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Biochemical Compositions and Biological Activities of Extracts from 3 Species of Korean Pine Needles

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Abstract Crude extracts of pine needles have long been used as a health-food and for cosmetics. In this study, we determined the biochemical composition and biological activities of extracts from three pine species: red pine (RP, *Pinus densiflora* S et Z), Keumkang pine (KP, *Pinus densiflora* for. erecta) and sea pine (SP, *Pinus thunbergii*). The SP extract had the highest levels of moisture, ash, crude protein, and lipids based on the dry weight. The SP extract also had the highest level of polyunsaturated fatty acids (PUFAs). The SP extract had much more β-pinene, β-caryophyllene, and germacrene-D than extracts from the other species. Total phenolic content was the highest in the 100% ethanol extract of SP, and the 50% ethanol extract of SP had the highest DPPH radical scavenging activity. The SP extract had the greatest antimicrobial effect. These results indicate that SP has the greatest potential as a natural antioxidant resource and raw material for cosmetic-goods.

Keywords: *pine needles, biochemical composition, nutritive value, polyphenol, physiological activity*

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1. Introduction

Research on substances in plant extracts has demonstrated that the wood and leaves of some plants have abundant bioactive agents. However, most of the plants used in this previous research were broadleaved trees. Research on extracts from coniferous tree barks is an emerging area in this field [1]. Pine trees are evergreen, coniferous, and resinous trees. There are more than 100 known species worldwide, and about 30-40 species are native to Asia and Europe. Pine species in Asia primarily grow in East Asia, including Korea, Japan, and China [2]. The major pines in Korea are red pine (RP; *Pinus densiflora* S. et Z.), which grows inland, pitch pines (*Pinus rigida* Mill), Keumkang pines (KP; *Pinus densiflora* for. erecta), which has a thin yet hard bark, and sea pines (SP; *Pinus thunbergii*), which grows in coastal regions [3]. Pine needles have a somewhat bitter taste, and can be harvested between spring and fall. Various parts of pines, including the needles, cones, barks, and pollens, have been used in traditional medicines and health foods [4]. Flavor compounds of pine needle tea have biological activities and may be effective in treating bronchial asthma, arteriosclerosis, and inflammation, and pine needle powder, tea, liquor, and soft drinks are commercially available as supplements or health foods [5]. The functional ingredients of pine extracts may promote metabolism by increasing the discharge of metabolic wastes, and they may also be effective in treatment of cardiovascular and skin diseases [5,6]. Pine barks have commonly been used in folk remedies because of their strong hemostatic, anti-inflammatory, and analgesic properties [7].

Pine needles are a traditional folk remedy in Korea and were used as a food source during famines. They are considered desirable due to their affordability and the presence of bioactive substances [8]. Pine needle extracts contain antioxidants that have may have anti-cancer effects, aide in heavy metal detoxification, and have antibacterial and anti-inflammatory effects [6]. They may also improve serum lipid metabolism and have cytotoxic and inhibit the effects of free radicals. Pine extracts are used in soaps, essential oils, hangover relief agents, and health drinks due to their antioxidant effects [1,9]. Moreover, pine needle extracts may be effective in treatment of arteriosclerosis and diabetes due to their high content of vitamins and iron, as well as high-quality proteins, lipids, and dietary fiber [3]. The high contents of unsaturated fatty acids (USFA) and aromatic agents in pine needles may be responsible for their antioxidant, and putative anti-aging, anti-microbial, and anti-cancer activities [10,11,12]. In particular, α-pinene, myrcene, and terpinene are aromatic hydrocarbons from pine needle extracts that have higher antioxidant activities than vitamin E [13], and may therefore be useful for functional foods and cosmetic products.

This study aim to study the nutritional values of pine needles from RP, KP, and SP that were growing near Uljin, Gyeongbuk (Korea). In particular, we analyzed the biochemical compositions of these pine needles, including

general components, fatty acids, and aromatic ingredients. We also examined pine needle extracts by conducting a comparative analysis of their bioactivities to assess their potential for use as ingredients in functional foods and cosmetic products.

2. Materials and Methods

2.1. Sample Collection and Crude Extract Preparation

Pine needle samples of RP, KP, and SP were collected near Uljin, Gyeongbuk (Korea). The needles were washed, freeze-dried, and then pulverized in the laboratory. Powdered samples (10 g) were extracted using water and organic solvents (50% and 100% ethanol). Water extraction was conducted at 65°C, and organic solvent extraction was conducted at room temperature. Each sample was mixed with 200 mL of extraction solvent (three times the volume of the sample) and stirred at 250 rpm for 24 h. The supernatant was separated by centrifugation at 5000 rpm for 5 min. The extracts were concentrated using a vacuum evaporator, and the powdered samples were stored at –20°C in darkness until use. Specific amounts of powdered samples were dissolved in extraction solvents for use in bioactivity assays.

2.2. Biochemical Compositions

Moisture content was determined by an infrared moisture analyzer (Mettler LJ 16, Greifensee, Switzerland) at 120°C, and is expressed as percentage by weight. Ash content was performed according to the AOAC [14]. Dried needles were placed in an electric oven (Robertshaw, Divisao Pyrotec) for 5 h at 525°C, and the ash content was determined gravimetrically. The crude protein content was calculated by measurement of nitrogen with a CHNS/O Analyzer (Perkin-Elmer 2400, Connecticut, USA) and multiplication by the Jones conversion factor (6.25). Crude lipids were extracted from the needle powder in a Soxhlet extractor (Soxtec System HT6, Tecator, Hoganas, Sweden) with chloroform:methanol (2:1, v/v). The crude lipid content was determined gravimetrically after overnight drying of the extract in an oven (80°C). Total dietary fiber and the soluble and insoluble fractions, were determined by the AOAC method [14]. The weight of total dietary fiber was corrected for ash and residual protein content by use of a blank.

2.3. Fatty Acids

Lipid extraction was performed based on the method of Folch et al. [15]. In particular, 5 mL of $CHCl_3$:MeOH (2:1) was added to 5 g of pine needle powder and the sample was sonicated for 20 min. Then, 5 mL of 0.58% sodium chloride was added and the sample was sonicated for an additional 10 min. The sample was then centrifuged for 5 min at 3000 rpm, the upper layer was removed, and the lower layer was transferred to another tube using a Pasteur pipette, followed by drying with nitrogen gas. A known amount of heneicosanoic acid (C21:0) was used as an internal standard. Next, 0.5 mL of toluene and 2 mL of 0.5 N NaOH were added to the dried sample, which was then incubated for 5 min in a heated bath and then cooled. BF_3MeOH was then added, and the sample was heated in the bath for 3 min and cooled again. In the final step, 15 mL of petroleum ether and 20 mL of H_2O were added, the sample was sonicated, and the supernatant was isolated and dried using nitrogen gas.

Fatty acid methyl esters (FAMEs) were analyzed by gas chromatography (GC) in a Varian CP-3800 that was equipped with an HP-Innowax silica capillary column (30 m × 0.25 mm id., 0.25 μm film thickness), using helium as the carrier gas [16]. Samples (1 mL) were injected and the column temperature was held at 50°C for 2 min, then increased at a rate of 5°C /min to 220°C, and finally maintained at 220°C for 30 min until all FAMEs of interest were eluted. The injector and flame ionization detector temperatures were both 250°C. FAMEs were identified by comparison of retention times with validated standards (Sigma-Aldrich Co., USA) and quantified using heneicosanoic acid as an internal standard.

2.4. Volatiles

Extraction and determination of volatiles from pine needle extracts were based on the method of Kim and Shin [17]. Volatile extracts were obtained by simultaneous distillation and extraction (SDE) using the Likens–Nickerson apparatus [18]. After circulating 300 mL of extraction solvent (redistilled diethyl ether) through the apparatus at 36°C, 50 g of pine needles were ground in a Waring blender (Waring, New Hartford, Connecticut, USA) and mixed with 1000 mL of distilled water in a round-bottomed flask. The SDE times were 0.5, 1.0, 1.5, and 2.0 h at pH 3.6 (control pH). Then the pH was increased to 4.6, 5.6, or 6.6, and each mixture (including the control pH mix) was extracted for 1.5 h. Anhydrous sodium sulfate (~15 g) was then added to remove the water. The ether mixture was then cooled to -20°C for 12 h, and evaporated to 1 mL using nitrogen gas. Then, 10 μL of 1-pentanol (n-amyl alcohol) was added to the extracts as the internal standard. The extracts were tested for antibacterial activity and their volatile components were analyzed.

Volatile components were analyzed by a GC/mass selective detector (MSD) using an HP-Innowax silica capillary column (30 m × 0.25 mm id., 0.25m film thickness). Helium was used as the carrier gas (flow rate: 1 mL/min). The GC oven temperature was maintained at 70°C for 5 min, then increased to 240°C at a rate of 3°C/min, and then held at 240°C for 15 min. The temperature of the injector was 250°C and that of the FID detector was 280°C. The GC split ratio was 1:50 and 0.5 mL of extract was injected per run. The mass spectra ranged from *m/e* 25 to 450. The extracted compounds were identified by use of a mass spectrum library (Wiley NBS 139) and by comparison of retention times with known standards.

2.5. Phenolics

The phenol content was measured using the Folin-Ciocalteu reagent (FCR) according to the method of Capannesi and Palchetti [19]. A 0.5 mL pine needle extract was mixed with FCR, then 1 mL of 7.5% Na_2CO_3 was added. The solution was then diluted with 8 mL of distilled water and left to stand at 65°C for 20 min. The reaction was measured at 765 nm using a spectrophotometer with gallic acid as

the standard. The phenol content is expressed as gallic acid equivalent (GAE).

2.6. DPPH-free Radical Scavenging Capacity

DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging capacity was measured based on a modification of the method of Lu and Foo [20]. DPPH solution was prepared by dissolving 100 mM of DPPH in 80% methanol. Then, 0.1 mL of pine needle extracts was added to the freshly prepared solution, followed by mixing. The solution was left to stand in a dark place at 25°C for 10 min, after which the absorbance was measured at 517 nm. The percent DPPH free radical scavenging capacity was calculated as $[(A_0-A_1)/A_0]\times100$, where A_0 is the absorbance of the control group and A_1 is the absorbance of the experimental group.

2.7. Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity was assessed according to the method of Kim et al. [21]. 0.2 mL of mushroom tyrosinase (100 unit/ml), 0.2 mL of 60 mM potassium phosphate buffer (pH 6.8), and 0.4 mL of 10 mM dihydroxyphenylalanine (DOPA) were mixed. Then 0.2 mL of pine needle extract was added to the mixture, after which the absorbance was measured at 475 nm. The percent tyrosinase inhibitory activity was calculated as $(1-A_1/A_0)\times100$, where A_0 is the absorbance of the control group and A_1 is the absorbance of the experimental group.

2.8. Antimicrobial Activity

Antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) using the broth dilution method [22]. The bacterial strains were *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. The bacteria were grown in nutrient broth for 6 h. Then, 100 µL of 106 cells/mL was added to tubes with nutrient broth that was supplemented with different concentrations (200 µL) of the extracts. After incubation at 37°C for 24 h, the MIC of each sample was determined by measuring the optical density at 620 nm in a spectrophotometer. The absorbance of each sample was compared with non-inoculated nutrient broth.

2.9. Data Analysis

Statistical analysis was performed using SPSS statistical software. Normality and homogeneity of data were verified using analysis of variance (ANOVA), and differences among experimental groups were evaluated using one-way ANOVA and Duncan's multiple range tests. All experiments were performed in triplicate and results are presented as means ± SDs.

3. Results

3.1. Biochemical Compositions

Table 1 shows the basic biochemical compositions of pine needles from RP, KP, and SP. SP had the highest levels of moisture (16.1%), ash (3.1%), protein (9.1%), and lipid (9.0%) ($p < 0.05$ for all comparisons). In contrast, KP had the highest carbohydrate content (42.0%), and RP had the highest fiber content (27.2%). The biochemical components of pine needles did not vary significantly among these 3 species; however, moisture, ash, protein, and lipid content were higher in SP extracts than in RP and KP extracts (Table 1).

3.2. Fatty Acid Contents

The fatty acid compositions of the pine needles from RP, KP, and SP were shown in Table 2. The total saturated fatty acid content was highest in RP (45.9%), and the levels of lauric acid (C12:0) and myristic acid (C14:0) were much higher in RP than in KP and SP. The total monounsaturated fatty acid content was the highest in KP (18.0%), and the levels of oleic acid (C18:1n-9) was high in the pine needles of all 3 species (8.2-13.1%). The total polyunsaturated fatty acid (PUFA) content was high in all 3 species (41.8-50.8%), especially in SP (50.8%). The levels of linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) were higher than those of other PUFAs ($p < 0.05$ for both comparisons). The results of fatty acid analysis showed that RP had the highest saturated fatty acid content, KP had the highest monounsaturated fatty acid content, and the PUFA content was about 8-9% higher in SP and KP than in RP.

Table 1. Nutritional composition (% dry weight ± SD) of pine needle extracts from red pine (RP), Keumkang pine (KP), and sea pine (SP)

Component	RP	KP	SP
Moisture	13.1±1.02	14.6±0.87	16.1±2.01
Ash	2.5±0.05	2.2±0.07	3.1±0.04
Crude protein	8.1±0.51	8.5±0.19	9.1±0.91
Crude lipid	7.4±0.71	8.9±0.58	9.0±0.12
Carbohydrate	41.7±5.28	42.0±8.12	40.9±5.21
Dietary fiber	27.2±2.28	23.8±5.21	21.8±3.28

Table 2. Fatty acid composition (% dry weight ± SD) of pine needle extracts from red pine (RP), Keumkang pine (KP), and sea pine (SP)

Fatty acid	RP	KP	SP
C12:0	16.5±4.31	7.5±1.11	8.5±2.32
C14:0	6.8±0.09	2.3±0.09	5.1±1.32
C15:0	0.4±0.02	0.2±0.02	0.4±0.09
C16:0	15.6±1.22	11.1±2.29	17.6±3.09
C17:0	1.8±0.01	0	1±0.09
C18:0	2.7±0.33	1.6±0.12	1.9±0.15
C20:0	0	8.6±3.19	0
C22:0	1.9±0.19	1.5±0.12	1.1±0.09
SFA	45.9±0.88	32.8±0.99	35.6±1.02
C14:1	0	1.1±0.25	0.5±0.11
C16:1	2.0±0.15	0.8±0.11	2.9±0.55
C17:1	1.8±0.19	2.5±0.71	2±0.15
C18:1n9c	9.1±2.23	13.1±3.15	8.2±1.19
C20:1n9	0	0.5±0.05	0
MUFA	12.9±0.85	18±0.85	13.6±0.51
C18:2n6c	12.1±2.15	15.8±3.15	20.6±4.12
C18:3n6	1.3±0.15	0	3.4±0.75
C18:3n3	27.8±3.17	25.9±4.15	26.5±5.15
C20:2	0	1±0.15	0.3±0.05
C20:3n6	0	5.3±1.15	0
C20:3n3	0	0.8±0.15	0
C20:5n3	0	0.4±0.15	0
PUFA	41.8	49.2	50.8

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: poly-unsaturated fatty acids.

3.3. Aromatic Contents

Table 3 shows the identity of all aromatic compounds that accounted for at least 1% of the total. The main compounds were β-pinene (2.04-19.57%), germacrene-D (2.26-15.46%), β-caryophyllene (5.43-10.78%), α-pinene (6.72-9.99%), α-cardinene (1.82-5.55%), β-phellandrene (2.51-5.31%), and terpinolene (0.95-4.94%). These compounds accounted for 28.56-59.16% of the total peak area, depending on the species. The main compounds in RP were germacrene-D (9.94%), α-pinene (9.88%), β-caryophyllene (6.51%), and α-cardinene (5.55%); the main compounds in KP were α-pinene (9.99%), β-caryophyllene (5.43%), β-phellandrene (5.31%), and germacrene-D (2.26%); and the main compounds in SP were β-pinene (19.57%), germacrene-D (15.46%), β-caryophyllene (10.78%), and α-pinene (6.72%). All 3 species had abundant α-pinene, β-pinene, germacrene-D, and β-caryophyllene. RP and KP had the highest levels of α-pinene, but SP had the highest level of β-pinene. Notably, the levels of β-pinene, β-caryophyllene, and germacrene-D were much higher in SP than in RP and KP ($p < 0.05$ for all comparisons). Moreover, α-terpineol, an irritant and volatile compound present in pines, was not detected in KP (data not shown). Bornyl acetate, which gives a strong forest aroma, was present in the pine needles of all 3 species.

Table 3. Aromatic compounds (% dry weight) of pine needle extracts from red pine (RP), Keumkang pine (KP), and sea pine (SP)

Compound	RP	KP	SP
α-Pinene	9.88	9.992	6.724
Camphene	2.4	1.112	0.425
β-Pinene	2.95	2.036	19.573
Myrcene	2.68	3.384	0.669
D-Limonene	2.5	1.101	1.871
β-Phellandrene	2.51	5.31	2.99
Terpinolene	4.94	1.712	0.946
Bornyl acetate	3.66	2.118	1.695
β-Caryophyllene	6.51	5.432	10.776
α-Amorphen	1.8	-	-
Germacrene-D	9.94	2.258	15.464
α-Cardinene	5.55	1.819	2.686
1,6-Germacradien-5-ol	3.14	1.243	-
Benzoic acid	1.17	2.958	0.252
Thunbergol	1.57	7.661	-
Phytol	1.19	0.494	3.395

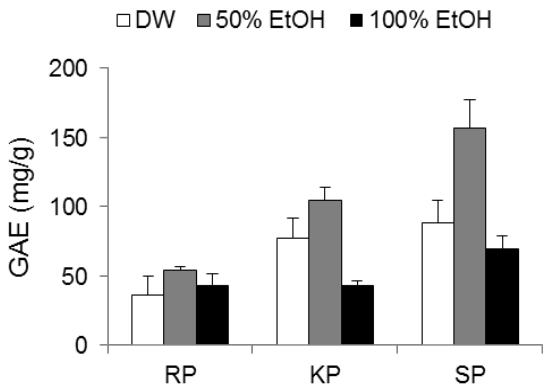

Figure 1. Total phenolic content (gallic acid equivalents, mg/g) of pine needle extracts from red pine (RP), Keumkang pine (KP), and sea pine (SP) following extraction by hot-water, 50% ethanol, and 100% ethanol

3.4. Phenolic Contents and Biological Activities

Figure 1 shows the total phenolic content of pine needles from all 3 species based on extraction in hot water (65°C), 50% ethanol, and 100% ethanol. In all 3 species, the phenolic content was highest in the 50% ethanol extracts. For the 50% ethanol extracts, the phenolic content was highest in SP (156.6 mg/g), followed by KP (104.8 mg/g) and RP (53.8 mg/g).

3.5. DPPH-free Radical Scavenging, Tyrosinase Inhibitory and Antimicrobial Activity

We also compared the antioxidant, tyrosinase inhibitory, and antimicrobial activities of pine needle extracts of the 3 species based on different methods of extraction. Based on DPPH radical scavenging capacity assay (Figure 2A), SP and KP extracts had the greatest antioxidant activities (over 90%). The antioxidant activities of RP extracts were lower and varied according to the different extraction methods (50% ethanol: 82.36%, hot water: 63.12%, 100% ethanol: 53.01%). Analysis of the tyrosinase inhibitory activity indicated large differences according to the extraction method and species. When hot water extraction was used, SP had the highest tyrosinase inhibition (69.44%), but when 50% ethanol extraction was used, KP had the highest tyrosinase inhibition (76.02%) (Figure 2B). Figure 3 shows that correlation between total phenolic content and tyrosinase inhibitory activities in the pine needle extracts. These results indicate the strong association between total phenolic content and tyrosinase inhibitory activities of RP extracts (R^2=0.9661, y=2.8758x+97.938) and KP extracts (R^2=0.9619, y=0.5904x+11.838), suggesting that phenolic compounds play an important role in the tyroxinase inhibitory activities of pine needle extracts. In addition, high correlation was observed between tyrosinase inhibitory activity and DPPH activity (R^2=0.9097, y=0.50444x-416.69) in SP extracts.

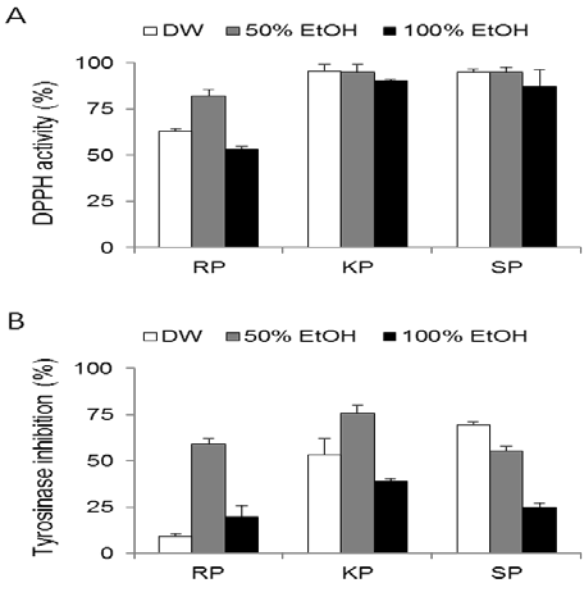

Figure 2. (A) DPPH radical-scavenging activity and (B) tyrosinase inhibitory activity of pine needle extracts from red pine (RP), Keumkang pine (KP), and sea pine (SP) following extraction by hot-water, 50% ethanol, and 100% ethanol

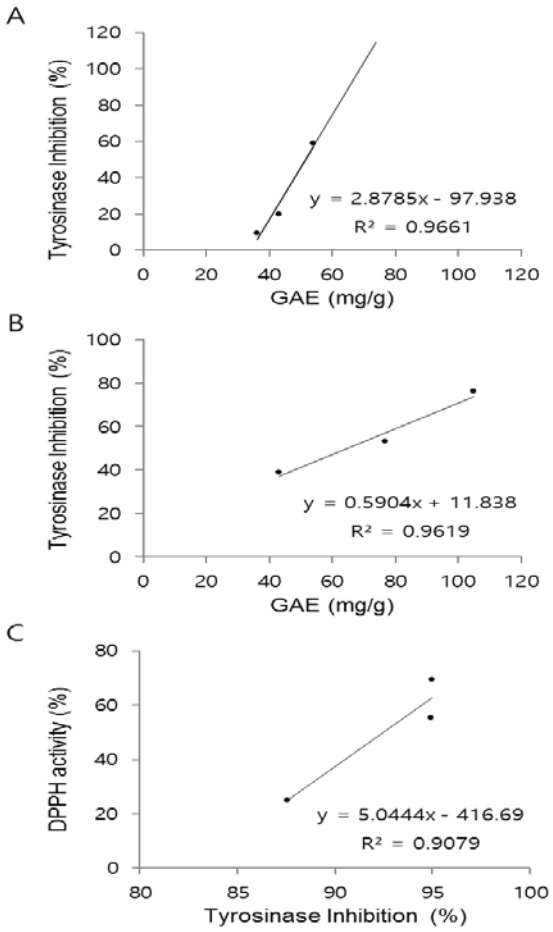

Figure 3. Correlation between phenolic content and tyrosinase inhibitory activity of red pine (A), Keumkang pine (B). Correlation between tyrosinase inhibitory activity and DPPH activity of sea pine (C)

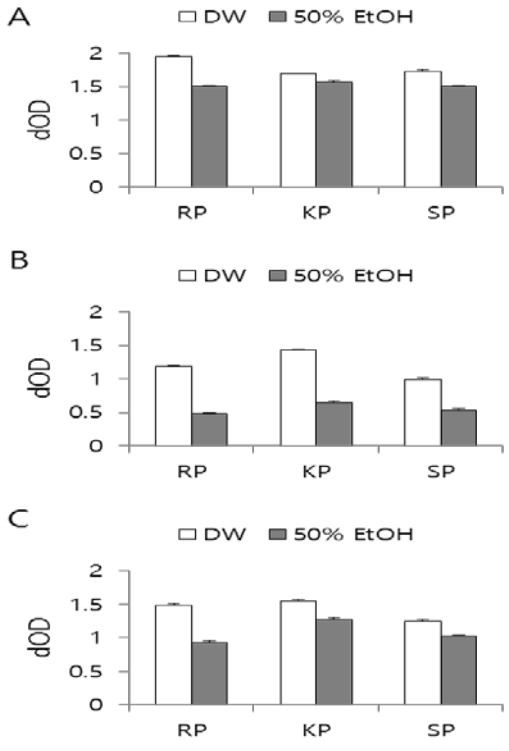

Figure 4. Antibacterial activities (ΔA620 nm) of pine needle extracts from red pine (RP), Keumkang pine (KP), and sea pine (SP) following extraction by hot-water and 50% ethanol. A, *E. coli*. B, *S. aureus*. C, *B. subtilis*. A lower ΔA620 nm value indicates greater inhibition

We examined the antimicrobial activity of the pine needle extracts by use of two Gram-positive species (*B. subtilis, S. aureus*) and a Gram-negative species (*E. coli*) (Figure 4). In all cases, the antimicrobial effect was greater (smaller ΔA620 nm) in the 50% ethanol extracts than in the hot water extracts. In general, SP had the strongest antimicrobial activity and RP had the weakest antimicrobial activity. Although the antimicrobial effect on E. coli and B. subtilis did not vary greatly among species, the antimicrobial effect on S. aureus was notably stronger in 50% ethanol extracts than in water extracts. All 3 species had strongest inhibition of *S. aureus*, and weakest inhibition of *E. coli*.

4. Discussion

This study was conducted to examine the potential uses of pine species growing near Uljin, Gyeongbuk as functional foods, cosmetics, and fragrances. We compared the extracts of pine needles from RP, KP, and SP with respect to biochemical components, fatty acids, and aromatic compounds. The results of fatty acid analysis showed that RP had the highest saturated fatty acid content, KP had the highest monounsaturated fatty acid content, and the PUFA content was about 8-9% higher in SP and KP than in RP (Table 2). Cosmetic products containing unsaturated fatty acids have skin elasticating and softening effects, and enhance the skin due to their antioxidant properties and stability [23]. The C18 series of PUFAs are important fatty acids because they are not synthesized in humans [24]. Pine needles have large quantities of α-linolenic acids and these prevent blood clots, function as a key component of cell membranes, and also function in coagulation, arterial wall contraction and relaxation, and inflammation [25]. Therefore, pine needles are great candidates for use in functional foods and pharmaceutical products.

Aromatic compounds vary significantly among pine species, and the contents of key aromatic compounds may also vary according to collection period, site, and environmental conditions [17]. In the present study, we compared the aromatic compounds of pine needle extracts by GC-MS, and found clear variations in the contents of the key aromatic compounds. Contents of α-pinene, β-pinene, germacrene-D, and β-caryophyllene were abundant in all three species of pine needles (Table 3). This result is consistent with another study which reported that the key aromatic compounds of P. nigra pine needles were α-pinene, germacrene-D, β-caryophyllene, and β-pinene [26]. In particular, the levels of major aromatic compounds such as β-pinene, β-caryophyllene, and germacrene-D were much higher in SP than in RP and KP. Therefore, pine needle extracts from SP may best suited as an ingredient in cosmetics and fragrance products due to their high content of pinene, a bioactive substance.

This study identified bioactive agents present in the pine needles of 3 species, specifically the polyphenol content, antioxidant effect, tyrosinase inhibitory activity, and antimicrobial activity. Although components extracted by hot water had bioactivity, 50% ethanol extracts had the highest levels of polyphenols, antimicrobial activity, and tyrosinase inhibitory activity. Samples had the lowest

bioactivity following extraction with 100% ethanol. Although 50% ethanol extracts had higher activity, organic solvent extraction methods are unlikely to be commercially viable because extraction with organic solvents is more expensive than extraction using water. We also found higher levels of polyphenols and antioxidant, tyrosinase inhibitory, and antimicrobial activities in SP and KP than in RP (Figure 1, Figure 2 and Figure 4). In general, SP is likely to have greater utility than KP and RP due to its higher bioactivity. The results of this study provided an important contribution to development of potential ingredients for cosmetics and foods.

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