

# Evaluation of *In Vitro* Antioxidant Activity of the Water Extract Obtained from Dried Pine Needle (*Pinus densiflora*)

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**ABSTRACT:** Antioxidant activities of water extracts obtained from dried pine needle (*Pinus densiflora*) were measured at 0, 4, 20, 100, 500, 1,000, and 1,200 ppm and compared with those of phenolic compounds of butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butylhydroquinone, ferulic acid, and  $\alpha$ -tocopherol. The activity was determined as the ability to scavenge 1,1-diphenyl-2-picrylhydrazyl radical and hydrogen peroxide, reductive power, and inhibition of lipid peroxidation in a linoleic acid system using the ferric thiocyanate method and thiobarbituric acid method, respectively. Pine needle water extract (PNWE) exhibited antioxidant activity in a concentration-dependent mode at the same parameters mentioned above, and a significant difference ( $P < 0.05$ ) was observed at 1,000 ppm. The protective activity of PNWE as a potent antioxidant in a non-cellular system was compared with that of phenolics at 150.67  $\mu\text{g/mL}$  in the two assays using biological cellular systems, namely 2,2'-azobis(2-amidinopropane) dihydrochloride-initiated hemolysis and  $\text{Fe}^{2+}$ -induced lipid peroxidation, using rat red blood cells and rat brain homogenate, respectively. The PNWE showed a strong power comparable to those of commercial phenolic compounds in biological systems. These results indicated that the protective activity of PNWE could be due to the presence of naturally-occurring phenolic compounds, which act as potent *in vitro* antioxidants in both non-cellular and cellular systems.

**Keywords:** antioxidant activity, *Pinus densiflora*, water extract, lipid peroxidation, phenolics

## INTRODUCTION

Among traditional medicinal plants containing naturally-occurring antioxidants, *Pinus densiflora*, known as Japanese red pine in western countries, is a species widely distributed in Korea (1-3), and the evergreen tree with needle leaves, belonging to the Pinaceae family (2), is indigenous to Eastern Asia. Pine needles have been widely used as food ingredients, food additives, and folk medicines in pine-based products due to their characteristic aroma and taste, and health-promoting properties (3,4). They have been shown to possess a wide spectrum of biological and pharmacological actions such as antioxidant, anti-mutagenic, antitumor, anti-inflammatory, antibacterial effects as well as protection against oxidative DNA damage, and apoptosis induced by hydroxyl radicals (4-6), which may induce preventive and therapeutic effects against degenerative diseases associated with aging. The effective components in pine needles are chlorophyll, carotenes, dietary fiber, terpenoids, phenolics, tannins, and alkaloids (6).

Generative diseases are associated with free radicals, because oxidative damages to DNAs, proteins, and macromolecules accumulate with age and have been postulated to be a major type of endogenous damage leading to aging (7,8). The oxidative damage depends on a common initiating step, the peroxidation of polyunsaturated fatty acid components in biological cellular system (9). Such oxidative damage by lipid peroxidation in biological cellular systems can be inhibited by antioxidants. Clinical, experimental, and epidemiological studies have shown that dietary antioxidants are important in preventing the degenerative diseases are increasing (10-12). Lipid peroxidation is also a major cause of deterioration in food, which affects color, flavor, texture, and the nutritional quality of food (7). In order to minimize lipid peroxidation in cellular and non-cellular systems, the use of naturally-occurring antioxidants provides protection against degenerative diseases as well as food deterioration. Such protection can be explained by the antioxidant effects of phenolic compounds to scavenge free radicals, which are responsible for lipid peroxidation. Phenolic compounds,

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naturally in plants, are secondary plant metabolites (13). They have been reported to play important roles as antioxidants by potential pathways such as free-radical scavenging, oxygen radical absorbance, and the chelating of metal ions (13,14). These compounds are a part of the everyday diet and also used as medicines and supplements. Research has shown that many plants including medicinal fruits and herbs contain other antioxidant nutrients, in addition to vitamins C and E, flavonoids, and carotenoids, which significantly contribute to their total antioxidant capacity (13-15).

Synthetic phenolic antioxidants have been used in stabilizing lipids in foods to prevent oxidative deterioration. The most commonly used synthetic phenolic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butyl hydroquinone (TBHQ) (16). However, interest has increased considerably in finding naturally occurring antioxidants for use in foods and as additives and medicinal materials to replace synthetic antioxidants, which are being restricted due to their inherent carcinogenicity including tumor-initiating and tumor-promoting actions (7,17). Utilization of natural by-products based on medicinal plants such as pine needle powder presents an opportunity to provide the antioxidant potential of plants by reducing the risks of degenerative diseases and deterioration in biological and food systems, respectively, which may result from the primary and secondary oxidation products by lipid peroxidation occurring in both cellular and non-cellular systems. Therefore, the objective of the present study is to provide baseline data on the antioxidant activity of pine needle in a water matrix, a commonly used method in home cooking. *In vitro* antioxidant activities of the water extract obtained from dried pine needle, including free radical scavenging, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging, reducing power, and inhibition of lipid peroxidation in a linoleic acid system, were determined at different concentrations and compared to those of commercially available phenolic compounds, BHT, BHA, TBHQ, ferulic acid, and  $\alpha$ -tocopherol, in biological cellular systems using rat blood cells and rat brain tissues.

## MATERIALS AND METHODS

### Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), linoleic acid, polyoxyethylene-sorbitan monolaurate (Tween 20), ammonium thiocyanate, ferrous chloride, ferric chloride, potassium ferricyanide, H<sub>2</sub>O<sub>2</sub>, trichloroacetic acid (TCA), 2-thiobarbutric acid (TBA), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), BHT, BHA, TBHQ, ferulic acid,  $\alpha$ -tocopherol, ascorbic acid, Folin-Ciocalteu reagent, tannic acid, Na<sub>2</sub>CO<sub>3</sub>, NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>, NaCl, Fe<sub>2</sub>SO<sub>4</sub>, and

Tris were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade.

### Preparation of pine needle water extract (PNWE)

Dried pine needles (*Pinus densiflora*), produced at Cheonan, South Korea, were purchased from a Jegidong market in 2015, kept in a -20°C freezer, and a 20 g sample was extracted with distilled water (700 mL, reflux, 1 h×2) according to the method described by Tang et al. (18) with a slight modifications. The aqueous solution was filtered with Whatman filter paper and evaporated under vacuum at 40°C, and then lyophilized to obtain the water extract (PNWE 30.73%: 6.06±0.24 g, n=3). The water extract obtained from the dried pine needle was diluted with distilled water to prepare the tested samples at different concentrations of 0, 4, 20, 100, 500, and 1,000 ppm for estimation of antioxidants.

### Antioxidant estimation of PNWE

**DPPH free radical scavenging activity:** The scavenging activity at different concentrations [2 to 1,400 ppm ( $\mu$ g/mL)] of the water extract on the DPPH radicals was estimated using the method outlined by Kim (16) based on the method of Hatano et al. (19). Specifically, the water extract (4 mL) was dissolved in water and added to a solution of DPPH (1 mL, 0.2 mM) radical in methanol. The mixture was reacted for 30 min prior to the analysis, and the absorbance of the reaction mixture (0.1 mL) was measured at 517 nm to show the degree of free radical scavenging activity. Low absorbance of the reaction mixture indicates high free radical scavenging activity. The DPPH radical scavenging activity was calculated using the following equation [1]:

$$\text{DPPH scavenging effect (\%)} = \frac{A_c - A_t}{A_c} \times 100 \quad [1]$$

where  $A_c$  is the absorbance of the control reaction (blank sample treated with no added water extract) and  $A_t$  is the absorbance in the presence of the water extract.

**H<sub>2</sub>O<sub>2</sub> scavenging activity:** The H<sub>2</sub>O<sub>2</sub> scavenging capacity of the water extract at different concentrations [4 to 1,200 ppm ( $\mu$ g/mL)] was determined by the method of Atmani et al. (20) with slight modifications. Test tubes were prepared with 2 mL extract and 1.2 mL H<sub>2</sub>O<sub>2</sub> (20 mM) in phosphate buffer (pH 7.4). A blank solution was prepared the same way but without H<sub>2</sub>O<sub>2</sub>. After incubation of the mixture for 10 min, the absorbance was recorded at 230 nm. The H<sub>2</sub>O<sub>2</sub> scavenging activity was calculated using equation [2]:

$$\text{H}_2\text{O}_2 \text{ scavenging effect (\%)} = \frac{A_c - A_t}{A_c} \times 100 \quad [2]$$

where  $A_c$  is the absorbance of the control and  $A_t$  is the absorbance of the extract.

**Reducing power of PNWE:** The reducing power of the water extract at different concentrations [0, 4, 20, 100, 500, and 1,000 ppm ( $\mu\text{g}/\text{mL}$ )] was determined using the method described by Gülçin et al. (21). To 0.1 mL of sample solution, phosphate buffer (0.25 mL, 0.2 M, pH 6.6), and potassium ferricyanide [ $\text{K}_3\text{Fe}(\text{CN})_6$ ] (0.25 mL, 1%) were mixed and incubated at  $50^\circ\text{C}$  for 20 min. Aliquots (0.25 mL) of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged for 10 min at 1,700 g ( $25^\circ\text{C}$ ). The upper layer of the solution (0.25 mL) was mixed with distilled water (0.25 mL) and ferric chloride ( $\text{FeCl}_3$ ) (0.05 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates increased reducing power.

#### Determination of total antioxidant activity of PNWE in a linoleic acid system

**Ferric thiocyanate (FTC) method:** The antioxidant activity at different concentrations [0, 4, 20, 100, 500, and 1,000 ppm ( $\mu\text{g}/\text{mL}$ )] on the inhibition of linoleic acid peroxidation was assayed with a thiocyanate method modified by Kim (16) based on the method of Yen et al. (22). A sample solution (0.5 mL) of the water extract at different concentrations was mixed with 2.5 mL of 0.02 M linoleic acid emulsion at pH 7.0 and 2 mL of 0.2 M phosphate buffer at pH 7.0. The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as an emulsifier, and 50 mL of phosphate buffer. The reaction mixture was incubated at  $37^\circ\text{C}$  for 5 days. To 0.1 mL of the reaction mixture, 75% EtOH (4.7 mL), 30% ammonium thiocyanate (0.1 mL), and 0.02 M ferrous chloride in 3.5% HCl (0.1 mL) were added at 24-h intervals. The peroxide value was determined to evaluate the degree of oxidation by reading the absorbance at 500 nm. The percent inhibition of linoleic acid peroxidation by each water extract was calculated by equation [3]:

$$\% \text{ Inhibition} = 100 - \frac{A_t}{A_c} \times 100 \quad [3]$$

where  $A_c$  and  $A_t$  are the absorbances of the control (without sample) and the experimental (with sample) reactions, respectively.

**TBA method:** The TBA test was conducted on the final incubation day (6 d) using a method modified by Kim (16) based on the method of Kikuzaki and Nakatani (23) to determine the malonaldehyde formation from linoleic acid peroxidation. The same sample preparation method as described in the FTC method was used. To 1 mL of sample solution, 20% trichloroacetic acid (2 mL) and TBA solution (2 mL) were added. The mixture was boiled

and then cooled. After centrifugation of the sample mixture, the absorbance of the supernatant was measured at 532 nm.

#### Determination of total phenolics

The concentration of total phenolic compounds in the water extract of dried pine needle was determined spectrophotometrically using the Folin-Ciocalteu reagent (24) according to the method of Lim et al. (25). The water extract (0.1 mL) was diluted with deionized water (7.9 mL), followed by addition of the Folin-Ciocalteu phenol reagent (0.5 mL) and the contents were mixed thoroughly. After 1 min, 1.5 mL of 20% sodium carbonate solution was added, and the mixture was mixed thoroughly. The mixture was allowed to stand for 1 h. The absorbance of the blue color produced was measured with a spectrophotometer at 750 nm. The phenolic content was expressed as milligrams per gram of dry weight based on a standard curve of tannic acid, which was expressed as milligrams per gram of tannic acid equivalent (TAE).

#### Comparison of antioxidant activities between the PNWE and commercial phenol compounds

**Scavenging activity against  $\text{H}_2\text{O}_2$ :** The  $\text{H}_2\text{O}_2$  scavenging capacity of the aqueous extract [1,000 ppm ( $\mu\text{g}/\text{mL}$ )] with relatively higher antioxidant activity in the previous experiment was determined and compared with those of the commercial phenol antioxidants (BHA, BHT, TBHQ, ferulic acid, and  $\alpha$ -tocopherol) using the same method mentioned earlier. The concentration of commercial phenol compounds used for comparison in this experiment was 150.67 ppm ( $\mu\text{g}/\text{mL}$ ), because 1,000 ppm of PNWE contained 150.67  $\mu\text{g}$  of phenolic compounds.

**Hemolysis inhibition using rat red blood cells (RBC):** Hemolysis inhibition activity of the aqueous extract [1,000 ppm ( $\mu\text{g}/\text{mL}$ )] was compared with those of the commercial phenol antioxidants (BHA, BHT, TBHQ, ferulic acid, and  $\alpha$ -tocopherol) based on the method of Cheung et al. (7). RBCs were obtained from male Sprague-Dawley rats (200 g). RBC were centrifuged at 1,000 g for 5 min and washed three times with 10 mL of 10 mM phosphate-buffered saline (PBS) at pH 7.4. The plasma and buffy coat were removed by aspiration after each wash. After the 3rd wash, RBC were obtained by centrifugation at 1,000 g for 10 min and resuspended in PBS to obtain a 20% RBC suspension. Hemolysis of RBC mediated by AAPH, a peroxy radical initiator, was measured according to the method of Miki et al. (26). A portion of the RBC suspension (0.1 mL) was mixed with 0.1 mL of PBS solution containing either the water extract or a commercial phenol compound. An aliquot of 200 mM AAPH (0.2 mL, in PBS) was then added to the mixture. The reaction mixture was shaken and incubated in a water bath at  $37^\circ\text{C}$  for 3 h. After incubation, one of the reaction

mixtures was diluted with 8 ml of PBS (A), and the other was diluted with 8 mL of distilled water (B) to induce hemolysis. Both reactions were centrifuged at 1,000 g for 10 min. The absorbance of the supernatants from A and B ( $A_{\text{abs}}$  and  $B_{\text{abs}}$ , respectively) at 540 nm was measured, and the percent hemolysis inhibition was calculated according to the following equation [4]:

$$\% \text{ Inhibition} = \frac{B_{\text{abs}} - A_{\text{abs}}}{B_{\text{abs}}} \times 100 \quad [4]$$

**Lipid peroxidation inhibition using rat brain homogenate:** The inhibition activities of the water extract [1,000 ppm ( $\mu\text{g/mL}$ )] and the commercial phenolic antioxidants on the rat brain homogenate induced by  $\text{Fe}^{2+}$ /ascorbate-stimulated lipid peroxidation were assayed by the method of Cheung and Cheung (9) based on the method of Liu et al. (27). The brain was removed quickly from male Sprague-Dawley rats, each weighing 200 g and washed with ice-cold 20 mM Tris-HCL buffer (pH 7.4). The brain was homogenized in 2 volumes of ice-cold Tris-HCl buffer using a Polytron PT 3000 homogenizer (Brinkmann Instruments, Inc., Westbury, NY, USA) followed by centrifugation at 3,000 g for 10 min. The supernatant was used for the study of lipid peroxidation. Lipid peroxidation was determined using the formation of malondialdehyde (MDA) as an indicator. The reaction mixture was composed of 0.1 mL of brain homogenate, 0.1 mL of 10  $\mu\text{M}$   $\text{FeSO}_4$ , 0.1 mL of 0.1 mM ascorbic acid, and 0.2 mL of experimental sample (water extract or commercial phenolic compounds). The mixture was incubated at 37°C for 1 h. The reaction was stopped by adding TCA and TBA. The mixture was then heated at 80°C for 20 min. After cooling, centrifugation was carried out for 10 min. The absorbance of the MDA-TBA complex in the supernatant was determined at 532 nm by a spectrophotometer. The percent lipid peroxidation was calculated according to equation [5]:

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100 \quad [5]$$

where  $A_1$  is the absorbance of the test sample, and  $A_0$  is the absorbance of the control.

#### Statistical analysis

All analyses for the determination of antioxidant activity in the samples were performed in triplicate, and the experimental data were reported as mean  $\pm$  standard deviation. Experimental data were analyzed by the analysis of variance (ANOVA) using the General Linear Model (Minitab Inc., State College, PA, USA). Significant differences ( $P < 0.05$ ) among means for each treatment were determined using the Tukey's test at a level of  $\alpha = 0.05$ .

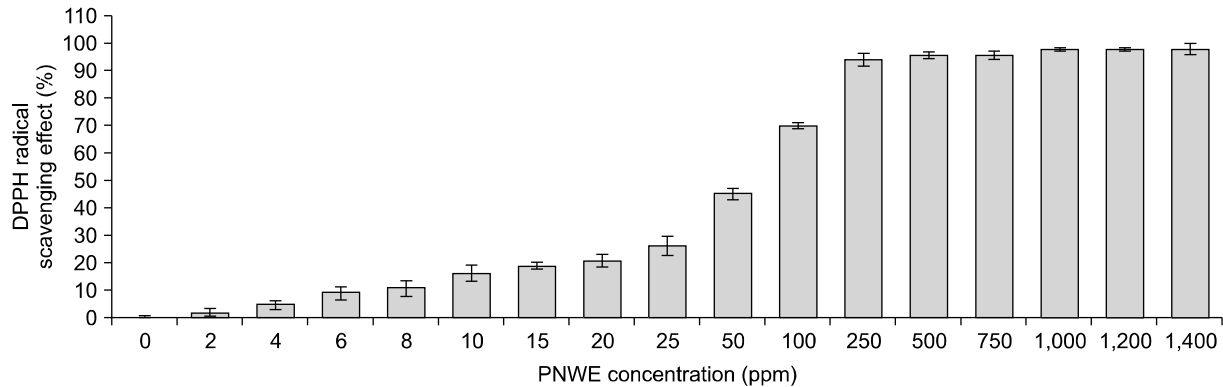
## RESULTS AND DISCUSSION

The DPPH radical scavenging capacity,  $\text{H}_2\text{O}_2$  scavenging capacity, and reducing power of PNWE obtained from dried pine needle were evaluated at different concentrations. Total antioxidant activity on lipid peroxidation was determined to evaluate the primary and secondary oxidation products generated during incubation of the linoleic acid system containing the PNWE at different concentrations. Total phenolic content of PNWE was also determined using the Folin-Ciocalteu phenol reagent to compare its antioxidant activity with those of commercial phenolic compounds at the same phenolic contents. Total phenolic content of PNWE was 150.67 mg/g of dry weight. Three assays including scavenging of  $\text{H}_2\text{O}_2$ , inhibition of rat RBC hemolysis, and suppression of lipid peroxidation using rat brain homogenates were used to compare the antioxidant activity of PNWE with those of commercially available phenolic compounds, namely, BHA, BHT, TBHQ, ferulic acid, and  $\alpha$ -tocopherol, at the concentration of 150.67  $\mu\text{g/mL}$ .

#### Antioxidant activity of the PNWE fraction at different concentrations

**DPPH radical and  $\text{H}_2\text{O}_2$  scavenging effect of the PNWE:** The DPPH radical scavenging test is a short and simple method for investigating the hydrogen-donating potential. It is theoretically accepted that the electron-donating ability of chemical substances results in antioxidant activity on lipid oxidation (28,29). The scavenging effects of the different concentrations of PNWE on DPPH radicals were determined by measuring the decrease in absorbance at 517 nm due to the DPPH radical reduction, which indicates the antioxidant activity of a compound. As shown in Fig. 1, the DPPH radical-scavenging capacity of test concentrations increased in a concentration-dependent mode, but was not significantly different ( $P > 0.05$ ) in the range of 500 to 1,200 ppm (Table 1). PNWE at 1,000 ppm showed a relatively higher scavenging activity of 97.765% (Table 1) on the DPPH radicals.

$\text{H}_2\text{O}_2$  can be generated in foods and biological systems. Being a non-radical oxygen-containing reactive agent, it can form the hydroxyl radical, the most highly reactive oxygen radical, in the presence of transition metal ions and participate in free radical reactions (30,31). Table 2 shows a similar concentration dependent trend as shown in the DPPH radical scavenging. The  $\text{H}_2\text{O}_2$ -scavenging effect of PNWE was found to increase with increasing concentrations up to 1,000 ppm (Table 2) and at 1,200 ppm, showed a noticeable scavenging effect of 95.220%, but was not significantly increased ( $P > 0.05$ ) at higher concentrations. The present study shows that the PNWE is not only a good scavenger of DPPH and  $\text{H}_2\text{O}_2$  but also is a primary antioxidant in non-cellular systems, which dem-



**Fig. 1.** Scavenging activities of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals at different concentrations of pine needle water extract (PNWE).

**Table 1.** Antioxidant activities of the pine needle water extract (PNWE) fractions against DPPH radicals at different concentrations

PNWE fraction (ppm)	Absorbance at 517 nm	DPPH radical scavenging effect (%) <sup>1)</sup>
4	0.499±0.003	4.525±1.485 <sup>a</sup>
20	0.414±0.014	20.778±2.241 <sup>b</sup>
100	0.156±0.006	70.108±1.214 <sup>c</sup>
500	0.023±0.004	95.653±1.072 <sup>d</sup>
1,000	0.011±0.003	97.765±0.787 <sup>d</sup>
1,200	0.010±0.002	97.974±0.596 <sup>d</sup>

Each value is the mean±standard deviation (n=3).

Values with different letters (a-d) are significantly different ( $P < 0.05$ ).

<sup>1)</sup>Radical scavenging effect (%) = [(absorbance of control at 517 nm - absorbance of sample at 517 nm) / absorbance of control at 517 nm] × 100.

**Table 2.** Antioxidant activities of the pine needle water extract (PNWE) fractions against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) at different concentrations

PNWE fraction (ppm)	Absorbance at 230 nm	H <sub>2</sub> O <sub>2</sub> radical scavenging effect (%) <sup>1)</sup>
4	0.535±0.001	0.372±0.186 <sup>a</sup>
20	0.526±0.005	1.986±0.880 <sup>b</sup>
100	0.449±0.004	16.449±0.753 <sup>c</sup>
500	0.059±0.004	88.951±0.654 <sup>d</sup>
1,000	0.027±0.003	94.910±0.599 <sup>e</sup>
1,200	0.026±0.004	95.220±0.775 <sup>e</sup>

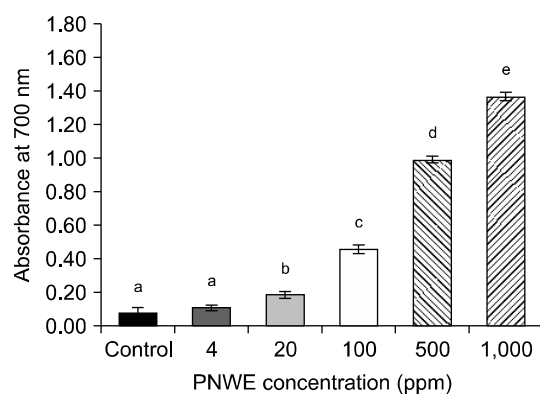
Each value is the mean±standard deviation (n=3).

Values with different letters (a-e) are significantly different ( $P < 0.05$ ).

<sup>1)</sup>Radical scavenging effect (%) = [(absorbance of control at 230 nm - absorbance of sample at 230 nm) / absorbance of control at 230 nm] × 100.

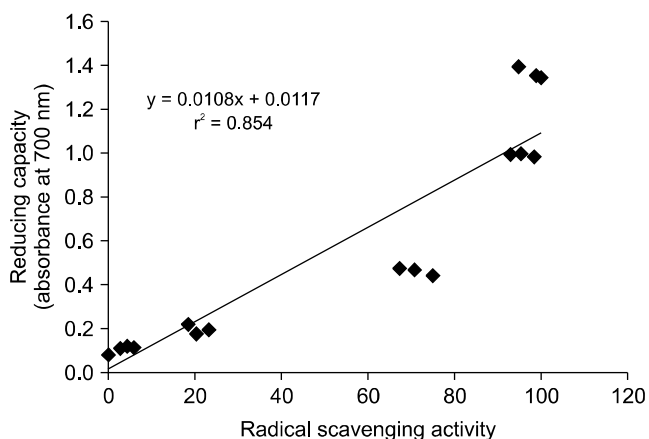
onstrates that the PNWE contains naturally occurring components with a noticeable effect on scavenging radicals generated during oxidation.

**Reducing power of the PNWE:** Fig. 2 shows the reductive capability of PNWE as a function of its concentrations. For the measurements of the reductive ability, the Fe<sup>3+</sup>-

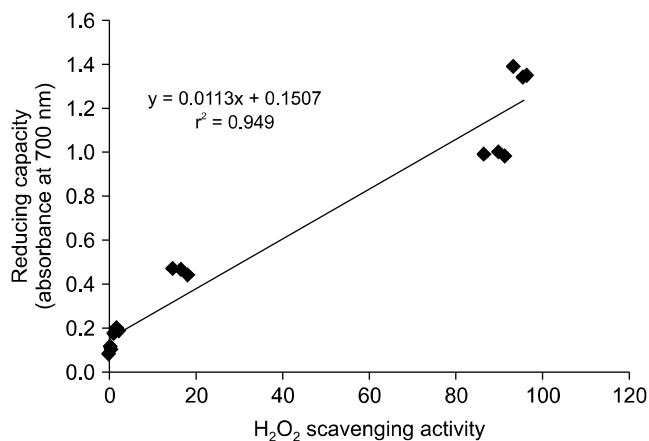


**Fig. 2.** Reducing powers of pine needle water extract (PNWE) at different concentrations. Bars with different letters (a-e) are significantly different at  $P < 0.05$  (n=3).

Fe<sup>2+</sup> transformation in the presence of different concentrations of PNWE was investigated. The reducing power of PNWE increased with increasing concentration of PNWE up to 1,000 ppm in a concentration-dependent mode and a statistical difference of the reducing power was found among the different concentrations of the PNWE ( $P < 0.05$ ) (Fig. 2). The presence of a reducer (i.e. antioxidant) induces the reduction of the Fe<sup>3+</sup>/fericyanide complex to the ferrous form (32). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (33). As shown in Fig. 3 and 4, the high correlations between the DPPH radical scavenging activity or H<sub>2</sub>O<sub>2</sub> scavenging activity and reducing capacity of the PNWE were observed with a correlation coefficient of determination ( $r^2$ ) of 0.854 and 0.949, respectively, revealing that the reducing capacity of PNWE is positively correlated with the activities to scavenge free radicals or H<sub>2</sub>O<sub>2</sub>. It can be stated that a compound with anti-radical or H<sub>2</sub>O<sub>2</sub> scavenging activity is a strong reducer, which may act as an antioxidant in non-cellular and cellular systems. The antioxidative activity of a compound has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition



**Fig. 3.** The relationship between DPPH radical scavenging activity and reducing capacity of pine needle water extract at different concentrations.

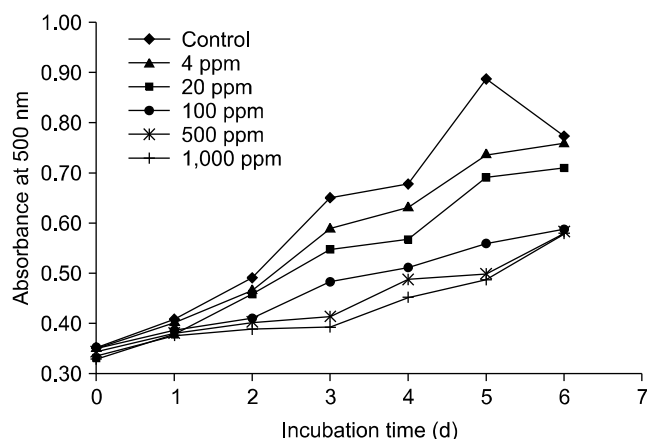


**Fig. 4.** The relationship between H<sub>2</sub>O<sub>2</sub> scavenging activity and reducing capacity of pine needle water extract at different concentrations.

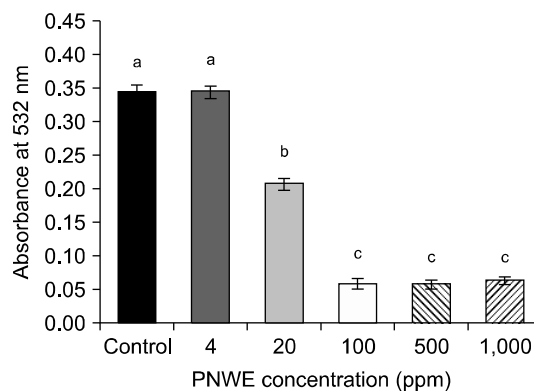
of peroxides, prevention of continued hydrogen abstraction, reductive, and radical scavenging capacities (16). The reducing power of pine needle could be due to its hydrogen-donating ability resulting from phenolic compounds including flavonoids and phenolic non-flavonoids (34). Accordingly, the water extract obtained from pine needle powder could contain higher amounts of phenolic compounds, which may react with free radicals to stabilize and block radical chain reactions.

**Total antioxidant activity of the PNWE in a linoleic acid system**

The total antioxidant activity of PNWE in linoleic acid peroxidation at different concentrations (0, 4, 20, 100, 500, and 1,000 ppm) was determined using the FTC and TBA methods, and the data are plotted in Fig. 5 and 6. The FTC method is used to measure the peroxide level at the initial stage of linoleic acid peroxidation, whereas the TBA method measures the secondary stage (16,35). As shown in Fig. 5, the individual activity of the PNWE



**Fig. 5.** Antioxidative activity at different concentrations of pine needle water extract (PNWE) against linoleic acid peroxidation as measured by the thiocyanate method.



**Fig. 6.** Antioxidative activity at different concentrations of pine needle water extract (PNWE) as measured by thiobarbituric acid method. Bars with different letters (a-c) are significantly different at  $P < 0.05$  ( $n = 3$ ).

test samples showed relatively lower absorbance values than that of the control, an indication of high antioxidant activity. In general, the antioxidant activity of PNWE is exhibited in a concentration-dependent mode, which showed a more effective antioxidant activity with increasing concentration and is significantly ( $P < 0.05$ ) different from the control. For example, the antioxidant activities of PNWE at 5 days of linoleic acid peroxidation were 16.788, 22.219, 37.139, 43.682, and 45.149% when the concentrations of PNWE were 4, 20, 100, 500, and 1,000 ppm, respectively (Table 3). In the early stage, the autoxidation of linoleic acid without PNWE (control) was accompanied by a rapid increase of peroxide levels up to 5 days of incubation. Significant differences ( $P < 0.05$ ) were found between the control and linoleic acid systems with PNWE, in which a slowed rate of peroxide formation was observed.

The absorbance values obtained by the TBA method reflect total peroxide values produced by the oxidation of linoleic acid (Fig. 6). During the oxidation of linoleic acid, peroxides are gradually decomposed to lower mo-

**Table 3.** Inhibition percentage of peroxide formation in the presence of the pine needle water extract (PNWE) fraction at 4 days after linoleic acid peroxidation

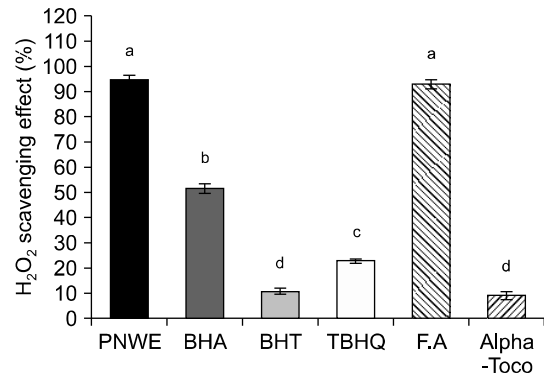
PNWE fraction (ppm)	Inhibition of peroxide formation (%)
4	16.788±0.956 <sup>a</sup>
20	22.219±0.561 <sup>b</sup>
100	37.139±0.820 <sup>c</sup>
500	43.682±0.678 <sup>d</sup>
1,000	45.149±1.375 <sup>d</sup>

Each value is the mean±standard deviation (n=3). Values with different letters (a-d) are significantly different ( $P < 0.05$ ).

lecular weight compounds (35) and one such compound is malonaldehyde, which was measured by the TBA method on the final day of the incubation period. The higher the absorbance values, the lower the level of antioxidant activity (16). As shown in Fig. 6, the control showed the highest absorbance value followed by increasing concentration of PNWE up to 100 ppm in a concentration-dependent mode; however, no significant difference was observed ( $P > 0.05$ ) after 100 ppm of PNWE. The FTC data indicated the control reached a maximum level of peroxide formation at day 5 and finally dropped at day 6 (Fig. 5). This reduction of the peroxide level is due to the accumulation of malonaldehyde from linoleic acid oxidation, which is not stable. Further oxidation causes malonaldehyde to be converted to secondary products such as alcohols and acids that cannot be detected.

#### Comparison of the antioxidant activity between the PNWE and commercial phenol compounds

***H<sub>2</sub>O<sub>2</sub> scavenging activity:*** The  $H_2O_2$  scavenging ability of PNWE at 1,000 ppm containing 150.67  $\mu\text{g}/\text{mL}$  (total phenolics calculated by a method for the determination of total phenolics described in MATERIALS AND METHODS) of phenolic compounds was compared with those of commercial phenolic antioxidants such as BHA, BHT, TBHQ, ferulic acid, and  $\alpha$ -tocopherol at a concentration of 150.67  $\mu\text{g}/\text{mL}$ . As shown in Fig. 7, the PNWE showed a significant scavenging capacity ( $P < 0.05$ ) for  $H_2O_2$  compared with commercial phenolic antioxidants. The order of the antioxidant power by the  $H_2O_2$  scavenging test was as follows: PNWE  $\geq$  ferulic acid  $>$  BHA  $>$  TBHQ  $>$  BHT  $\geq$   $\alpha$ -tocopherol (Fig. 7). Thus, the PNWE fraction could be an effective scavenger against  $H_2O_2$ , one of reactive oxygen species (ROS).  $H_2O_2$  is highly important due to its ability to penetrate biological membranes. Although  $H_2O_2$  itself is not very reactive, it can sometimes exert toxicity to cells, because it may give rise to the hydroxyl radical, the strongest ROS, in the cells (36). There is increasing evidence that increased production of ROS is involved in the progress of many human diseases resulting from oxidative cellular damage (37).

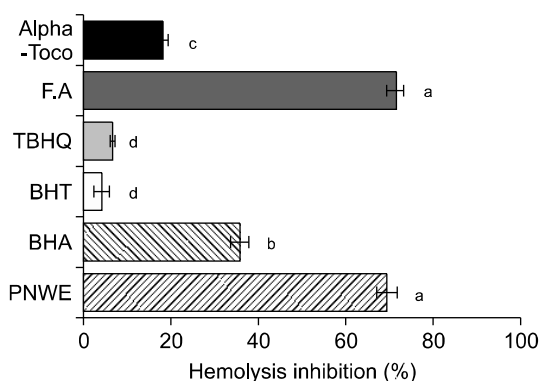


**Fig. 7.** Comparison of scavenging activities between pine needle water extract (PNWE) and commercial phenol compounds against hydrogen peroxide. BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, *tert*-butyl hydroquinone; F.A, ferulic acid. Values with different letters (a-d) are significantly different ( $P < 0.05$ ).

Recently, much attention has been focused on the protective function, especially the antioxidative effect of naturally occurring compounds in our diet. Phenolic compounds in plant-derived foods have been considered to play an important role as dietary antioxidants for the prevention of oxidative cellular damage in biological systems. The data of the present study show that PNWE is a good scavenger of  $H_2O_2$  *in vitro* and could play a role in the decomposition of peroxy radicals generated during the oxidative process.

#### Antioxidant activity in biological systems

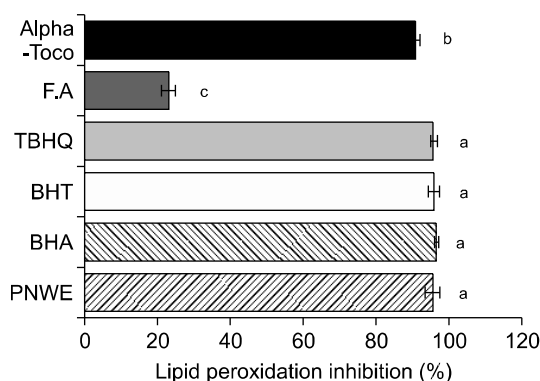
***Assay for rat RBC hemolysis mediated by peroxy free radicals:*** AAPH is a peroxy radical initiator that generates free radicals by its thermal decomposition, allowing the radicals to attack RBC, which induces the chain oxidation of lipids and proteins by disturbing the cell membrane and eventually leading to hemolysis (7,26). In the present study, the protective effect of PNWE on hemolysis by peroxy radical scavenging activity was investigated and compared with those of commercial phenolic antioxidants. Fig. 8 shows the inhibition percentage of hemolysis induced by AAPH as a result of the protection against the oxidative damage of RBC membranes obtained from rats. The order of the inhibition activity on hemolysis was as follows: PNWE  $\cong$  ferulic acid  $>$  BHA  $>$   $\alpha$ -tocopherol  $>$  TBHQ  $\geq$  BHT. In the present study, PNWE appeared to possess relatively higher antioxidant activity ( $P < 0.05$ ) compared to the commercial phenolic antioxidants at a concentration of 150.67  $\mu\text{g}/\text{mL}$ , which suggest that the antioxidant activity of phenolic compounds could depend on the number and position of hydrogen-donating hydroxyl groups on the aromatic cycles of the phenolic molecules (38). The data in the present study shows that PNWE behaves as a potent scavenger of peroxy radicals, which may lead to hemolysis in cellular systems. Moreover, the ability of PNWE to counteract



**Fig. 8.** Comparison of inhibition activities between pine needle water extract (PNWE) and commercial phenol compounds on hemolysis of rat red blood cells induced by 2,2'-azobis(2-amidinopropane) dihydrochloride. BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, *tert*-butyl hydroquinone; F.A, ferulic acid. Values with different letters (a-d) are significantly different ( $P < 0.05$ ).

the hemolysis mediated by peroxy radicals confirms the previous observations on its antioxidant activity in non-cellular systems. According to a study by Tedesco et al. (39), when intact human RBC were pre-incubated with plant extracts rich in phenolic compounds, a strong protective effect against  $H_2O_2$ -generated hemolysis was observed. In addition, an increasing body of evidence supports the hypothesis that a number of dietary phenolic compounds labeled as "antioxidants" have a beneficial role in retarding or reversing the course of chronic degenerative diseases.

**Assay for lipid peroxidation of rat brain homogenate:** MDA, which is formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of lipid peroxidation (27). Lipid peroxidation has long been recognized as a potential mechanism of cell injury, and  $Fe^{2+}$  is well-known to stimulate lipid peroxidation in cellular systems (40). The inhibition of  $Fe^{2+}$ /ascorbate-induced lipid peroxidation in rat brain homogenates by PNWE was measured by the color intensity of the MDA-TBA complex and compared with those of commercial phenolic antioxidants at a concentration of 150.67  $\mu\text{g}/\text{mL}$ . As shown in Fig. 9, PNWE was found to be comparable and not significantly different ( $P > 0.05$ ) in inhibiting  $Fe^{2+}$ /ascorbate-induced lipid peroxidation in rat brain homogenates with the synthetic phenolic antioxidants such as TBHQ, BHT, and BHA. The result of lipid peroxidation suggest that PNWE has a preventive effect against metal-induced lipid peroxidation in a cellular system, which may be due to some bioactive compounds involved in metal-induced lipid peroxidation. Some flavonoids such as myricetin, quercetin, and fisetin, effectively suppressed lipid peroxidation induced by Fe ion (41) which thus opens up the possibility to support the hypothesis that the chelating



**Fig. 9.** Comparison of inhibition activities between pine needle water extract (PNWE) and commercial phenol compounds by  $Fe^{2+}$ /ascorbate-induced lipid peroxidation in rat brain homogenate. BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TBHQ, *tert*-butyl hydroquinone; F.A, ferulic acid. Values with different letters (a-c) are significantly different ( $P < 0.05$ ).

of metal ion by phenolic compounds present in PNWE could prevent lipid peroxidation by restricting the access of the metal ions toward lipid hydroperoxide in a rat brain cell line. Based on the results of the two assays performed in biological systems, PNWE could have a positive and comparable antioxidant power in inhibiting both the hemolysis induced by peroxy radicals and metal-induced lipid peroxidation as compared to commercial phenolic antioxidants.

In conclusion, the antioxidant activity in PNWE obtained from dried pine needle was observed at different concentrations (4, 20, 100, 500, and 1,000 ppm), and the optimum concentration at which the strongest antioxidant power could be obtained was selected and compared to those of commercial phenolic compounds at 150.67  $\mu\text{g}/\text{mL}$ . The optimum antioxidant power tested in the concentration-dependent mode was observed in the samples containing 1,000 ppm of PNWE at which a significant power in DPPH radical and  $H_2O_2$  scavenging activities as well as reductive capability were shown. PNWE also effectively inhibited the formation of the primary and secondary oxidation products generated during linoleic acid peroxidation. Interestingly, the PNWE fraction obtained from dried pine needle exhibited high antioxidative activity comparable to those of the widely used commercial phenolic compounds in biological systems, which inhibited rat RBC hemolysis mediated by peroxy free radicals and suppressed metal-induced lipid peroxidation of rat brain homogenates. The strong antioxidative activity of PNWE could be due to the presence of naturally-occurring phenolic compounds including flavonoids and derivatives, which are attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging.



## AUTHOR DISCLOSURE STATEMENT

The author declares no conflict of interest.

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