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Volatile components and antibacterial effects of pine needle (*Pinus densiflora* S. and Z.) extracts

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Abstract

The antibacterial effects of the volatile components extracted from *Pinus densiflora* S. and Z., by simultaneous steam distillation and solvent extraction (SDE), were examined on six foodborne bacteria using Bioscreen C (a computer-controlled shake–incubator–reader). The SDE extracts of *P. densiflora* obtained after 1.5 or 2.0 h at pH 3.6 exhibited a strong growth inhibitory effect on *Escherichia coli* O157:H7 and their overall antibacterial activities against the various bacteria tested tended to increase with increased extraction time and with a lower extraction pH. The major volatile components of the SDE extracts obtained at pH 3.6 and 1.5 h, as determined by gas chromatography, were α-ocimene (29.3%), sabinene (10.9%), β-myrcene (9.6%), β-caryophyllene (8.0%), β-cadinene (7.3%), α-terpinolene (4.9%), 2-hexanal (4.5%), and β-pinene (4.3%). The addition of 8% or 10% (v/v) of the SDE extracts to culture broth completely inhibited the growths of *Bacillus cereus*, *Salmonella* Typhimurium, and *Staphylococcus aureus*. The intracellular adenosine triphosphate (ATP) concentration of *S.* Typhimurium treated with *P. densiflora* extract reduced to 0.165 μM from 0.595 μM, whereas the ATP concentration in culture supernatants was increased to 0.469 μM form 0.065 μM.

Keywords: *Pinus densiflora*; Antibacterial activity; Volatile components; SDE; Foodborne bacteria

1. Introduction

Since materials added to prolong shelf-life should not remain in the food, the use of volatile antibacterial materials as food preservatives and as a means of the preventing micro-organism development has become the subject of study (Kim and Shin, 2003).

Pinus densiflora S. and Z. belongs to the Pinaceae family. It is an evergreen needle-leafed tree indigenous to East Asia, and has bitter tasting leaves, which are gathered between spring and autumn (Korea Food & Drug Administration, 1997). Its leaves contain an essential oil (0.3–1.3%), which contains α-pinene, β-pinene, camphene, phellandrene, limonene, borneol (6.8%), and bornyl acetate (3.8%) (Im, 1998). The fresh needles untreated as a folk medicine. Apparently, the medical affects of the needles are strongest in the winter, and they said to expel pathogenic wind, remove dampness, and to stop bleeding (Korea Food & Drug Administration, 1997). Various parts of this tree, i.e., needles, cones, cortices, and pollen, have been widely used for health promoting purposes as a folk medicine or as a food (Dongeuhak Institute, 1994).

Recently, the composition and the various biological functions of the volatile fraction of *P. densiflora* were described: growth-inhibiting effects of the constituents of leaves on human intestinal bacteria (Jeon et al., 2001), components of the root or needles (Watanabe et al., 1991; Jung et al., 2001), volatile constituents and the extraction solvent used (Cho et al., 1999a), essential oils from needles and twigs (Koukos et al., 2000), and the antimicrobial effects of ethanol extracts on lactic acid bacteria (Lim et al., 2001).

Given the lack of research information in this field, we examined the antibacterial effects of the essential oil of the leaves of *P. densiflora* extracted under different

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conditions. In addition, the concentration of ATP in cells and in culture media treated with the simultaneous steam distillation and solvent extraction (SDE) extract of *P. densiflora* were determined and related to observed bactericidal effects of SDE on several bacteria. Specifically, efforts were made to identify and quantity the volatiles present, and to assess their antibacterial effects with a view to increasing the shelf lives of limited-storage instant foods.

2. Materials and methods

2.1. Micro-organisms and cultures

Six different foodborne bacteria were used. *Bacillus cereus* (ATCC 11778) and *Salmonella* Typhimurium (ATCC 14028) strains were grown at 30°C in nutrient broth or nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, England). *E. coli* O157:H7 (ATCC 43894) and *Staphylococcus aureus* (ATCC 25923) strains were grown at 37°C, and *Listeria monocytogenes* (ATCC 19111) at 30°C, in tryptic soy broth or tryptic soy agar (Difco, Detroit, Michigan, USA). *Vibrio parahaemolyticus* (ATCC 33844) strain was grown at 37°C in tryptic soy broth or tryptic soy agar supplemented with 3% (w/v) NaCl. All bacteria were grown for 24 h in sterilized broth medium. An aliquot of each culture (0.1 ml) was then transferred to a 9.9 ml new broth medium and culture for 18 h.

2.2. Extraction of volatile components

The leaves of *P. densiflora* were collected from the Jeonju Arboretum (Jeonju, Korea) in September 2001, washed and stored at −20°C. Volatile leaf extracts were obtained by SDE using the 'improved' Likens–Nickerson apparatus (Parliament, 1997). After circulating 50 ml of the extracting solvent (redistilled diethyl ether) through the apparatus at 36°C, 100 g of the leaves were ground in a Waring blender (Waring, New Hartford, Connecticut, USA) and mixed with 1000 ml of distilled water in a round-bottomed flask. The SDE times used were 0.5, 1.0, 1.5, and 2.0 h at pH 3.6 (the control pH), then the pH was increased to 4.6, 5.6, or 6.6 and these mixtures and the control pH mix were extracted for 1.5 h. Anhydrous sodium sulfate (~15 g) was then added to remove water. The ether mixture was then cooled to −20°C for 12 h, and evaporated to 1 ml using a nitrogen flow. Ten microliters of 1-pentanol (*n*-amyl alcohol) was then added to the extracts as the internal gas chromatography (GC) standard. The extracts obtained were tested for antibacterial activity and their volatile components were analysed.

2.3. Analysis and identification of volatile constituents

GC (GC-17A V3) and GC-MS (QP5050, Shimadzu Co., Kyoto, Japan) using Supelcowax 10 fused silica capillary column (60 m × 0.25 mm; 0.25 μm film thickness) were employed for the analysis. Helium was used as the carrier gas at a flow rate of 1 ml/min. The GC oven temperature was maintained at 50°C for 5 min, then increased to 230°C at the rate of 2°C/min and held for 10 min.The temperature of the injector was 250°C and that of the FID detector was 260°C. The GC split ratio was 1:60 and 0.5 μl of the extract was injected per GC run. The mass spectra ranged from *m/e* 28 to 400 and the ionizing voltage used was 70 eV. Extracts components were identified by comparing the spectra obtained with a mass spectrum library (Wiley NBS 139), and by comparing the GC retention indices against known standards.

2.4. Antibacterial activities of SDE extracts

The antibacterial activities of the SDE extracts were determined using a Bioscreen C Microbiology Reader (Labsystem, Helsinki, Finland). Bioscreen C is a computer-controlled shake–incubator–reader (Flower, 2001)

The extracts of *P. densiflora* leaves (4.38 μl from 100 g of fresh leaves) were obtained by completely evaporating off the diethyl ether with nitrogen. They were then resuspended in 0.5 ml water containing 10% Tween 80 (v/v) (Showa Chemical Co. Ltd., Tokyo, Japan). Extracts were sterilized by passing them through a membrane filter (0.2 μm) (Naigre et al., 1996; Kim and Shin, 2004). To determine antibacterial activity, 0.06 ml of bacteria (10^5–10^6 cfu/ml) were incubated in 5.82 ml media and 0.12 ml of either sterilized extract or the control containing 10% Tween 80. In addition, antibacterial experiments were conducted using different concentrations (2, 4, 8, or 10% (v/v) in 10% Tween 80 (v/v)) of the SDE extracts. Aliquots of these cultures (0.3 ml) were dispensed into Bioscreen C wells and incubated as described in the *Material and methods* section. The optical densities (600 nm) of the media were measured every 12 h for 3 days using Tween 80 as control.

2.5. Measurement of adenosine triphosphate (ATP)

Luciferase–luciferin (Sigma Chemical Co., Missouri, USA) stock was prepared by dissolving the luciferase-luciferin reagent in 5.0 ml of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) and stored at –20°C. Just before use, 500 μl of stock solution was mixed with 1.0 ml of HEPES. ATP was mixed with luciferase–luciferin stock and used as a standard.