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A dual antibacterial mechanism involved in membrane disruption and DNA binding of *2R,3R*-dihydromyricetin from pine needles of *Cedrus deodara* against *Staphylococcus aureus*

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ABSTRACT

The antibacterial activity and mechanism of *2R,3R*-dihydromyricetin (DMY) against *Staphylococcus aureus* were investigated. The minimum inhibitory concentration of DMY against *S. aureus* was 0.125 mg/ml, and the growth inhibitory assay also revealed that DMY showed a potent antibacterial activity against *S. aureus*. Massive nucleotide leakage and flow cytometric analysis demonstrated that DMY disrupted the membrane integrity of *S. aureus*. Morphological changes and membrane hyperpolarization of *S. aureus* cells treated with DMY further suggested that DMY destroyed cell membrane. Meanwhile, DMY probably interacted with membrane lipids and proteins, causing a significant reduction in membrane fluidity and changes in conformation of membrane protein. Moreover, DMY could interact with *S. aureus* DNA through the groove binding mode. Overall, the results suggested that DMY could be applied as a candidate for the development of new food preservatives as it achieved bactericidal activity by damaging cell membrane and binding to intracellular DNA.

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1. Introduction

Food-borne diseases, provoked by food pathogens, are important causes of morbidity and mortality worldwide. *Staphylococcus aureus*, a commensal and opportunistic pathogen, is one of the most common causes of food-borne diseases, causing an estimated 241,000 illnesses per year in the United States (Kadariya, Smith, & Thapaliya, 2014). *S. aureus* can grow in many food products and cause contamination due to the ability to survive in potentially dry and stressful environments (Le Loir, Baron, & Gautier, 2003). This ubiquitous bacterium has emerged as a significant pathogen for both nosocomial- and community-acquired infections due to a combination of toxin-mediated virulence, invasiveness and antibiotic resistance (Kadariya et al., 2014). The presence of *S. aureus* in ready-to-eat foods puts consumers at high risk and causes diverse food-borne diseases, ranging from relatively mild skin infections to more severe and invasive infections, including endocarditis, necrotizing fasciitis, osteomyelitis, and pneumonia (Rozemeijer et al., 2015). In this regard, food-borne disease caused by *S. aureus* is still an important issue facing the food industry and consumers.

Many strategies, such as use of synthetic chemicals, have been adapted to prevent the growth of pathogens in food. On the other hand, there is a strong debate about the safety of chemical preservatives, because they are considered to have carcinogenic and teratogenic attributes as well as residual toxicity (Ye et al., 2013). Thus, it is still necessary to find new methods to reduce or eliminate food-borne pathogens during the shelf life of food products. Meanwhile, the increasing demand of consumers for natural products and organic foods, resulting from the trend of 'green' consumerism, has led to a search for natural products as safer preservative alternatives. Plant secondary metabolites are known to play a promising role in the fight against pathogens, due to the fact that plants fight off infections successfully by producing a great number of small molecules (Klančnik, Možina, & Zhang, 2012). Many plant secondary metabolites, such as essential oils, tannins and flavonoids, are responsible for antibacterial activity (Gyawali & Ibrahim, 2012). Therefore, development of plant-derived compounds with antibacterial activities, to be used as safe and natural preservatives in food applications, is becoming increasingly attractive.

2R,3R-Dihydromyricetin (DMY), a secondary plant metabolite, enriched in the leaves of *Ampelopsis grossedentata* (Ye, Wang, Duncan, Eigel, & O'Keefe, 2015), is also commonly found in plants such as *Hovenia dulcis* (Chaturvedula & Ruo, 2013) and *Cedrus*

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deodara (Liang, Wu, Qiu, Zhong, & Gao, 2014). DMY was reported to possess a variety of biological activities, including hypoglycemic, hepatoprotective, antioxidant, antibrowning and antibacterial activities (Liang et al., 2014; Zhang et al., 2007). Interestingly, in our continuous study searching for natural food preservatives, it was found that DMY showed strong antibacterial activity against *S. aureus*. Nevertheless, to the best of our knowledge, there has been limited literature related to whether DMY has the potential to be used as a novel preservative in the food industry. Most importantly, the antibacterial mechanism of DMY still remains largely unknown.

Therefore, the present study was carried out to investigate, for the first time, the antibacterial activity and mode of action of DMY against *S. aureus*. After evaluating the antibacterial activity of DMY, we further elucidated the mechanism of DMY against *S. aureus* in terms of its interaction with the cell membrane, the first barrier of pathogenic bacteria that serves as a defence against antibacterial agents, and DNA, one of the most basic life molecules with a decisive effect on gene expression, heredity and variation.

2. Materials and methods

2.1. Materials

Staphylococcus aureus ATCC 6538 was obtained from the China Medical Culture Collection Center (Beijing, China). *2R,3R*-Dihydromyricetin (HPLC purity $\geqslant$ 98%) was obtained from pine needles of *Cedrus deodara* according to a previous report (Liang et al., 2014). A stock solution of DMY in 25% DMSO was diluted with nutrient broth or 0.85% saline solution before use to maintain the final concentration of DMSO in bacterial suspension below 0.5%. Epigallocatechin gallate (EGCG, HPLC purity $\geqslant$ 98%) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The LIVE/DEAD BacLight bacterial viability kit, bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Life Technologies Corporation (Shanghai, China). All other chemicals and reagents used were of analytical grade.

2.2. Detection of minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

The MIC of DMY against *S. aureus* was determined by broth microdilution assay, as recommended by the Clinical and Laboratory Standards Institute (NCCLS/CLSI – National Committee for Clinical Laboratory Standards, 2006). In brief, *S. aureus* was cultured in nutrient broth at 37 °C overnight to give the logarithmic phase bacteria and subsequently diluted in the same medium to about 1×10^6 CFU/ml. Then, 100 μl of a set of twofold serial dilutions of DMY in nutrient broth was added to 100 μl of the bacterial suspension together in a sterile 96-well microplate, giving final concentrations from 0.001 to 1 mg/ml. The plate was again incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration of sample, which prevented the visible growth. The MBC was defined as the lowest concentration at which no growth was observed after sub-culturing into fresh nutrient agar. As the positive control, the MIC and MBC of epigallocatechin gallate (EGCG) against *S. aureus* were similarly determined.

2.3. Growth curves

The effect of DMY on the growth of *S. aureus* was evaluated with a slightly modified method (Zeng, He, Sun, Zhong, & Gao, 2012). Briefly, logarithmic phase *S. aureus* was diluted to 10^7 CFU/ml with nutrient broth. Then, 2.5 ml of the bacterial suspension were inoculated into the flasks containing 50 ml of sterile nutrient broth. The filtered DMY (with 0.22 μm membrane) was added to the cultures to keep the final concentrations of 1/4 × MIC, 1/2 × MIC, 1 × MIC and 2 × MIC. The *S. aureus* culture containing DMSO vehicle without DMY was the control. And then, the culture was further incubated (120 rpm at 37 °C), and cell growth was monitored every 2 h at OD_{600nm}, using a microplate reader (Spectra MAX-190, Molecular Devices Co., California, USA.).

2.4. Nucleotide leakage

The leakage of nucleotide into supernatant was determined according to a modified method (Zhao, Zhang, Hao, & Li, 2015). Logarithmic phase *S. aureus* was collected by centrifugation at 3000 rpm for 5 min, washed twice and resuspended in 0.85% sterile saline to keep it at a final density of 10^7 CFU/ml. Then, the bacterial suspensions were incubated with DMY at concentrations of 1 × MIC, 2 × MIC and 4 × MIC at 37 °C. Negative control cells treated with DMSO vehicle were tested under the same conditions. The mixture was filtered through 0.22 μm membrane to remove bacteria cells. After being diluted 10 times with ultrapure water, the filtrate was determined at 260 nm, using the microplate reader described above.

2.5. Flow cytometric analysis

The membrane integrity of *S. aureus* following exposure to DMY was evaluated, using the LIVE/DEAD BacLight bacterial viability kit, as previously reported (Otto, Cunningham, Hansen, & Haydel, 2010). Logarithmic phase *S. aureus* was washed and resuspended in 0.85% sterile saline at a cell density of 10^9 CFU/ml. Following exposure to DMY at 37 °C for 3 h, cells were harvested by centrifugation, washed and resuspended in 0.85% sterile saline. After being stained with 10 μM SYTO9 and 60 μM propidium iodide (PI) for 15 min in the dark at room temperature, the cell suspensions were subjected to flow cytometric analysis immediately. The sample without DMY treatment was used as the negative control, and the sample treated with 70% isopropyl alcohol was used as the positive control.

Bacterial samples were analyzed on a BD FACSVerse flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 15 mW, 488 mM argon ion laser. The green fluorescent SYTO9-stained cells were detected in the FL1 channel with a 525 nm bandpass filter and the red fluorescent PI-stained cells were detected by FL3 channel with a 620 nm bandpass filter. In total, 50,000 events were acquired at a low flow rate (12 μl/min) for each sample.

2.6. Transmission electron microscope

The experiment was performed according to the previous reported method with a few modifications (Zeng et al., 2012). In brief, logarithmic phase *S. aureus* was incubated with DMY at a concentration of 1 × MIC for 12 h at 37 °C and 120 rpm. After incubation, the treated bacteria were centrifuged at 1000 rpm for 1 min, and washed with 0.85% sterile saline. The pellets were fixed with 3% buffered glutaraldehyde overnight at 4 °C. Then the cells were collected by centrifugation at 5000 rpm for 10 min. After being treated with 3% buffered glutaraldehyde, 1% osmium tetroxide, acetone and epoxy in sequence, the sample was cut into thin sections to perform transmission electron microscopy (Tecnai G2F20S-TWIN, FEI, USA).

2.7. Membrane potential

The effect of DMY on membrane potential was evaluated as previously reported with minor exceptions (Sánchez, García, &

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