

Antioxidant activity and analysis of proanthocyanidins from pine (*Pinus densiflora*) needles

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Abstract

In this study, we evaluated the antioxidant activity of pine needle extracts prepared with hot water, ethanol, hexane, hot water-hexane (HWH), and hot water-ethanol (HWE), using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical method. The hot water extract possessed superior antioxidant activity than the other extracts. We also compared the antioxidant activity of pine needle extracts through ROS inhibition activity in a cellular system using MC3T3 E-1 cells. The hot water extract exhibited the lowest ROS production. The pattern of HPLC analysis of each extract indicated that the hot water extract contained the highest proanthocyanidin level. The pine needle hot-water extract was then isolated and fractionated with Sephadex LH-20 column chromatography to determine the major contributor to its antioxidant activity. The No.7 and 12 fractions had high antioxidant activities, that is, the highest contents of proanthocyanidins and catechins, respectively. These results indicate that the antioxidant activity of proanthocyanidins from the hot water extract of pine needles is positively related to not only polymeric proanthocyanidins but also to monomeric catechins. Moreover, the antioxidant activity of the pine needle hot water extract was similar to well-known antioxidants, such as vitamin C. This suggests that pine needle proanthocyanidins and catechins might be of interest for use as alternative antioxidants.

Key Words: DPPH, ROS, HPLC analysis, proanthocyanidin, pine needle

Introduction

Free radicals and other reactive oxygen species are generated by exogenous chemicals or endogenous metabolic processes in food systems or the human body. ROS, such as superoxide radicals (O₂⁻), hydroxyl radicals (·OH), and hydrogen peroxide (H₂O₂), react very rapidly with DNA, lipids, and proteins causing cellular damage. A variety of enzymatic and nonenzymatic mechanisms have evolved to protect cells against ROS [1], including the superoxide dismutase, catalase, and glutathione peroxidase systems, which remove H₂O₂ [2]. The antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. Many phenolic compounds, particularly flavonoids, exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic, and vasodilatory actions [3]. Polyphenols are secondary metabolites and are widely distributed throughout the plant kingdom. Plants produce various antioxidant compounds to counteract ROS in order to survive. Almost all foods derived

from edible plants contain polyphenol compounds. Plant polyphenols are well-known natural antioxidants, and recent studies have reported that polyphenols in food play an important role in the prevention of geriatric disorders and cancers.

Pine trees (*Pinus densiflora*) belong to the family *Pinaceae* and are widely distributed around the world. In East-Asian countries such as Korea and China, various parts of pine trees, including the needles, cones, cortices, and pollen, are widely consumed as foods or dietary supplements to promote health [4]. Pine needles have been used to prepare drinks in Asia. In addition, pine needle drinks have been used as folk medicine, to treat hypertension for example [5]. Moreover, pine needles have been shown to inhibit leukemia cell growth [6] and protect against oxidative DNA damage and apoptosis induced by hydroxyl radicals [7]. For the remainder of the biological effects of pine needles, those from extracts of similar materials (i. e. pine bark) have pharmacological, antioxidant activity, antiproliferative, and antiinflammatory actions [8,10].

Proanthocyanidins, known as condensed tannins, belong to the

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oldest of plant secondary metabolites. These compounds are widespread in woody plants, but are also found in certain forages. Catechins and proanthocyanidins are strong antioxidants and are associated with many useful biological effects of tea and other plant products [11,12]. Proanthocyanidins are the major polyphenols in red wine as well as in grape seeds, and they have potent antioxidant activity [13,14]. More recently, a large number of reports have demonstrated that, besides anti-tumor action, proanthocyanidins can enhance the activity of chemotherapeutic agents and diminish normal cell toxicity [15,16]. Proanthocyanidins are a group of naturally occurring polyphenolic bioflavonoids, specifically taking the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (-)-epicatechin [17].

It is known that pine bark and grape seeds contain numerous proanthocyanidins and these materials have been actively studied; however, there have not been enough studies on pine needle proanthocyanidin contents and antioxidant ability. In this study, we extracted antioxidants from pine needles with various solvents in order to review the antioxidant activity of pine needles. The antioxidant activity of each extract was measured with the DPPH method. Oxidative stress regulates cellular functions in multiple pathological conditions, including bone formation by osteoblastic cells. The MC3T3-E1 pre-osteoblastic cell line is a well-accepted model of osteogenesis *in vitro* [18]. Therefore, we compared the antioxidant activity of pine needle extracts through a ROS inhibition activity measurement method in a cellular system using MC3T3 E-1 cells. Moreover, the proanthocyanidin content of the extracts was measured through HPLC and the extract with the highest level of antioxidants was isolated and fractionated by Sephadex LH-20 column chromatography in order to determine the major contributor to antioxidant activity.

Materials and Methods

Chemicals

2,2-diphenyl-1-picrylhydrazyl, L(+)-ascorbic acid, and (+)-catechin hydrate (Minimum 98%) were obtained from Sigma (St. Louis, MO). Acetonitril HPLC ultra Gradient and methanol HPLC solvent were obtained from JT Baker (New Jersey USA). Sephadex LH-20 was obtained from GE Healthcare (Stockholm Sweden). α -MEM, floated with Hank's balanced saline solution (HBSS), and FBS were obtained from Gibco BRL (Grand island, N.Y., USA). Phosphoric acid was obtained from JUNSEI (Tokyo Japan). DCF-DA was obtained from Invitrogen (California, USA).

Preparation of pine needle extracts

Pine needles were ground mechanically into a powder form using a mortar and pestle. They were extracted in a manner

similar to previous studies [19]. One kilogram of the pine needle powder was extracted with 2L of distilled water, ethanol, and hexane at 80°C for 12 h to obtain the hot water, hexane, and ethanol extracts, respectively. The water-insoluble fraction was collected and then extracted with ethanol (HWE) and hexane (HWH), respectively. After 12h, each extract was filtered with Whatman filter paper, the solvents were then evaporated under reduced pressure, and the solid residue was lyophilized. The extracts were stored at -20°C until used.

Analysis of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity in pine needle extracts

The scavenging effects of the samples for DPPH radicals were monitored according to the method for measuring activities of extracts developed by Duan *et al.* [20]. Each extract was dissolved and diluted in 99% methanol. One-hundred microliters of various concentrations of the extracts was mixed with 2,900 μ l of DPPH solution (120 μ M). The concentrations of the extracts were 1,000, 500, 250, 100, 50, and 10 μ g/ml and they were placed in a 96-well microplate. The mixture was incubated for 30 min at room temperature and then the absorbance was measured at 517 nm using a microplate reader. The inhibition of free radicals from DPPH as a percentage was calculated with the following equation:

DPPH radical scavenging activity (%) = $[(A_0 - A_1)/A_0] \times 100$
where A_0 is the absorbance of the control reaction, and A_1 is the absorbance of the test compound. The IC₅₀ value was defined as the concentration of 50% of DPPH radical scavenging activity. Proanthocyanidin was used as a positive control, and all tests were carried out in triplicate.

Intracellular ROS scavenging activity

MC3T3-E1 pre-osteoblastic cells were used for cell cultures. The cells were fed twice a week with α -MEM (Gibco BRL, Grand island, N.Y., USA) containing 10% fetal calf serum and antibiotics (Gibco BRL, Grand island, N.Y., USA). The cultures were grown in 5% CO₂ at 37°C. The medium was removed and the cell monolayer was gently washed twice with PBS, and then centrifuged with lysate at 1,100 rpm for 3 min. The cells were floated with Hank's balanced saline solution (HBSS) and DCF-DA. The solution was incubated at 37°C for 2 h. After incubation, HBSS was removed and PBS was added. The cells were seeded in a 96-well microplate. The MC3T3-E1 cells with DCF-DA were treated with 50 μ g/ml of the pine needle extracts (or 10 μ g/ml of standard proanthocyanidins) and H₂O₂ in each well. The fluorescence intensity was measured during excitation at 485 nm and emission was measured at 528 nm using a multi-detection microplate.

HPLC analysis of pine (*pinus densiflora*) needle proanthocyanidin content (HPLC-UV)

High-performance liquid chromatography (HPLC) was used to identify proanthocyanidin content in the hot water, ethanol, hexane, HWE, and HWH extracts. The extracts were dissolved in 1 ml/mg of methanol and then passed through a 0.45 μ m membrane filter. Reverse-phase separations were performed using a SupelcoSil LC-18 column (250 \times 4.6 mm i.d.) with a particle size of 5 mm (Supelco). The binary mobile phase consisted of (A) MeCN and (B) 0.3% phosphoric acid. A linear gradient elution was performed from 10% A to 20% A in 45 min, then to 60% A in 20 min. The flow-rate was 0.7 ml/min and the UV detector was set at 280 nm and the injection volume was 10 μ l.

Column chromatographic separation of hot water extract

The hot water extract (500 mg) was subjected to column chromatography (glass column 5 \times 30 cm) over sephadex LH-20 (50 g), using a 100% HPLC grade methanol solvent. Altogether, 30 fractions of 10 ml each were collected, which were then filtered through Whatman No. 2 filter paper. The fractions were used directly in the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical assay, and were then used to identify the individual antioxidant compounds in HPLC.

Statistical analysis

All the data are expressed as means \pm standard deviations (SD) of three determinations. Statistical comparisons were performed via one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. *P*-values of less than 0.05 were considered significant.

Results

DPPH radical scavenging activity

The DPPH method is based on the reduction of DPPH in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form of DPPH-H [21]. The stable radical DPPH has been widely used to determine the primary antioxidant activity of pure antioxidant compounds, plant and fruit extracts, and food materials [22]. The DPPH radical-scavenging activities of the extracts from pine needles are presented in Fig. 1. The highest scavenging effect was in the water extract, followed by the ethanol, HWE, hexane, and HWH extracts in order. The hot water extract at the 250–500 μ g/ml concentration exhibited the most powerful scavenging activity against DPPH radicals in a concentration dependant manner, and it exhibited about an 82% higher electron donating ability. The ethanol extract at about 1,000 μ g/ml, exhibited similar results as the hot water extract

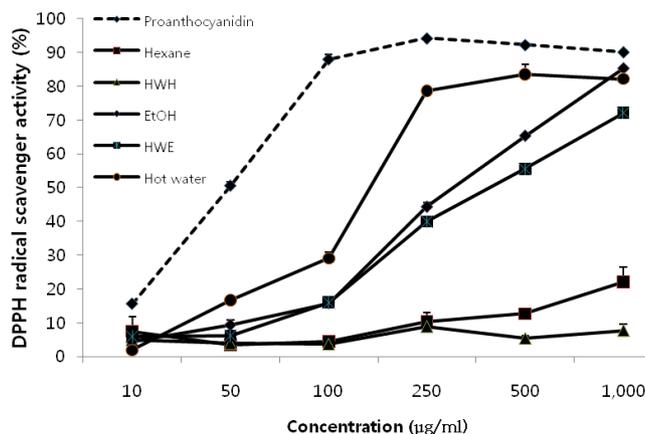


Fig. 1. DPPH radical scavenging activities of pine needle extracts

Table 1. DPPH radical scavenging activities (IC_{50}) of pine needle extracts at the 500 μ g/ml concentration

Pine needle extracts	IC_{50}^1 value (mg/ml)
Vitamin C	0.26 \pm 0.00 ^{2a3)}
Hot water	0.27 \pm 0.00 ^a
Ethanol	0.37 \pm 0.01 ^b
HWE	0.48 \pm 0.00 ^c
Hexane	1.00 \pm 0.01 ^d
HWH	2.49 \pm 0.04 ^e

¹⁾ IC_{50} value was defined as the concentration of 50% of DPPH radical scavenging activity.

²⁾ Values are means \pm SD of three determinations.

³⁾ Values with different letters are significantly different at *P* < 0.05 as analyzed by Duncan's multiple range test.

and it had the 2nd highest electron donating ability among the pine needle extracts. On the other hand, the hexane and HWH-extract had electron donating abilities below 20%, which were the lowest among the extracts. Moreover, the antioxidant activity of the pine needle hot water extract was similar to well-known antioxidants, such as vitamin C (Table 1).

Effects of pine needle extracts on reactive oxygen species (ROS) production

To investigate the ROS scavenging activity of pine needle extracts, we treated osteoblastic cells with each solvent fraction. The effects of the pine needle extracts were examined by comparing the results with those of a positive control, proanthocyanidins. As shown in Fig. 2, treatment with H_2O_2 resulted in 100% ROS formation after 30 min, which was significantly suppressed by treatment of most of the solvent extracts. Proanthocyanidins decreased H_2O_2 -stimulated ROS formation. In addition, the hot water extract decreased H_2O_2 -stimulated ROS formation even at the basal level. However, the addition of the HWH extract caused the highest ROS production (61.72%). These results confirmed that the ROS inhibition activity of the hot water extract compared to the other extracts.

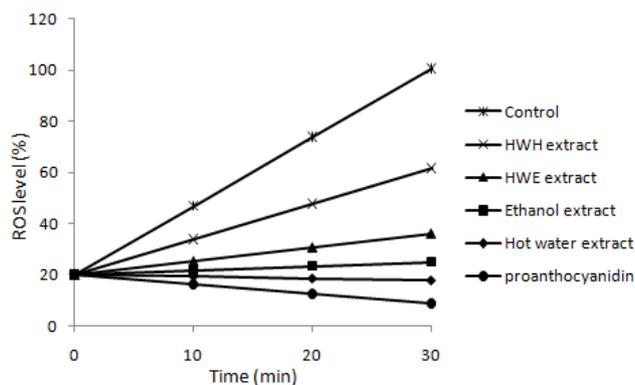
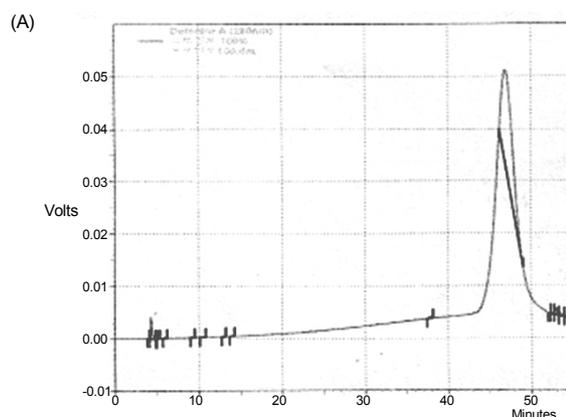


Fig. 2. Effects of pine needle extracts on H_2O_2 -induced reactive oxygen species (ROS) production in MC3T3-E1 osteoblastic cells. MC3T3-E1 cells loaded with DCF-DA were treated with or without pine needle extracts, followed by addition of 0,3 mM H_2O_2 in a cuvette for the quantitation of fluorescence intensity, which was detected by a multi-detection microplate.

Improvement of the HPLC analysis method for determination of proanthocyanidins

As a result of measuring the polymer proanthocyanidins by HPLC with the Federico Peterlongo method [23], the peak of the proanthocyanidins was not formed as a single peak, and the analysis method for proanthocyanidins was changed due to a low sensitivity and late analysis time. As a result of changing the concentration of the transition phase in proanthocyanidin HPLC analysis, from method (A) (Gradient : solvent system was a linear gradient using MeCN [solvent A] and 0.3% phosphoric acid [solvent B] at levels ranging from 10% A to 20% A during 45 min, then to 60% A during 20 min.) to method (B) (Gradient : solvent system was a linear gradient using MeCN [solvent A] and 0.3% phosphoric acid [solvent B] ranging from 10% A to 20% A during 35 min, then to 90% A during 20 min.), the proanthocyanidin analysis time decreased by about 13 min, from 60 min to 47 min. And this method showed the formation of a single peak and high sensitivity (Fig. 3). The content of the proanthocyanidins was measured using an HPLC analysis method developed similar to this.



Proanthocyanidin contents of pine needle extracts by HPLC analysis

Analysis was performed using HPLC in order to measure proanthocyanidin content among the pine needle extracts. The results of their proanthocyanidin contents are presented in Table 2. Measuring the proanthocyanidin contents of the pine needle extracts resulted in the hot water extract having the highest content at 30.54 mg/g and the ethanol extract having the second highest content at 30.11 mg/g. On the other hand, the hexane and HWH extract exhibited low contents of proanthocyanidin with 8.67 and 3.41 mg/g, respectively. These results indicate that the proanthocyanidin contents of pine needle extracts may be attributed to hydrophilicity. Following the extraction, the extract (hot water) with the highest antioxidant activity was isolated and fractionated by Sephadex LH-20 column chromatography to determine the major contributor to its antioxidant activity.

Antioxidant activity of hot water extract fractionated by Sephadex LH-20

The presence of complex components in an extract makes it difficult to quantify each antioxidant component separately [22]. Sephadex LH-20 has been one of the more widely used materials for proanthocyanidin chromatography since 1974 [24], both for size fractionation and more frequently for sample cleanup. The hot water extract with the highest antioxidant activity was refined using sephadex LH-20 column chromatography, and then the

Table 2. Concentrations of proanthocyanidin in pine needle extracts by HPLC

Pine needle extracts	Proanthocyanidin content (mg/g extract)
Hot water	30.54 ± 2.10 ^{1)a2)}
Ethanol	30.11 ± 3.05 ^a
HWE	27.11 ± 3.22 ^a
Hexane	8.67 ± 0.61 ^b
HWH	3.41 ± 0.01 ^c

¹⁾ Values are mean ± SD of three determinations.

²⁾ Values with different letters are significantly different at $P < 0,05$ as analyzed by Duncan's multiple range test.

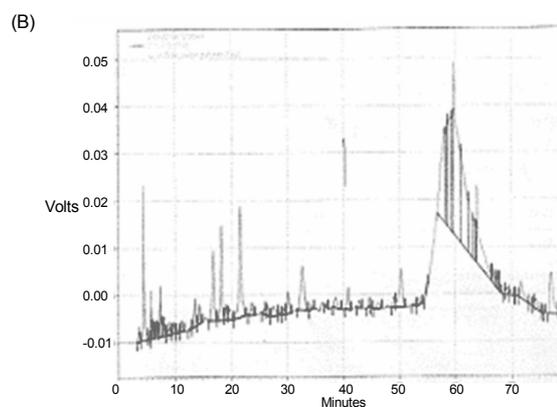


Fig. 3. HPLC chromatograms for proanthocyanidin standards of grape seed analyzed by the (B) Federico Peterlongo 1999 method and a (A) newly developed method

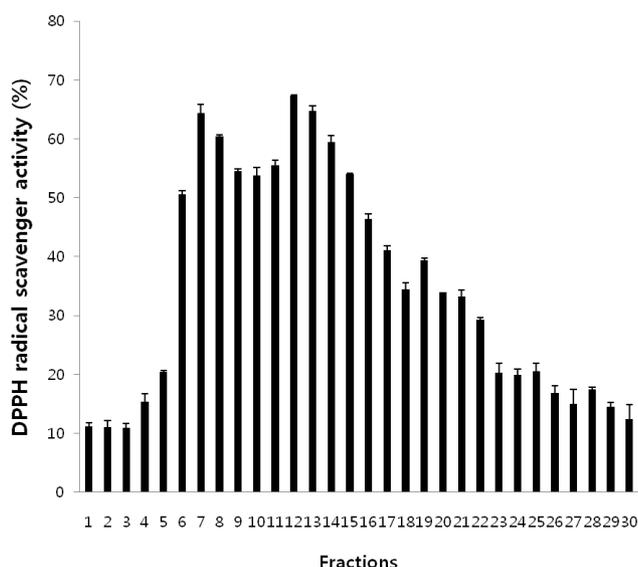


Fig. 4. DPPH radical scavenging activity of hot water extract fractions

antioxidant activity was compared to the DPPH method. These results are presented in Fig. 4. From fractions 6 to 21, antioxidant activities of more than 50% were maintained. Of these, fraction No. 7 in the first half of fractions with antioxidant activity had higher activity than fraction No. 12, which had the second highest antioxidant activity of the fractions. Thereafter, the substance that caused the high antioxidant activities of fractions No.7 and 12 was confirmed through HPLC analysis.

Characterization of procyanidins in hot water fractions by HPLC analysis

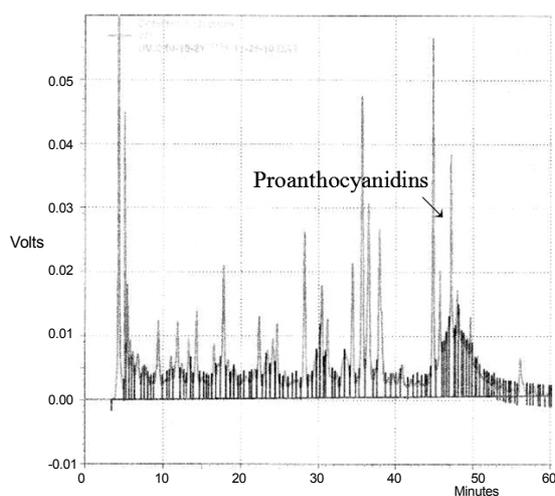
Procyanidinins are oligomers and polymers of flavan-3-ol monomer units [25]. The most common classes of procyanidinins consist of catechins, epicatechins, and/or their gallic acid esters.

To search for antioxidant substances that cause antioxidant behavior, procyanidinins were analyzed by HPLC. Two procyanidinins were detected and verified using standard compounds of catechins and procyanidinins (Fig. 5). The pattern of fraction No. 7 by HPLC analysis appeared to be the highest peak for procyanidinins and fraction No. 12 appeared to have the highest catechin content. These results indicate that the highest antioxidative effect of the hot-water extract might be due to the compositive effect of procyanidinins and catechins. Therefore, this reveals that hydrophilic polyphenols, such as catechins and procyanidinins, played important roles in the antioxidant activities of the hot water extract.

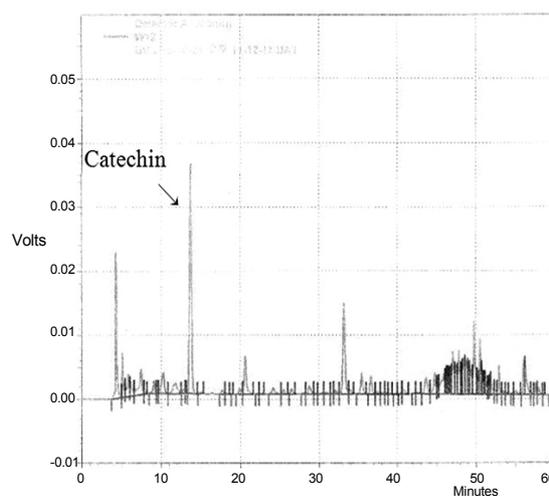
Discussion

Procyanidinins, known as condensed tannins, are among the oldest of plant secondary metabolites. These compounds are widespread in woody plants, but are also found in certain forages. Catechins and procyanidinins are strong antioxidants and are associated with many useful biological effects of tea and other plant products. The effects of bioflavonoids extracted from pine on free radical formation have already been investigated in murine macrophage cell lines, and strong scavenging activities against reactive oxygen species were exhibited [26]. We compared the antioxidant activity effects of pine needle extracts extracted via different solvents, using the DPPH method and at the cell level, in order to confirm the extraction method that would yield high antioxidant activity from pine needles.

The hot water extract exhibited the most powerful scavenging activity against DPPH radicals. On the other hand, the HWH extract exhibited the lowest DPPH radical activity. The antioxidant activity of the water extract from pine needles was high in this study and similar to that of pine needle hot water



(No. 7 fraction)



(No. 12 fraction)

Fig. 5. Analysis of a HPLC for fractions No. 7 and 12 separated from hot water extract

extract reported by Jeong *et al.* [7]. The results of the present study indicate that the DPPH radical-scavenging activity of pine needle extracts may be attributed to hydrophilic compounds. From the results of this experiment, the antioxidant component of pine needles is expected to be water-soluble, and it is also expected that any component that dissolves well in fat-soluble solvents like hexane would not exhibit active antioxidant activity.

Oxidative stress is caused by a disturbance of the balance between the antioxidant defense mechanisms of human organism and the level of reactive oxygen species (ROS), and it has been associated with many pathological disorders, such as atherosclerosis, diabetes, and cancer [27]. ROS include hydrogen peroxide (H₂O₂), hydroxyl radicals (.OH), and peroxynitrite (ONOO⁻). ROS are formed as by-products of mitochondrial respiration or by certain oxidases, such as nicotine adenine diphosphate (NADPH) oxidase, xanthine oxidase (XO), and a number of arachidonic acid oxygenases [28]. To investigate the ROS scavenging activity of pine needle extracts, we treated osteoblastic cells (MC3T3-E1) with each solvent fraction. The effects of the pine needle extracts were examined by comparing the results with those of a positive control, proanthocyanidin. Treatment with H₂O₂ resulted in 100% ROS formation after 30 min, which was significantly suppressed by treatment with most of the solvent extracts. Proanthocyanidins decreased H₂O₂-stimulated ROS formation. In addition, the hot water extract decreased H₂O₂-stimulated ROS formation even at the basal level. However, addition of the HWH extract resulted in the highest ROS production. These results confirm that the ROS inhibition activity of the hot water extract compared to the other extracts. As shown by this experiment, the water-soluble component of pine needles plays a positive role in the antioxidative activity.

Ku *et al.* [29] extracted proanthocyanidins from pine bark by sequencing the use of hot water, acetone, and hexane. We compared diverse extraction methods for proanthocyanidin extraction from pine needles as there are less systematic comparisons of these extraction methods. Souquet *et al.* [30] reported that six polymeric proanthocyanidins could be purified from grape skins by using size exclusion chromatography followed by normal phase HPLC, however, this procedure is too complicated and costly to be applied for commercial use of PA mass production. As a result of measuring the polymer proanthocyanidins by HPLC with the Federico Peterlongo method [23], the peak of the proanthocyanidins was not formed in a single peak, and the analysis method for proanthocyanidins was changed due to a low sensitivity and late analysis time. Therefore, we measured the content of proanthocyanidins using a newly developed HPLC analysis method. Fig. 4 shows proanthocyanidin amounts from the pine needles in reverse phase HPLC. The proanthocyanidin content of the hot water extract was higher than the contents of the other extracts. On the other hand, the HWH extract had the lowest proanthocyanidin content. From these results, water-soluble extraction is thought to be a more proper method than fat-soluble extraction to yield high proanthocyanidin

contents from pine needles and to extract highly concentrated proanthocyanidins.

The hot water extract is not harmful to the human body and seems to be the most appropriate for use as a functional food as opposed to using hexane as an extraction method. Furthermore, methanol and acetone are prohibited for use as extraction solvents due to oral usage by humans in Korea. In our study, the antioxidant activity of pine needle extracts agreed with their proanthocyanidin contents. In addition, the high antioxidant ability of the hot water extract is a complex synergy phenomenon between proanthocyanidins and catechins.

Consequently, the best extraction method to obtain high levels of proanthocyanidins and antioxidative extract from pine needles is hot water, and it would be possible to develop functional foods by proving the antioxidative ability of hydrothermal extract through animal testing.

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