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Essential Oil of Citrus Fruit Waste Attenuates LPS-induced Nitric Oxide Production and Inhibits the Growth of Skin Pathogens

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ABSTRACT

Citrus is an economically important fruit for Jeju Island, but its peel also is one of the major sources of agricultural waste. Due to its fermentability, this waste causes many economic and environmental problems. Therefore it is worthwhile to investigate ways to make use of this citrus waste generated by the juice industry. The aim of this study was to examine the chemical composition of the essential oil obtained from citrus peel waste by hydrodistillation and to subsequently test the efficacy of this essential oil against a diverse range of microorganisms comprising drug-susceptible and drug-resistant skin pathogens. The chemical composition of the essential oil was analysed by GC-MS, revealing that a total of 6 compounds comprised 94.5% of the total oil. The oil contained limonene (80.51%), γ-terpinene (6.80%), cymene (4.02%), β-myrcene (1.59%), α-pinene (1.02%) and α-terpinolene (0.56%). The oil exhibited promising antibacterial effects against drug-susceptible and drug-resistant skin pathogens as evidenced by the diameter of zones of inhibition and MIC values. The effects of citrus peel waste essential oil (CPWE) on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages were also examined in this study. The addition of CPWE (100 μg mL^{-1}) to the medium with LPS largely inhibited the production of NO by 42.5%. The number of viable activated macrophages was not altered by CPWE, as determined by MTT assays, indicating that the inhibition of NO synthesis by CPWE was not simply due to cytotoxic effects. In addition CPWE (6.125 to 100 μg mL^{-1}) inhibited NO production in a dose-dependent manner. Based on these results, we suggest the possibility that CPWE may be considered as an antibacterial and anti-inflammatory agent.

Key Word: Chemical composition; Citrus peel waste; Essential oil; Inflammation; Nitric oxide

INTRODUCTION

Hallabong (*Citrus unshiu*) is a popular citrus fruit in Korea. In 2008, 64,000 tonnes of *C. unshiu* were produced and used as a fresh food as well as raw material for juice and canned food. However the *C. unshiu* peel is one of the major sources of agricultural waste, with annual yields of more than 50,000 tonnes in Jeju Island alone from juice factories and households. Part of the peel is employed for a variety of uses, such as fodder at fisheries, activated carbon, raw materials for traditional paper, cosmetic materials and bio-ethanol (Song *et al*., 2002; Kim *et al*., 2007; Sharma *et al*., 2007b; Kim *et al*., 2008a). At the moment, however this waste is sent to waste-disposal locations, which involves substantial costs for handling and transport. Moreover places for waste disposal are becoming increasingly difficult to find. Additionally the prices of recycled materials often are not high enough to cover operating costs (Tripodo *et al*., 2004). Collectively these various disadvantages mean that most of the waste is incinerated in disposal yards and dumped into the ocean. Construction of new waste disposal yards is also very difficult in Korea and due to the London Dumping Convention, which will go into effect in the year 2012 it will also become impossible to dump the waste into the ocean. Therefore research into the utilisation of agricultural waste, including the *C. unshiu* peel, is important and urgently required in order to accomplish waste reduction.

Essential oils obtained from plants have a number of potential applications, including use as food additives, preservatives against spoilage and pharmaceuticals, due to their notable antimicrobial, antioxidant and anti-inflammatory properties (Baik *et al*., 2008; Kim *et al*., 2008b, c, & d; Oh *et al*., 2009; Imelouane *et al*., 2009a & b; Yoon *et al*., 2009a, b, & c). These natural properties are in line with the increasing demand from consumers to limit the use of synthetic additives, as these artificial chemicals have been established as potential health hazards in some

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instances, due to toxic impurities deriving from the synthetic pathways. Essential oils are much more acceptable to the end consumers than are synthetic substances and they do not cause bacterial resistance mainly, because they are comprised of a wide spectrum of compounds (Maggi *et al*., 2009).

The aim of this research was to elucidate the biological activities of citrus waste essential oil (CPWE), which would facilitate the conversion of this waste into high value-added products. This would then allow it to be recycled as a component of cosmetic materials. More specifically, the present study focused on whether CPWE has antibacterial and anti-inflammatory effects against skin pathogens.

MATERIALS AND METHODS

Plant material and oil extraction. The peel waste of *C. unshiu* after juice extraction (peel & attached membranes) was obtained from a local food processing company (Ilhae Corporation, Jeju, Korea), frozen and stored at -20°C until use. The frozen-dried citrus peel waste was subjected to water distillation for 6 h using a Clevenger-type apparatus. The obtained essential oil was dried over anhydrous sodium sulphate and after it was filtered, it was stored in a sealed vial at 4°C until tested. The essential oil yield was about 1.0% (v/w).

GC-MS analysis. An analysis of the principal components in the most active essential oil was carried out using an Agilent 6890 gas chromotragraph (GC) connected to an Agilent 5975 mass spectrometer (MS) (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.). The GC was equipped with a DB1-HT column with a 30 m × 0.32 mm × 0.1 μm film thickness. The oven temperature was programmed to increase from 40 to 100°C at a rate of 2°C per min, from 100 to 230°C at a rate of 5°C per min and then held at 230°C for 5 min (71 min analysis time). The injector and detector temperatures were 240 and 280°C, respectively. The flow rate of the carrier gas (He) was 1.5 mL per min and the split ratio was 1:10. For the injection (splitless), 10 μL of essential oil was diluted in 500 μL of CH_2Cl_2 and 1 μL of this diluted solution was injected. Identification of the compounds was achieved by comparison of their mass spectra with those of the Wiley libraries. The GC-MS retention indices were also calculated using a homologous series of *n*-alkanes C_6-C_{31}.

Microorganisms and medium. In all 7 bacterial pathogens, including drug-susceptible skin pathogenic bacteria such as *Propionobacterium acnes* CCARM 0081**,** *Staphylococcus epidermidis* CCARM 3709 and *Candida albicans* KCCM 11282, and drug-resistant skin pathogenic bacteria, namely, *S. epidermidis* CCARM 3710, *S. epidermidis* CCARM 3711, *P. acnes* CCARM 9009 and *P. acnes* CCARM 9010, were obtained from the Culture Collection of Antimicrobial Resistant Microbes (CCARM, Seoul, Korea) and the Korean Collection Center of Microorganisms (KCCM, Seoul, Korea). *Propionibacterium* strains were cultured at 37°C for 48 h in GAM broth (Nissui Pharmaceutical Co., Tokyo, Japan) under anaerobic conditions before the assay. *S. epidermidis* was cultured at 37°C for 24 h in Trypticase soy agar (TSA). *C. albicans* was grown at 30°C for 24 h.

Disc diffusion assay. The inhibitory effects on test bacteria were determined by the disc diffusion method. The cell suspension was adjusted with sterile saline solution to obtain turbidity comparable to that of a 0.5 McFarland standard (1.5×10^8 cells mL^{-1}). Sterile 7-mm paper discs (Becton Dickson, Sparks, Md., U.S.A.) were aseptically placed on the agar, 4 discs per plate. The CPWE treatments were aseptically pipetted onto the discs, 20 μL per disc. After a period of 30 min, during which the oils were allowed to absorb and diffuse, the plates were inverted and incubated at 37°C for 48 h under anaerobic conditions for *Propionibacterium* sp. (BBL GasPak System). Other pathogens were incubated at 37°C for 24 h under aerobic conditions. The diameters of zones of inhibition were measured in millimetres and recorded.

Determination of the minimum inhibitory concentration. The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of CPWE against the test organisms as recommended by the National Committee for Clinical Laboratory Standards. A stock solution of essential oil was prepared in 10% dimethyl sulphoxide (DMSO) and serially diluted to 0.13 to 20 μL per mL. The 96-well plates were prepared by dispensing 95 μL of culture broth, 100 μL of essential oil and 5 μL of the inoculants into each well. A positive control (containing the inoculum but no essential oil) and a negative control (containing essential oil but no inoculum) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at the proper temperature and incubation times. The MIC was defined as the lowest concentration of the compound that inhibited microorganism growth.

Cell culture. The mouse macrophage-like cell line, RAW 264.7, was purchased from the Korean Cell Line Bank (KCLB; Seoul, Korea). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS; GIBCO Inc., NY, USA) and 100 U per mL of penicillin/streptomycin. The cells were incubated at 37°C in a humidified, 5% CO_2 atmosphere. To stimulate the cells, the medium was regularly replaced with fresh DMEM and LPS was added in the presence or absence of various concentrations of CPWE.

MTT assay for cell viability. Cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells were cultured in 96-well plates for 18 h, followed by treatment with LPS (1 μg mL^{-1}) in the presence of various concentrations of CPWE. After a 24 h incubation, MTT was added to the medium for 4 h. Finally, the supernatant was removed and the formazan crystals were dissolved in DMSO. The absorbance was measured at 540 nm. The percentage of cells showing cytotoxicity was determined relative to the control group.

Measurement of the nitric oxide concentration. Nitric Oxide (NO) production was assayed by measuring nitrite levels in the supernatants of cultured RAW 264.7 cells. The cells were seeded at 2.5×10^5 cells per mL in 24-well culture plates and cultured for 18 h. The cells were stimulated with LPS (1 μg mL^{-1}) and various concentrations of CPWE for 24 h and briefly centrifuged. The supernatant was mixed with an equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride & 2.5% phosphoric acid) and incubated at room temperature for 10 min. The concentrations of nitrite were measured by reading the absorbance at 540 nm. Nitrite production was determined by comparing the optical density with the standard curve obtained with sodium nitrite. All experiments were performed in triplicate.

Statistical analysis. All data were obtained in triplicate and are represented as the mean ± standard error (SE). Significant differences between treatments were determined by the Student's *t* test in a one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Hydrodistillation of CPWE yields about 1.0% (w/w) essential oil according to wet weight. A total of 6 compounds (Table I) were identified in CPWE, representing 94.5% of the total essential oil. The main constituents in CPWE were quantified to be limonene (80.51%), γ-terpinene (6.80%), cymene (4.02%), β-myrcene (1.59%), α-pinene (1.02%) and α-terpinolene (0.56%) according to the results obtained from GC-MS and GC-FID analyses.

Data from the evaluations of the antibacterial activity of CPWE against different microorganisms are tabulated in Table II. The oil exhibited antibacterial activity at 20 μL (about 1.6 mg) against the test microorganism with zone sizes ranging from 9 to 11 mm. The activity of CPWE against drug-susceptible and resistant Gram-positive skin pathogens was less than that of standard antibiotic erythromycin. The most susceptible bacteria were *C. albicans* KCCM 11282 and *S. epidermidis* CCARM 3709 with each having 11 mm zones of inhibition and MIC value of 5 μg per mL. The antibacterial activity present in the CPWE can be due to the presence of limonene, γ-terpinene, cymene, β-myrcene, α-pinene and α-terpinolene. It has been reported previously that the monoterpene composition of the essential oil is responsible for the antibacterial activity. These compounds destroy the cellular integrity of microbial cells by inhibiting the respiration process (Helander *et al*., 1998; Pavithra *et al*., 2009). However the antibacterial activity of the essential oil may also be correlated to a synergistic effect of all the chemical components present in the oil (Dorman & Deans, 2000).

NO is an endogenous free radical species that is synthesised from L-arginine by nitric oxide synthase (NOS) in various animal cells and tissues. Small amounts of NO are important regulators of physical homeostasis, whereas

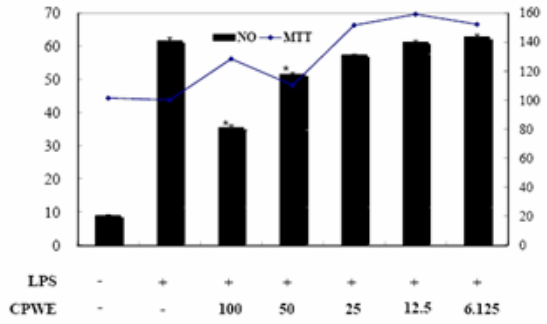
Table I. Chemical composition (%) of CPWE

Retention time (min)	RI	Constituents	Peak area (%)	Identification (%)
5.282	917.8	α-pinene	1.02	97
7.767	977.9	β-myrcene	1.59	91
8.868	1003.3	cymene	4.02	97
9.595	1016.0	limonene	80.51	97
10.918	1039.2	γ-terpinene	6.80	96
12.488	1066.7	α-terpinolene	0.56	98
			(94.5)	

The GC-MS retention indices were calculated using a homologous series of *n*-alkanes C_6-C_{31}. Components were characterised based on library searches and only those components showing matches exceeding 90% were selected. RI, retention indices; RT, retention time

Fig. 1. Effect of CPWE on nitric oxide production in LPS-stimulated RAW264.7 cells

The cells were stimulated with 1 μL per mL of LPS only or with LPS plus various concentrations of CPWE (100, 50, 25, 12.5, 6.125 μg mL^{-1}) for 24 hr. Nitric oxide production was determined by the Griess reagent method. Cell viability was determined from the 24 h culture of cells stimulated with LPS (1 μL mL^{-1}) in the presence of CPWE. The data represent the mean ± SD of triplicate experiments. **P<0.05,**P<0.01* versus LPS alone.



large amounts of NO have been closely correlated with the pathophysiology of a variety of diseases and inflammation (Tripathi, 2007; Sharma *et al*., 2007a). After exposure to inducers, such as lipopolysaccharide (LPS) from Gram-negative bacteria, inducible NOS (iNOS) can be induced in various cells such as macrophages, smooth muscle cells and hepatocytes, to trigger tissue damage, inflammation sepsis and stroke (Murakami & Ohigashi, 2007; Murakami, 2009; Pytel & Alexander, 2009). Thus, measuring NO production may be one useful method for assessing the anti-inflammatory effects of essential oils. As for the inhibitory effects of CPWE on NO production, data in Fig. 1 show that CPWE had a strong inhibitory effect. CPWE (6.125 to 100 μg mL^{-1}) inhibited NO production in a dose-dependent manner. In addition the MTT assay revealed that the inhibition of NO synthesis by CPWE was not due simply to cytotoxic effects.

In summary, the manipulation of food processing waste is now becoming a very serious environmental issue. Therefore we sought to make use of *C. unshiu* waste to high value-added materials such as essential oil. In this study we elucidated that CPWE showed excellent antibacterial

Table II. Antimicrobial activity of the CPWE

Strains	Drug-resistance patterns of skin pathogens (MIC; μg mL^{-1})	Disc diffusion assay (mm)	MIC values	
			Erythromycin (μg mL^{-1})	CPWE (μL mL^{-1})
S. epidermidis CCARM 3709	Susceptible	11	0.13	5
S. epidermidis CCARM 3710	Erythromycin (>32), Clindamycin (>16), Chloramphenicol (64)	10	> 100	>20
S. epidermidis CCARM 3711	Tetracycline (>32)	9	0.13	>20
P. acnes CCARM 0081	Susceptible	10	0.13	>20
P. acnes CCARM 9009	Clindamycin (64)	10	4.00	>20
P. acnes CCARM 9010	Clindamycin (64)	10	> 100	>20
C. albicans KCCM 11282	-	11	-	5

For determination of the MIC, the microdilution broth susceptibility assay was used. The MIC was defined as the lowest concentration of the compound that inhibited microorganism growth. Erythromycin was used as a control

activities against acne-causing skin pathogens. In addition CPWE reduced the NO production induced by LPS in RAW264.3 cells, indicating anti-inflammatory properties. Based on these results, we suggest that CPWE could be considered as an attractive antibacterial and anti-inflammatory material for topical application.

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