

## Evaluation of Antioxidant Activities and Active Compounds Separated from Water Soluble Extracts of Korean Black Pine Barks

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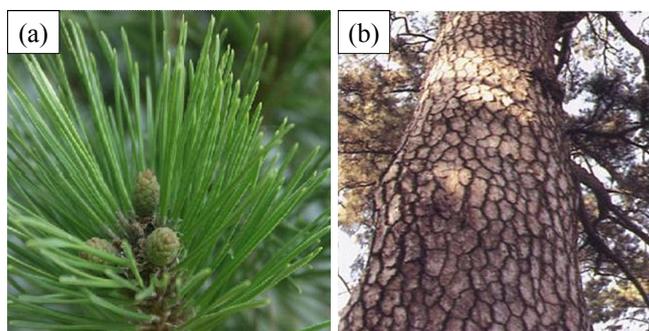
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Black pine barks from the southern region of Korea were extracted using pressurized hot water and the water soluble extracts were then separated in a stepwise fashion using a variety of solvents, column chromatography (CC), thin layer chromatography (TLC), and high pressure liquid chromatography (HPLC). The antioxidant activities of each fraction and the active compounds were determined based on the radical scavenging activities of 2,2-diphenyl-1-picrylhydrazyl (DPPH), reductive potential of ferric ion, and total phenol contents. A DPPH test showed that the half maximal effective concentration (EC<sub>50</sub> value : 6.59 ± 0.31 µg/mL) of the ethyl acetate fraction (ca. 0.67%) was almost the same as that of the control compounds and inversely proportional to the value of the total phenol contents. The cell viability of the water extracts was confirmed by methyl thiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) with enzyme linked immune sorbent assay (ELISA). Catechin, epicatechin, quercetin and ferulic acid were isolated from the ethyl acetate fraction as active compounds and identified by nuclear magnetic resonance. The antioxidant activity as value of DPPH of each of the separated compounds was lower than the ethyl acetate fraction, and ferulic acid was the lowest among these compounds.

**Key Words:** Black pine bark, Antioxidant activity, Total phenol content, Reductive potential, DPPH

### Introduction

Korean black pine (*Pinus thunbergiana*; Fig. 1) is a medium sized, irregularly shaped pyramidal tree that can resist the effects of salt spray and saline soil in the shore line better than most other plants. For centuries, Koreans have eaten the needles and bark of pine trees in the spring lean season and prepared medicinal extracts from the endodermis of black pine bark.<sup>1</sup> Over the past few years, waste barks or solids of pine trees have attracted a great deal of attention in the research arena due to their high potential for use as bio-active compounds, antioxidants, and biomedical resources.<sup>2-5</sup> Pine bark extracts contain numerous phenol compounds such as flavonoid condensates (proanthocyanidin),<sup>6</sup> natural antioxidants,<sup>7</sup> and anti-aging substances.<sup>8</sup> Recently, procyanidin-rich extracts (flavonoids mixture) from pine (*Pinus maritima*) bark, known as pycnogenol, were reported to have strong antioxidant effects,<sup>9,10</sup> maintain human circulatory health,<sup>11</sup> induction of human mammary cancer cell (cancer chemoprevention),<sup>12,13</sup> and to have the potential for use as an herbal medication with a diverse clinical pharmacology.<sup>14-16</sup> As a result, pycnogenol is now widely utilized as a nutritional supplement and phytochemical remedy for a variety of diseases.<sup>17,18</sup> The extract from the bark of *Pinus radiata* containing vitamin c, Enzogenol<sup>®</sup>, was reported to have beneficial clinical effects such as preventing cardio vascular risk and use as a dietary supplement.<sup>19</sup> Several studies comparing and evaluating the active compounds in bark from pine have been reported, including: characterization of the components of bark from Japanese pine trees,<sup>20</sup> phenol composition and antioxidant activities of pine needles,<sup>21</sup> extraction of poly flavonoids from



**Figure 1.** A part photograph of Korean black pine (a): needles, (b): barks.

*Pinus radiata* bark,<sup>22</sup> comparison of the antioxidant activity and procyanidin composition,<sup>23</sup> and relationships between structure (composition) and antiradical activity.<sup>24</sup>

Pine trees native to different soil environments appear to contain different biological active substances, and may contain compounds that have not yet been described. Therefore, detailed studies of the activity of antioxidants in the active compounds obtained from the barks of Korean black pine to determine if they are useful as herbal medications and functional foodstuffs are warranted. In this study, the barks of Korean black pine were extracted using pressurized hot water to avoid solvent toxicity. Stepwise separation of the active compounds was then performed by TLC, CC, and HPLC. The antioxidant activities of single active compounds and water crude extracts were then determined based on a DPPH test, reductive potential power, and total phenol assay (TPA). In addition, the effects on cell

viability were assessed by a MTT assay. Three polyphenols and one phenolic acid were separated and identified by Fourier transform nuclear magnetic resonance spectroscopy (FT-NMR). The order of the antioxidant power was as follows: catechin  $\approx$  epicatechin > quercetin  $\gg$  ferulic acid.

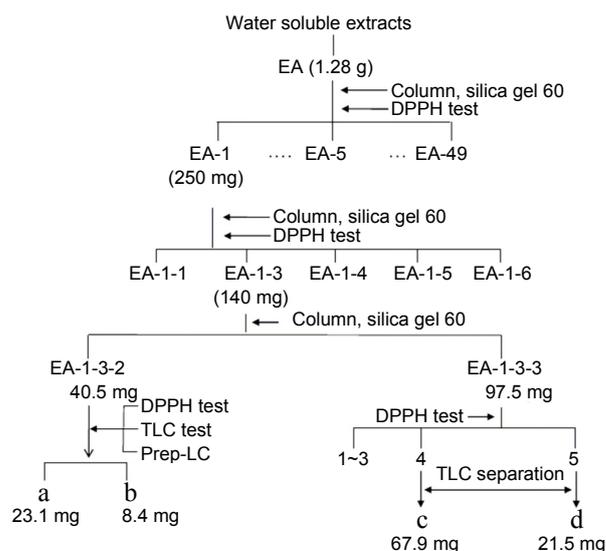
## Experimental

**Chemicals and materials.** The bark of black pine was collected from the mountains of the Iksan city at South Korea. Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tris (hydroxyl methyl) aminomethane hydrochloride (Tris-HCl), trichloro acetic acid, Folin-Ciocalteu reagent, ascorbic acid, sodium hydroxide (NaOH), potassium dihydrogen phosphate, potassium ferricyanide, iron (II) chloride (FeCl<sub>2</sub>), iron (III) chloride (FeCl<sub>3</sub>), hydro chloric acid (HCl), sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>), vitamin E, MTT, and DPPH were purchased from Sigma-Aldrich Co. (St. Louis, MO., USA).

The organic solvents, such as acetonitrile (AN), dichloromethane (DCM), ethyl ether (EE), ethyl acetate (EA), ethanol (EtOH), chloroform (CHCl<sub>3</sub>), butanol (BuOH), and dimethyl sulfoxide (DMSO) were used without further purification as purchased from Sigma-Aldrich Co. Silica gel 60 (0.04 - 0.063 mm) and silica gel 60 F<sub>254</sub> TLC sheets (20 × 20 cm) were purchased from Merck & Co., Inc. (Whitehouse Station, NJ., USA).

**Instruments.** A BioTek PowerWave microplate spectrometer (Winooski, VT., USA) equipped with a 96 well plate and the Gen 5 Data Analysis software was used for the DPPH tests by ELISA. A Hanil centrifuge (Hm 150 IV, Seoul, Korea) and a revolving evaporator with an EYELA digital water bath (SB-1000, Tokyo Rikakikai Co., LTD.) were used for extraction of the effective compounds in black pine barks. A Fourier-transform nuclear magnetic resonance (FT-NMR) spectrometer (JEOL Ltd. Eclipse 500, Tokyo, Japan) was used to identify the structure of the active compounds. Preseparation was conducted by column (id. 4 cm, L. 40 cm) chromatography using organic solvents and a Waters preparative chromatography system (Delta Prep, Milford, MA., USA) equipped with a photodiode array detector, prep LC controller, YMC Hydrosphere C<sub>18</sub> column (20 × 250 mm, particle size 5  $\mu$ m, pore size 120 Å), and Empower 2 Build 2154 software was used for separation of the final active compounds.

**Extraction and separation.** Chopped and dried barks of Korean black pine were ground into a powder for complete extraction. Water soluble crude extracts were obtained from the powder (2.0 kg) using pressurized hot water (80 °C), which was then evaporated under vacuum to yield 76 g (3.8%) of water crude extracts. Next, the water extracts were dissolved in distilled water and partitioned with *n*-hexane, ethyl acetate, and *n*-butanol successively in order of their polarity. The yields of the solvent partition from the crude extract were as follows: *n*-hexane fraction 0.016 g (0.021%), ethyl acetate fraction 13.3 g (17.5%), *n*-butanol fraction 20.9 g (27.5%), and water layer 38.9 g (51.2%). The ethyl acetate fraction showed the highest antioxidant activity upon DPPH assay. A portion (1.28 g) of the EA fraction (13.3 g) was taken and further subjected to separation by silica gel 60 column chromatography. Stepwise gradient elution with EE: MeOH (9:1  $\rightarrow$  0:1, v/v) gave 49 fractions, with fraction



**Scheme 1.** Stepwise separation processes for active compounds from ethyl acetate layer.

EA-1 (250 mg, 19.5%) showing strong antioxidant activity. The EA-1 fraction was chromatographed on a silica gel 60 column (L:30 cm, I.D. 1 cm) with a mixed eluant EE: CHCl<sub>3</sub> (9:1  $\rightarrow$  0:1, v/v) to give six fractions. Next, the obtained fraction EA-1-3 (140 mg) was processed again on the same column using the mixed eluant, EE: CHCl<sub>3</sub> (7:3), which yielded three fractions, EA-1-3-1, EA-1-3-2, and EA-1-3-3. The stepwise separation of EA-1-3-2 and EA-1-3-3 is outlined in detail in Scheme 1 except for the fraction EA-1-3-1 of a small quantity and low antioxidant activity.

**Isolation of the active compounds.** A Prep LC system was used to separate fraction EA-1-3-2 (40.5 mg), which had the greatest antioxidant activity among the three fractions, using a mixture of H<sub>2</sub>O : AN (9:1  $\rightarrow$  5:5, v/v) applied at a flow rate of 4.0 mL/min through a Hydrosphere C<sub>18</sub> column. The separated peaks observed at wavelengths of 215 nm and 254 nm were inspected by TLC at EA : CHCl<sub>3</sub> (1:1) mixture system. The separation of fraction EA-1-3-3 (97.5mg) was conducted by TLC. Fraction EA-1-3-3-4 which had the greatest antioxidant activity among the five fractions of EA-1-3-3 was concentrated in methanol and developed using a carrier solvent system (AN : DCM = 1:1). The fraction EA-1-3-3-5 was concentrated in methanol and loaded on to a TLC plate, and developed using a solvent mixture system (AN : DCM = 85:15).

**Free radical scavenging activity (DPPH test).** The reaction mixture consisted of 1.0 mL of 0.5 mM DPPH and 1.0 mL of 0.05 M Tris-HCl buffer (pH 7.4) in 1.0 mL of ethanol.<sup>25</sup> The absorbance of the mixture was measured at 517 nm exactly 30 min after the addition of different concentrations of extracts (concentration ranging from 0.5  $\mu$ g/mL to 100  $\mu$ g/mL). The DPPH radical scavenging activity of the extracts was expressed as the percentage decrease in the absorbance compared with the control.

$$\text{Free radical concentration (\%)} = (A_1/A_0) \times 100 \quad (1)$$

where  $A_0$  is the absorbance of the control reaction and  $A_1$  is the

absorbance of the sample. The uncompensated equation (1) was simple and matched the experimental data.

**Reductive potential test.** The reductive potential of the extracts was measured using a modification of the method described by Oyaizu.<sup>26</sup> A 250  $\mu\text{L}$  sample containing different concentrations of pine bark was mixed with 250  $\mu\text{L}$  phosphate buffer (0.2 M, pH = 6.6) and 250  $\mu\text{L}$  potassium ferricyanide (1%). The mixture was then incubated at 50  $^{\circ}\text{C}$  for 20 min. Next, a portion (250  $\mu\text{L}$ ) of trichloroacetic acid (10%) was added to the mixture, which was subsequently centrifuged for 10 min at 3000 rpm. The upper layer of the solution (600  $\mu\text{L}$ ) was mixed with  $\text{FeCl}_3$  (120  $\mu\text{L}$ , 0.1%), and the absorbance was measured at 700 nm in a 96 wells microplate. A higher absorbance of the reaction mixture indicated a greater reductive potential power.

**Total phenol content measurements.** The total amount of phenol compounds in the pine bark extracts was measured using a modification of the method described by Spanos and Wroldstad.<sup>27</sup> Briefly, 150  $\mu\text{L}$  of the extracts were appropriately diluted to different concentrations. Each sample was then oxidized with 750  $\mu\text{L}$  of freshly diluted Folin-Ciocalteu reagent, which is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenol and polyphenol antioxidants.<sup>28</sup> Folin-Ciocalteu reagent works by measuring the amount of the substance being tested that is required to inhibit the oxidation of the reagent.<sup>29</sup> This reaction was neutralized by adding 600  $\mu\text{L}$  of 7.5% w/v sodium carbonate and then vortexing the samples for 20 sec. Next, the samples were incubated at 45  $^{\circ}\text{C}$  for 20 min and the absorbance of the resulting blue color was measured at 765 nm using a spectrophotometer. The results were expressed as the gallic acid equivalence (GAE).

**MTT assay.** Cell viability (cytotoxicity) was measured in a 96-well plate using an enzyme linked immunosorbent assay (ELISA).<sup>30</sup> RAW 264.7 cells were mechanically scraped and plated at  $1 \times 10^5$  cells/well in 96 well plates containing 100  $\mu\text{L}$ /well of Dulbecco's modified eagle medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS). Samples were incubated overnight. The concentrated water extract was dissolved in DMSO to give a final concentration not exceeding 0.1%. After overnight incubation, the test material was added and the plates were incubated for 24 h. The cells were then washed once before adding 50  $\mu\text{L}$  of FBS-free medium containing 5 mg/mL MTT. After 4 h of incubation at 37  $^{\circ}\text{C}$ , the medium was discarded and the formazan blue formed in the cells was dissolved in 100  $\mu\text{L}$  of DMSO. The optical density of the untreated control cells measured at 540 nm was taken as 100% viability.

**Statistical analysis and data evaluation.** All data are expressed as the mean  $\pm$  S.E.M. of at least three experiments and were analyzed statistically using Microsoft Excel's ToolPak to acclimate the t-test with equal variances. Data were considered to be statistically significant if the probability value was 0.05 or less ( $p \leq 0.05$ ). Some data treatments and figures were processed using the Origin Pro 7.5 data analysis and graphing software.

## Results and Discussion

**Antioxidant activities of solvent layers.** The antioxidant activities of solvent layers separated from the water soluble extracts of Korean black pines were measured using the DPPH method

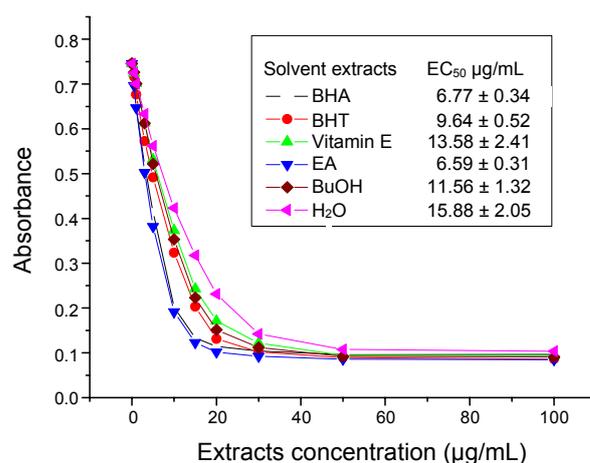


Figure 2. The radical scavenging activities (DPPH test) of solvent extracts.

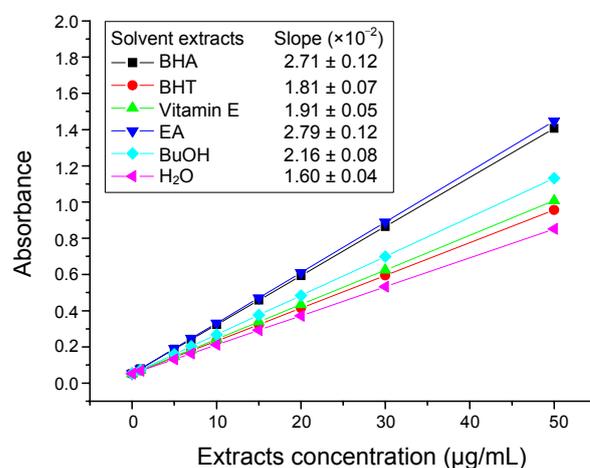


Figure 3. The Fe(III) reductive potential power of solvent extracts.

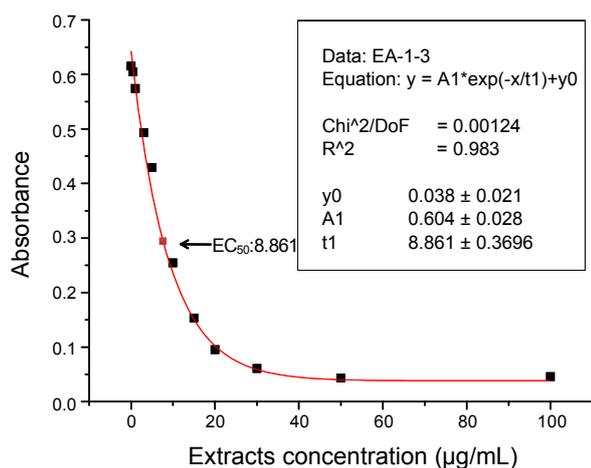
and reductive potential tests. The DPPH results are shown at Fig. 2 with the EC<sub>50</sub> values and compared with the control standards (BHA, BHT and vitamin E). The DPPH tests revealed that the hexane layer (0.021%) separated from the water extracts showed only a very low activity (EC<sub>50</sub> > 200  $\mu\text{g/mL}$ ). Conversely, the ethyl acetate (EC<sub>50</sub> = 6.59  $\pm$  0.31  $\mu\text{g/mL}$ ) and butanol (EC<sub>50</sub> = 11.56  $\pm$  1.32  $\mu\text{g/mL}$ ) layers showed the antioxidant activity of the same level as the control standards (BHA, EC<sub>50</sub> = 6.77  $\pm$  0.34  $\mu\text{g/mL}$ ; BHT, EC<sub>50</sub> = 9.64  $\pm$  0.52  $\mu\text{g/mL}$ ; vitamin E, 13.58  $\pm$  2.41  $\mu\text{g/mL}$ ). Evaluation of the reductive potential results revealed that the activity effect and concentration of the sample were well correlated at concentrations of less than 50  $\mu\text{g/mL}$ . Additionally, deposition of  $\text{Fe}^{2+}$  at concentrations greater than 50  $\mu\text{g/mL}$  influenced determination of the activity. The reductive potential power was determined from the linear slope shown in Fig. 3. The EA and BuOH layers showed a higher slope value of  $2.79 \times 10^{-2}$  and  $2.16 \times 10^{-2}$  than the standard BHA ( $2.71 \times 10^{-2}$ ). Based on these results, the order of antioxidant activities was EA > BuOH > H<sub>2</sub>O, and pressurized hot water extraction was sufficient to collect the antioxidants from bark of black pine.

**Antioxidant activities of fractions obtained by stepwise separation.** The antioxidant activities of the fractions separated step-

**Table 1.** An evaluating data for the antioxidant activities of stepwise separation fractions and control standards

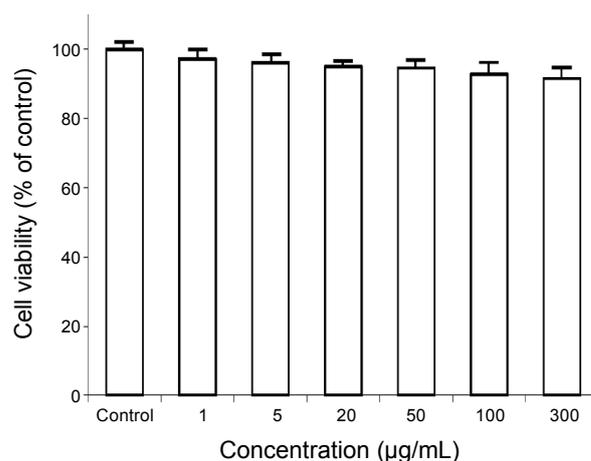
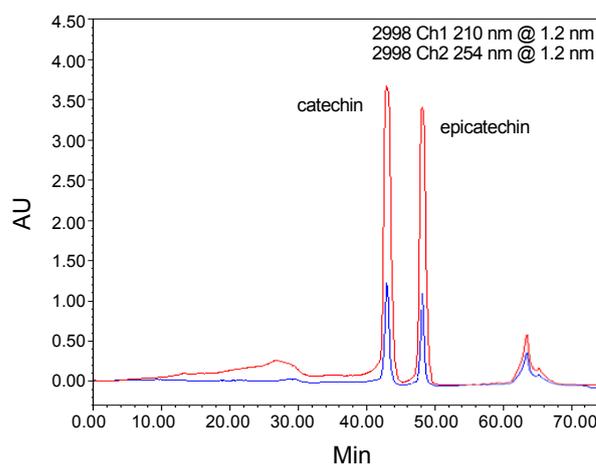
Fractions	DPPH method	Reductive potential	Total phenol
	EC <sub>50</sub> (μg/mL)	slope (A/10 <sup>2</sup> )	(GAE* mg/g)
EA-1	11.26 ± 1.40	3.82 ± 0.01	428.31 ± 0.83
EA-2(5~30)	34.72 ± 5.77	< 0.3	64.24 ± 0.90
EA-3(31~49)	> 100	< 0.5	32.81 ± 0.58
EA-1-1	46.83 ± 1.23	0.81 ± 0.09	112.42 ± 0.15
EA-1-2	14.52 ± 1.62	1.04 ± 0.08	156.00 ± 0.41
EA-1-3	8.86 ± 0.37	3.23 ± 0.01	395.05 ± 0.45
EA-1-4~6	> 50	< 0.3	13.21 ± 0.28
EA-1-3-2	8.97 ± 0.45	2.57 ± 0.04	178.28 ± 0.99
EA-1-3-3	7.66 ± 0.43	2.89 ± 0.07	150.26 ± 1.26
BHA	6.77 ± 0.34	2.71 ± 0.02	39.21 ± 0.09
BHT	9.64 ± 0.52	2.81 ± 0.07	77.16 ± 0.16
Vitamin E	13.58 ± 2.41	1.91 ± 0.05	20.17 ± 0.39

\*GAE: gallic acid equivalence (mg/g).

**Figure 4.** Plot of free radical scavenging activity (DPPH) vs. concentration of fraction EA-1-3 obtained from black pine bark.

wise by silica gel column chromatography were measured by DPPH, reductive potential (RP), and total phenol tests. As shown in Table 1, the EA-1 (DPPH EC<sub>50</sub> = 11.26 ± 1.4 μg/mL, RP slope = 0.0382 ± 0.0001, TP GAE = 428.31 ± 0.83 mg/g) and EA-1-3 (DPPH EC<sub>50</sub> = 8.86 ± 0.37 μg/mL, RP slope = 0.0323 ± 0.0001, TP GAE = 395.05 ± 0.45 mg/g) fractions showed almost the same antioxidant activity as the control standards BHA, BHT and Vitamin E. Though the control standards BHA, BHT and Vitamin E have small GAE values of total phenol (BHA: 39.21 ± 0.09, BHT: 77.16 ± 0.16), they are acting as good antioxidant of lower DPPH EC<sub>50</sub> values (BHA: 6.77 ± 0.34, BHT: 9.64 ± 0.52, Vitamin E: 13.58 ± 2.41).

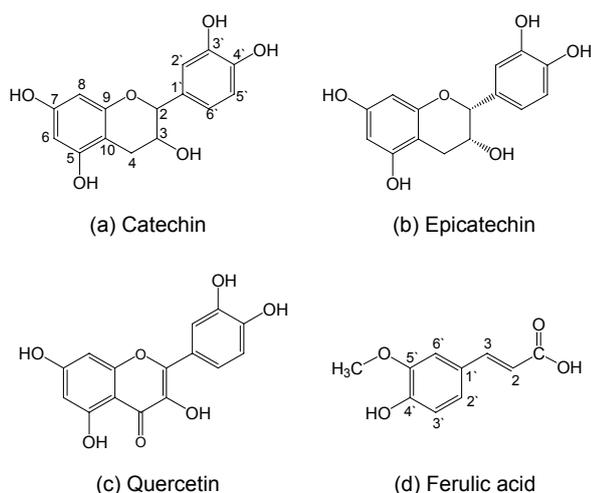
Even if the control standards, synthetic BHA and BHT are good antioxidants of low DPPH values, natural antioxidants such as EA-1 and EA-1-3 of high GAE values are more desirable antioxidants containing polyphenols depending on the quantity of hydroxyl (-OH) group on the phenyl rings. The variation in

**Figure 5.** Effect of water soluble extracts on the viability of RAW 264.7 cells (48 hrs). The viability of the cells was measured by MTT assay. The results are expressed as the mean ± S.E.M. of at least three experiments. (\*p < 0.05).**Figure 6.** The HPLC chromatogram of catechin and epicatechin obtained from fraction EA-1-3-2.

absorbance as a function of the concentration of fraction (EA-1-3) are also shown in Fig. 4.

**The MTT assay in Raw 264.7 cells.** The cytotoxic effect of the water soluble extracts was determined based on the optical density (OD) value of the formazan after addition of the MTT solution. Fig. 5 shows the OD values as a percentage of the control (distilled water instead of sample) at different concentrations range (1 μg/mL ~ 300 μg/mL). The cell viability was held with a high existent probability: 96.7% at 5 μg/mL; 93.2% at 50 μg/mL; 91.8% at 300 μg/mL. The water soluble extracts had relatively no cytotoxic effects on cells at concentrations less than 300 μg/mL.

**Identification of the active compounds.** The two substances were separately collected from fraction EA-1-3-2 (40.5 mg), which was found to have the most intense antioxidant activity by Prep LC. The two strong peaks shown in Fig. 6 were detected using a dual wavelength (215 nm, 254 nm) UV detector and identified as catechin (a) and epicatechin (b) by NMR. The broad



**Figure 7.** The structures of isolated antioxidation active compounds from ethyl acetate fractions (EA-1-3-2, EA-1-3-3-4, EA-1-3-3-5).

band of 26 - 28 min and small sharp peak of 62 - 64 min shown in Fig. 6 were considered to be impurities.

The separation of fraction EA-1-3-3 (97.5 mg) was not easy due to the long tail of its developing band; therefore, separate collection was conducted by TLC. A TLC separation method was used for EA-1-3-3-4, which had the greatest activity among the five spots on the TLC plate. The spot EA-1-3-3-4 was concentrated in methanol and then loaded onto a TLC plate and developed using a carrier solvent system (AN : DCM = 1:1) in the Latch-Lid developing chamber. The separated compound (c), was confirmed to be quercetin by NMR. The spot EA-1-3-3-5 was concentrated in methanol and developed using a solvent mixture system (AN : DCM = 85:15) in the developing chamber. The isolated compound (d) was confirmed to be ferulic acid by NMR.

The structures of the isolated compounds were confirmed by comparison with previously published data.<sup>31-36</sup> The authentic data obtained from the reference standard compounds were as follows. Catechin:<sup>32</sup> 4.54 d (C-2), 3.99 m (C-3), 2.44 dd (C-4), 2.76 dd (C-4), 5.95 d (C-6), 5.82 d (C-8), 6.81 d (C-2'), 6.75 d (C-5'), 6.68 dd (C-6'). Epicatechin:<sup>32</sup> 4.74 s (C-2), 3.22 m (C-3), 2.44 - 2.71 m (C-4), 5.89 d (C-6), 5.73 d (C-8), 6.68 d (C-2'), 6.89 d (C-5'), 6.68 d (C-6'). Quercetin:<sup>33,34</sup> 6.18 d (C-6), 6.40 d (C-8), 7.67 d (C-2'), 6.87 d (C-5'), 7.53 dd (C-6'). Ferulic acid:<sup>35,36</sup> 6.22 d (C-2), 7.64 d (C-3), 3.87 s (-OCH<sub>3</sub>), 6.99 m (C-2').

The confirmed structures of four active substances (three flavonoids and one phenolic acid) are shown in Fig. 7. Catechin (a), epicatechin (b), and quercetin (c) belong to a large class of flavonoids that have been shown to protect against a variety of diseases, including tumors. Studies have demonstrated that the potent peroxy radical scavenging abilities of flavonoids contribute to inhibition of lipid peroxidation and the oxidation of LDL.<sup>37,38</sup> Several flavonoids including the epicatechin family of flavonols are powerful inhibitors of nitration and DNA deamination in vitro.<sup>39</sup> Feeding rats with quercetin during either the initiation or promotion stage inhibited carcinoma formation in the tongue.<sup>40</sup> Additionally, quercetin contributed to the protection

of neuronal cells from oxidative stress-induced neurotoxicity, such as occurs in Alzheimer's disease.<sup>41</sup>

In this study, water soluble extract, EA, and BuOH fractions with high antioxidant activity were obtained from the barks of the Korean black pine. The water extract and EA fraction of plentiful polyphenol obtained from black pine barks which represent an inexpensive and diverse source of catechin, epicatechin, and quercetin, are expected to be useful as chemopreventive agents.

## Conclusion

The ethyl acetate fraction of the water soluble extract from the barks of Korean black pine showed almost the same antioxidant activity as the control synthetic compounds (BHT, BHA, and Vitamin E). Substance analysis and antioxidant activity tests conducted via stepwise separation revealed that the effective compounds with antioxidant activity were identified as catechin, epicatechin, quercetin, and ferulic acid. The order of the antioxidants power by DPPH test was as follows: catechin  $\approx$  epicatechin > quercetin  $\gg$  ferulic acid. This suggested that the derivatives of poly hydroxyl phenols (catechin, epicatechin, and quercetin) were strong and carboxylated phenol (ferulic acid) was weak antioxidant. The total phenol content of the effective fractions was inversely proportional to their EC<sub>50</sub> values; the compounds with lower EC<sub>50</sub> values for antioxidant activity showed higher total phenol content. It is desirable that doses of the concentrated mixture from the water crude extracts were more effective in antioxidation than doses of single substances. These findings indicate that the polar-hydrophilic properties of these antioxidants allow them to be easily absorbed in vivo. Furthermore, some mixtures of polyhydroxy phenols in the EA fractions showed synergistic increases in antioxidant activity. Because these fractions and mixtures contained abundant antioxidants and showed a lack of toxicity, water-soluble extracts of black pine bark could be applied to a wide range of uses, including cosmetics, food additives, therapeutic materials (chemopreventive agents), and health drinks.

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