R]-3-hydroxy-3-methylpent-4-enyl}-2,5,5,8a-tetramethyl-3,4,4a,6,7,8-hexahydro-1H-naphthalene-2-ol) is a fragrant chemical compound (Fig. 1) found in Salvia sclarea [15]. It is classified as a bicyclic diterpene alcohol that is primarily used in cosmetics, such as decorative cosmetics, fine fragrances, shampoos, and toilet soaps [16]. Sclareol has potent pharmacological activities, including antimicrobial [15], anti-inflammatory and antitumor activities [17]. Miski et al. [15] reported that sclareol has limited activity against S. aureus. However, no studies have focused on the effects of sclareol on Hla secretion in S. aureus. In this study, we evaluated the effect of sclareol on the inhibition of Hla secretion in S. aureus USA300, using the hemolysis assay, western blotting, RT-PCR, and cell experiments.

Materials and Methods

Bacterial Strains, Cell Line, and Reagents

The CA-MRSA S. aureus strain BAA-1717 (USA300), a Hla-producing strain, used in this study was purchased from the American Type Culture Collection (ATCC). The human alveolar epithelial cell line (A549, ATCC CCL 185) was also obtained from ATCC. Sclareol (purity ≥ 98%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (China). The chemical structure of sclareol is presented in Fig. 1. Sclareol was dissolved in dimethyl sulfoxide (DMSO, ≥99.5%; Sigma-Aldrich, USA) to make a stock solution (40.96 mg/ml). Defibrinated rabbit blood was purchased from Zheng Zhou Jiu Long Biological Products Co. Ltd. (China), and no animals were directly used in the experiments.

Preparation of Bacterial Cultures

For in vitro hemolysis assays, western blotting, and real-time PCR, S. aureus USA300 was cultured with the addition of varying amounts of sclareol in tryptic soy broth (TSB; Oxoid, UK) at 37°C until the cultures reached the post-exponential phase (OD₆₀₀nm = 2.5). For cytotoxicity assays, S. aureus USA300 was cultured at 37°C in TSB to OD₆₀₀nm = 0.5. Five milliliters of bacterial culture was pelleted (1 min, 1,000 × g) and washed with sterile phosphate-buffered saline (PBS). The pellet was then resuspended in 10 ml of Dulbecco’s modified Eagle’s medium (DMEM).

Susceptibility Testing

The broth microdilution method was used to determine the minimal inhibitory concentration (MIC) of sclareol against S. aureus USA300 and was performed according to the Clinical and Laboratory Standards Institute [18]. The MIC was defined as the lowest concentration of the agent that inhibited bacterial growth. The MIC experiment was repeated three times.

Growth Curve Assay

S. aureus USA300 was grown in 500 ml of TSB at 37°C to OD₆₀₀nm = 0.3. The cell culture was divided into five flasks (250 ml), and sclareol was added to a final concentration of 0, 1, 2, 4, or 8 μg/ml. DMSO (0.04%) was used for the solvent control group. The flasks were incubated at 37°C with constant shaking (200 rpm) and bacterial growth was determined by measuring the absorbance of the cultures at OD₆₀₀nm at intervals ranging from 0 to 390 min after treatment.

Hemolysis Assay

Sclareol was investigated for its ability to inhibit the hemolytic activity of S. aureus culture supernatants. Bacterial cultures (1 ml) were centrifuged (5,000 × g, 20°C, 2 min) and sterilized by filtration through a 0.2 μm filter. One-hundred microliters of culture supernatant was transferred to a sterile tube, to which 875 μl of sterile PBS and 25 μl of defibrinated rabbit red cells were added and then mixed. The mixture was incubated at 37°C for 15 min. All samples were then centrifuged (10,000 × g, 20°C, 1 min). Hemolytic activity was determined by measuring the OD₅₄₀nm values of the supernatant of the mixtures. A negative control (without sclareol) served as 100% hemolysis, and all the percentage hemolysis of the test groups were compared with that of the drug-free control.

Western Blotting

Secretion of Hla in the bacterial cultures was detected by western blotting. Bacterial cultures (1 ml) were centrifuged (5,000 × g, 20°C, 2 min) and filtered (0.2 μm) to remove residual bacteria. Samples (25 μl) were boiled with Laemmli sodium dodecyl sulfate (SDS)