Antibacterial effect of citrus press-cakes dried by high speed and far-infrared radiation drying methods

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Abstract

In this study, the antibacterial effect was evaluated to determine the benefits of high speed drying (HSD) and far-infrared radiation drying (FIR) compared to the freeze drying (FD) method. Citrus press-cakes (CPCs) are released as a by-product in the citrus processing industry. Previous studies have shown that the HSD and FIR drying methods are much more economical for drying time and mass drying than those of FD, even though FD is the most qualified drying method. The disk diffusion assay was conducted, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined with methanol extracts of the dried CPCs against 11 fish and five food-related pathogenic bacteria. The disk diffusion results indicated that the CPCs dried by HSD, FIR, and FD prevented growth of all tested bacteria almost identically. The MIC and MBC results showed a range from 0.5-8.0 mg/mL and 1.0-16.0 mg/mL respectively. Scanning electron microscopy indicated that the extracts changed the morphology of the bacteria cell wall, leading to destruction. These results suggest that CPCs dried by HSD and FIR showed strong antibacterial activity against pathogenic bacteria and are more useful drying methods than that of the classic FD method in CPCs utilization.

Key Words: Citrus press-cakes, fish and food-related pathogenic bacteria, high speed drying, far-infrared radiation drying, antibacterial agent

Introduction

The prevalence of antibiotic resistance is a continual problem due to the evolution of a potent defense mechanism against antibiotics [1]. One of the main concerns is spread of various diseases by fish and food-related pathogenic bacteria, which can affect both the aquaculture and agriculture sectors leading to economic losses. Therefore, it is necessary to exploit and develop a novel inhibitory agent against those bacteria. An increasing number of studies have considered plant extracts for immune enhancement and antimicroorganism activities [2,3]. Essential oils as well as ethanol and methanol extracts of plants possess antimicrobial activity [4,5], which can be used as antibacterial agents.

The appearance and development of fish and food-related diseases are the result of the interaction among pathogens, hosts, and the environment. Prophylactic chemo-therapeutants have been commonly used in the fish and food industries for controlling opportunistic bacteria. Hence, nonchemotherapeutic methods for microbial control, such as the use of vaccine, probiotics, immune-stimulants, and natural therapeutics from plants have been considered [6,7].

Finding new naturally active components from plants or plant-based agricultural products has been of interest to many researchers. Hence, a great deal of attraction has been paid to the antibacterial activity of citrus as a potential and promising source of pharmaceutical agents [8-11]. Furthermore, citrus fruit peel has been used in traditional Asian medicines for centuries to treat indigestion and to improve bronchial and asthmatic conditions [12]. Ghasemi *et al.* [13] and Johann *et al.* [14] have shown that citrus varieties are considered and containing a rich source of secondary metabolites with the ability to produce a broad spectrum of biological activities. Yao *et al.* [15] reported that seven individual polymethoxylated flavones can be obtained from *Citrus sinensis* peel. Moreover, different parts of *Citrus reticulate* (peel, pith, endocarp, pulp, and seeds) have been used

to identify five kinds of flavonoids, including hesperidin, naringin, didymin, tangeretin and nobiletin by high performance liquid chromatography-photodiode array [16]. In addition, shoots of germinated citron seeds (*Citrus junos* Sieb. ex Tanaka) have significant antioxidant and antilipidemic activities due to their high content of flavonoids in methanol extracts [17]. Some significant components are abundantly available in citrus peel, including ascorbic acid, phenolic acids, polyphenols, and dietary fiber [18]. Constituents with antioxidant, antiviral, antibacterial, antifungal, and anticancer activities have also been reported in citrus [19-21]. Numerous studies have described the inhibitory activities of citrus against human pathogens, fungi, and yeasts. However, only a few studies have reported on the antibacterial effects against fish [22] and food pathogens [23].

Our previous studies have revealed that citrus by-products (CBPs) from citrus peel, which are comprised of flavonoid compounds, might be a useful antimicrobial agent [24,25]. Senevirathne *et al*. [26] have shown that enzymatic digests from CBPs dried by high speed drying (HSD) or freeze drying (FD) are responsible for the antioxidant properties in the *in vitro* assays conducted. In addition, citrus essential oils, including citrullene and limonene from CBPs have antimicrobial effects against a broad range of microorganisms [27]. Therefore, the aim of the present study was to investigate the antibacterial activity of a dried CPC methanol extract by HSD and far-infrared radiation drying (FIR) against pathogenic bacteria by comparing the results with those of FD dried CPC. We used 11 fish and five food-related pathogenic bacteria to evaluate the effectiveness of the HSD and FIR drying systems compared to that of the qualified FD method using maximum inhibitory concentrations (MIC), minimum bactericidal concentration (MBC), and scanning electron microscopy (SEM) techniques in accordance with the antibacterial activity of dried CPCs, and to describe the alternative beneficial value of processing and utilizing CPCs.

Materials and Methods

Material and chemicals

CPCs were obtained from Jeju Provincial Development Co. in Korea. Nutrient agar (NA), nutrient broth (NB), brain heart infusion agar (BHIA) and brain heart infusion (BHI) were purchased from Difco (Sparks, MD, USA). Authentic reference compounds, including narirutin, quercetagetin, sinesetin, 3',4',7,8-tetramethoxyflavone, 5,6,7,3',4',5'-hexamethoxyflavone, and scutelarin tetramethyl ether were purchased from Extrasynthase (Genay, France). Hesperidin and neohesperidin were purchased from Sigma Co. (St. Louis, MO, USA). Nobiletin and tangeritin were purchased from Wako Pure Chemicals (Osaka, Japan), and 3,5,6,7,8,3'4', heptamethoxyflavone was provided by the Pharmacy Department of Tokyo University. All other chemicals used were of analytical grade.

Table 1. Fish and food-related pathogenic bacteria used in this study

Bacteria	Strain	Gram stain	Shape	Temperature
Fish pathogenic bacteria				
Vibrio salmonicida	KCCM 41663	(-)	Rod	30℃
Vibrio vulnificus	KCTC 2662	(-)	Rod	30℃
Vibrio fluvialis	KCTC 2692	(-)	Rod	30℃
Vibrio alginolyticus	KCCM 40513	(-)	Rod	30℃
Vibrio logei	KCTC 2721	(-)	Rod	25℃
Vibrio ichthyoenteri	KCCM 40870	(-)	Rod	26℃
Vibrio anguillarum	KCTC 2711	(-)	Rod	25℃
Vibrio ratiferianus	KCTC 12125	(-)	Rod	25℃
Vibrio parahemolyticus	KCCM 1965	(-)	Rod	30℃
Streptococcus parauberis	KCTC 3651	(+)	Coccui	25℃
Streptococcus iniae	KCTC 3657	(+)	Coccui	25℃
Food-related pathogenic bacteria				
Bacillus subtilis	KCCM 40820	(+)	Rod	30℃
Salmonella thyphimurium	KCCM 4025	(-)	Rod	30℃
Escherichia coli	KCCM 40880	(-)	Rod	37℃
Staphylococcus oureus	KCTC 1916	(+)	Coccui	37℃
Listeria monocytogens	KCCM 40307	(+)	Bacilli	30℃

Appropriate temperatures used to culture each fish and food-related pathogenic bacteria and adjusted standard inoculums to 1×10^6 (CFU)/mL.

Microorganisms and culture

Fish and food-related pathogenic bacteria were kindly provided by the Marine Microbes Laboratory at Jeju National University, Korea. The bacterial strains were cultured at an appropriate temperature as given in Table 1. The optical densities of each working culture were adjusted to 0.1 mg/mL at 625 nm using fresh broth to give standard inoculums of 1×10^6 colony-forming units (cfu) per mL.

HSD or FIR of CPCs

CPCs stored at -50℃ were converted to a dried form by HSD (Okadora, Incheon, Korea) or FIR drying (TOURI-Q, Seoul, Korea) as explained in our previous studies [24,28]. Then, the dried CPCs were pulverized into fine powder using a grinder (MF 10 basic mill, GMBH & Co., Staufen, Germany) and passed through a 300 mm standard testing sieve (no. 50).

Extraction of bioactive compounds from dried CPC

A 20 gram portion of the ground dried CPC powder was mixed with 100% methanol and kept in a shaking incubator at 25℃ for 1 day, and then it was filtered in a vacuum using Whatman no. 1 (Whatman Ltd., Maidenstone, England) filter paper. The methanol was evaporated in a rotary evaporator, and the extracts were dissolved in 1% dimethyl sulfoxide (DMSO).

Disc diffusion assay

The CPC extract was screened for antibacterial activity against 16 bacterial strains using agar diffusion technique [29,30]. The

bacterial strains were grown in nutrient agar (NA) and brain heart infusion agar (BHIA) (Difco Laboratories, Detroit, MI). Sterilized paper disks (Whatman no. 1, 6 mm diameter) containing 50 µL of each extract were applied to the surface of agar plates that were previously seeded by spreading 0.1 mL of culture overnight. Ampicillin and 1% DMSO was used as positive and negative controls, respectively. The plates were incubated overnight at respective temperatures, and the diameter of the clear zone was measured in millimeters. The measurement scale was the following: > 20 mm clear zone was strong inhibitory activity; < 12-20 mm clear zone was moderate/mild inhibitory activity; and < 12 mm was low inhibitory activity.

Determination of MIC

MIC was defined as the minimum concentration of the test extract that was required to limit turbidity (growth) to < 0.05 absorbance units. The MICs of the extracts from the dried CPCs were assessed by a modified microdilution broth method described by Cai and Wu [31]. Briefly, bacteria from overnight cultures were adjusted to 1×10^6 CFU/mL. The extracts were serially diluted with broth to give concentrations of 0.5, 1, 2, 4, 8, and 16 mg/mL. A 100 µL aliquot of the diluted extracts was added to wells containing 100 µL of bacterial suspension. A parallel series of uninoculated broth samples were used as blanks. After 24 h incubation at an appropriate temperature under anaerobic conditions, optical density was measured at 600 nm using a microtiter plate reader (Tecan, Vienna, Austria).

Determination of MBC

The MBC was defined as the lowest concentration of the extract that allowed < 0.1% of the original inoculum treated with the extracts to survive and grow on the surface of the medium used. A method described by Patrick [32] in the ASM Pocket Guide to Clinical Microbiology was slightly modified to determine MBC in this study. Briefly, a 50 µL aliquot of the mixture was withdrawn from the MIC assay wells where no visible turbidity (growth) was observed and spread on freshly prepared BHIA/NA plates. The plates were incubated at an appropriate temperature for 24 h to determine the MBC.

SEM observations

Bacterial cells were suspended in NA or BHI broth and incubated overnight at 37℃ or 30℃ respectively. Both suspensions were divided equally into four tubes accordingly. The extracts from the dried CPCs (8 mg/mL) were added to the tubes and incubated at 30℃ for 4 h. One tube was used as a control for each (no extract). Later, bacterial cells were harvested and prefixed with 2.5% glutaraldehyde solution at 4℃ overnight followed by resuspension in 0.1 M Na-cacodylate buffer. The mixture was dehydrated rapidly in an ethanol series (30, 50, 70, 90, and 100%). Then, bacterial cells were dried with liquid CO2 at "critical point" (Balzers CPD 030) under 95 bars of pressure and gold-covered by cathodic spraying (Edwards S 150 B). Finally, bacterial cell morphology was observed by SEM (JEOL, JSM 6700F, Tokyo, Japan).

Statistical analysis

All experiments were conducted in triplicate (n = 3) and an analysis of variance (using SPSS 11.5 statistical software, Chicago, IL, USA) was used to compare the mean values of each treatment. Significant differences among the means of parameters were determined using Duncan's test. A $P < 0.05$ was considered significant.

Results

Growth inhibitory activity

Eleven fish pathogenic bacteria (nine Gram-negative and two Gram-positive) and five food-related pathogenic bacteria (two Gram-negative and three Gram-positive) were tested for their sensitivity against methanol extracts from CPCs prepared using different drying methods. Antibacterial potency was initially determined by the disc diffusion assay. Table 2 presents the inhibition zone diameters (clear zones around discs) formed by the various extracts against the bacteria. The CPC extracts dried by HSD or FIR showed strong inhibitory activity against fish pathogenic bacteria strains, as > 21 mm clear zones were observed with respect to *Streptococcus iniae, Vibrio ichioenteri*, and *S. parauberis*. In addition, those extracts showed higher inhibitory activities against other fish and food-related bacterial strains, and the activities were almost the same as with the CPC extract dried by FD (inhibition zone, 9-20 mm). The inhibition zones were very prominent when compared with that of ampicillin, which is a commercial antimicrobial agent (positive control). *Vibrio salmonicida* was more susceptible to the extracts and showed the smallest inhibition zones (9-11 mm). However, the methanol extracts from CPCs dried by HSD or FIR showed antibacterial activity in both Gram-negative and Gram-positive bacteria.

Susceptibility test

A quantitative evaluation of the antibacterial activity of the extracts from the dried CPCs was carried out against selected bacteria according to the method described by Cai and Wu [31]. The MICs of the extracts to the fish and food-related pathogenic bacteria are shown in Table 3. The MIC values were 2-8 mg/mL for the fish pathogenic bacteria and both extracts from CPCs dried by HSD or FIR showed strong activities against *S. iniae, V. anguilarum, S. parauberis*, and *V. parahemolyticus*. These

Table 2. Growth inhibition zones (mm) showing antibacterial activity of the dried citrus press-cakes (CPCs) against fish and food-related pathogenic bacteria

Bacteria	Diameter of clear zone (mm)							
	Amp[1]	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Fish pathogenic bacteria								
Vibrio salmonicida	27 ± 2^c	9 ± 0.5^a	10 ± 0.5^{ab}	11 ± 1^b	10 ± 0.5^{ab}	9 ± 0.5^a	9 ± 0.5^a	10 ± 0.5^{ab}
Vibrio vulnificus	25 ± 1.5^e	19 ± 1^d	17 ± 1^c	12 ± 1^{ab}	12 ± 1^{ab}	11 ± 1^a	13 ± 0.5^b	13 ± 0.5^b
Vibrio fluvialis	15 ± 1^c	15 ± 1^c	14 ± 1^{bc}	15 ± 1^c	15 ± 1^c	12 ± 1^a	13 ± 0.5^{ab}	12 ± 0.5^a
Vibrio alginolyticus	-	15 ± 1^{cd}	14 ± 1^{bc}	16 ± 1^d	15 ± 1^{cd}	12 ± 1^a	13 ± 1^{ab}	13 ± 0.5^{ab}
Vibrio logei	-	13 ± 1^c	11 ± 1^{ab}	12 ± 1^{bc}	11 ± 1^{ab}	10 ± 1^a	10 ± 0.5^a	12 ± 1^b
Vibrio ichthyoenteri	24 ± 1^b	22 ± 1.5^{ab}	22 ± 1.5^{ab}	23 ± 1.5^b	22 ± 1.5^{ab}	20 ± 1.5^a	20 ± 1^a	21 ± 1^a
Vibrio anguillarum	24 ± 2^c	17 ± 1^{ab}	16 ± 1^a	19 ± 1^b	17 ± 1^{ab}	15 ± 1^a	16 ± 1^a	16 ± 1^{ab}
Vibrio ratiferianus	25 ± 1.5^d	20 ± 1.5^c	17 ± 1^b	16 ± 1^b	16 ± 1^b	12 ± 1^a	13 ± 1^a	17 ± 1^b
Vibrio parahemolyticus	24 ± 1.5^b	19 ± 1.5^a	19 ± 1^a	18 ± 1^a	18 ± 1^a	18 ± 1^a	19 ± 1^a	20 ± 2^a
Streptococcus parauberis	46 ± 2^d	27 ± 2^c	20 ± 1.5^a	21 ± 1.5^a	21 ± 1.5^a	18 ± 1^a	20 ± 1^a	24 ± 2^b
Streptococcus iniae	49 ± 2^d	24 ± 2^b	19 ± 1^a	29 ± 2^c	28 ± 2^c	24 ± 2^b	24 ± 1^b	19 ± 1^a
Food-related pathogenic bacteria								
Bacillus subtilis	38 ± 0.5^d	14 ± 0.5^b	13 ± 0.5^a	19 ± 0.5^c	18 ± 0.5^{ab}	15 ± 0.5^a	16 ± 0.5^a	16 ± 0.5^a
Salmonella thyphimurium	32 ± 2^d	16 ± 1^b	14 ± 0.5^{ab}	15 ± 0.5^{bc}	15 ± 1^b	15 ± 1^{bc}	13 ± 0.5^a	13 ± 0.5^a
Escherichia coli	23 ± 1.5^b	12 ± 0.5^a	13 ± 0.5^a	12 ± 0.5^a	13 ± 0.5^a	13 ± 0.5^a	13 ± 0.5^a	12 ± 0.5^a
Staphylococcus oureus	47 ± 3^b	11 ± 0.5^a	11 ± 0.5^a	11 ± 0.5^a	11 ± 0.5^a	11 ± 0.5^a	10 ± 0.5^a	11 ± 0.5^a
Listeria monocytogens	30 ± 2^e	12 ± 0.5^a	13 ± 0.5^{ab}	15 ± 0.5^c	14 ± 0.5^b	17 ± 1^d	14 ± 0.5^{bc}	12 ± 0.5^a

Diameter of inhibition zone includes the disc diameter of 6 mm.
[1] The concentration of the extract and ampicillin was 10 mg/disc and 2 mg/disc, respectively. All data are means of three determinations. Significant differences at $P < 0.05$ indicated with different letters along the row.
FD, freeze dried; HSD, high speed dried; FIR-40, FIR-50, FIR-60, FIR-70, FIR-80, far-infrared radiation dried at 40℃, 50℃, 60℃, 70℃, and 80℃ temperatures, respectively.

Table 3. Minimum inhibitory concentration (MIC) of methanol extracts from dried citrus press-cakes (CPCs) against fish and food-related pathogenic bacteria

Bacteria	Minimum inhibitory concentration (mg/mL)							
	Amp[1]	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Fish pathogenic bacteria								
Vibrio anguillarum	80	2^a	2^a	2^a	4^b	2^a	2^a	4^b
Vibrio alginolyticus	-	8^a	8^a	8^a	8^a	8^a	8^a	8^a
Vibrio ichthyoenteri	160	8^a	8^a	8^a	8^a	8^a	8^a	8^a
Vibrio parahemolyticus	80	4^a	4^a	4^a	4^a	4^a	4^a	4^a
Streptococcus iniae	160	2^a	2^a	4^b	2^a	4^b	2^a	2^a
Streptococcus parauberis	80	2^a	4^b	4^b	4^b	4^b	4^b	4^b
Food-related pathogenic bacteria								
Bacillus subtilis	80	2^a	2^a	2^a	2^a	2^a	2^a	2^a
Salmonella thyphimurium	40	4^a	4^a	4^a	4^a	8^b	4^a	8^b
Escherichia coli	40	4^a	4^a	8^b	8^b	8^b	8^b	8^b
Staphylococcus aureus	80	1^{ab}	2^c	2^c	0.5^a	1^{ab}	1^{ab}	1^{ab}
Listeria monocytogens	40	0.5^a	1^b	1^b	0.5^a	1^b	1^b	0.5^a

[1] Concentrations of ampicillin are given in μg/mL. Significant differences at $P < 0.05$ are indicated with different letters along the row.
FD, freeze dried; HSD, high speed dried; FIR-40, FIR-50, FIR-60, FIR-70, FIR-80, far-infrared radiation dried at 40℃, 50℃, 60℃, 70℃, and 80℃ temperatures, respectively.

values were almost the same as those of CPC extracts dried by FD. Furthermore, all extracts showed moderate MIC values against the *V. alginolyticus* and *V. ichyienter* strains tested. In contrast to the food-related pathogenic bacteria, MIC values were 0.5-8 mg/mL. Strong activity was evident against *L. monocytogens*, *S. aureus*, and *Bacillus subtilis* at 0.5-2 mg/mL, which was significant. These values were also the same as those of the HSD and FIR extracts from dried CPCs with the extract from CPCs dried by FD. All extracts showed moderate MIC values against *Salmonella thyphimurium* and *E. coli*. The MBC results against

fish and food-related pathogenic bacteria are shown in Table 4. The MBC results were 1-16 mg/mL. In contrast to the food-related pathogenic bacteria, strong inhibitory activities were shown against *Listeria monocytogens* and *S. aureus* at 1-4 mg/mL. Moreover, moderate MBC values were exhibited at 4-16 mg/mL against all other tested fish and food-related pathogenic bacteria. These MBC values were the same for all extracts dried by HSD and FIR compared to that of those dried by FD. Therefore, these results further confirmed the potential activities of the CPC extracts.

Table 4. Minimum bactericidal concentration (MBC) of methanol extracts from dried citrus press-cakes (CPCs) against fish and food-related pathogenic bacteria

Bacteria	Minimum bactericidal concentration (mg/mL)							
	Amp[1]	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Fish pathogenic bacteria								
Vibrio anguillarum	160	4[a]	4[a]	4[a]	4[a]	4[a]	4[a]	8[b]
Vibrio alginolyticus	-	16[a]	16[a]	16[a]	16[a]	16[a]	16[a]	16[a]
Vibrio ichthyoenteri	320	16[a]	16[a]	16[a]	16[a]	16[a]	16[a]	16[a]
Vibrio parahemolyticus	160	4[a]	4[a]	4[a]	4[a]	4[a]	4[a]	4[a]
Streptococcus iniae	320	4[a]	4[a]	8[b]	4[a]	8[b]	4[a]	4[a]
Streptococcus parauberis	160	4[a]	4[a]	4[a]	4[a]	4[a]	4[a]	8[b]
Food-related pathogenic bacteria								
Bacillus subtilis	160	4[a]	4[a]	4[a]	4[a]	4[a]	4[a]	4[a]
Salmonella thyphimurium	80	4[a]	4[a]	4[a]	4[a]	8[b]	4[a]	8[b]
Escherichia coli	80	4[a]	4[a]	8[b]	8[b]	8[b]	4[a]	8[b]
Staphylococcus oureus	160	2[ab]	4[b]	4[b]	1[a]	2[ab]	2[ab]	2[ab]
Listeria monocytogens	80	1[a]	2[b]	2[b]	1[a]	2[b]	2[a]	1[a]

[1] Concentrations of ampicillin are given in μg/mL, Significant differences at *P* < 0.05 are indicated with different letters along the row.
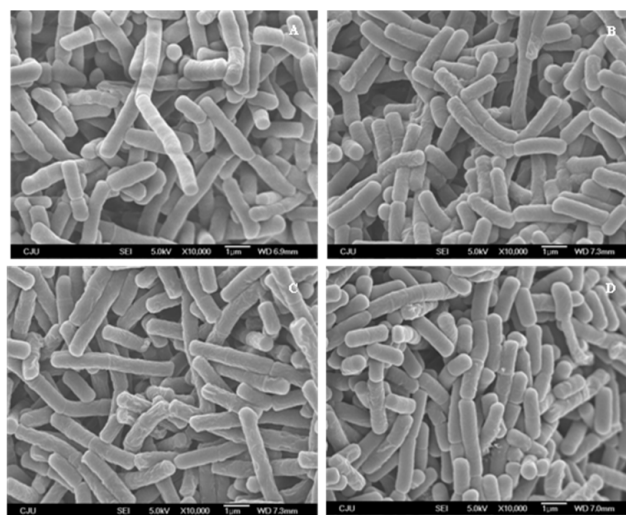FD, freeze dried; HSD, high speed dried; FIR-40, FIR-50, FIR-60, FIR-70, FIR-80, far-infrared radiation dried at 40℃, 50℃, 60℃, 70℃, and 80℃ temperatures, respectively.
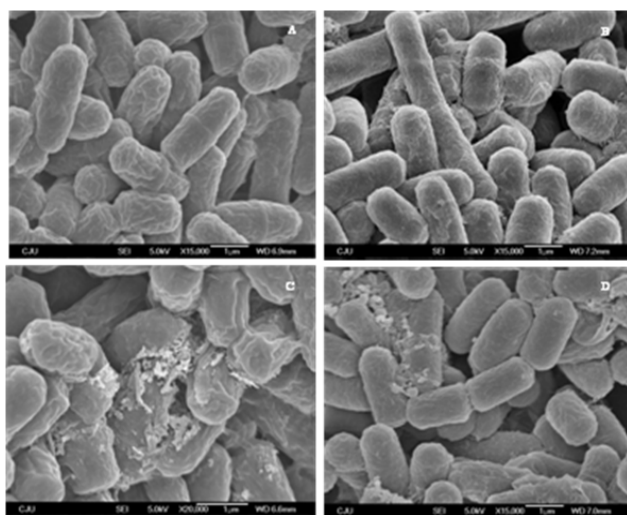
Fig. 1. Scanning electron micrographs of *Streptococcus iniae* treated with the dried citrus press-cake (CPCs) extracts. A, Negative control; B, treated with 8 mg/mL CPC extract dried by high speed drying (HSD); C, treated with 8 mg/mL CPC extract dried by far-infrared radiation drying (FIR) at 50℃; D, treated 8 mg/mL CPC extract dried by freeze drying (FD)


Fig. 2. Scanning electron micrographs of *Bacillus subtilis* treated (8 mg/mL) with the dried citrus press-cake (CPC) extracts. A, Negative control; B, treated with 8 mg/mL extract from CPCs dried by high speed drying (HSD); C, treated 8 mg/mL extract from CPCs dried by far-infrared radiation drying at 50℃; D, treated 8 mg/mL extract from CPCs dried by freeze drying (FD)

SEM observations

The CPC extracts dried by HSD, FIR, and FD were added to *S. iniea* and *B. subtilis* bacteria strains and observed by SEM (Figs. 1 and 2 respectively). The SEM results suggested that the CPC extracts caused morphological changes in the surface of the treated bacteria compared to those of the control. Untreated bacterial cells (control) remained intact and showed a smooth surface, whereas the treated bacterial cells showed damage or distinctive changes in cell membranes.

Discussion

Efficient, fast, and economically reliable drying methods were used to prepare HSD and FIR extracts from CPCs and was compared with that of FD in our previous studies [24,25]. Furthermore, total flavonoid contents in the methanol extracts from the CPCs, dried by HSD, FIR, and FD were determined to be 477.4 mg/100 g, 463 mg/100 g, and 624 mg/100 g, respectively. All extracts from dried CPCs had hesperidin and narirutin in large quantities as compared to other flavonoids [24,25]. We focused on the antibacterial activity of some of the different flavonoid compounds found in the extracts (Table 5). Hence, the antibacterial activities shown in this study might be

Table 5. Flavanone glycosides; A and polymethoxylated flavones; B found in the extracts from citrus press-cakes prepared by high speed drying (HSD) or far infrared drying (FIR).
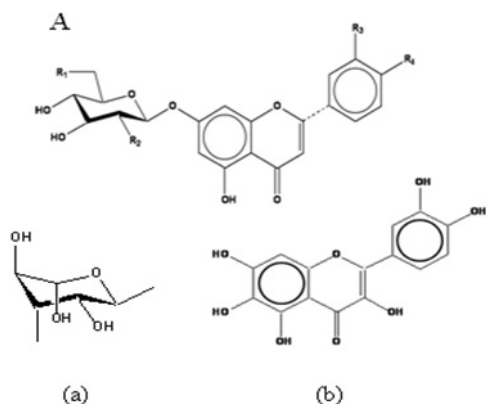
Name	R₁	R₂	R₃
Nobiletin	OCH₃	H	OCH₃
Tangeritin	OCH₃	H	H
Sinesetin	H	H	OCH₃
Hexamethoxyflavone	H	OCH₃	OCH₃
Heptamethoxyflavone	OCH₃	OCH₃	OCH₃
Scutelarin	H	H	H

a: Tetramethoxy flavones structure
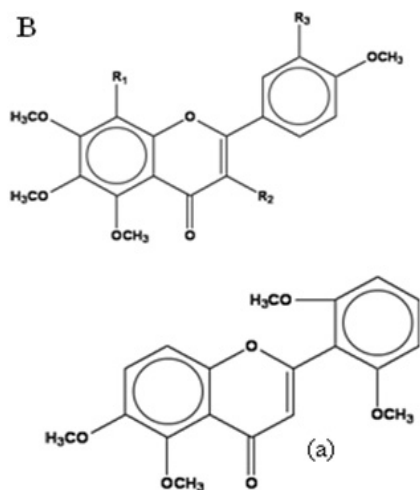
Name	R₁	R₂	R₃	R₄
Hesperidin	a	H	OH	OCH₃
Narirutin	a	H	H	OH
Neohesperidin	H	a	OH	OCH₃

b: Quercetagetin structure

due to the presence of a variety of flavonoid or phenolic compounds. The synergistic effects of those flavonoids may have resulted in higher antibacterial activities, and they are thermostable, as they showed activity even after being dried at very high temperatures during HSD compared to those of FIR. However, the FIR drying process is shorter drying time than that of FD

[26]. Moreover, differences in the activities of the CPC extracts could be partially explained by variations in flavonoid content and strain sensitivity. Yi *et al.* [9] showed that hesperidin is more active against bacterial growth than that of polymethoxylated flavones such as tangeritin and nobiletin, whereas various flavanones such as hesperitin and naringenin are active against *Helicobacter pylori* [33]. The antimicrobial activity of purified flavonoids has also been described [34].

The reason for the lower sensitivity of the Gram-negative bacteria compared to that of Gram-positive bacteria could be due to differences in their cell wall composition. Gram-positive bacteria contain an outer peptidoglycan layer, which is an effective permeability barrier [35], whereas Gram-negative bacteria have an outer phospholipidic membrane that makes the cell wall impermeable to lipophollic solutes, and porins act as a selective barrier to hydrophilic solutes [36].

The SEM results showed that the active components of the extracts destroyed the bacterial cell walls and changed their morphology. Several mechanisms are responsible for those changes. The phospholipid bilayer of the cytoplasmic membrane in the bacterial cell wall may be sensitized and inhibit membrane-bound enzymes by the biologically active compounds in the CPC crude extracts. In fact, those bind to the surface of bacterial cells, preventing target points, and changing biochemical processes in the bacterial cells. These processes may lead to uncoupling of oxidative phosphorylation, restraints from on active transport, reductions in metabolites, and disruption of nucleic acid, protein, lipid, and polysaccharide synthesis [37-39]. The bacterial cells loses its structural integrity, and membrane permeability due to the damage to the cell wall and cytoplasmic membrane [40]. This has been shown in many cases with an increase in altered membrane fluidity in the hydrophilic and hydrophobic regions. Therefore, a reduction in membrane fluidity of bacterial cells may have led to the reduced number of CFUs in the viable counts [41]. In addition, perturbing the lipid bilayer by membrane fusion by leakage of intramembranous materials, including ions, ATP, nucleic acid, and amino acids that cause aggregation, may have led to a disruption in activity [42,43].

There may be a relationship between antibacterial activity and the chemical structures of the major phenolic compounds in the extracts [44]. However, the different groups of phytochemicals may target different components and functions of bacterial cells [45]. The inhibitory action on DNA and RNA synthesis could be affected by creating a hydrogen bond with the B ring of flavonoids [46]. Moreover, inhibiting the enzyme's ATPase activity which is related to DNA gyrase, has been coupled in cases of antibacterial activity [47].

In conclusion, we showed that the major active components from dried CPCs possessed significant *in vitro* antibacterial properties against 11 fish and five food-related pathogenic bacteria. CPCs as a citrus peel by-product from the citrus processing industry can be recovered as a value-added source. The HSD and FIR drying systems were confirmed as economical

and time effective alternative techniques compared to the qualified FD technique. Taken together, our results demonstrate that drying CPCs by HSD and FIR could provide beneficial antibacterial components for the fish and food preservative industries. However, further studies on improving food safety by controlling or eliminating these pathogenic bacteria is needed for future prospective applications.

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