Antioxidant Activity and Protection from DNA Damage by Water Extract from Pine (*Pinus densiflora*) Bark

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**Abstract**

Water extract from *Pinus densiflora* (WPD) was investigated for its antioxidant activity and its ability to provide protection from DNA damage. A series of antioxidant assays, including a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay, a reducing power assay, a metal-chelating assay, a superoxide radical scavenging assay, and a nitrite scavenging ability, as well as a DNA damage protection assay were performed. Total phenolic content was found to be 211.32 mg Tan/g WPD. The extract scavenged 50% DPPH free radical at a concentration of 21.35 μg/mL. At that same concentration, the reducing power ability of WPD was higher than that of α-tocopherol. The extract chelated 68.9% ferrous ion at the concentration of 4 mg/mL. WPD showed better nitrite scavenging effect at the lower pH. Meanwhile, WPD exhibited a strong capability for DNA damage protection at 1 mg/mL concentration. Taken together, these data suggest water extract from *Pinus densiflora* could be used as a suitable natural antioxidant.

**Key words:** antioxidant, DNA damage, phenolic content, reactive oxygen species

**INTRODUCTION**

Reactive oxygen species (ROS) are formed *in vivo* and raise the incidence of more than 30 different diseases by damaging cell structures, DNA, lipids, and proteins (1); therefore, it is necessary to employ exogenous antioxidants to balance the ROS in human body (2). The large amount of evidence suggesting that oxidative stress is involved in the pathogenesis of various disorders and diseases, has inspired both scientists and the general public to pay attention to the role of antioxidants in maintaining the health of human body and preventing and treating diseases (3). A medical revolution that has been induced as a result of the discovery of the effect of free radicals in cancer, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders, aging, and other diseases, and this revolution is promising a new outlook for healthcare (4).

Antioxidants have been used as food supplements for a very long time, but people still pay close attention to the safety of chemical or artificial antioxidants (5). The antioxidants used currently, such as tocopherol, tertiary-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been reported for the toxicity and carcinogenicity and other adverse effects (6). Therefore, natural antioxidants from plant extracts are gaining increasing interest because of consumer’s concern about the security of the synthetic antioxidants that are used as food supplements. Natural antioxidants such as tocopherols, flavonoids, and rosemary (*Rosmarinus officinalis* L.) extracts avoid the disadvantages of synthetic antioxidants. The synthetic antioxidants such as BHA, BHT, and propyl gallate (PG) may cause toxicity problems (7). Plants are the main source of natural antioxidants, such as vitamin E (α-tocopherol), vitamin C (ascorbate) and phenolic compounds. The antioxidant effects of some plants are mainly due to the phenolic component (8), and phenolic compounds found in food also have been demonstrated to have potential antioxidant activity (9). Some extracts from fruits, vegetables, cereals, and their by-products exhibit valid antioxidant activity in a model system and researchers have also tested natural antioxidants in food systems (10).

*Pinus* (*P.*) *densiflora* belongs to the Pinaceae family and is mainly distributed in Northeastern China (Heilongjiang, Jilin, Liaoning, Shandong), the extreme Southeast of Russia (Southern Primorsky Krai), Korea and Japan. The forest area is more than 65% of total area in South Korea and *P. densiflora* accounts for about 87% of coniferous forests as the most abundant conifer (11). *P. densiflora* has been used as an herbal medicine to treat stroke, atherosclerosis, hypertension, and diabetes mellitus in the Orient (12). Pine bark accounts for about 10~15% of the total tree weight and could be an atten-
tion-getting and significant biomass resource (13). Pine bark extract is reported to be rich in bioflavonoids, pro-
cyans and phenolic acids and has been shown to pos-
sess the ability to decrease the level of blood glucose and decrease the undesirable complications of the dia-
abetes (14). Our main work focuses on the antioxidant ac-
vity of water extract from *P. densiflora* (WPD), as determined by a series of antioxidant assays.

**MATERIALS AND METHODS**

**Preparation of extract**

The *P. densiflora* bark was harvested from the *P. den-
siflora* tree grown in Chuncheon, Korea. *P. densiflora*
bark was dried and immersed in ten times the weight 
of distilled water at room temperature for 12 hr. The 
extract was filtered and dried by evaporation of water. 
Dry extract was stored in a refrigerator before analysis. 
The WPD was dissolved in distilled water with the con-
centration of 10 mg/mL and kept at -20°C for stock.

**Chemicals**

1,1-Diphenyl-2-picrylhydrazyl (DPPH), BHA, α-toco-
pherol, Folin-Ciocalteu reagent, ethylenediamine tetra-
acetic acid (EDTA), 3-(2-pyridyl)-5,6-bis(4-phenyl-sul-
fonic acid)-1,2,4-triazine (ferrozine), tannic acid, querce-
tin, trichloroacetic acid (TCA), aluminium chloride hex-
hydrate (AlCl₃), phenazonium methosulphate (PMS), β-
nicotinamide adenine dinucleotide reduced disodium salt 
(NADH), and nitro blue tetrazolium tablet (NBT) were 
purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Determination of total phenolic content**

The concentration of WPD was determined at 1 mg/ 
ml and 0.1 mL of solution or tannic acid (20, 40, 80, 
160, and 320 μg/mL) was mixed with 0.5 mL of Folin-
Ciocalteu reagent (10%). After shaking, 0.4 mL of 7.5%
sodium carbonate solution was added and the mixture 
was kept at room temperature for 30 min. The absorb-
ance was measured at 750 nm using an absorbance mi-
croplate reader (ELX800, Bio-Tek, Vermont, IL, USA). 
Tannic acid was used as a standard to present the total 
phenolic content of WPD. The results were expressed 
as mg Tan/g WPD.

**DPPH free radical scavenging activity**

DPPH was dissolved in methyl alcohol and the sol-
tion was prepared at concentration of 0.1 mM. A volume 
of 0.5 mL WPD at five different concentrations (3.125, 
6.25, 12.5, 25, and 50 μg/mL) and 0.5 mL of DPPH sol-
tion were mixed in a 1.5 mL test tube. After shaking 
adequately, then allowed to react at room temperature 
in the dark for 30 min, absorbance was measured at 
515 nm. BHA and α-tocopherol were used as positive 
controls. For calculating the capability to scavenge the 
DPPH free radical, the following formula was used:

\[
I (\%) = \left[1 - \frac{A_b - A_i}{A_c - A_i}\right] \times 100
\]

Where \(A_b\) is absorbance of extract without DPPH sol-
tion (0.5 mL of extract plus 0.5 mL of methanol); \(A_c\) 
is absorbance of DPPH solution without extract (0.5 mL 
of DPPH solution plus 0.5 mL of methanol); \(A_i\) is ab-
sorbance of extract with DPPH solution (0.5 mL of ex-
tract plus 0.5 mL of DPPH solution).

**Reducing power assay**

The reducing power of WPD was evaluated by the 
method of Hu et al. (15). Five different concentrations 
(0.1, 0.2, 0.4, 0.8 and 1 mg/mL) of extract were mixed 
with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) 
and 2.5 mL of 0.1% potassium ferricyanide. The mixture 
was incubated at 50°C for 30 min, then 2.5 mL of 10%
TCA solution was added. Next, the mixture was cen-
trifuged at 3,000 rpm for 10 min at room temperature. 
After that, 2.5 mL of the upper layer solution was re-
moved and put into a new test tube. 2.5 mL of distilled 
water and 0.5 mL of 0.1% ferric chloride were added 
and then the mixture was mixed evenly. The absorbance 
was measured at 700 nm against a reagent blank. α-
Tocopherol was used as a positive control.

**Metal chelating activity**

Various concentrations (0.5, 1, 2, and 4 mg/mL) of 
WPD and 2 mM FeCl₂ (in methanol surroundings) were 
prepared. 0.2 mL of WPD and 0.02 mL of FeCl₂ were 
mixed and 0.04 mL of 5 mM ferrozine was added. The mixture 
was vigorously shaken then left at room temper-
ature for 10 min. The absorbance was then measured 
at 562 nm. EDTA was used as a positive control. The 
following formula was used to calculate the metal chelat-
ing activity:

\[
\text{Metal chelating activity} (\%) = \left[1 - \frac{A_i}{A_c}\right] \times 100
\]

Where \(A_i\) is the absorbance of WPD or EDTA against 
the blank and \(A_c\) is the absorbance of the control.

**Superoxide radical scavenging assay**

The superoxide radical scavenging activity of WPD 
was assessed using the model PMS-NADH described by 
Singh and Rajini (16). 0.1 mL of the extract solution 
was mixed with 0.1 mL of 150 μM NBT and 0.1 mL 
of 468 μM NADH in 1 mL of 0.1 M phosphate buffer 
(pH 7.4). Then 0.02 mL of 60 μM PMS was added. The reaction 
was carried out at room temperature for 8 min. 
The absorbance was measured at 560 nm and gallic 
acid was used as a positive control. The superoxide rad-
cial scavenging ability of WPD was evaluated by calcu-
lating the inhibition of superoxide radical generation ac-
cording to the following formula:
Inhibition (%) = \left[ 1 - \left( \frac{A_i}{A_c} \right) \right] \times 100

Where \( A_i \) is the absorbance of samples against the blank and \( A_c \) is the absorbance of the control.

Measurement of nitrite scavenging ability
200 \( \mu \)L of 1 mM nitrite sodium was added to 200 \( \mu \)L of extract solution, then 1.6 mL of 0.1 M HCl buffer (pH 1.2 adjusted with NaOH) or 0.2 M citrate buffer at pH 4.2 or 6.0 was mixed. The mixture was incubated at 37°C for 1 hr and then 200 \( \mu \)L of solution was taken out and mixed with 400 \( \mu \)L of 2% acetic acid. Then, 80 \( \mu \)L of Griess reagent [the mixed solution of 1% sulfanilamide (in 5% phosphoric acid) and 0.1% aqueous solution of naphthyl-ethylenediamine dihydrochloride] was added. The mixture was held at room temperature for 15 min after shaking. The absorbance was measured at 515 nm. The calculation was performed according to the method in superoxide radical scavenging assay.

DNA damage protection assay
Genomic DNA was isolated from RAW 264.7 cells. 0.5 \( \mu \)g of DNA was mixed with 3 \( \mu \)L of 50 mM phosphate buffer (pH 7.4) and 3 \( \mu \)L of 1 mM FeSO4. Then, 10 \( \mu \)L of WPD was added. After adding 4 \( \mu \)L of 0.1 mM H2O2 the mixture was incubated at 37°C for 30 min. After that, 5 \( \mu \)L of gallic acid with high concentration was added immediately to end the reaction. Finally, the DNA was analyzed with 1% agarose gel electrophoresis. A solution of hydrogen peroxide and a ferrous iron catalyst is called Fenton’s reagent. Fenton’s reagent is usually used to oxidize organic contaminants or waste waters. It was used to destroy the DNA in this assay.

RESULTS AND DISCUSSION
Determination of total phenolic content
Plant phenolic compounds with reducing and antioxidant properties can inhibit the formation of superoxide anion radicals, which has been reported by Bursal et al. (17); therefore, the total phenolic content can be used as a useful indicator of antioxidant potential. In this study, the yield of \textit{P. densiflora} bark extract was 3.6% and the total phenolic content of this sample was 211.32 mg Tan/g WPD (Fig. 1). Compared to similar research, the total phenolic content of WPD reported in this paper is high. For example, Li et al. (18) reported that the total phenolic content of \textit{Crataegus pinnatifida} Bunge methanol extract was 101.56 Tan/g. 80% methanol extract of \textit{Pinus cembra} L. bark has higher content of total phenolic compounds (299.3 mg gallic acid equivalents/g extract), however, the content of total phenolic compound of \textit{Pinus cembra} L. needle extract was low (78.22 mg gallic acid equivalents/g extract), as presented by Apetrei et al. (19).

DPPH free radical scavenging activity
The DPPH free radical scavenging activity of WPD is presented in Table 1. WPD showed an IC50 (the concentration at which WPD scavenges 50% of the DPPH free radical) at a concentration of 21.35 \( \mu \)g/mL, which is significantly higher than BHA and \( \alpha \)-tocopherol. The significantly lower IC50 value of BHA or \( \alpha \)-tocopherol indicates that the free radical scavenging effects of BHA and \( \alpha \)-tocopherol are better than that of WPD. However, the DPPH free radical scavenging activity of WPD was found to be strong compared to the water and methanol extracts of \textit{P. densiflora} needles, which showed an IC50 value of 25.1 \( \mu \)g/mL and 32.5 \( \mu \)g/mL (20), respectively.

Reducing power assay
Results of the reducing power assays are presented in Fig. 2. The higher absorbance of the reaction mixture reflects a stronger reduction capability. The extract showed a dose-dependent reducing power. At 1 mg/mL, the reducing power of WPD was the strongest. With higher concentration (0.8 and 1 mg/mL) the reducing power of WPD was higher than that of \( \alpha \)-tocopherol. Ustun et al. (21) reported the reducing power ability of needle acetone extracts from four Turkish \textit{Pinus} species
which include Pinus brutia, Pinus nigra, Pinus halepensis, and Pinus sylvestris. The reducing power (absorbance at 700 nm) of these extracts at a concentration of 1 mg/mL was 0.889, 0.893, 0.941, and 1.015, respectively, while the reducing power of WPD was 1.087 at the same concentration.

**Metal chelating activity**

The extract was able to chelate ferrous ion, as shown in Fig. 3, in a concentration-dependent fashion. WPD did not show an obvious metal-chelating effect when the concentration was low. WPD exhibited 68.9% chelating ability of ferrous ion at concentration of 4 mg/mL. On the other hand, EDTA exhibited 70.0% inhibition at 0.1 mg/mL concentration. Similar research was carried out by Lantto et al. (22) who showed that Siberian pine seeds chelated 50% of the iron (II) at a concentration of 20.1 mg/mL. Chelating agents could be used as an effective secondary antioxidants due to reducing their redox potential and steadying the oxidized form of the metal ion (23). The results in this study showed that WPD had an available activity for metal chelating.

**Superoxide radical scavenging assay**

Data in Fig. 4 shows the superoxide radical scavenging capacity of WPD compared with gallic acid. The inhibition of WPD was enhanced with increased concentration. However, the inhibitory effect of WPD was nearly the same at 250 and 500 µg/mL, which means that WPD has achieved a maximum effect on scavenging superoxide radical at concentration of 250 µg/mL. In addition, at that concentration, the superoxide radical scavenging activity of extract was lower than that of gallic acid. Joo et al. (24) have reported the superoxide anion radical scavenging activity of 70% ethyl alcohol extract from Pinus densiflora root to be 50% of the superoxide radical at a concentration of 149.7 µg/mL. WPD exhibits 62.8% of superoxide radical scavenging activity at a concentration of 62.5 µg/mL.

**Measurement of nitrite scavenging ability**

As shown in Fig. 5, the extract scavenged nitrite in a concentration-dependent fashion under a condition of pH 1.2. WPD exhibited 34.28% of NO radical scaveng-
free radical scavenging activity, reducing power, metal-antioxidant activities, such as exhibiting stronger DPPH which is the most effective antioxidant currently in use. Reducing power of WPD even higher than against DNA damage. In the reducing power assay, the protective ability of water extract from 
P. densiflora against DNA damage protection assay
The stability of the genome and the normal life cycle of the cell are affected by DNA damage, which has been associated with cell cycle regulation, repair pathways, and cell death through a variety of mechanisms (25). Fig. 6 shows the agarose gel electrophoretic pattern of the damage induced by hydroxyl radicals on DNA in the presence and the absence of the various concentrations of pine bark water extracts (200, 500, 1000 μg/mL). The DNA derived from RAW 264.7 cells that was not incubated with Fenton’s reagent showed up as a bright band on agarose gel electrophoresis (lane 1), but a definite DNA band was not found in either lane 2, which contained Fenton’s reagent, or lane 3, which had the same Fenton’s reagent plus 250 μg/mL extract. The lack of DNA in these lanes implies that it has been thoroughly degraded. As the concentration of WPD was increased in lanes 4 and 5 (500 and 1000 μg/mL, respectively), the presence and brightness of the DNA band increased, indicating WPD can confer a protective effect against Fenton’s agent-induced DNA damage.

CONCLUSION
This study demonstrates the antioxidant activity and protective ability of water extract from 
P. densiflora against DNA damage. In the reducing power assay, the reducing power of WPD even higher than α-tocopherol, which is the most effective antioxidant currently in use. WPD showed some advantages, such as the advantage of abundant resources and the security and low-cost advantages of extraction with water. WPD showed strong antioxidant activities, such as exhibiting stronger DPPH free radical scavenging activity, reducing power, metal-chelating activity, and superoxide radical scavenging ability when compared with other samples including 
P. densiflora needles, needle acetone extracts from four Turkish 
Pinus species (Pinus brutia, Pinus nigra, Pinus halepensis, and Pinus sylvestris), Siberian pine seeds, and 
P. densiflora root in previous publication. These research results suggest 
P. densiflora could be used as an antioxidant source.

Fig. 6. Visualization of the damage induced by hydroxyl radicals on genomic DNA in the presence and absence of water extract from 
P. densiflora by agarose gel electrophoresis. Lane 1, DNA incubated without Fenton’s reagent; Lane 2, DNA incubated with Fenton’s reagent; Lanes 3 ~ 5, DNA incubated with Fenton’s reagent in the presence of 250 μg/mL, 500 μg/mL, and 1000 μg/mL of WPD, respectively.

| Fe²⁺/H₂O₂ | – | + | + | + | + |
| WPD (μg/mL) | – | – | 250 | 500 | 1000 |

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