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Antibacterial effects of cinnamon (*Cinnamomum zeylanicum*) bark essential oil on *Porphyromonas gingivalis*

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ABSTRACT

The objective of this study was to investigate the antibacterial effects of cinnamon (*Cinnamomum zeylanicum*) bark essential oil (CBEO) and its principal constituent cinnamaldehyde against *Porphyromonas gingivalis* and to elucidate the antibacterial mechanism. GC-MS analysis showed that cinnamaldehyde was the major constituent in CBEO (57.97%). The minimum inhibition concentrations (MICs) of CBEO and cinnamaldehyde were 6.25 μg/mL and 2.5 μM for *P. gingivalis*, respectively. Nucleic acid and protein leakage was observed with increasing concentrations of CBEO and cinnamaldehyde. Additionally, propidium iodide uptake assays revealed CBEO and cinnamaldehyde at 1 × MIC impaired *P. gingivalis* membrane integrity by enhancing cell permeability. Morphological changes in *P. gingivalis* cells were observed by scanning electron microscopy, which indicated cell membrane destruction. To further determine the *anti*-biofilm effect, relative biofilm formation and established biofilms were examined, which demonstrated that both CBEO and cinnamaldehyde at sub-MIC levels inhibited *P. gingivalis* biofilm formation by 74.5% and 67.3% separately, but only CBEO slightly decreased established biofilms by 33.5% at 4 × MIC. These results suggest the potential of CBEO as a natural antimicrobial agent against periodontal disease. Furthermore, cinnamaldehyde was confirmed to be the antibacterial substance of CBEO with inhibitory action against *P. gingivalis*.

1. Introduction

Infection and inflammation in the oral cavity range from mild and reversible gingivitis to aggressive periodontitis. Periodontitis is associated with an exaggerated immune response that leads to progressive destruction of the periodontal ligament and alveolar bone [1]. Over the last decade, evidence has accumulated to suggest that periodontal disease represents a risk factor for systemic complications such as cardiovascular disease, type 2 diabetes, pneumonia, and preterm low birth weight [2].

Porphyromonas gingivalis (*P. gingivalis*), a Gram-negative anaerobic bacterium that is one of the key causative pathogens in chronic periodontitis, is an aggressive periodontal pathogen that persists in mixed-species plaque biofilms on tooth surfaces. *P. gingivalis* expresses a variety of virulence factors to cripple the immune system and causes tissue destruction [3]. Oral pathogenic bacteria comprise multiple bacterial species and trigger common oral diseases such as caries, gingivitis, and periodontitis [4]. Another major role of *P. gingivalis* is to mediate biofilm formation by attaching to saliva-coated surfaces and gingival epithelial cells with other pathogenic bacteria [5]. This co-adhesion event leads to the development of *P. gingivalis* biofilms, which provokes periodontal inflammatory responses and results in the release of high amounts of cytokines by lymphocytes, macrophages, and other cell lineages [6]. Due to the toxicological effect of synthetic pharmaceutical products, alternative prevention and treatment options for antibacterial agents are to avoid side effects of antibiotics with increased antibacterial resistance of bacteria. Hence, natural herbs and drugs have become a subject of importance with their unique attributes.

Some herbs have been reported to be useful in the prevention, treatment and maintenance of periodontal and other oral diseases [7]. As a traditional aromatic herbal medicine, *Cinnamomum cassia* Presl, which belongs to the *Lauraceae* family, has long been associated with anti-inflammatory and antimicrobial properties with many applications in indigenous medicines [8]. *Cinnamomum* bark essential oil (CBEO) typically contains a very high concentration of cinnamaldehyde and a small concentration of eugenol, among many other aromatic compounds. CBEO and cinnamaldehyde have been studied for their antibacterial, antifungal, anti-inflammatory and anticancer activities [9–11]. However, their effects on oral pathogenic bacteria have rarely been examined in detail.

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In this study, we aimed to investigate the antibacterial impact of CBEO and evaluate whether its major constituent, cinnamaldehyde, is responsible for its antibacterial effects against pathogenic bacteria, as exemplified by *P. gingivalis*. This study will provide fundamental understanding of the mode of antibacterial action and help to determine how to exert beneficial effects of CBEO and cinnamaldehyde in antibacterial applications.

2. Materials and methods

2.1. Microorganisms and chemicals

Gram-negative *P. gingivalis* strain (*Porphyromonas gingivalis* ATCC33177) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Tryptic Soy Broth (TSB) supplemented with hemin (5 μg/mL) and vitamin K_1 (1 μg/mL) at 37 °C. The strain was cultured in TSB at 37 °C for 24 h under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). All other chemicals used in the study were of analytical grade.

2.2. Preparation of CBEO

The Cinnamon Bark Essential Oil 708922 was commercially available from Kerry Ingredients and Flavours (CLARK, NJ, US). It was extracted by steam distillation. The specimens are deposited in Shanghai Institute of Technology (Shanghai, China). The stock CBEO was dissolved in Dimethyl sulfoxide (DMSO) (1%) to a concentration of 10 mg/mL and added to sterile TSB medium at a final concentration of 500 μg/mL. Serial 2-fold dilutions were performed to obtain serial CBEO concentrations ranging from 2 to 500 μg/mL for the evaluation.

2.3. GC-MS analysis

GC-MS analysis of CBEO was performed on an Agilent 7890A GC apparatus with a 5975C mass spectrometer detector (Agilent, Santa Clara, CA, United States). A DB-5MS capillary column (30 m × 0.25 mm, 0.25-μm film thickness) was used. The operating conditions were as follows: carrier gas: helium, at a flow rate of 1 mL/min; split ratio 1:50; injector and detector temperatures: 250 °C; sample size: 1 mL of CBEO, manual injection; oven temperature program: 60 °C as an initial temperature, 1 min isothermal raised to 160 °C at a rate of 2 °C/min, then isothermal at 295 °C for 30 min; ion source temperature: 230 °C; energy ionization: 70 eV; and electron ionization spectra with a mass scan range of m/z 29–450. The constituents of CBEO were determined by using the NIST 11.LIB mass spectrometry database of GC-MS (https://www.nist.gov/srd). The relative content of individual compounds was analyzed by the percentage of their peak area to the total oil peak area.

2.4. Minimum inhibitory concentration (MIC) assay

CBEO and cinnamaldehyde susceptibility was estimated by 2-fold serial dilution method with slight modification. Serial 2-fold dilutions of CBEO or cinnamaldehyde were added to the wells of a 96-well round-bottomed microtiter plate, then *P. gingivalis* suspensions with a final concentration of approximately 2×10^6 colony-forming units (CFU)/mL were added. As a control, TSB without bacteria was used. Plates were incubated at 37 °C for 60 h. Untreated bacteria was used as the negative control, and tinidazole was added as the positive control. The MIC was determined as the lowest concentration of CBEO or cinnamaldehyde that inhibited visible growth of *P. gingivalis*. Each experiment was performed in triplicates.

2.5. Determination of cell membrane integrity

2.5.1. DNA and RNA leakage through the bacterial membrane

The integrity of the bacterial cell membrane can be monitored by the release of nucleic acids from the cell. Bacteria were incubated anaerobically at 37 °C for 24 h. Bacteria in logarithmic growth phase (concentration 1×10^8 CFU/mL) were treated with CBEO or cinnamaldehyde at 1 × MIC, 2 × MIC, and 4 × MIC. Phosphate-buffered saline (PBS) was used as a control. The samples were then incubated at 37 °C for 0, 1, 2, and 4 h. Bacteria were then centrifuged at 6000 rpm for 10 min at 4 °C. To determine the amounts of DNA and RNA released from the cytoplasm, the supernatant was used to measure the optical density at 260 nm (OD_{260}). Each experiment was performed in triplicates.

2.5.2. Protein leakage through the bacterial membrane

Cell integrity was also examined by determining the release of proteins into the supernatant. The concentrations of proteins in supernatants were determined by the Bradford method [12]. Bacteria in logarithmic growth phase (concentration of 1×10^8 CFU/mL) were treated with CBEO or cinnamaldehyde at 1 × MIC, 2 × MIC, and 4 × MIC. PBS was used as a control. All samples were then incubated at 37 °C for 0, 1, 2, and 4 h. Bacteria were then separated by centrifugation at 2500 rpm for 5 min at 4 °C. To determine the concentrations of proteins released from the cytoplasm, the supernatant was used to measure OD_{595}. Each experiment was performed in triplicates.

2.5.3. Propidium iodide (PI) uptake assay

PI, a fluorochrome, intercalates with DNA, giving off increased fluorescence. Extracellular PI can enter the bacterial cell membrane only when it has been permeabilized. Fluorescence microscopy was used to observe the uptake of extracellular PI by permeabilized *P. gingivalis* cells following exposure to CBEO or cinnamaldehyde. *P. gingivalis* cells were grown to the mid-logarithmic phase, harvested, washed, and adjusted to a final concentration of 1×10^7 CFU/mL in PBS. Untreated bacteria were used as the control. The cells were incubated at 37 °C with CBEO at 1 × MIC, except for the PBS control. After treatment, the cells were washed with PBS and incubated with PI (1.3 μg/mL) at 37 °C for 10–15 min in the dark. PI fluorescence was then measured at excitation and emission of 535 nm and 615 nm, respectively, through an inverted fluorescence microscope (Nikon Ti, Tokyo, Japan).

2.6. Scanning electron microscope (SEM) observation

SEM was used to observe morphological changes of *P. gingivalis*. *P. gingivalis* cells in logarithmic growth phase (concentration of 1×10^7 CFU/mL) were treated with CBEO or cinnamaldehyde at 1 × MIC, except for the PBS control. Bacteria were incubated at 37 °C for 2 h, centrifuged at 5000 rpm for 5 min at 4 °C, fixed with 2.5% (v/v) glutaraldehyde for 2 h at 4 °C, and washed with 0.1 M PBS (pH 7.2) three times. After centrifugation, the cells were fixed with 1% osmic acid for 1.5 h at 4 °C and washed with 0.1 M PBS (pH 7.2). The cells were then dehydrated in a graded series of ethanol (30%, 40%, 50%, 70%, 90%, 100%). Finally, the samples were sputter-coated with gold under vacuum, followed by microscopic examination using an SEM (S-3400, Hitachi, Tokyo, Japan).

2.7. Evaluation of biofilms

2.7.1. Effect of CBEO on biofilm formation

Biofilm formation was examined using the crystal violet staining method with minor modification [13]. Bacteria in logarithmic growth phase (concentration of 1×10^6 CFU/mL) were inoculated into 96-well flat-bottom microplates, treated with CBEO or cinnamaldehyde at 2/3 × MIC, 1/2 × MIC, and 1/4 × MIC, and cultured anaerobically at 37 °C for 3 d. Untreated bacteria were used as the control. The culture