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A comparison of Pycnogenol[®] and bark extracts from *Pinus thunbergii* and *Pinus densiflora*: Extractability, antioxidant activity and proanthocyanidin composition

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Hot water extracts (HWEs) from *Pinus densiflora* and *Pinus thunbergii*, two major *Pinus* species in Korea, and Pycnogenol[®] (PYC), a standardized *Pinus pinaster* bark extract, were fractionated to yield ethyl acetate (EAF) and proanthocyanidin (PAF) fractions for determination of total phenolic (TP) and proanthocyanidin (PA) content, and antioxidant activity. The TP and PA contents, the PAF/TP ratio, and the antioxidant activity from the HWE, EAF and PAF were all in the order of *P. densiflora* > *P. pinaster* > *P. thunbergii*. The mean degree of polymerization (mDP) by acid catalysis in the presence of excess phloroglucinol were similar in all three pine bark EAFs (3.51 to 3.69), but were quite variable in the other fractions (in PAFs, 5.56 (*P. thunbergii*) to 7.49 (*P. densiflora*); in HWEs, 4.85 (*P. densiflora*) to 6.14 (*P. pinaster*)). However, the PA structures determined by ¹³C NMR analysis with PAFs were similar for all three *Pinus* species with respect to the ratio of procyanidin and prodelphinidin and C2-C3 stereochemistry. From the aforementioned results, we could suggest that the bark from *P. densiflora* had more beneficial characteristics than the well-known Pycnogenol[®].

Key words: Antioxidant activity, pine bark, *Pinus densiflora*, *Pinus thunbergii*, proanthocyanidin, Pycnogenol[®].

INTRODUCTION

Pine bark is removed prior to the chipping process and is generally viewed as an inconvenient residue for the wood industry due to its high lignin/polyphenol content which causes difficulties in processing (Kofujita et al., 1999). However, pine bark contains high levels of natural polyphenols with various biological activities, making this material an attractive source of raw materials used in the fields of nutrition, health, cosmetics and medicine (Ku

and Mun, 2008). One example is Pycnogenol[®] (PYC), a well-known commercial product from Horphag Research that is extracted with water from the bark of the maritime pine (*Pinus pinaster*) and now utilized throughout the world as a nutritional supplement and as a phytochemical remedy for several diseases (Parker et al., 1999; Rohdewald, 2002; Maimoona et al., 2011). A number of pharmaceutical functions have been reported for PYC, including antioxidant, anti-diabetic, pro-cardiovascular and anti-inflammatory effects (Virgili et al., 1998; Liu et al., 2004; Maimoona et al., 2011). The most obvious action of PYC is its free radical scavenging activity. Many of the radicals that play roles in human health and disease, such as superoxide radical anion (O₂⁻), hydroxyl radical (HO[•]), lipid peroxy radical, and reactive nitrogen species (NO, ONOO⁻), can be quenched by phenolic compounds *in vitro* or *in vivo* by the formation of resonance-stabilized phenoxyl radicals (Maimoona et al., 2011).

The main constituents of pine bark extract have been identified as phenolic compounds, broadly divided into

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Abbreviations: ABTS, 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid); CE, (+)-catechin equivalents; DP, degree of polymerization; EAF, ethyl acetate fraction; HWE, hot water extract; mDP, mean degree of polymerization; mMW, mean molecular weight; NMR, nuclear magnetic resonance; PA, proanthocyanidin; PAF, proanthocyanidin fraction; PC, procyanidin; PD, prodelphinidin; PYC, Pycnogenol[®]; TP, total phenolic.

monomers (e.g., catechin, epicatechin, and taxifolin) and condensed flavonoids (e.g., oligomeric to polymeric proanthocyanidins (PAs)) (Jerez et al., 2009). The procyanidins (PCs), one subclass of PAs, consist of flavan-3-ols units: (+)-catechin and/or (-)-epicatechin units linked mainly through C4→C8 and/or C4→C6 bonds (Yanagida et al., 2003). In the case of PYC, oligomers ranging from five to seven flavan-3-ol units are the main constituents of PAs (Parker et al., 1999). The radical scavenging actions take place within the aromatic rings bearing one or more hydroxyl groups and the scavenging capacities are increased according to the increase in number of flavan-3-ols units and hydroxyl groups in the B-ring (Rice-Evan et al., 1996). However, the absorption and bioavailability of PAs in the human digestion system is inversely proportional to the length of flavan-3-ol unit (Schäfer and Högger, 2006). Therefore, some modifications of natural PAs have been attempted to improve the oligomeric PA content via acid-catalyzed cleavage of polymeric PAs (Fujii et al., 2007). Meanwhile, multiple phenolic hydroxyl groups in PAs can also form complexes with proteins, metal ions, and other macromolecules like polysaccharides (Schofield et al., 2001).

Analysis of PAs is difficult because of the structural complexity of these compounds; however, structural information on PAs is needed to extend the utilization of PAs in the future. Currently used analytical methods include oxidative depolymerization of PA, reactions of the A ring with an aromatic aldehyde, and oxidation-reduction reactions (Schofield et al., 2001). In addition, acid cleavage reactions, precipitation reactions, and enzyme inhibition reactions have also been used as analytical methods (Schofield et al., 2001). Each of these methods has advantages and disadvantages. For instance, the vanillin method is effective for the quantification of PAs with a standard, typically (+)-catechin. However, this method lacks specificity for PAs, and the reaction can vary depending on the subunits of PAs and the solvents used (Sun et al., 1998).

The number of flavan-3-ol subunits, expressed as degree of polymerization (DP), is one of the most important properties of PAs (Matthews et al., 1997). The most common method for the estimation of the DP in a PA extract is based on acid-catalyzed degradation in the presence of nucleophiles such as phloroglucinol, toluene- α -thiol, or cysteamine hydrochloride (Kennedy and Jones, 2001). Unlike colorimetric methods, including the vanillin method, this method can give information about compositional structure derived from the PA-nucleophile cleavage reaction and it avoids interferences with other constituents in the extract.

We have explored major pine tree species in Korea as suitable sources for PA. In this study, hot water extracts (HWEs) were prepared from the barks of black pine (*Pinus thunbergii*) and red pine (*Pinus densiflora*) and their ethyl acetate (EAF) and water (PAF) fractions were

obtained. The extraction yields, total phenolic (TP) content, PA contents, and radical scavenging activities were determined and compared with fractions from commercial PYC. The ^{13}C NMR method was used for the structural determination of PAF, and products from the acid-catalyzed cleavage of PAF in the presence of phloroglucinol were analyzed via reverse-phase HPLC. Based on these results, we could understand more about the chemical properties of barks from two pine species in Korea and suggest that the HWE from *P. densiflora* can be a good natural resource of PA for biological and pharmaceutical applications.

MATERIALS AND METHODS

Plant materials and chemicals

The outer bark of *P. thunbergii* was collected from Sacheon Beach, Ganeneung, Korea on September 20, 2010 and that of *P. densiflora* was collected from the forest on the grounds of the KIST Gangneung Institute on September 21, 2010. The bark was dried in the shade for one week and ground using an electric grinder. Commercial PYC was purchased from Jupiter International (Seoul, Korea). Phloroglucinol, ascorbic acid, and sodium acetate were purchased from Sigma-Aldrich (St Louis, MO). All solvents used for chromatography and extraction were of analytical grade and purchased from Daejung (Gyeonggi, Korea). All HPLC grade solvents were purchased from Fischer Scientific (Pittsburgh, PA, USA). Methanol- d_4 was purchased from Cambridge Isotope Laboratory (Andover, MA, USA).

Extraction and fractionation of HWE

Dried bark powder (100 g) was extracted with 1000 ml of boiling water for 3 h and the extracted solution was cooled to room temperature. After filtration through 3 M filter paper, the solution was frozen at -70°C and lyophilized for 2 days to obtain the HWE. The HWE was further fractionated by partitioning and Sephadex LH-20 chromatography. Four grams of HWE was resuspended in 100 ml of 70% aqueous acetone and extracted two times with 100 ml *n*-hexane to remove lipids. The aqueous layer was further extracted five times with 100 ml ethyl acetate to obtain EAF. The remaining aqueous layer was evaporated under vacuum at 60°C and dissolved in 50% aqueous methanol. This PA-rich material was further fractionated by Sephadex LH-20 chromatography (3 \times 50 cm). The sample loaded onto the column was washed five times with five column volumes of 50% aqueous methanol and then eluted with three column volumes of 70% aqueous acetone. The collected aqueous acetone solution was vacuum-dried to yield the PAF. PYC was isolated using the same procedure to make EAF and PAF. All samples were analyzed by HPLC.

Determination of total phenolics

Total phenolic contents from HWEs, EAFs, and PAFs from two pine trees and PYC was determined according to the Folin-Ciocalteu method using (+)-catechin as the standard (Ku et al., 2007). For each sample, 100 μl of 1 mg/ml solution was mixed with 100 μl of 50% Folin-Ciocalteu reagent. The mixture was incubated for 5 min, followed by the addition of 1 ml of 20% Na_2CO_3 . After 10 min of incubation at room temperature, the absorbance of the solution was measured at 730 nm. (+)-Catechin solution of different concen-

tration were serially prepared and used for construction of a calibration curve ($R^2 = 0.995$). The total phenolic content was expressed as (+)-catechin equivalents (CE) in mg/g of sample.

Determination of PA contents

PA contents from HWEs, EAFs, and PAFs from two pine trees and from PYC were determined by the vanillin- H_2SO_4 assay as described previously (Ku et al., 2007). (+)-Catechin was used as the standard ($R^2 = 0.995$ for the calibration curve) and the results were expressed as mg CE/g of sample.

ABTS free radical scavenging assay

The antioxidant activity of HWEs, EAFs, and PAFs from the two pine trees and the PYC was measured by their ability to donate hydrogen to 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radicals. A 2 mM ABTS radical stock solution containing 3.5 mM potassium persulfate was prepared in water and diluted 8-fold in water. This solution was incubated overnight in darkness at room temperature for radical stabilization and then used for reaction with sample solution (5 μ g/ml). A 100 μ l volume of sample solution was mixed with the same volume of ABTS solution and reacted for 5 min at room temperature. The absorbance of the sample and blank were recorded at 734 nm and the results were expressed using the following equation:

$$\text{ABTS free radical scavenging activity (\%)} = [1 - (\text{absorbance of sample}/\text{absorbance of blank})] \times 100$$

Phloroglucinol degradation of PA

The HWEs, EAFs, and PAFs from the two pine trees and from PYC were subjected to the acid-catalyzed cleavage reaction in the presence of excess phloroglucinol to investigate their degradation products, the mean degree of polymerization (mDP), and the mean molecular weight (mMW). A 10 mg sample was dissolved in 2 ml 0.1% hydrochloric acid in methanol containing 50 mg/ml phloroglucinol and 10 mg/ml ascorbic acid. The mixture was heated at 50°C for 30 min and then 10 ml sodium acetate (40 mM) was added to stop the reaction. The depolymerized reaction mixtures were immediately analyzed by HPLC. The PA cleavage products were estimated using their response factors relative to (+)-catechin, which was used as the quantitative standard, as described previously (Kennedy and Jones, 2001). To calculate mDP, the sum of all subunits (flavan-3-ol monomer and phloroglucinol adduct, in moles) was divided by the sum of all flavan-3-ol monomers (in moles).

High performance liquid chromatography

All fractions and phloroglucinol adducts were analyzed by reverse-phase HPLC. General HPLC analysis was performed with an Agilent 1200 analysis HPLC system (Agilent Technologies) consisting of a binary pump (G1312A), auto sampler (G1367B), diode array detector (G1315D) and degasser (D1379B). Separation of each sample with a reversed-phase YMC Pack Pro C_{18} RS column [150 mm length \times 4.6 mm i.d. and 3 μ m particle size (YMC Co., Kyoto, Japan)] was performed under ambient conditions. The mobile phase consisted of acetonitrile (A) and 0.1 % trifluoroacetic acid in water (B) at a 1 ml/min flow rate. The solvent gradient program was as follows: 10% of solvent A was held first for 10 min, and then increased from 10 to 40% over 40 min. The chromatograms were recorded at 280 nm.

^{13}C Nuclear magnetic resonance (NMR) spectroscopy

The PAFs from the two pine trees and PYC were subjected to ^{13}C NMR spectroscopy to obtain more information about individual subunits of PA. The NMR spectra were recorded on a Varian 500 MHz NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 125 MHz for ^{13}C in CD_3OD . A 100 mg sample of PAF was dissolved in 1 ml CD_3OD and the chemical shifts were expressed as δ values based on tetramethylsilane.

Statistical analysis

All analytical data were generated from at least three independent experiments. Mean values and standard deviations were calculated with Microsoft Excel software and the data were expressed as mean \pm standard deviation.

RESULTS AND DISCUSSION

Characterization of hot water extracts and their fractions from pine barks

The most commonly used method for the extraction of pine bark is hot water extraction, which can efficiently remove phenolic compounds, mainly PA. However, the extraction yields among pine tree species are quite variable and their TP and PA contents are highly dependent on the *Pinus* species used. In a previous publication, the yields from the barks of 11 *Pinus* species varied widely from 2.4 to 23.2% in the dry weight after hot water extraction (Ku et al., 2007). In particular, the HWE yield was relatively higher for *P. radiata* (23.3%) than for the other species. In the present study, the extraction yields from *P. thunbergii* and *P. densiflora* were 1.9 and 8.9%, respectively, based on dry weight, at a liquor ratio of 1:10 (Table 1). The yield for *P. thunbergii* was similar to reported literature values (2.5%), but the yield for *P. densiflora* was substantially higher (5.1%) (Ku et al., 2007). Increasing the liquor ratio to 1:50 increased the extraction yields to 3.7 and 8.2% for *P. thunbergii* and *P. densiflora*, respectively. In general, higher yields of HWE are obtained for *P. densiflora* than *P. thunbergii*. The extraction yield for PYC was reported as 6.4% (Vázquez et al., 2001).

The solvent fractionation of HWE can separate monomeric and oligomeric PAs from polymeric PAs. The EAF contains mainly monomeric flavonoids and short oligomeric PAs (DP 2-5), while the PAF obtained after Sephadex LH-20 chromatography contains mainly polymeric PA consisting of flavan-3-ol hexamers and longer (Ku and Moon, 2007). The relative ratio of EAF and PAF from three HWE samples was quite variable (Table 1). Based on the dried HWE, the highest percentage of PAF was found for *P. densiflora* (39.3%) and of the lowest for *P. thunbergii* (15.6%). The PYC showed the highest value (52.2%) for EAF, while the value for *P. thunbergii* was only 15.6% of HWE. This indicated that the HWE of *P. densiflora* contains more

Table 1. Characteristics of barks from *P. densiflora*, *P. thunbergii*, and Pycnogenol® (*P. pinaster* bark).

Parameter	<i>P. thunbergii</i>			<i>P. densiflora</i>			Pycnogenol®		
	HWE ^a	EAF ^b	PAF ^b	HWE ^a	EAF ^b	PAF ^b	HWE ^a	EAF ^b	PAF ^b
Extraction yields (%)	1.9 ± 0.23	15.66 ± 1.3	15.66 ± 0.9	8.9 ± 1.3	32.3 ± 2.1	39.3 ± 1.3	6.4 ^f	52.2 ± 3.4	30.4 ± 1.7
Total phenolics (mg/g) ^c	192.9 ± 13.4	379.4 ± 32.9	570.9 ± 62.3	524.7 ± 76.2	614.8 ± 49.5	611.4 ± 38.6	440.2 ± 23.4	404.2 ± 39.5	573.2 ± 64.3
Proanthocyanidins (mg/g) ^d	111.2 ± 4.8	166.8 ± 21.6	348.6 ± 42.5	382.2 ± 47.1	419.3 ± 39.6	483.2 ± 28.4	266.1 ± 32.1	198.8 ± 9.6	390.7 ± 28.4
PA/TP ratio (%)	57.6 ± 3.2	44.0 ± 3.9	61.1 ± 3.7	72.8 ± 7.3	68.2 ± 7.2	79.0 ± 6.4	60.4 ± 5.4	49.2 ± 2.2	68.2 ± 4.1
ABTS radical scavenging activity (%) ^e	15.6 ± 1.5	32.9 ± 1.32	59.7 ± 3.8	51.5 ± 3.8	57.9 ± 3.3	63.9 ± 4.3	40.7 ± 3.7	37.2 ± 3.6	59.2 ± 3.6

HWE, Hot water extract; EAF, ethyl acetate soluble fraction; PAF, proanthocyanidin fraction. ^{a,b}HWE was based on dried bark whereas EAF and PAF were based on dry HWE. ^c, Values determined by Folin-Ciocalteu assay, in units of milligrams (+)-catechin equivalent per gram sample. ^d, Values determined by vanillin-H₂SO₄ assay, in units of milligrams (+)-catechin equivalent per gram sample. ^e, Concentration of samples in reaction solution were 5 µg/ml. This value was from the published data (Vázquez et al., 2001)

polymeric PA than do the HWEs of the other two *Pinus* species and that the PYC HWE contains relatively high levels of monomeric or oligomeric PAs. In addition, the bark of *P. thunbergii* showed the lowest extractability with hot water, and the lowest portion of EAF and PAF among three *Pinus* species.

The HWE and their fractions were characterized in more depth by determining their TP and PA contents and ratios. As shown in Table 1, the HWE of *P. densiflora* contained the highest levels of TP and PA (524.7 mg CE/g HWE for TP and 382.2 mg CE/g HWE for PA), indicating that the bark component of *P. densiflora* can be more readily extracted with water compared to the other two pine barks. In addition, the PA/TP ratio of this sample was 72.8%, which implied that most of the TP consisted of PA. The PA/TP ratios were in the order of PAF > HWE > EAF in each *Pinus* species and *P. densiflora* > *P. pinaster* > *P. thunbergii* in HWEs and their fractions. The PAFs from all three pine barks showed relatively similar TP contents (570.9 to 611.4 mg CE/g sample) even though their PA/TP ratios varied from 61.1% in *P. thunbergii* to 79.0 in *P. densiflora*.

The ABTS radical scavenging activity determined with a 5 µg/ml solution ranged from 15.6% for the HWE of *P. thunbergii* to 63.9% for the PAF of *P. densiflora*. As reported in many other research studies, the antioxidant activity of pine bark is significantly dependent on the content of PA in HWE (Ku and Mun, 2007). This finding was also supported by the R^2 value of 0.9079 between PA content and ABTS radical scavenging activity (Figure 1). However, the TP content showed a better correlation with antioxidant activity than did the PA content. The R^2 value between TP content and ABTS radical scavenging activities was 0.9787, which was higher than that of PA content and ABTS radical scavenging activities. This finding was opposite to previous results that showed TP content to have a low correlation with antioxidant activity (Burns et al., 2000; Ku and Mun, 2007). This discrepancy may be explained by the high correlation between TP and PA ($R^2 = 0.8912$) in HWEs and their fractions from three pine bark samples used in this study and some impurities, such as oxidizable nonphenolics, reducing polar impurities, lipids, and free sugars, in HWEs which do not have

radical scavenging function, but are removed by the fractionation step. If a correlation is constructed only with HWEs, these impurities in HWEs would have a significant influence on the antioxidant activity, thereby giving a low correlation between HWEs and antioxidant activity. However, since these impurities were removed in the EAFs and PAFs, the TP content might show a greater proportionality with ABTS radical scavenging activities. Meanwhile, it was found that the antioxidant activities of PAFs were similar (59.7 to 63.9%) each other regardless of *Pinus* species.

Taken together, compared to commercial PYC, the bark of *P. densiflora* demonstrated better characteristics with respect to extraction yield, TP and PA content, and antioxidant activity, suggesting its potential application in the pharmaceutical field. However, the bark of *P. thunbergii* did not show better characteristics to those of PYC. Meanwhile, it is obvious that pine bark extracts also contain several compounds other than PAs such as gallic acid, caffeic acid, and ferulic acid which are known to have various biological activities (D'Andrea, 2010). In addition,

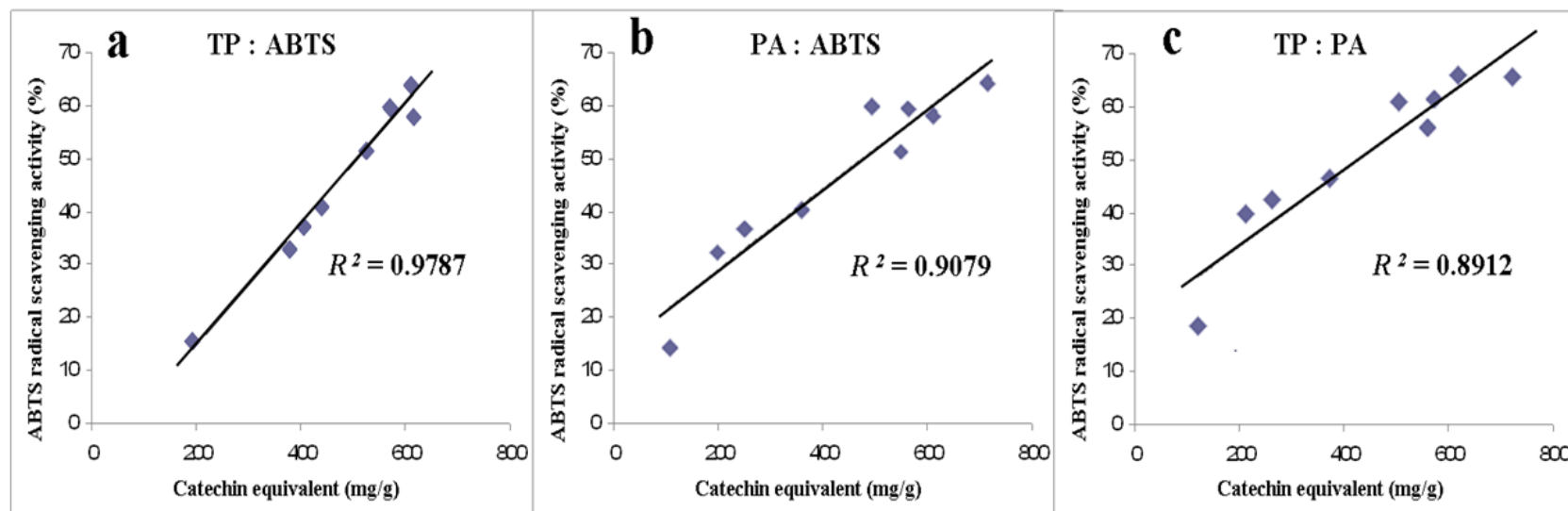


Figure 1. Correlation between total phenolics and ABTS radical scavenging activity (a), proanthocyanidins and ABTS radical scavenging activity (b), and total phenolics and proanthocyanidins (c).

these compounds were identified as major constituents in the plasma of eleven volunteers who were kept on flavonoid free diet for 24 h and given a single dose (300 mg) of pine bark extract (Grimm et al., 2006). Therefore, these compounds should be considered importantly as biological constituents of pine bark extracts. However, since the most obvious feature of pine bark extracts is the presence of PAs and their ability to quench free radicals, the deeper characterization of PAs were investigated in this study as shown subsequently.

Characterization of proanthocyanidin from hot water extracts and their fractions from pine barks

Figure 2 shows the reverse-phase HPLC chromatograms of HWE, EAF, and PAF from *P.*

densiflora. Due to the presence of polymeric PA, the baselines of chromatograms of HWE from the pine barks are often not linear and the separation of large polymer ($DP > 4$) is not possible (Lazarus et al., 2001). This was observed in the HPLC chromatogram of *P. densiflora* HWE. Some monomeric or oligomeric phenolic compounds could be detected as peaks, while polymeric PAs in HWEs were detected as broad peaks (Figure 2a). Fractionation of HWE separated monomeric and oligomeric phenolic compounds from polymeric PAs, as shown in Figures 2b and c. The EAF chromatogram showed better separation of each peak and a more linear baseline. In contrast, the PAF chromatogram did not show any separated peaks. These chromatograms indicated that the fractionation step was adequate for separation of monomeric or oligomeric phenolics from polymeric PAs. The compositional structure

of polymeric PA from HWEs and their fractions was analyzed following acid-catalyzed depolymerization in the presence of phloroglucinol. The phloroglucinol-depolymerization reaction converts flavan-3-ol extension units into corresponding phloroglucinol adducts, and releases the terminal units as monomeric flavan-3-ols. The hydrolyzed subunits can then be analyzed, with respect to their nature, proportion of the constitutive units of PAs, and mDP (Qa'dan et al., 2006). Structures of PAs and their phloroglucinol adduct from the HWEs and their fractions from pine barks in the current study are illustrated in Figure 3. After the cleavage reaction, the product was subjected to RP-HPLC and each peak was identified by retention order and comparison with published data (Cortés et al., 2010). Major reaction products were identified as (+)-catechin-phloroglucinol (C-P) and (-)-epicatechin-phloroglucinol (EC-P). The

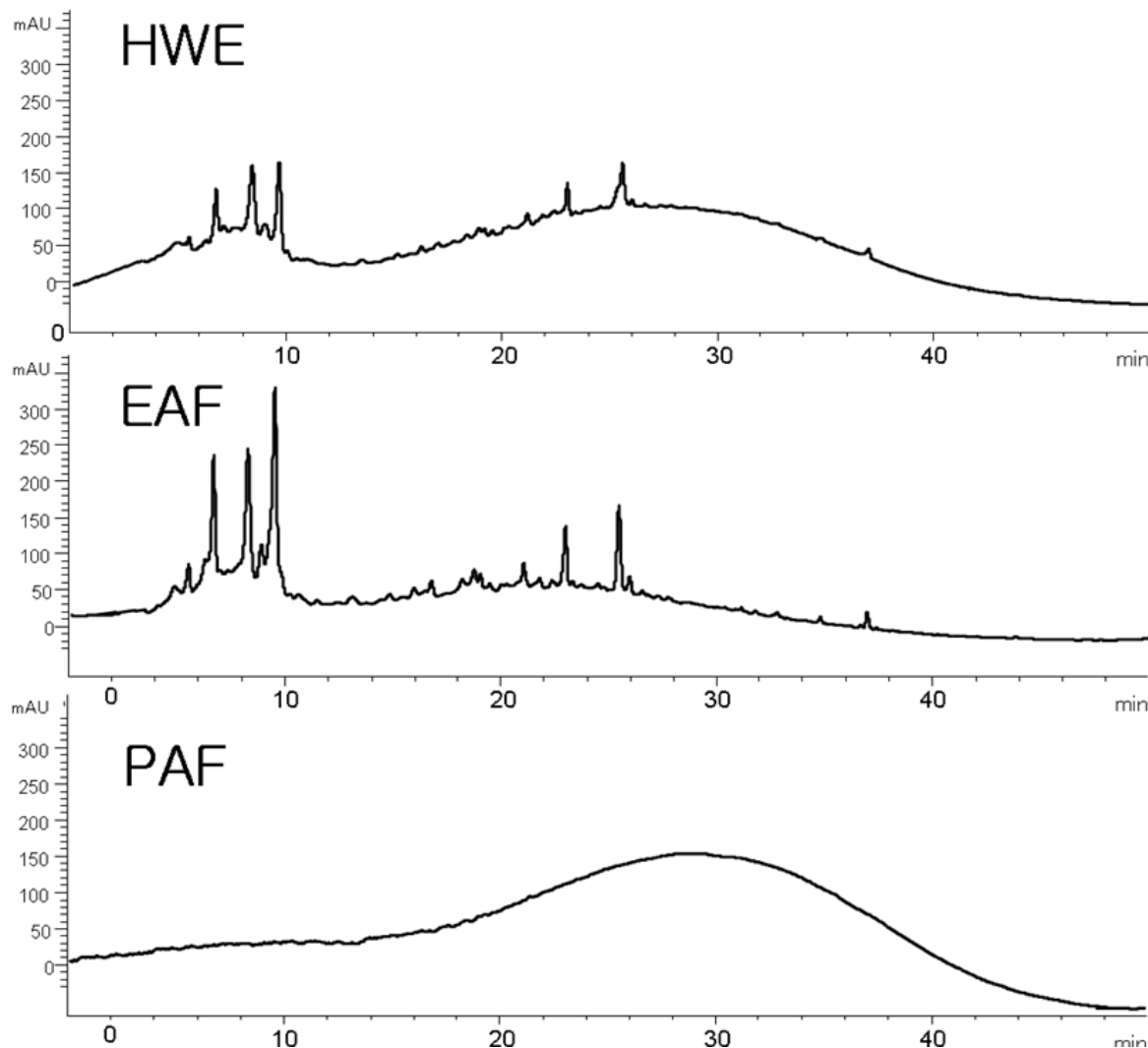


Figure 2. HPLC chromatograms of hot water extract (HWE), ethyl acetate fraction (EAF), and proanthocyanidin fraction (PAF) from *P. densiflora*. The HWE was fractionated to yield EAF and PAF by solvent partitioning and Sephadex LH-20 chromatography, and then all samples were analyzed by reverse-phase HPLC. The chromatograms were detected at 280 nm.

monomer (+)-catechin was detected as a major product, but other flavan-3-ols could not be detected. This implied that the major terminal unit of PAs from pine barks is (+)-catechin (Figure 4).

In general, other phloroglucinol adducts containing (+)-gallo catechin-phloroglucinol, (-)-epigallocatechin-phloroglucinol, (-)-epigallocatechin-gallate-phloroglucinol, as well as C-P and EC-P, have been detected in the plant kingdom after acid-catalyzed depolymerization by phloroglucinol (Qa'dan et al., 2006). In the grape seed, (-)-epigallocatechin-phloroglucinol and (-)-epigallocatechin were the main product and terminal unit after phloroglucinol depolymerization (Kennedy and Jones, 2001). However, the HWEs from various pine barks, including those in the present study, showed C-P and EC-P as the main adducts, which means that (+)-

catechin and (-)-epicatechin were main structural components of PAs from pine barks (Cortés et al., 2010).

The relative ratios of each product in terms of peak area varied among the HWEs and their fractions in the *Pinus* species examined here. From these ratios, the mDP and mMW were calculated, as shown in Table 2. The mDP of *P. densiflora* showed the lowest value (3.54 ± 0.09) and that of *P. densiflora* PAF showed the highest value (7.49 ± 0.03), whereas EAFs of three pine barks showed similar mDP values (3.51 to 3.69). In a previous report, the mDPs of PYC and its EAF were determined as 7.0 and 3.0, respectively, by acid cleavage in the presence of toluene- α -thiol, and as 7.6 and 2.3, respectively, by acid cleavage in the presence of cysteamine (Jeres et al., 2007). In case of *P. radiata*, mDPs of HWE and EAF were determined as 5.3 and 2.9,

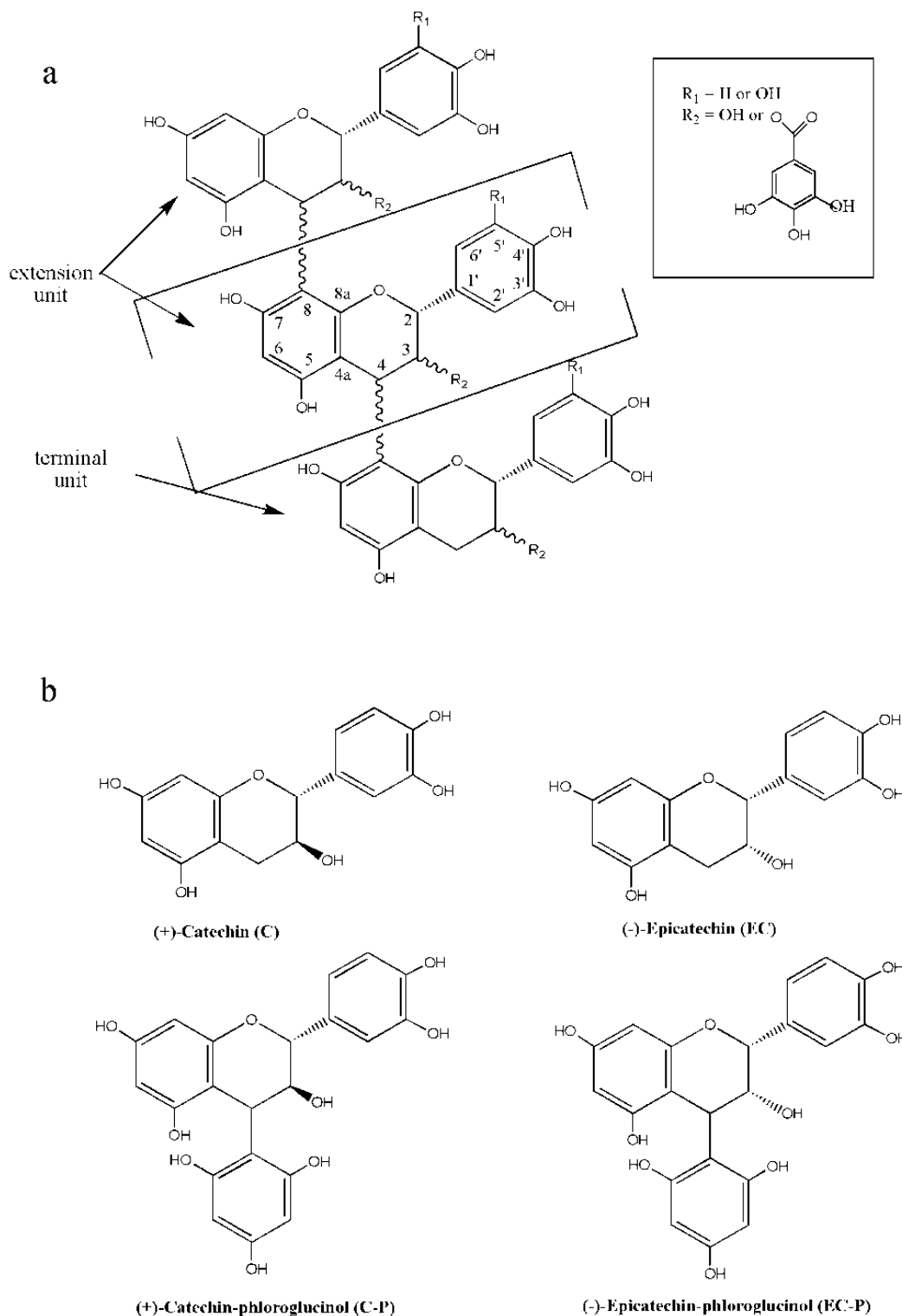


Figure 3. Generalized proanthocyanidin structure (a) and subunit products found in pine tree barks following acid-catalysis in the presence of phloroglucinol (b).

respectively, by acid cleavage in the presence of cysteamine, whereas the mDP of PAF was estimated as 13 by a ^{13}C NMR method (Ku and Mun, 2007). However, to the best of our knowledge, data for acid-catalyzed

depolymerization products and mDP from *P. thunbergii* and *P. densiflora* have not yet been reported. As mentioned previously, the bioavailability of PAs is highly dependent on the subunit length; a ten-fold lower

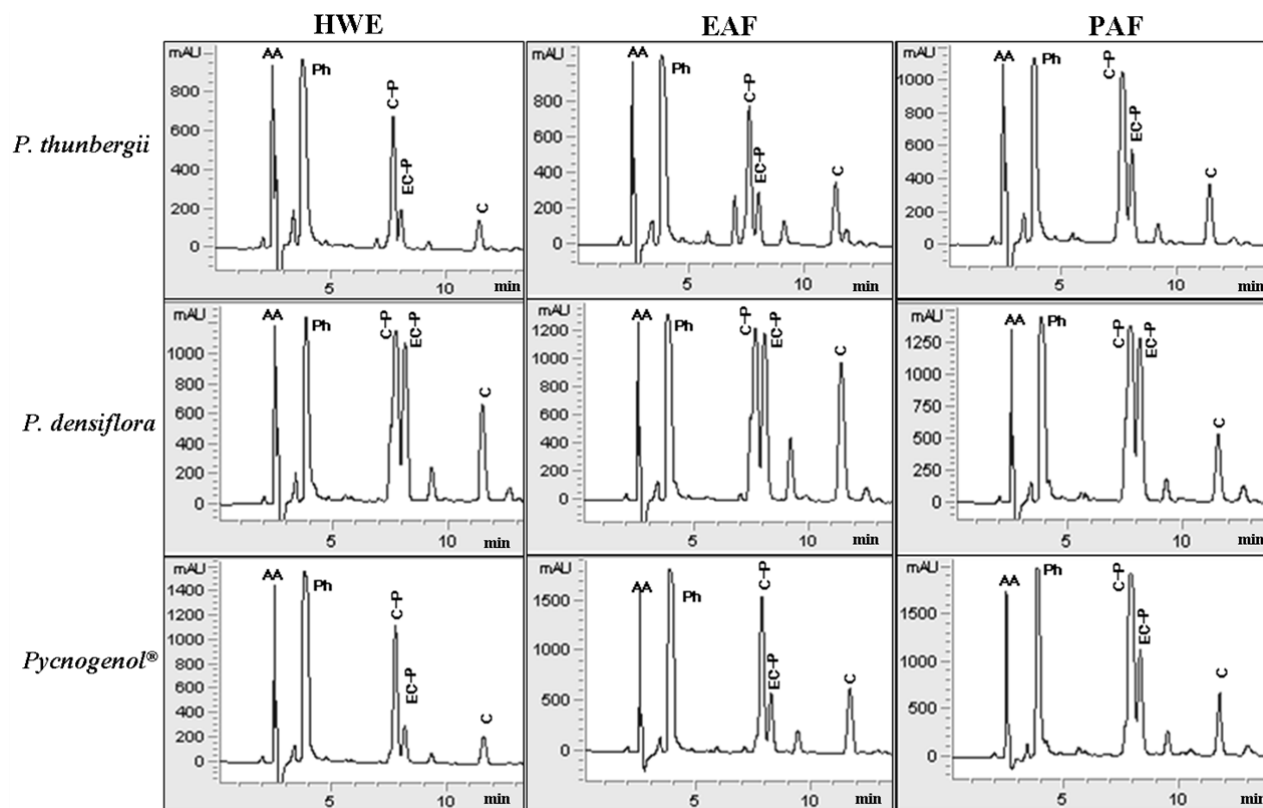


Figure 4. HPLC chromatograms of depolymerization products by the acid-catalysis in the presence of phloroglucinol. All products were filtered after reaction and analyzed by reverse-phase HPLC. HWE: Hot water extract; EAF, ethyl acetate fraction; PAF, proanthocyanidin fraction; AA, ascorbic acid; Ph, phloroglucinol; C-P, (+)-catechin-phloroglucinol; EC-P, (-)-epicatechin-phloroglucinol; (+)-catechin.

Table 2. The mean degree of polymerization and mean molecular weight of the barks and their fractions from *P. densiflora*, *P. thunbergii*, and Pycnogenol® (*P. pinaster* bark).

Sample	Fraction	mDP	mMW
<i>P. thunbergii</i>	HWE	5.55 ± 0.21	1579 ± 59
	EAF	3.51 ± 0.25	1012 ± 73
	PAF	5.65 ± 0.09	1654 ± 26
<i>P. densiflora</i>	HWE	4.85 ± 0.11	1403 ± 32
	EAF	3.54 ± 0.09	1015 ± 26
	PAF	7.49 ± 0.03	2619 ± 7
Pycnogenol®	HWE	6.14 ± 0.07	1784 ± 19
	EAF	3.69 ± 0.12	1090 ± 33
	PAF	6.42 ± 0.09	1873 ± 25

absorption was reported for six unit oligomers compared to monomers and dimers in Caco-2 cells (Hackman et al., 2008). In addition, only dimeric B1 and B2 were detected in human plasma after the consumption of grape seed extract (Sano et al., 2003). In humans and rats, only dimers seem to be absorbed intact, but with much lower efficiency than the monomers (Manach et al., 2005). The

slightly absorbed dimers were reported not to be conjugated or methylated in the small intestine of rats (Appeldoorn et al., 2009b). The dimers were also reported to be metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)- γ -valerolactone (Appeldoorn et al., 2009a). As one of the methods overcoming absorption

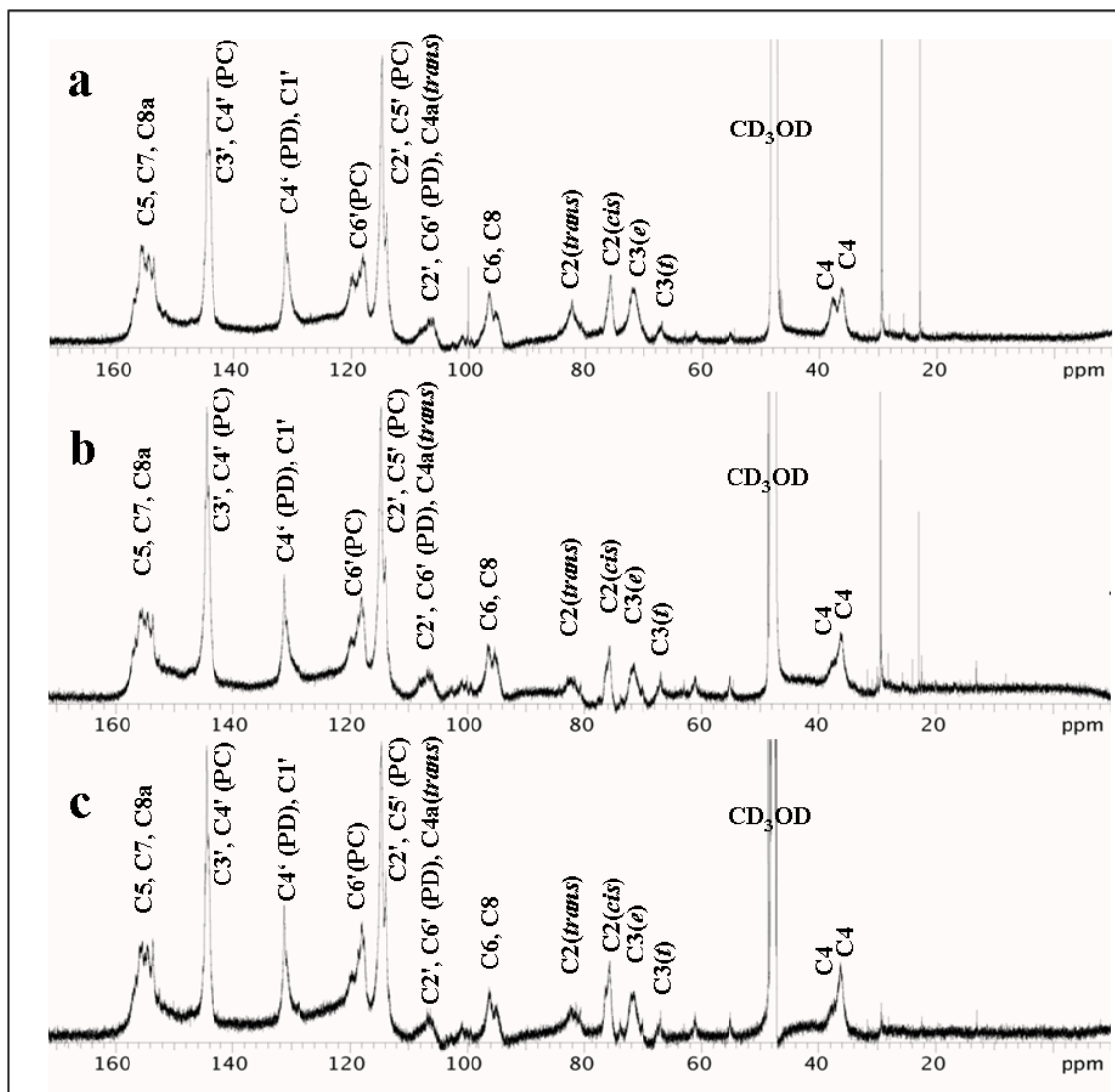


Figure 5. ^{13}C NMR spectra from proanthocyanidin fractions from three pine bark (a: *Pinus densiflora*, b: *Pinus thunbergii*, c: *Pinus pinaster*). NMR spectra were recorded on 500 MHz NMR spectrometer operating at 125 MHz for ^{13}C in CD_3OD .

problem of polymeric PA, technical process containing a mild depolymerization reaction with L-cystein has been applied to PA-rich extract from lychee fruit to produce Oligonol[®], which contains monomer or lower oligomers of PA (Fujii et al., 2008). If this method is applied to the bark of *Pinus* species, the bioavailability would be increased due to the decrease of mDP.

The nature of polymeric PAs can be determined using ^{13}C NMR spectroscopy, which can provide major properties for the elucidation of PA structure including the ratio of procyanidin (PC) and prodelphinidin (PD) extension units, and the stereochemistry of the heterocyclic ring (C2 - C3). Figure 5 shows the ^{13}C NMR spectrum of each PAF from the three pine barks. The signal assignment was made based on literature data (Czochanska et al., 1979). The typical signals of PC and

PD units were detected in the spectrum and the three spectra were very similar each other even though each PA was isolated from different pine species. The PC/PD ratio, usually determined from the relative ratio of the peak areas at 145 ppm (C3' and C4' of PC) and 146 ppm (C3' and C5' of PD), could be determined from the ratio of peak heights at 116 and 107 ppm instead of peak areas since the higher proportion of PC led to low resolution at 145 to 146 ppm (Kraus et al., 2003). The ratios of peak heights at 116 and 107 ppm from three spectra were all over 3.3, which mean that the extension unit of PAs mostly consisted of PC units. Peak areas at 73.6 (for the *cis* isomer) and 82.6 ppm (for the *trans* isomer) were used to determine stereoisomers, and all PA samples were found to contain both *cis* and *trans* isomers in similar ratios. These results were well matched with the

literature data from *P. radiata*, in which a 94/6% ratio was reported for PC/PD ratio and both the presence of both stereoisomers was confirmed (Ku and Mun, 2007). However, when samples from different plant families and different tissues are analyzed for these characteristics, the values were entirely different. For instance, the peak height ratio at 116 and 107 ppm from foliage of different plant species ranged from 0.00 to 3.96 (Kraus et al., 2003). For example, the % PC ratios of foliage from *Pinus consorta* spp. Bolanderi and *Pinus muricata* were 35 and 19%, respectively.

Conclusion

In summary, the barks from three *Pinus* species demonstrated different extraction yield, TP and PA content, and ABTS radical scavenging activity. The degree of polymerization also varied with species. However, the PA structure, especially with respect to terminal and extension units, PC/PD ratio, and stereochemistry at C2-C3, was very similar within the three species. Comparing the results, the HWE from *P. densiflora* appears to be a good natural resource for biological and pharmaceutical applications, with phenolic composition equivalent to that of commercial PYC.

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