Phytochemical, antioxidant, and antibacterial activities of fermented *Citrus unshiu* byproduct

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Abstract *Citrus unshiu* is an economically important fruit on Jeju Island, Korea, but byproducts are a major source of agricultural waste. The aim of this study is to examine changes in phytochemical, antioxidant, and antibacterial activities of four *C. unshiu* byproducts: citrus flesh byproduct (CFB), fermented citrus flesh byproduct (FCFB), citrus peel byproduct (CPB), and fermented citrus peel byproduct (FCPB). Fermented citrus byproducts (FCFB and FCPB) exhibited greater inhibition effect on radical scavenging abilities of 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid)-diammonium salt, and superoxide anion compared with those of CFB and CPB. Total polyphenol contents of FCFB and FCPB were significantly increased compared to those of CFB and CPB. Significant bioconversion of the flavonoid glucosides into corresponding bioactive aglycones during citrus byproduct fermentation was observed. Fermented citrus byproducts exhibited antibacterial activity against *Listeria monocytogenes* and *Escherichia coli* suggesting that FCFB and FCPB are potent antioxidant and antibacterial sources due to the remarkable contents of bioactive compounds in them.

Keywords: *Citrus unshiu*, antioxidant activity, antibacterial activity, fermented citrus byproducts, bioactive compounds

Introduction

Vitamin C, B6, and polyphenols are nutrients that are widespread throughout the plant and comprise a large group of naturally occurring compounds found in vegetables and fruits, especially in the *Citrus* genus (1). *Citrus unshiu*, commonly known as satsuma mandarin, is mainly used in the food industry to produce fresh juice and citrus-based foods. However, during production, large amounts of byproducts and agricultural waste are generated. The disposal of byproducts from *C. unshiu*, with annual yields of more than 60,000 tons from juice factories, creates a major problem on Jeju Island in the Korea Strait between Japan and South Korea. Handling, transportation, and disposal of this waste are costly, and the value of recycled materials is often sufficient to cover operating costs (2,3). However, citrus byproducts potentially present a rich source of natural flavonoids, owing to the large amount of peels produced that contain high concentrations of narirutin, naringenin, hesperidin, and nobletin (4-6).

Citrus-derived flavonoids offer several health benefits, including antioxidative, anti-inflammatory, anticancer, antiviral, antibacterial, and cardiovascular protective activities (7-9). Citrus flavonoids are found mainly as *O*-glycosides, that are mostly bound to glucose, but also to other sugars, such as galactose, rhamnose, arabinose, and

xylose. The *O*- β -Glycosidic bonds of flavonoids are hydrolyzed in the gut mainly by microbial α -glycosidase to produce aglycon (10). The enzymatic de-glycosylation of flavonoids has been reported as a viable alternative mechanism to increasing the bioavailability of these compounds (11,12) and increasing the antioxidant activity of kaempferol (13) and the anti-inflammatory activity of naringin (14,15).

Nuruk is a Korean traditional fermentation starter that is naturally inoculated by airborne microorganisms, such as *Aspergillus* sp., *Rhizopus* sp., *Saccharomyces cerevisiae*, *Bacillus subtilis*, and lactic acid bacteria (16,17). Microbial fermentation is known to have improved digestibility of starch and protein. Through the process of fermentation, it produces diverse and numerous bioactive compound such as organic acids and flavor (18).

The aim of the present study is to elucidate the biological activities of fermented *C. unshiu* byproducts to facilitate the conversion of this waste into highly value-added products.

Materials and Methods

Sample preparation *Nuruk* was purchased from a local market located in Seogwipo, Korea. Citrus byproducts were collected in

October 2014 from the Citrus Research Institute, (Jeju, Korea). The citrus byproducts were dried and then ground into a powder suitable for fermentation. Citrus byproduct (100 g), *nuruk* (50 g), and sugar (100 g) were added to 1 L of water to produce a mash that was mixed thoroughly and fermented at 25°C for 15 days. Fermented citrus byproducts were extracted using 70% ethanol at room temperature for 24 h. Then, the extracted solutions were filtered, the solvents were evaporated, and the obtained extracts were dried and stored at 4°C.

Antioxidant activities

Free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH): The reaction was carried out in 100% ethanol containing 0.1 mM DPPH and the sample (19). The scavenging effect against DPPH radicals was assessed at room temperature for 10 min. Changes in absorbance were measured at 517 nm using a SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). All samples were analyzed in triplicate.

Radical scavenging activity of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS): For the ABTS assay, 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution were prepared as stock solution. The working solution was then prepared by allowing equal quantities of the two stock solutions to react for 15 h at room temperature in the dark. The solution was diluted to obtain an absorbance of 0.70 ± 0.02 AU at 734 nm using microplate reader. Samples (20 µL) were allowed to react with 180 µL of the ABTS solution and absorbance at 734 nm was read after 15 min. The ABTS scavenging activity of each sample was compared with that of vitamin C. All samples were tested in triplicate.

Superoxide anion radical scavenging activity: Superoxide anion radicals were assayed by the reduction of nitro blue tetrazolium (NBT) to form blue formazan (20). In this experiment, superoxide anion radicals were generated in 880 µL of Tris-HCl buffer (20 mM Tris-HCl, pH 8.0) containing NADH (98 µM), 20 µL of NBT (62 µM) solution, 100 µL of phenazine methosulfate (33 µM). The sample solution was mixed with water. The reaction mixture was incubated at 25°C for 10 min, and the absorbance was measured at 560 nm. Ascorbic acid was used as a control. Decreased absorbance of the reaction mixture indicated increased superoxide anion radical scavenging activity. All measurements were performed three times.

Protective effect against hydrogen peroxide induced oxidative stress on human keratinocyte, HaCaT cells: Human skin keratinocyte HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin (100 U/mL of penicillin) and streptomycin (100 U/mL) under 95% air/5% CO₂ humidified atmosphere at 37°C. The keratinocytes were stimulated with H₂O₂ (500 µM) and then treated with 100 µg/mL of the sample for 24 h. Cell viability was determined using a colorimetric assay that used Cell Proliferation Reagent WST-1 (Roche, Mannheim, Germany). Briefly, HaCaT cells were washed twice with phosphate-buffered saline and then incubated with serum-free DMEM containing WST-1

at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 1-2 h. After the reaction was completed, the medium was discarded and 200 µL of dimethyl sulfoxide (Amresco, Solon, OH, USA) was added to each well of the cell culture plate. Formazan was eluted and 200 µL was transferred to each well of a 96-well plate. Samples were read at a wave length of 570 nm using a microplate reader.

High-Performance Liquid Chromatography (HPLC) The citrus byproduct samples were filtered through 0.22 µm polyvinylidene difluoride membrane filter. The following flavonoid compounds were analyzed in the citrus byproduct sample: rutin, naringin, hesperidin, neohesperidin, neohesperidin dihydrochalcone, quercetin, naringenin, and hesperitin. A 10 µL aliquot of filtered sample was analyzed using an HPLC system (Waters, Milford, MA, USA) with a YMC Pro C18 RS column (250x4.6 mm, 5-5 µm, 8 nm; YMC Co., Kyoto, Japan) and a Waters 2489 UV visible detector. The mobile phases for the HPLC system were acetonitrile (A) and water containing 0.1% acetic acid (B) at a flow rate of 1 mL/min.

Disc diffusion assay Antibacterial activity was determined using the agar diffusion method as described by Yoon *et al.* (21). Briefly, a disc paper (8 mm) was impregnated with 20 µL of a 100 mg/mL solution of each citrus byproduct and FCB dissolved in distilled water. The disc was placed on a plate of sensitivity testing agar inoculated with *Escherichia coli* and *Listeria monocytogenes*. Petri dishes were left at room temperature for approximately 1 h to allow the extract from the disc to infuse into the medium and were subsequently incubated at 37°C for 24 h. The clear zones showing no growth were then noted and the diameter of each was recorded as the zone of inhibition.

Analysis of organic acid Analysis of organic acids was performed using an ion chromatography (IC) system (DIONEX ICS-3000; Dionex Corporation, Sunnyvale, CA, USA), which consisted of an eluent pump, sample injector, auto-sampler, column oven, conductivity detector, and an ion exclusion column (IonPac® ICE-AS6 9x250 mm; Dionex Corporation). The mobile phase for the IC system was 0.4 mM heptafluorobutyric acid at a flow rate of 1 mL/min. The concentrations of oxalic, malic, citric, and lactic acid were analyzed in the citrus byproduct sample.

Statistical analysis All data are expressed as means ± standard deviation. Significant differences among the groups were determined using the unpaired Student's *t*-test. A probability (*p*) value <0.05 was considered statistically significant.

Result and Discussion

Total phenolic and antioxidative activities of fermented citrus byproducts The total polyphenolic contents increased from 1.49

Fig. 1. Total polyphenol contents of citrus flesh byproduct (CFB), fermented citrus flesh byproduct (FCFB), citrus peel byproduct (CPB), and fermented citrus peel byproduct (FCPB) (A). Antioxidative activities of CFB, FCFB, CPB, and FCPB. Free radical scavenging activity of (B) DPPH, (C) ABTS. The data are presented as mean±standard deviation.

and 6.37 g gallic acid equivalence (GAE)/100 g dry extract in unfermented citrus flesh byproduct (CFB) and citrus peel byproduct (CPB) to 8.61 and 16.34 g GAE/100 g dry extract in fermented citrus flesh byproduct (FCFB) and fermented citrus peel byproduct (FCPB) with *nuruk*. These results demonstrate that levels of bioactive compounds can be modified during fermentation by the metabolic activity of micro-organisms. All samples showed a significant amount of total phenols and effective antioxidant activity (Fig. 1). The antioxidant activity increased in most cases during fermentation. The citrus byproduct was fermented for 15 days and its ability to scavenge DPPH, ABTS, and superoxide anion radicals was determined. Fermentation of citrus byproducts with *nuruk* also showed enhanced radical scavenging activity of DPPH and ABTS. A dose-dependent activity was observed when all samples were tested at concentrations

of 250, 500, and 1,000 µg/mL with the DPPH and ABTS radical scavenging assay. Among the samples, FCFB and FCPB showed particularly high antioxidant activities, which increased approximately two-fold compared with CFB and CPB.

Superoxides are anion radicals with an unpaired electron located on an oxygen that indirectly initiates lipid oxidation as a result of superoxide and H_2O_2 serving as precursors of singlet oxygen and hydroxyl radicals, respectively (22). The non-fermented CFB and CPB exhibited a scavenging effect on superoxide anion radicals of 5.4 and 56.05% whereas fermented CFB and CPB resulted in a significantly enhanced superoxide anion scavenging effect of 67.14 and 96.77% depending on the *nuruk* used (Fig. 2A). The protective effect of CFB, CPB, FCFB, and FCPB on the survival of H_2O_2 -treated HaCaT cells was measured. Cells were treated with CFB, CPB, FCFB, and FCPB at

Fig. 2. Antioxidative activities of citrus flesh byproduct (CFB), fermented citrus flesh byproduct (FCFB), citrus peel byproduct (CPB), and fermented citrus peel byproduct. (A) Free radical scavenging activity of superoxide. (B) Protective effect of CFB, FCFB, CPB, and FCPB against oxidative stress induced by hydrogen peroxide in HaCaT keratinocytes. The data are presented as mean±standard deviation.

100 µg/mL for 24 h, followed by stimulation with 500 µM H₂O₂. Cell viability was determined 24 h later using the colorimetric assay. As shown Fig. 2B, treatment with FCFB and FCPB induced an increase in the cell survival rate (15% at 100 µg/mL).

HPLC analysis of fermented citrus byproducts components

Flavanone glycosides of citrus byproduct were transformed into corresponding aglycones by *nuruk* fermentation. To identify the chemical profiles of the flavonoid glycosides and hydrolysis products, CFB and CPB before and after bioconversion were characterized by HPLC (Table 1). The results of quantitative analyses of the glucosidic flavones rutin, narirutin, and hesperidin and the aglyconic flavones quercetin, naringenin, and hesperetin are shown in Table 1. After 15 days, the *nuruk*-fermented citrus byproducts (FCFB and FCPB) contained quercetin, naringenin, and hesperetin. The concentration

of aglycones in FCFB and FCPB were significantly increased (130.396 and 50.757 ppm, respectively), while the concentration of hesperidin and narirutin were decreased.

It has been demonstrated that flavanone aglycones such as hesperetin and naringenin have a greater potential for antioxidant activity than the corresponding glycosides hesperidin and naringin (23). The phenolic hydroxyl groups of flavonoids are reported to play a pivotal role in scavenging activities of DPPH, ABTS, and superoxide, which was also observed in the present study given that FCPB and FCFB exhibited more potent activities than CPB and CFB (24).

Antibacterial activities of fermented citrus byproducts The disk diffusion method was used to assess the antibacterial activity of CFB, FCFB, CPB, and FCPB against *E. coli* and *L. monocytogenes*. As shown in Table 2, FCFB and FCPB exhibited significant susceptibility with

Table 1. Change of flavonoids in citrus byproduct fermented with *nuruk* for 15 days at 25°C

Sample	Flavonoid content (ppm)					
	Glucosides				Aglycones	
	Rutin	Narirutin	Hesperidin	Quercetin	Naringenin	Hesperetin
CPB ¹⁾	231.552±18.76	422.361±46.22	169.351±18.33	12.44±1.35		
FCPB	200.015±25.5	169.843±12.49	46.06±5.02	50.262±6.23	66.299±7.08	13.835±1.56
CFB	24.071±1.92	192.96±20.52	33.701±2.89	5.141±0.36		
FCFB	18.342±0.97	44.03±6.12	16.583±0.87	11.031±1.12	32.739±4.11	6.987±0.54

¹⁾CPB, citrus peel byproduct; FCPB, fermented citrus peel byproduct; CFB, citrus flesh byproduct; FCFB, fermented citrus flesh byproduct

Table 2. Antibacterial effects of CFB, FCFB, CPB, and FCPB against target strains

Sample	Diameter of inhibition zone (mm)	
	<i>L. monocytogenes</i>	<i>E. coli</i>
CFB	8	8
FCFB	9	12
CPB	8	8
FCPB	9	11

inhibition diameters >10 mm against *E. coli*. According to the literature on *nuruk*, an organic acid is considered to be responsible for the inhibitory effect against a number of microbes. Organic acids are weak acids that are commonly found in fruit, vegetables and synthesized by microorganisms as a result of fermentation. The antibacterial activity of organic acids is attributed to pH reduction (25). It has been proposed that a low external pH causes acidification of the cell cytoplasm, while an undissociated acid flow through the cell membrane of microorganisms (26). The undissociated acid acts by collapsing the electrochemical proton gradient, or by altering cell membrane permeability, which results in disruption of substrate transport systems (27).

The contents of oxalic, malic, citric, and lactic acids were measured as the organic acids of citrus byproducts and FCBs. In the FCFB and FCPB, the concentrations of citric acid and lactic acid increased with the amount of added *nuruk* (Table 3). Especially, lactic acid contents of FCFB and FCPB were 7.625 and 7.571 mg%, respectively, which were greater than those of the other samples. Lactic acid may be produced during *nuruk* fermentation by the proliferation of lactic acid bacteria. Lactic acid bacteria isolated from FCFB and FCPB were incubated anaerobically on de Man, Rogosa, and Shape (MRS) media. The isolated strains were subjected to 16S rRNA gene sequence analysis, which showed 99% identity with that of *Weissella cibria* (data not shown).

Fermentation can enhance the levels of bioactive compounds in citrus byproducts and can be used to improve product properties by changing the ratio of nutritive and antinutritive components of plants. Fermentation can potentially influence the bioactive constituents of citrus byproducts, by altering the degree of modification of most bioactive compounds. The finding of the present study showed that capacity to scavenge DPPH, ABTS, superoxide radicals was increased by *nuruk* fermentation of citrus byproducts, and the increased capacity was due to the increased content of flavonoid aglycones bioconverted during fermentation. However, more detailed studies of the changes and activities of relevant enzymes of microorganism

population during fermentation of citrus byproducts are required to establish precise mechanisms that improve the nutritional value of FCBs.

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Disclosure The authors declare no conflict of interest.

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Table 3. Content of organic acid in CFB, FCFB, CPB, and FCPB (%)

Sample	Oxalic acid	Citric acid	Malic acid	Lactic acid	Total organic acid
CFB	0.8402	1.6164	0.2763		2.7331
FCFB	1.2033	5.2054		7.6175	14.0262
CPB	0.8638				0.8638
FCPB	1.6213	1.6033		7.5708	10.7954

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