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A Comparison of the Biological Activities of *Citrus sudachi* Hort. ex Shirai Peels Grown in Japan and Korea

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Abstract

This study evaluates the *Citrus sudachi* Hort. ex Shirai (sudachi) peel grown in Japan and Korea as an antioxidant, an antimicrobial, and *α*-glucosidase inhibitory agent. In this investigation, 80% methanol (MeOH) partitioned into four different fractions—*n*-hexane, ethyl acetate (EtOAc), *n*-butanol, and aqueous, were used as solvents for sudachi. It was found that the *n*-butanol fraction was the highest among the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assays for both countries. For the reducing power assays, the EtOAc fraction showed the highest reducing power for both countries. The highest phenol and flavonoid content was found in the EtOAc fraction in the samples from both Korea and Japan. For the *α*-glucosidase inhibitory activity, Japanese sudachi demonstrated greater inhibitory activity than Korean sudachi. For the minimum inhibitory concentration (MIC) assay, gram-positive bacteria were more sensitive than gram-negative bacteria, and the EtOAc fraction showed greater inhibitory activity in samples from both countries; however, Korean sudachi exhibited the greatest inhibitory activity. The combined data from all assays indicated that sudachi can be used effectively as a natural antioxidant, antimicrobial, and *α*-glucosidase inhibitory agent.

Keywords: Sudachi peel, Biological activities, Polyphenolics, Different solvents

1. Introduction

Reactive oxygen species (ROS), such as the superoxide anion (O^-), the peroxyl radical (ROO), nitric oxide (NO), and the hydroxyl radical (ROC) are constantly produced endogenously through biological activity (Menković *et al*., 2014). Although antioxidants are also produced in the body (Hwang *et al*., 2013), the over production of ROS results in antioxidative stress, which is expressed as an imbalance of ROS and antioxidant concentrations (Kücükakin *et al*., 2009). These unstable molecules lack one electron in their outer shell and seek to stabilize by stealing an electron from healthy cells around them (Jiménez-Monreal *et al*., 2009). The cells that lose the electron are damaged causing adverse effects for humans, including asthma, diabetes, and cancer (Sindhi *et al*., 2013). These diseases are somehow induced by ROS. Protection against ROS can be accelerated by the intake of antioxidants and the most commonly used synthetic antioxidants are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are used as food additives, or to slow the deterioration of cosmetics (Sindhi, *et al*., 2013). However, Anbudhasan *et al*. (2014) suggested that the BHT and BHA could have negative health effects. Accordingly, consumer interests shifted to antioxidants from natural foods, which have no reported adverse side effects. Many studies have described the beneficial antioxidants in foods such as cereals, herbs, vegetables, fruits, and medicinal plants (Singh and Kumari, 2015; Djordjevic *et al*., 2011; Jayanthi and Lalitha, 2011; Akdaş and Bakkalbaşı, 2017; Jamuna *et al*., 2010). Ascorbic acid and *α*-tocopherol, commonly known as vitamin C and vitamin E, respectively are two of the most well-known natural antioxidants. In recent years, much more attention has been focused on citrus fruits due to their high concentration of antioxidants and their anticancer properties (Karsheva *et al*., 2013; Entezari *et al*., 2009).

Citrus sudachi Hort. ex Shirai (sudachi) belongs to the Rutaceae family and is originally from Japan. Sudachi is mostly known in the southern parts of Japan. Unlike the orange, its fruit is harvested before it has completely ripened. The taste of sudachi is relatively bitter and gives off a unique aroma (Kim and Kim, 2016). This strong aroma is one of the key characteristics of sudachi, as also is its high levels of vitamin C (Lee *et al*., 2015). Recent reports suggest that the constituents of sudachi reduce the risk of hyperglycemia and lower the serum glucose level in obese individuals (Akaike *et al*., 2014). Sudachitin, a unique component found only in sudachi peel, increases energy expenditure and weight loss by increasing metabolism and stimulating mitochondrial biogenesis (Tsutsumi *et al*., 2014). The ability of sudachi to inhibit bacterial growth has also been investigated (Tomotake *et al*., 2006).

Sudachi is generally consumed as an acidulant in savory foods or juice. In both cases, the fruits are used, and

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the peel is generally discarded as industrial waste by the factories that process them. In Japan, the discarded sudachi peel gives off an unpleasant odor, and this has become a significant problem, both financially and environmentally, for the producers and processors (Tsukayama *et al*., 2010). In contrast, sudachi is much less popular in Korea, and it is consumed in very small amounts. Although some studies describe the antioxidative, antidiabetic, and antimicrobial activity of sudachi, there are no reports that compare sudachi from Japan with sudachi from Korea. Thus, the aim of this study is to investigate the efficacy of sudachi peel as a natural antioxidant by comparing the sudachi from these two countries. Strong antimicrobial and *α*-glucosidase inhibitory activity has also been reported with regard to sudachi peel (Ali *et al*., 2017; Vasu *et al*., 2017), and these inhibitory activities were also evaluated.

2. Materials and Methods

2.1. Chemicals

Potassium ferricyanide, trichloroacetic acid, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric chloride, sodium carbonate, and BHT were purchased from Wako Pure Chemical Industries Ltd. (Japan). Quercetin, gallic acid, arbutin, and 4-Nitrophenyl-β-D-glucopyranoside (*p*NPG) were obtained from Sigma Chemical Co. (USA). Other reagents were obtained from Daejung Chemical and Metal Co., Ltd. (Republic of Korea). All reagents were of analytical grade.

2.2. Plant Material and Extraction

Two samples from Japan and Korea were collected for this study. The sudachi grown in Japan was obtained from the Tokushima prefecture on September 10th, 2014. Another sample was collected from Jeju Island in Korea on September 22nd, 2014. Both samples were peeled, and the peels were dried under ambient temperature conditions. After drying, the samples were crushed by a mixer to make a powder. Each sample was extracted with 900 mL of 80% methanol (MeOH) solution with 64 g of pulverized Japanese sudachi peel (145 g of pulverized Korean sudachi peel) and were put in an ultrasonic bath (Power Sonic 520, Hwashin, Co., Korea) for ninety minutes. This process was continued three times. The solution was then filtered to remove impurities and the extracts were evaporated by a rotary vacuum evaporator (Hei-VAP Precision 280 rpm, Heldolph, Germany). The concentrate was set aside, and the remnants were dissolved by water and partitioned with *n*-hexane, ethyl acetate (EtOAc), *n*-butanol, and distilled water (aqueous) sequentially. The samples were stored in a refrigerator at -20ºC until further analysis.

2.3. Analysis of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) Radical Scavenging Activity

A DPPH assay was performed using Hyun *et al*.'s method (2014), with slight modification. Briefly, each diluted sample (100 µL) was placed in a test tube. The MeOH solution was added to each sample test tube to adjust the total volume to 4 mL. One hundred µL of DPPH (0.15 mM) was added to each mixed solution and incubated in a dark room for thirty minutes. After thirty minutes, the absorbance was measured with a UV-Spectrophotometer at 517 nm (UV-1800, Shimadzu Co., Japan) against MeOH as the blank. Lower UV-Spectrophotometer readings indicate greater DPPH radical scavenging activity. The scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = (1 - A_S / A_C) \times 100$$

(where A_C is the absorbance of the negative control [without sample extract], and A_S is the actual absorbance value of each sample and the positive control [BHT, *α*-tocopherol, and ascorbic acid]). Also, BHT, ascorbic acid, and *α*-tocopherol were used as positive controls.

2.4. Measurement of Reducing Power Assay

The reducing power assay was performed using the method described by Nakamura *et al*. (2017). The samples (10, 20, and 30 µL) were each dispensed to a test tube, and the total volume of each sample was adjusted to 100 µL with distilled water. Then, 500 µL of 1% potassium ferricyanide and 0.2 M of sodium phosphate buffer (pH = 6.6) were added to the mixed solution. The samples were allowed to react at 50°C for twenty minutes. The resulting solution was spiked with 2.5 mL of 10% trichloroacetic acid to stop the reaction. The supernatant (500 µL) was transferred into the test tube and mixed with 500 µL of distilled water. Finally, 0.1% ferric chloride was added to the mixed solution. The absorbance was measured at 700 nm. Higher absorbance rates indicate a greater reducing power. Alpha-tocopherol and BHT were used as standards.

2.5. Determination of Total Phenol and Flavonoid Contents

The total phenol content was measured using the Folin–Ciocalteu method employed by Nakamura *et al*. (2016). One hundred µL of each sample or a standard solution of gallic acid to draw the calibration curve was mixed thoroughly with 50 µL of Folin–Ciocalteu reagent for five minutes. Then, 300 mL of 20% sodium carbonate was added to the solution, and the mixture was allowed to react for twenty minutes at ambient temperature. Prior to measuring the absorbance rate at 725 nm by UV-Spectrophotometer, 1 mL of distilled water was added. The data were expressed as milligrams of gallic acid equivalent (GAE) per gram of the sudachi extract, based on the calibration curve of gallic acid (mg GAE/g of sudachi).

The total flavonoid content was estimated using the aluminum chloride colorimetry method described by Nakamura *et al*. (2016). In brief, to draw the calibration curve, a 6-fold serial diluted solution with quercetin was prepared (3.125, 6.25, 12.5, 25, 50, 100 mg/L). Simultaneously, the 20 µL of samples were diluted with 180 µL of 80% EtOH. Each 200 µL diluted sample and standard solution was transferred to a test tube and mixed with 100 µL of 10% aluminum nitrate. Then, 100 µL of 1 M potassium acetate and 4.6 mL of 80% ethanol were added to the solutions. After keeping the solution at room temperature for forty minutes, the maximum absorbance of each mixture was measured by UV-Spectrophotometer at 417 nm, and a comparison with the standard curve obtained from quercetin was done. The total flavonoid content was described as milligrams of quercetin equivalent (QE) per gram of sudachi extract, based on the calibration curve of quercetin (mg QE/g of sudachi).

2.6. Assay for α-Glucosidase Inhibitory Activity

The *α*-glucosidase inhibitory activity assay employed the method used by Yang *et al*. (2011) with a minor modification. Fifty µL of the sample solution and sodium phosphate buffer (0.2 M, pH = 6.8) were added to each

microtube. Then, prior to incubating for fifteen minutes at 37°C, 50 µL of *α*-glucosidase (0.5 U/mL) was mixed with each sample solution. The substrate *p*NPG (100 µL, 0.3 mM) was allowed to react with the reaction mixture for ten minutes at 37°C. The reaction was forced to terminate by adding 750 µL of Na_2CO_3 (0.1 M). Finally, the absorbance was measured at an absorbance intensity of 405 nm. Alpha-glucosidase inhibitory activity was calculated using the equation:

$$\% \text{ of inhibition} = (1 - (As / Ab) / Ac) \times 100$$

The control was prepared by including all reagents without a sample solution, whereas the blank was all reagents without *p*NPG. As a positive control, acarbose was used. The absorbance of the test samples was presented with the absorbance of the blank solution (Ab) and the absorbance of the control without samples (Ac).

2.7. Determination of Minimum Inhibitory Concentration

To determine the minimum inhibitory concentration (MIC) of each extract and each fraction, we performed a serial two-fold dilution method, adapted from Jeong *et al.* (2010), using 96 well microtiter plates. First, 180 µL of an appropriate medium with bacteria and 20 µl of sample were added to the first row. Then, 100 µL of the medium was spiked from the second row to the last row. Finally, the mixed medium was diluted sequentially. The inhibition activity was evaluated for tardiness with the naked eye after twenty hours. In the current study, six different kinds of bacteria were used—3 gram-positive bacteria (*Bacillus subtilis* subsp. *spizizenii*, *Staphylococcus epidermidis*, and *Micrococcus luteus*) and 3 gram-negative bacteria (*Salmonella enterica* subsp. *enterica*, *Escherichia coli*, and *Klebsiella pneumonia*). The *Salmonella enterica* subsp. *enterica* samples were incubated at 30°C with nutrient agar, and the other bacteria samples were incubated at 37°C in PP-Medium. All the strains of bacteria used in this study were distributed by the Korean Agricultural Culture Collection (KACC) in Korea.

2.8. Phenolic Compound Evaluation by HPLC

HPLC analysis was performed with a Shimadzu (LC-10ADvp) Liquid Chromatography System using an SPD-10A UV-Vis detector for the extracts. The column (Luna 5µ C18 [2] 100A 205 x 460 mm) was used at 40°C. The elution solvents used were (A) acetonitrile and 0.5% acetic acid and (B) water and 0.5% acetic acid. The solvent gradient was carried out as follows: 0 min, 20–80; 14 min, 20–80; 19 min, 40–60; 33 min, 40–60; 38 min, 70–30; 47 min, 70–30; 50 min, 20–80; and 60 min, 20–80, and the injection volume was 10 µL. The phenolic compounds were identified by the retention time and UV spectra of a standard measured from the peak area at 280 nm.

2.9. Correlation and Data Analysis

All determinations were expressed as the mean ± standard deviation of an average triplicate analysis. The difference between the treatment and control groups were analyzed using ANOVA with Duncan's multiple range test. Significance was expressed by P values, and ANOVA values were considered to be statistically significant when the P value was less than 0.05. Data analysis was performed using the SPSS program (Statistical Package for Social Science, Ver. 20.0 [SAS Institute Inc., Cary, NC, USA]).

3. Results and Discussion

3.1. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) Radical Scavenging Activity

Table 1 shows that the Japanese sudachi exhibits greater DPPH radical scavenging activity than the Korean sudachi. The *n*-butanol fraction from both countries (45.3±2.3 µg/mL for the Korean sudachi and 32.0±2.01 µg/mL for the Japanese sudachi) demonstrated the greatest ability to reduce DPPH radicals, whereas significant DPPH radical scavenging activity was not observed in the *n*-hexane fractions or the aqueous fractions (with the exception of the aqueous fraction from Japan). In addition, only the EtOAc and *n*-butanol fractions in Japanese sudachi were superior to BHT, but they did not show greater antioxidative activity than ascorbic acid and *α*-tocopherol. Kim (2014) performed the DPPH scavenging activity assay to determine the antioxidant ability of *Maesa japonica* (Thunb.) leaves and twigs, and found that the *n*-butanol fraction exhibited the greatest antioxidant activity, while the EtOAc fraction showed a slightly lower value than the *n*-butanol fraction for leaves. His results exhibited a similar trend to the results of the present study. Comparing the Korean and Japanese sudachi, the Japanese sudachi exhibited a greater radical scavenging activity than the Korean sudachi for each of the extracts and fractions. Furthermore, the RC_{50} value of the aqueous fraction in Korean sudachi was much higher than 100 µg/mL. Conversely, the Japanese sudachi exhibited a value less than 100 µg/mL (72.7±13.5 µg/mL). This might explain why the Japanese sudachi has slightly more DPPH radical scavenging activity than the Korean sudachi. Also, it is widely thought that the antioxidant activity is closely related to the total phenolic content (Orhan and Üstün, 2011). According to the current data, the fractions and extracts that contained greater quantities of phenolic compounds also showed a greater radical scavenging ability, but this correlation was not always consistent. In Tables 1 and 2, the EtOAc fractions exhibited the highest phenolic content. In contrast, the *n*-butanol fraction demonstrated the highest DPPH radical scavenging activity, followed by the EtOAc fraction. This small discrepancy might be attributed to the different response to the Folin–Ciocalteu reagent by each of the phenolic compounds (Yu *et al.*, 2002). It may be that the total phenol content assays did not fully reflect the actual total phenolic content in the samples, excluding certain antioxidants such as vitamin C or vitamin E, for example (Saha and Paul, 2014).

Table 1. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity in the extract and fractions from *Citrus sudachi* Hort. ex Shirai.

Extract and fractions	RC_{50} (µg/mL)[z]	
	Korea	Japan
80% MeOH extract	60.0±0.9	46.1±0.4
n-Hexane fraction	> 100	> 100
EtOAc fraction	49.3±3.3	37.5±1.4
n-Butanol fraction	45.3±2.3	32.0±2.01
Aqueous fraction	> 100	72.7±13.5
Ascorbic acid	0.6±0.01	
a-Tocopherol	1.2±0.02	
BHT	37.6±0.7	

[z] The amount of RC_{50} required for a 50% reduction of DPPH after 30 min; each value is the mean±standard deviation of triplicate experiments.

3.2. Reducing Ability Assay

The capacity of sudachi to reduce Fe^{3+} to Fe^{2+} is shown in Figure 1. As is evident, among the extracts and fractions, the greatest reducing power was observed for the EtOAc fraction, followed by the *n*-butanol fraction, the 80% MeOH extract, the aqueous fraction, and the *n*-hexane fraction. There is agreement between the samples from both countries, and, according to these data, there is no significant difference between Korean and Japanese sudachi, except for the EtOAc fraction; in this case, Japanese sudachi exhibited a greater reducing ability than Korean sudachi. Although the EtOAc fractions from both countries exhibited the highest reducing ability relative to the other fractions, their values were not higher than the reference compounds (*α*-tocopherol and BHT). However, the 30 µg/mL concentration of the EtOAc fraction for the Japanese sudachi exhibited a similar reducing ability to that of the 20 µg/mL concentration of *α*-tocopherol and BHT. Venkatachalam and Muthukrishnan (2012) indicated that inherent reductive components in the sample serve as useful indicators of potential antioxidative activity. In the EtOAc fraction, there might be increased reduction due to vitamin C, for example, which has a ketone and enediol structure.

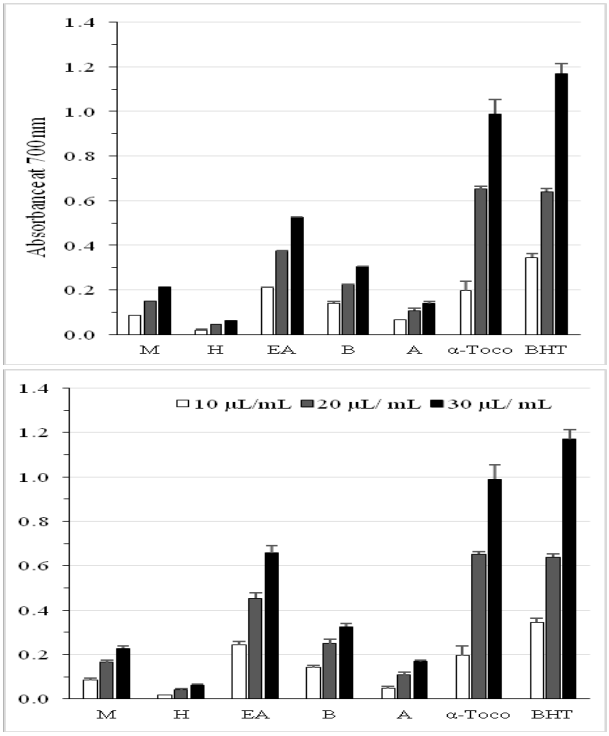

Figure 1. The reducing power of *Citrus sudachi* Hort. ex Shirai in Korea (up) and Japan (down). M: 80% MeOH; H: *n*-hexane; EA: EtOAc; B: *n*-butanol; A: aqueous; *α*-Toco: *α*- tocopherol.

3.3. Total Phenol and Flavonoid Content

According to Table 2, the highest phenol concentrations were in the EtOAc fractions (2,420.0±17.5 mg GAE/g for the Korean sudachi and 2,041.6±96.0 mg GAE/g for the Japanese sudachi). The *n*-hexane fraction exhibited the lowest phenol content, with values of 204.9±5.3 mg GAE/g for the Korean sudachi and 192.6±12.8 mg GAE/g for the Japanese sudachi. Comparing Korean and Japanese sudachi, Korean sudachi showed a higher total phenol content in 80% MeOH extract (1,157.1±17.5 mg GAE/g), the EtOAc fraction (2,420.0±17.5 mg GAE/g), and the *n*-butanol fraction (2,011.0±105.1 mg GAE/g), whereas the Japanese sudachi showed a higher total phenol content in the aqueous fraction (707.1±32.0 mg GAE/g). For the flavonoid content in the Korean sudachi, the values ranged from 12.4±0.0 mg QE/g for the aqueous fraction to 445.9±12.5 mg QE/g for the EtOAc fraction. For the Japanese sudachi, the flavonoid content ranged from 63.2±3.1 mg QE/g for the aqueous fraction to 496.8±15.6 mg QE/g for the EtOAc fraction. Both Korean and Japanese sudachi demonstrated the highest total flavonoid concentration in the EtOAc fraction and the lowest in the aqueous fraction. Interestingly, although the *n*-hexane fraction exhibited the lowest total phenol content, it showed the second highest total flavonoid content. Overall, Japanese sudachi exhibited a slightly higher flavonoid content. As illustrated in Table 2, these trends are in agreement with the report from Ao *et al*. (2008). In addition, these data (2,420.0±17.5 mg GAE/g for the Korean sudachi and 2,041.6±96.0 mg GAE/g for the Japanese sudachi in the EtOAc fraction; and 204.9±5.3 mg GAE/g for the Korean sudachi and 192.9±12.8 mg GAE/g for the Japanese sudachi in the *n*-hexane fraction) were higher than those reported by Jan *et al*. (2013) (31.5±2.4 mg GAE/g for the EtOAc fraction and 16.7±1.3 mg GAE/g for the *n*-hexane fraction). The higher phenolic content might be attributed to their hydroxyl groups, which are directly related to the antioxidant activity. Hydroxyl groups have the important role of eliminating ROS. However, these special structures are capable of chelating with metal ions, which are responsible for producing ROS, thus revealing greater antioxidant activity (Heijne *et al*., 2001). The fractions showing a higher total phenol content in Table 2 might contain high levels of hydroxyl groups.

For the total flavonoid content, Ghasemi *et al*. (2009) investigated the peel and tissue of thirteen citrus fruits, including lemon, grapefruit, and oranges, and pointed out that the peel had a higher flavonoid content. However, the flavonoid content obtained in the present investigation was higher than their result, except for the aqueous fraction of Korean sudachi. In addition, Khatiwora *et al*. (2010) determined the total flavonoid content of *Ipomoea carnea* leaves, stems, and flowers. The results indicated that all parts had a relatively high flavonoid content. The flowers had the highest flavonoid content (422 mg QE/g); these values are similar to values from the present study for the EtOAc fractions in Korean and Japanese sudachi.

Table 2. Total phenol and flavonoid content of *Citrus sudachi* Hort. ex Shirai extract and its fractions.

Extract and fraction	TPC[z] (mg GAE/g)		TFC[zz] (mg QE/g)	
	Korea	Japan	Korea	Japan
80% MeOH extract	1,157.1±17.5	1,091.6±103.7	123.0±6.3	125.2±3.1
n-Hexane fraction	204.9±5.3	192.9±12.8	169.4±3.1	213.7±3.1
EtOAc fraction	2,420.0±17.5	2,041.6±96.0	445.9±12.5	496.8±15.6
n-Butanol fraction	2,011.0±105.1	2,002.0±121.6	138.5±9.4	169.4±9.4
Aqueous fraction	464.3±10.5	707.1±32.0	12.4±0.0	63.2±3.1

[z] TPC: Total phenolic content. Total phenolic content analyzed as gallic acid equivalent (GAE) mg/g of extract; values are the average of triplicates.

[zz] TFC: Total flavonoid content. Total flavonoid content analyzed as quercetin equivalent (QE) mg/g of extract; values are the average of triplicates.

3.4. α-Glucosidase Inhibitory Activity Assay

In this study, to evaluate the degree to which the samples exhibited the release of *p*-nitrophenol from *p*NPG, the IC_{50} value was calculated. As shown in Table 3, the inhibitory range is from 356.6±2.5 µg/mL to 41.9±0.5 µg/mL for Korean sudachi and 346.8±8.8 µg/mL to 40.0±0.7 µg/mL for Japanese sudachi. The *n*-butanol fraction exhibited the highest *α*-glucosidase inhibitory activity (41.9±0.5 µg/mL for Korean sudachi and 40.0±0.7 µg/mL for Japanese sudachi), while the 80% MeOH extract showed the lowest inhibitory activity (356.6±2.5 µg/mL for the Korean sudachi and 346.8±8.8 µg/mL for the Japanese sudachi). The *n*-hexane and aqueous fractions from both countries did not show any significant activity under the set environment. Japanese sudachi demonstrated greater inhibition than Korean sudachi. The *n*-butanol and EtOAc fractions of Korean and Japanese sudachi showed greater inhibition than that of acarbose (90.8±1.8 µg/mL), but the other extracts and fractions showed lower values than that of the positive reference. Jeong *et al*. (2013) performed *α*-glucosidase activity assays with *Rehmannia glutinosa* tuberous roots, discovering that acetone and EtOAc fractions showed poor *α*-glucosidase activity. These same fractions possessed the highest and second highest phenol (flavonoid) content. Their study suggested that phenol and flavonoid probably do not interact with the activity of *α*-glucosidase, and may contain non-polyphenolic active compounds such as polysaccharide (Chen *et al*., 2009). However, the present study exhibited results that were more similar to those described by Wongsa *et al*. (2012), as the EtOAc or *n*-butanol fraction demonstrated greater phenol and flavonoid content and *α*-glucosidase activity. Through this study, it is predicted that a higher phenol and flavonoid content leads to a greater potential *α*-glucosidase activity.

Table 3. The IC_{50} values of *α*-glucosidase inhibitory activity assay of the extracts and fractions**.**

Extract and fractions	IC_{50} (µg/mL)[z]	
	Korea	Japan
80% MeOH extract	356.6±2.5	346.8±8.8
n-Hexane fraction	> 500.0	> 500.0
EtOAc fraction	55.1±1.3	52.5±1.5
n-Butanol fraction	41.9±0.5	40.0±0.7
Aqueous fraction	> 500.0	> 500.0
Acarbose	90.8±1.8	

[z] IC_{50}: The concentration required to inhibit 50% *α*-glucosidase activity under the study condition.

3.5. Minimum Inhibitory Concentration (MIC) Determination

The MIC of the extracts and fractions against the tested strains are presented in Table 4. As shown, the 80% MeOH extract, the *n*-butanol fraction, and the aqueous fraction exhibited low inhibitory activity, whereas the *n*-hexane and EtOAc fractions showed some inhibitory activity. Inhibitory activity was found to be particularly high with the *n*-hexane and EtOAc fractions against *Staphylococcus epidermidis*. Also, the gram-positive bacteria exhibited more sensitivity than the gram-negative bacteria. Overall, Korean sudachi exhibited greater inhibition activity compared to the Japanese sudachi, and the inhibitory effect of Korean sudachi for the EtOAc fraction against *Bacillus subtilis* subsp. *spizizenii* and *Staphylococcus epidermidis* were twice that of Japanese sudachi. According to Blanco *et al*. (2005), antimicrobial activity mostly relies on phenol content, such as epigallocatechin gallate; however, the current study indicates otherwise. Table 2 shows that the EtOAc fraction had the highest total phenol content followed by the *n*-butanol fraction; however, the *n*-butanol fraction demonstrated a low antimicrobial effect against all tested bacterial strains, while the *n*-hexane fraction inhibited *Staphylococcus epidermidis* at a concentration of 500 µg/mL. Perhaps other compounds that could not be measured in the total phenol and flavonoid assay may also work as antimicrobial agents (Hyun *et al*., 2014). Also, it is believed that gram-positive bacteria are more susceptible to antimicrobial effects due to differences in their cellular mechanisms compared to gram-negative bacteria (Malanovic and Lohner, 2016). Though Çördük *et al*. (2017) previously reported that *Digitalis trojana* Ivanina plant extracts effectively inhibited the growth of gram-negative bacteria, sudachi may contain compounds that interfere with the antimicrobial defense of gram-positive bacteria. Singh *et al*. (2016) also reported the more inhibition of gram-negative bacteria from the *Sapindus mukorossi* plant extracts. Furthermore, Tomotake *et al*. (2006) reported that sudachi favorably constrained eight species of *Vibrio* strains multiplication when using disk diffusion methods (a clear zone of >5 mm in diameter for *V. alginolyticus* 6624 and *V. anguillarum* NCMB 829). These findings support the conclusion that most effective antimicrobial compounds are in the EtOAc fraction.

Table 4. The minimum inhibitory concentration (MIC) of each extract and fraction against selected strains of bacteria.

	Extracts and fractions	MIC (μg/mL)					
		B.S (+)	S.Ep (+)	M.L (+)	S.En (-)	E.C (-)	K.P (-)
Korea	80% MeOH extract	-	-	-	-	-	-
	n-hexane fraction	-	500.0	-	-	-	-
	EtOAc fraction	62.5	125.0	500.0	-	-	-
	n-butanol fraction	-	-	-	-	-	-
	Aqueous fraction	-	-	-	-	-	-
Japan	80% MeOH extract	-	-	-	-	-	-
	n-hexane fraction	-	500.0	-	-	-	-
	EtOAc fraction	125.0	250.0	500.0	-	-	-
	n-butanol fraction	-	-	-	-	-	-
	Aqueous fraction	-	-	-	-	-	-

B.S: *Bacillus subtilis* subsp. *spizizenii* KACC 14741; S.Ep: *Staphylococcus epidermidis* KACC 14822; M.L: *Micrococcus luteus* KACC 14819; S.En: *Salmonella enterica* subsp. *enterica* KACC 10769; E.C: *Escherichia coli* KACC 14818; K.P: *Klebsiella pneumoniae* KACC 14816; - : > 1,000 μg/mL.

3.6. Analysis of Phenolic Compounds by HPLC

Four phenolic compounds were identified from the retention time and UV spectra of standard as summarized in Table 5. The four phenolic compounds identified by HPLC were rutin, naringin, hesperidin, and hesperetin. The retention time for each of the flavonoids was: rutin, 7.73 minutes for the Korean sudachi and 7.52 minutes for the Japanese sudachi; naringin, 13.57 minutes for both countries; hesperidin, 16.78 minutes for both countries; and hesperetin, 27.95 minutes for the Korean sudachi and 27.98 minutes for the Japanese sudachi. There was no significant difference in the naringin content between the Korean and Japanese samples. Hesperidin showed the highest content of all four flavonoids identified. Korean sudachi contained more hesperidin and hesperetin (33.6±3.2 μg/mg and 3.5±0.2 μg/mg, respectively), whereas Japanese sudachi contained more rutin (14.6±0.4 μg/mg). Nakagawa *et al*. (2006) isolated twenty-seven known compounds from the peel of sudachi—including naringin, hesperidin, hesperetin, and five new compounds—and analyzed their structural composition. In the present study, however, rutin was detected. It can be assumed that this is related to a difference in fruit quality, variety, harvest season, or the sample preparation method (Kumazawa *et al*., 2007). Nogata *et al*. (2006) isolated compounds from forty-two citrus fruits, and found that the peel of sudachi contained greater amounts of rutin (121 mg/100g fresh weight), naringin (70.7 mg/100g fresh weight), and hesperidin (38.9 mg/100g fresh weight). Nonetheless, the current study shows that hesperidin had the highest amount (33.6±3.2 μg/mg DW for the Korean sudachi and 25.2±0.2 μg/mg DW for the Japanese sudachi), followed by naringin (15.1±0.9 μg/mg DW for Korean sudachi and 15.1±0.3 μg/mg DW for the Japanese sudachi), rutin (10.6±0.6 μg/mg DW for the Korean sudachi and 14.57±0.42 μg/mg DW for Japanese sudachi), and hesperetin (3.5±0.2 μg/mg DW for the Korean sudachi and 1.8±0.1 μg/mg DW for the Japanese sudachi).

Rutin is known as a strong antioxidant that attenuates senescence. According to Yang *et al.* (2008), rutin demonstrated greater DPPH radical scavenging activity and reductive capability than vitamin C and BHT. In their study, at a concentration of 50 μg/mL, rutin showed a slightly lower value than vitamin C (92.8% and 90.4%, respectively) but a much higher value than BHT (58.8%). These data clearly demonstrate that rutin has significant properties as a radical inhibitor or scavenger. Through the HPLC analysis in this study, rutin was found to be 10.6±0.6 μg/mg DW for the Korean sudachi and 14.6±0.4 μg/mg DW for the Japanese sudachi, revealing that Japanese sudachi contains 28% more rutin than Korean sudachi. This fact may be one of the reasons that Japanese sudachi exhibited greater DPPH radical scavenging activity and reducing ability in the current investigation. Furthermore, hesperetin, which has a low concentration in citrus fruits, may play an important role as a potent antimicrobial agent. Moon *et al*. (2013) performed a disk diffusion assay with three strains of *H. pylori* and showed more than 20 mm inhibition at concentrations ranging from 5 to 20 mM. This study showed that Korean sudachi demonstrated marginally greater inhibitory activity against *Bacillus subtilis* subsp. *spizizenii* and *Staphylococcus epidermidis*. Hesperetin may be partially responsible for the antimicrobial activity in the present study.

Table 5. Analysis of flavonoid for *Citrus sudachi* Hort. ex Shirai by HPLC.

	Compounds	Retention time (min)	Content μg/mg DW
Korea	Rutin	7.73	10.6±0.6
	Naringin	13.57	15.1±0.9
	Hesperidin	16.78	33.6±3.2
	Hesperetin	27.95	3.5±0.2
	Total		62.7
Japan	Rutin	7.52	14.6±0.4
	Naringin	13.57	15.1±0.3
	Hesperidin	16.78	25.2±0.2
	Hesperetin	27.98	1.3±0.1
	Total		56.7

3.7. Correlation among Phenolic Contents, Antioxidative, and α-Glucosidase Inhibitory Activity

To understand the relationships between the phenolic compound content and DPPH radical scavenging activity, reducing power, and α-glucosidase inhibitory activity, correlation analyses were performed. Table 6 shows the intercorrelation between the phenolic compound content and each assay. As displayed in Table 6, the total phenolic compound content is strongly correlated with DPPH radical scavenging activity (-0.762, $P < 0.001$), reducing power (0.879, $P < 0.001$), and α-glucosidase inhibitory activity (-0.97, $P < 0.001$). These negative correlations indicate that the extracts and fractions of sudachi with a

greater total phenolic compound content exhibit greater antioxidative and α-glucosidase inhibitory activity; conversely, the extracts and fractions with the lowest phenolic compound content showed the lowest reducing ability. As for the total phenolic compound content, the total flavonoid content was also highly correlated with the reducing power (0.806, $P < 0.001$) and the α-glucosidase inhibitory activity (-0.613, $P < 0.010$). However, the total flavonoid content and the DPPH radical scavenging activity (-0.15, $P > 0.050$) demonstrated a poor correlation. These results showed that the total content of phenol and flavonoid is strongly correlated with the inhibition of α-glucosidase, and in particular the total phenol content influences the antioxidative activity, as shown by Beretta *et al*. (2017) and Ngamdee *et al*. (2016). These findings, which correlated linearly between each parameter, are similar to the results obtained from pressurized methanolic extracts of oenological wood (Alanon *et al*., 2015), indicating that the linear coefficient may be considered as a useful indicator to evaluate the antioxidative activity.

Table 6. The correlation between antioxidative, α-glucosidase inhibitory assay, and total phenol and flavonoid**.**

	DPPH	RP[z)]	TPC	TFC	IC_{50}[zz)]
DPPH	1.000	-0.659**[zzz)]	-0.762***[zzzz)]	-0.150	0.646**
RP		1.000	0.879***	0.806***	-0.850***
TPC			1.000	0.593**	-0.970***
TFC				1.000	-0.613**
IC_{50}					1.000

[z)] RP: The value of the reducing power assay.

[zz)] IC_{50}:The value of α-glucosidase inhibitory assay.

[zzz)] **: Significance $P < 0.010$ compared to control.

[zzzz)] ***: Significance $P < 0.001$ compared to control.

4. Conclusions

This study presented a promising prediction regarding the effective use of sudachi peel in terms of its antioxidative effects, antimicrobial properties, and the inhibition of α-glucosidase. To compare the sudachi from Korea with that of Japan, the researchers investigated various *in vitro* assays—the DPPH radical scavenging assay, reducing power assay, total phenol and flavonoid assays, α-glucosidase inhibitory activity assay, and MIC assay. The findings in this study suggest that the EtOAc and *n*-butanol fractions demonstrated higher activities than the MeOH extract and other fractions. Also, the Japanese sudachi exhibited greater antioxidative and α-glucosidase inhibitory activities than Korean sudachi, while Korean sudachi demonstrated greater microbial inhibition. These activities mostly rely on phenol compounds. This is the first report that compares sudachi from Korea to that of Japan. The results of this study provide data about the potentially beneficial compounds contained in sudachi and could be used to help develop effective methods for utilizing the abundant sudachi peel waste coming from many processing facilities.

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References

Akaike M, Aihara K, Yanagawa H, Iwase T, Yoshida S, Sato C *et al*. 2014. Efficacy and safety of *Citrus sudachi* peel in obese adults: A randomized, double-blind, pilot study. *Funct Food Health Dis.*, **4**:276-284.

Akdaş ZZ and Bakkalbaşı E. 2017. Influence of different cooking methods on color, bioactive compounds, and antioxidant activity of kale. *Int J Food Prop.*, **20**: 877-887.

Alanon ME, García-Ruíz A, Díaz-Maroto MC, Perez-Coello MS and Moreno-Arribas MV. 2015. Antimicrobial and antioxidant activity of pressurized liquid extracts from oenological woods. *Food Control*, **50**:581-588.

Ali J, Das B and Saikia T. 2017. Antimicrobial activity of lemon peel (*Citrus Limon*) extract. *Int J Curr Pharm Res.*, **9**:79-82.

Anbudhasan P, Surendraraj A, Karkuzhali S and Sathishkumaran P. 2014. Natural antioxidants and its benefits. *Int J Food Nutr Sci.*, **3**:225-232.

Ao C, Li A, Elzaawely AA, Xuan TD and Tawata S. 2008. Evaluation of antioxidant and antibacterial activities of *Ficus Microcarpa L. Fil*. extract. *Food Control*, **19**:940-948.

Blanco AR, Roccaro AS, Spoto GC, Nosro A and Rusciano D. 2005. Epigallocatechin gallate inhibits biofilm formation by ocular staphylococcal isolates. *Antimicrob Agents Chemother.*, **49**: 4339–4343.

Beretta HV, Bannound F, Insani M, Berli F, Hirscheger P, Galmorini CR *et al*. 2017. Relation between bioactive compound content and the antiplatelet and antioxidant activities of six Allium vegetable spices. *Food Technol Biotechnol.*, **55**:266-275

Chen H, Qu Z, Fu L, Dong P and Zhang X. 2009. Physicochemical properties and antioxidant capacity of 3 polysaccharides from green tea, oolong tea, and black tea. *J Food Sci.*, **74**:469-474.

Çördük N, Demirbas S and Dogru NH. 2017. A comparative study of the antimicrobial properties and antioxidant enzyme activities of field-grown and *in vitro*-propagated plants of endemic *Digitalis trojana* Ivanina. *Arch Biol Sci.*, **69**:603-610

Djordjevic TM, Šiler-Marinkovic SS and Dimitrijevic-Brankovic SI. 2011. Antioxidant activity and total phenolic content in some cererals and legumes. *Int J Food Prop.*, **14**:175-184.

Entezari M, Majd A, Falahian F, Mehrabian S, Hashemi M and Lajimi AA. 2009. Antimutagenicity and anticancer effects of *Citrus medica* fruit juice. *Acta Med Iranica*, **47**: 373-378.

Ghasemi K, Ghasemi Y and Ebrahemzadeh MA. 2009. Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. *Pak J Pharm Sci.*, **22**:277-281.

Heijne CGM, Haenen GRMM, van Acker FAA, van der Vijgh WJF and Bast A. 2001. Flavonoids as peroxidynitrite scavengers: the role of the hydroxyl groups. *Toxicol In Vitro*, **15**: 3-6.

Hwang JH, Park KY, Oh YS and Lim SB. 2013. Phenolic compound content and antioxidant activity of citrus peels. *J Korean Soc Food Sci Nutr.*, **42**:153-160.

Hyun TK, Kim HC and Kim JS. 2014. Antioxidant and antidiabetic activity of *Thymus quinquecostatus* Celak. *Ind Crop Prod.*, **52**:611-616.

Jamuna KS, Ramesh CK, Srinivisa TR and Raghu KL. 2010. Comparative studies on DPPH and reducing power antioxidant properties in aqueous extracts of some common fruits. *J Pharm Res.*, **3**:2378-2380.

Jan S, Khan MR, Rashid U and Bokhari J. 2013. Assessment of antioxidant potential, total phenolics and flavonoids of different solvent fractions of *Monotheca Buxifolia* fruit. *Osong Public Health Res Perspect.*, **4**:246-254.

Jayanthi P and Lalitha P. 2011. Reducing power of the solvent extracts of *Eichhornia crassipes* (Mart.) Solms. *Int J Pharm Pharm Sci.*, **3**:126-128.

Jeong HJ, Kim JS, Hyun TK, Yang J, Kang HH, Cho JC *et al*. 2013. *In vitro* antioxidant and antidiabetic activities of *Rehmannia glutinosa* tuberous root extracts. *Sci Asia*, **39**:605-609.

Jeong JH, Lee JW, Kim KS, Kim JS, Han SN, Yu CY *et al*. 2010. Antioxidant and antimicrobial activities of extracts from a medical plant, sea buckthorn. *J Korean Soc Appl Biol Chem.*, **53**:33-38.

Jiménez-Monreal AM, Garcia-Diz L, Martínez-Tomé M, Mariscal M, Marcia MA. 2009. Influence of cooking methods on antioxidant activity of vegetables. *J Food Sci.*, **74**: 97-113.

Karsheva M, Kirova E, Alexandrova S and Georgieva S. 2013. Comparison of citrus peels as a source of variable components-polyphenol and antioxidant. *J Chem Technol Metallurgy*, **48**: 475-478.

Khatiwora E, Adsul VB, Kulkarni MM, Deshpande NR and Kashalkar RV. 2010. Spectroscopic determination of total phenol and flavonoid contents of *Ipomoea carnea*. *Int J Chem Tech Res.*, **2**:1698-1701.

Kim JH and Kim MY. 2016. Apoptotic properties of *Citrus sudachi* Hort, ex Shirai (Rutaceae) extract on human A549 and HepG2 cancer cells. *Trop J Pharm Res.*, **15**: 1167-1174.

Kim JS. 2014. Antioxidant, α-glucosidase inhibitory and antimicrobial activities of extracts from *Maesa japonica* (Thunb.). *Korean J Med Crop Sci.*, **22**:289-294.

Kücükakin B, Gögenur I, Reiter RJ and Rosenberg J. 2009. Oxidative stress in relation to surgery: Is there a role for the antioxidant melatonin? *J Surg Res.*, **152**:338-347.

Kumazawa S, Ikenaga M, Usui Y, Kajiya K, Miwa S, Endo J *et al*. 2007. Comprehensive analysis of polyphenols in fruits consumed in Japan. *Food Sci Technol Res.*, **13**:404-413.

Lee JE, Kim JH and Kim MY. 2015. Changes in phenolic composition, antioxidant and antidiabetic properties of Jeju *Citrus sudachi* as influenced by maturity. *J Life Sci.*, **25**: 1311-1318.

Malanovic N and Lohner K. 2016. Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *Biochim Biophys Acta*, **1858**: 936-946.

Menković N, Živkovic J, Šavikin K, Gođevac D and Zdunić G. 2014. Phenolic composition and free radical scavenging activity of wine produced from the Serbian autochthonous grape variety Prokupac-A model approach. *J Serb Chem Soc.*, **79**:11-24.

Moon SH, Lee JH, Kim KT, Park YS, Nah SY, Ahn DU *et al*. 2013. Antimicrobial effect of 7-*O*-Butylnaringenin, a novel flavonoid and various natural flavonoids against *Helicobacter pylori* strains. *Int J Env Res Pub He*, **10**:5459-5469.

Nakagawa H, Takahashi Y, Tanaka N, Tsuchiya K, Shibata H and Higuti, T. 2006. Chemical constituents from the peel of *Citrus sudachi*. *J Nat Prod.*, **69**:1177-1179.

Nakamura M, Ra JH and Kim JS. 2016. The comparative analysis of antioxidant and biological activity for the *Dendropanax morbifera* LEV. leaves extracted by different ethanol concentrations. *Yakugaku Zasshi*, **136**:1285-1296.

Nakamura M, Ra JH, Jee Y and Kim JS. 2017. Impact of different partitioned solvents on chemical composition and bioavailability of *Sasa quelpaertensis* Nakai leaf extract. *J Food Drug Anal.*, **25**:316-326.

Ngamdee P, Wichai U and Jiamyngyuen S. 2016. Correlation between phytochemical and mineral contents and antioxidant activity of black glutinous rice bran, and its potential chemopreventive property. *Food Technol Biotechnol.*, **54:**282-289

Nogata Y, Sakamoto K, Shiratsuchi H, Ishii T, Yano M and Ohta H. 2006. Flavonoid composition of fruit tissues of citrus species. *Biosci Biotechnol Biochem.*, **70**:178-192.

Saha D and Paul S. 2014. Evaluation of antioxidant and free radical scavenging activities of different fractions of *Pterospermum suberifolium* leaf extract. *Thai J Pharm Sci.*, **38**:28-35.

Sindhi V, Gupta V, Sharma K, Bhatnagar S, Kumari R and Dhaka N. 2013. Potential applications of antioxidants – A review. *J Pharm Res.*, **7**:828-835.

Singh R and Kumari N. 2015. Comparative determination of phytochemicals and antioxidant activity from leaf and fruit of *Sapindus mukorrossi* Gaertn. – A valuable medicinal tree. *Ind Crops Prod.*, **73**:1-8

Singh R, Kumari N and Nath G. 2016. Free radical scavenging activity and antimicrobial potential of leaf and fruit extracts of *Sapindus mukorossi* Gaertn. against clinical pathogen. *Int J Phytomed.***, 8:**22-28

Tomotake H, Koga T, Yamamoto M, Kassu A and Ota F. 2006. Antibacterial activity of citrus fruit juices against vibrio species. *J Nutr Sci Vitaminol.*, **52**: 157-160.

Tsukayama M, Sasaki T, Yamamoto K, Kawamura Y and Ichikawa R. 2010. Microwave-assisted extraction and methylation of useful flavones from waste peels of *Citrus sudachi*. *J Jpn Soc Food Sci.*, **57**:427-433.

Tsutsumi R, Yoshida T, Nii Y, Okahisa N, Iwata S, Tsukayama M *et al*. 2014. Sudachitin, a polymethoxylated flavone, improves glucose and lipid metabolism by increasing mitochondrial biogenesis in skeletal muscle. *Nutr Metab.*, **11**:1-14.

Orhan I and Üstün O. 2011. Determination of total phenol content, antioxidant activity and acetylcholinesterase inhibition in selected mushrooms from Turkey. *J Food Comp Anal.*, **24**: 386-390.

Vasu P, Khan ND, Khan ZH and Mular SM. 2017. *In Vitro* antidiabetic activity of methanol extract of *Citrus limon, Punica granatum, Musa acuminata* peel. *Int J Appl Res.*, 3:804-806

Venkatachalam U and Muthukrishnan S. 2012. Free radical scavenging activity of ethanolic extract of *Desmodium gangeticum*. *J Acute Med.*, **2**:36-42.

Wongsa P, Chaiwarit J and Zamaludien A. 2012. In vitro screening of phenolic compounds, potential inhibition against α-amylase and α-glucosidase of culinary herbs in Thailand. *Food Chem.*, **131**:964-971.

Yang J, Guo J and Yuan J. 2008. In vitro antioxidant properties of rutin. *LWT-Food Sci Technol.*, **41**:1060-1066.

Yang J, Kim JS, Sa JY, Kim MO, Jeong HJ, Yu CY *et al*. 2011. Antioxidant, antibacterial and α-glucosidase inhibitory activities of different extracts of Cortex Moutan. *Afr J Biotechnol.*, **10**: 9438- 9444.

Yu L, Perret J, Davy B, Wilson J and Melby CL. 2002. Antioxidant properties of cereal products. *J Food Sci.*, **67**:2600-2603.