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Chemical composition, antibacterial activity of *Cyperus rotundus* rhizomes essential oil against *Staphylococcus aureus* via membrane disruption and apoptosis pathway

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ABSTRACT

The chemical composition, antibacterial activity and mechanism of essential oil from *Cyperus rotundus* rhizomes against *Staphylococcus aureus* were investigated in this study. Results showed that α-cyperone, cyperene and α-selinene were the major components of the essential oil. The essential oil exhibited strong antibacterial activity against *S. aureus* with the minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) were 10 and 20 mg/mL respectively, and the antibacterial effects increased with increasing essential oil concentrations and treatment time. The electric conductivity, cell membrane integrity, NPN uptake, and membrane potential assays demonstrated that essential oil disrupted the membrane integrity of *S. aureus*. Electron microscope observations further confirmed that essential oil destroyed cell membrane. Moreover, we found that essential oil could induce cells death of *S. aureus* through apoptosis pathway based on apoptosis analysis. These findings suggested that essential oil mainly exerted antibacterial activity by damaging cell membrane and membrane-mediated apoptosis pathway.

1. Introduction

Food poisoning and spoilage caused by microorganisms results in enormous losses of foods, and has been of vital concern to public health (Burt, 2004; Scallan et al., 2011). Many strategies, such as use of synthetic chemicals, have been adapted to prevent the microbial growth in food resulting in reduced incidences of foodborne illnesses and an extension in the shelf-life of food products. However, their use is being questioned due to perceived possible health concerns and potential increases in microbial resistance (Ye et al., 2013). Meanwhile, because of consumers' growing concerns over the safety of foods containing synthetic chemicals, much attention has been paid to use antibacterial agents from natural resources as safer preservative alternatives (Delgado-Adámez, Fernández-León, Velardo-Micharet, & González-Gómez, 2012; Lanciotti et al., 2004). There has been increasing realization in recent years that many essential oils from various plants including edible and medicinal plants, herbs, and spices have been reported to be safe and possessed strong antimicrobial effects (Bajpai, Sharma, & Baek, 2013; Diao, Hu, Zhang, & Xu, 2014).

Cyperus rotundus L. (Cyperaceae) is widely distributed in tropical and warmer temperate regions worldwide. Some studies reported the biological and pharmacological activities of *C. rotundus* extracts (Kilani-Jaziri et al., 2011; Kumar, Razack, Nallamuthu, & Khanum, 2014; Nam, Nam, & Lee, 2016; Peerzada et al., 2015) and chemical composition of the essential oil from *C. rotundus* rhizome (Sonwa & König, 2001; Lawal & Oyedeji, 2009; Aghassi, Naeemy, & Feizbakhsh, 2013). The essential oil of *C. rotundus* rhizome possessed the antioxidant (Essaidi et al., 2014; Kilani et al., 2008), antibacterial activity (Duarte et al., 2007; Essaidi et al., 2014), insecticidal activity (Liu, Lu, Liu, & Liu, 2016), as well as antiradical and antimutagenic properties (Kilani et al., 2005, 2007). Interestingly, it was found that *C. rotundus* rhizome essential oil had strong antibacterial activity against *S. aureus*, *Staphylococcus epidermidis* and *Bacillus subtilis* in our previous study. However, to the best of our knowledge, although the antimicrobial activity of essential oil from *C. rotundus* rhizome has been reported earlier, there have been few reports of its antibacterial mechanism of action against

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foodborne pathogens. *S. aureus* is widely distributed in many food products and is a kind of common pathogenic bacteria which can lead to food poisoning and purulent diseases in human body (Xu et al., 2017). Therefore, *S. aureus* was selected as the model organisms to investigate the antibacterial activities of *C. rotundus* rhizome essential oil against *S. aureus*, and to further elucidate its mode of action against *S. aureus* in terms of its interaction with the cell membrane in the present study.

2. Materials and methods

2.1. Plant materials, microbial strain and culture

The rhizomes of *C. rotundus*, which were harvested in the region of Taian County of Shandong Province, China in 2015, were obtained as commercial products from the local market in March 2016. *Staphylococcus aureus* ATCC 25923 was provided by the College of Life Science, Shanxi Normal University. The strain was cultured at 37 °C on nutrient agar (NA) or nutrient broth (NB) media.

2.2. Extraction of essential oil

The dried rhizomes of *C. rotundus* were ground with a micro plant grinding machine to a powder and then hydrodistilled for 6 h using a Clevenger-type apparatus. The oil was separated from water and dried over anhydrous sodium sulfate and stored in tightly closed dark vials at 4 °C until use.

2.3. GC-MS analysis

The analysis of the essential oil was performed using a Hewlett-Packard 5890 II GC, equipped with a HP-5 MS capillary column (30 m × 0.25 mm; film thickness, 0.25 μm) and a HP 5972 mass selective detector for the separation. The mass selective detector was operated in electron-impact ionization (EI) mode with a mass scan range from *m*/*z* 30 to 550 at 70 eV. Helium was the carrier gas at a flow rate of 1 mL/min. The initial temperature at 50 °C, held for 1 min, ramped at 5 °C/min to 280 °C and held for 1 min. Injector and MS transfer line temperatures were set at 230 and 300 °C, respectively. A sample of 1 μL of 1% essential oil was injected manually using a 1:10 split ratio. Most components were identified by comparing their GC retention indices which were determined in relation to a homologous series of *n*-alkanes (C8–C24) under the same operating conditions, NIST mass spectral search program, and mass spectra with publish data. Component relative percentages were calculated based on GC peak areas without using correction factors.

2.4. Antibacterial activity

The in vitro antibacterial activity of the tested sample was carried out by disc diffusion method. The inoculum suspension containing 10^7 CFU/mL of bacteria was spread on nutrient agar medium uniformly. The sample was dissolved and was loaded on 6 mm sterile individual discs. The loaded discs were placed on the surface of medium and the diameter of zone of inhibition (ZOI) was measured after 24 h of incubation at 37 °C. Minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) of sample were determined according to the method as previously described (Diao et al., 2014).

2.5. Growth curves

The effect of essential oil 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. The essential oil was added to the cultures to keep the final concentrations of 0.5 × , 1 × and 2 × MIC. The cultures were incubated in NB at 37 °C and 120 rpm. At selected time intervals, samples from test culture were taken and the absorbance at 600 nm was measured.

2.6. Antibacterial mechanism

2.6.1. Permeability and integrity of cell membrane

The permeability of bacteria membrane was expressed in the relative electric conductivity and the cell integrity of *S. aureus* strains was examined by determining the release of cell constituents into supernatant. They were determined as previously reported (Diao et al., 2014).

2.6.2. NPN uptake

The *N*-phenyl-1-naphthylamine (NPN) uptake assay was conducted following the method reported by Helander and Mattila-Sandholm (2000) with some modifications. Logarithmic phase bacteria were harvested by centrifugation at 5000*g* for 10 min, washed three times and resuspended in 0.5% NaCl solution, and the concentration was adjusted to Abs600 = 0.5. The bacterial suspension was separated and treated with the different concentrations essential oil (0.5, 1 and 2 MIC) for 1 h. Suspension was centrifuged and washed by 0.5% NaCl, and resuspended. The 200 μL Bacterial suspension was added to 96-well plates then mixed with 1.5 μL of 100 mM NPN. The fluorescence value was measured immediately by a fluorescence spectrophotometer at the excitation and emission wavelengths of 350 and 420 nm, respectively.

2.6.3. Membrane potential

The effect of essential oil on membrane potential was evaluated according to the method of Zhang, Liu, Wang, Jiang, and Quek (2016). The bacterial suspension was prepared following NPN uptake assay and were incubated with different concentrations of essential oil at 37 °C for 30 min at dark, washed and resuspended with 0.5% NaCl, and stained with Rhodamine 123, and then its fluorescence value was immediately measured at the excitation and emission wavelengths of 480 and 530 nm, respectively, using the fluorescence spectrophotometer.

2.6.4. Scanning electron microscope (SEM) and transmission electron microscope (TEM)

The bacterial cells were incubated in NB at 37 °C for 10 h, and then the suspensions were added 1 × MIC of essential oil. The suspension was incubated at 37 °C for 4 h, and then the suspension was centrifuged at 5000 rpm for 10 min. The treatment of bacterial cells and observations of SEM and TEM were respectively performed according to the method described by Diao, Hu, Feng, Li, and Xu (2013).

2.6.5. Apoptosis analysis

Logarithmic phase bacteria were collected by centrifugation at 6000 × g for 5 min, washed several times, and resuspended in PBS (pH 7.4). Tested bacteria were treated with different concentrations of samples. After 2 h, cells containing approximately 1×10^8 CFU/mL were harvested by centrifugation at 6000 × g for 5 min and stained for 15 min with fluoresced inisothio-cyanate (FITC)-Annexin V and propidium iodide (PI) in the dark at room temperature, according to the manufacturer's recommendations (BD Biosciences). The flow cytometer (FACScan, BD Biosciences) equipped with a CellQuest software (BD Biosciences) were used to analyze 1×10^4 cells. Cells were sorted into living, necrotic, early apoptotic,