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Exploitation of polyphenol-rich pine barks for potent antioxidant activity

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Abstract The phenolic composition of pine bark from a variety of *Pinus* species was estimated by measuring Klason lignin, acid-soluble lignin, and a 1% NaOH extract. Polyphenol contents of hot water extracts from pine bark were determined by the Folin-Ciocalteu assay and the vanillin–H_2SO_4 assay. Among the pine bark varieties investigated, *Pinus radiata* bark showed the highest polyphenol content and potent antioxidant activity. *Pinus rigida* bark was also a usable polyphenol-rich source, whereas *Pinus densiflora* bark had a low yield (5.1%) of hot water extract, although it showed potent antioxidant activity. Correlations between proanthocyanidin content in pine bark and antioxidant activity were observed. The results suggested that proanthocyanidin was the crucial contributor to potent antioxidant activity in pine bark.

Key words Pine bark · Polyphenol · Antioxidant activity · Proanthocyanidin

Introduction

Pine wood is commonly used for producing pulp and board, but all bark is removed prior to the chipping process due to the high lignin/polyphenol content, which causes difficulties in processing. The bark removed from logs is mostly used as boiler fuel, but a huge surplus is still discarded as a waste residue.[1] In fact, pine bark is an important biomass resource, amounting to about 10%–15% of the total tree weight.[2] In the past few years, the awareness for effective and value-added utilization of these bark wastes has increased. From a commercial point of view, simultaneous utilization of wood and waste bark is required, but, in particular, *Pinus* species with its thick and polyphenol-rich bark is of significant interest due to the high cost of the extraction process. Various trials to cultivate the polyphenol-rich *Pinus* species have been conducted on the Iberian Peninsula or in Korea, although no successful results were gained due to geographical and environmental conditions.[3] However, the sufficiently high value of pine bark for commercial application has been proven by many biological studies. Pycnogenol, the patented trade name of *Pinus maritima* bark extract, is a typical example of a commercially successfully product that incorporates bark.[4]

Proanthocyanidin (PA) in pine bark is composed of flavan-3-ol subunits linked mainly through C4-C8 bonds. Recently, PA has received considerable attention for not only its various physiological activities such as antioxidant activity,[5] antimicrobial effect,[6] antiinflammatory property,[7] and antiallergy activity,[8] but also due to the fact that it is still used therapeutically as a dietary supplement in Europe.[3] PA in pine bark has been recognized as a significantly effective material for these activities.[9] In terms of antioxidant activity, PA shows not only antioxidant capacity dependent on the number of hydroxyl groups in the B-ring and its oligomerization,[10,11] but also unique structural behavior such as abstraction of the susceptible hydrogen at C-2 after proton donation of the B-ring, leading to transformation of B-type into A-type PA.[12] Hagerman et al.[13] suggested that the potential health benefit of PA is to stabilize prooxidants from exogenous or endogenous oxidation reaction during digestion. However, PA exists in pine bark as both solvent-extractable and nonextractable types depending on whether it is combined with a carbohydrate matrix. The quantitative ratio of these types in pine bark has been considered to be due to climatic conditions. The amount of extractable PA is a significant factor in estimating the suitability of bark among *Pinus* species.

In the present study, we collected the bark from 11 *Pinus* species, which are being used in the wood industry or/and autogenous or experimentally planted in South Korea, in

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order to screen pine bark containing a great deal of extractable PA. Phenolic components of these pine bark samples were estimated because it is the most basic factor in determining the bark's potential utilization. The target component of bark polyphenols was also determined by correlation with antioxidant activity.

Materials and methods

Pine bark

The outer bark of *Pinus densiflora*, *Pinus thunbergii*, *Pinus banksiana*, *Pinus contorta*, *Pinus rigida*, *Pinus taeda*, *Pinus rigida* × *taeda*, *Pinus serotina*, *Pinus radiata*, *Pinus koraiensis*, and *Pinus parviflora* were separated by hand peeling or with a debarker. The bark was dried in a convection oven at 60°C for 48 h and ground using a Wiley mill.

Chemical composition

Contents of cold and hot water extracts (CWE and HWE), ethanol–benzene (1:2, v/v) extracts, 1% NaOH extracts, and ash were determined by the Technical Association of the Pulp and Paper Industry (TAPPI) standards (1992) T 207 om-88, T 204 os-76, T 212 om-88, and T 211 om-85, respectively.[14] In particular, HWE was prepared at a liquor ratio of 1:50. Determination of acid-insoluble lignin (Klason lignin) content in the bark residues after ethanol–benzene and 1% NaOH extraction was determined according to TAPPI standard T 222 om-83.[14] Acid-soluble lignin content was estimated from the absorbance at 240 nm, and the absorptivity used was $106\,l \cdot g^{-1} \cdot cm^{-1}$ at 205 nm. Total lignin content was expressed as the sum of Klason lignin and acid-soluble lignin contents. The contents of total lignin, compensated total lignin, and polyphenols were calculated by the formula noted in Table 1.

Polyphenol content

Pine bark (20–80 mesh) was extracted with 100 ml of boiling water for 1 h at a liquor ratio of 1:10. The extract was filtered by a 2G3 glass filter. The residues were washed with 220 ml of hot water. The filtrate was evaporated at 65°C under reduced pressure, freeze-dried, and then vacuum-dried for 1 day.

Total phenolic contents of each HWE were determined by the Folin-Ciocalteu method based on complex formation of molybdenum-tungsten blue. Briefly, 2 ml of Folin-Ciocalteu phenol reagent was mixed with 1 ml of a diluted sample solution (800 μg/100 ml) in a Teflon-capped vial. After an interval of 3 min, 2 ml of 10% aqueous sodium carbonate (Na_2CO_3) was added to the vial, and the mixture was allowed to stand for 2 h at ambient temperature. The absorbance of the chromogen formed was read at 750 nm. The results were expressed as (+)-catechin equivalent based on the flavan-3-ol structure of PA. The regression coefficient of (+)-catechin was 0.99951.

PA contents of the HWEs were determined by vanillin–H_2SO_4 assay. Briefly, 1.0-ml aliquots of HWE (3 mg in 10 ml absolute methanol) were mixed with 2.5 ml of 1.0% (w/v) vanillin in absolute methanol and then with 2.5 ml of 25% (v/v) sulfuric acid in absolute methanol to undergo vanillin reaction with polyphenol in the HWEs. The blank solution was prepared in the same procedure without vanillin. The vanillin reaction was carried out in a 25°C water bath for 15 min. The absorbance at 500 nm was read and the results were expressed as (+)-catechin equivalent per gram of HWE. In the vanillin reaction, the regression coefficient of (+)-catechin was 0.99951.

Table 1. Contents of phenolic components and water extracts of various pine bark varieties

Leaf number	*Pinus* species	KL (%)	ASL (%)	CTL[a] (%)	PP[b] (%)	CWE (%)	HWE[c] (%)
2	*P. densiflora*	61.5[d] (51.2)[e]	1.3 (0.5)	30.0	29.6	2.1	8.2
	P. thunbergii	56.0 (48.2)	0.9 (0.6)	31.4	22.8	0.5	3.7
	P. banksiana	60.1 (46.5)	1.1 (0.7)	20.4	29.2	1.7	7.0
	P. contorta	58.5 (39.7)	1.9 (0.5)	14.2	39.4	2.3	10.2
3	*P. rigida*	67.5 (52.8)	2.3 (0.4)	23.4	43.4	5.4	15.1
	P. taeda	64.6 (54.5)	0.7 (0.4)	29.8	32.9	0.3	4.3
	P. rigida × *taeda*	60.9 (48.7)	0.6 (0.4)	26.9	32.2	0.9	4.8
	P. serotina	69.6 (52.8)	1.5 (0.4)	21.4	47.9	0.1	5.6
	P. radiata	70.4 (46.5)	9.6 (0.6)	16.6	55.0	10.6	23.4
5	*P. koraiensis*	50.5 (40.8)	1.2 (0.5)	21.8	26.5	0.6	7.7
	P. parviflora	47.6 (39.5)	1.4 (0.5)	15.0	23.7	2.7	9.4

KL, Klason lignin; ASL, acid-soluble lignin; CTL, compensated total lignin; CWE, cold water extract; HWE, hot water extract; PP, polyphenol

[a] Values are compensated total lignin content (Klason lignin + acid-soluble lignin) of the residues after 1% NaOH extraction based on amount of bark

[b] Values are calculated by the following equation: polyphenol (%) = [total lignin (g) in the residues after ethanol–benzene extraction – compensated total lignin (g)]/bark (g) × 100

[c] HWE was experimented at a liquor ratio of 1:50

[d] Values are lignin content calculated from the residue after ethanol–benzene (1:2, v/v) extraction

[e] Values are lignin content calculated from the residues after 1% NaOH extraction

DPPH free radical scavenging assay

Antioxidant activity was estimated by the hydrogen-donating ability to 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. The sample solution was prepared with a stock solution (5 mg in 10 ml absolute methanol) and diluted to 12.5 and 25 μg/ml. One milliliter of the sample solution was mixed with 2 ml of 0.1 mM DPPH and reacted in a water bath at 25°C for 30 min. The absorbance of the sample (As) and the control (Ac) solutions were read at 518 nm. The control was a DPPH solution, containing absolute methanol instead of the sample.

DPPH free radical scavenging activity (%)
$= [1 - (As/Ac)] \times 100$

Results and discussion

Chemical composition of pine bark

The pine bark used was previously extracted with ethanol–benzene cosolvent using a Soxhlet apparatus. Phenolic components of 11 pine bark samples are presented in Table 1. From these results, clear differences in the amounts of lignin and polyphenol could be found even within *Pinus* species. In fact, it is difficult for pine bark samples to give a meaningful chemical composition due to the great complexity of the components. The bark samples demonstrated mostly high extract contents, and, in particular, the 1% NaOH extracts ranged from 35% to 65% of the dry weight, indicating high phenolic content. The high total lignin contents ranging from 50% to 70% were assumed to be overestimated due to the condensation reaction between lignin and polyphenol in the presence of sulfuric acid. In fact, the interference in proximate chemical analysis between lignin and polyphenol present in pine bark may be why the analysis methods developed for wood cannot be directly applied to bark. In this study, the overestimation of lignin content due to the condensation reaction could be avoided by using the bark residues after 1% NaOH extraction.[2] Lignin in pine bark was considered as a significantly influencing determinant in estimating polyphenol content due to its solvent (ethanol-benzene)-resistant properties and, to a lesser degree, polyphenol relationship with structure–antioxidant activity. Polyphenols as extracts in pine bark are very important components because polyphenols are directly related to the quantitative estimation of antioxidant activity. The polyphenol contents among the varieties were widely varied in the range from 22.8% to 55.0%. *Pinus radiata* bark contained the highest polyphenol (55.0%) content. In addition, this pine bark had relatively high contents of CWE (10.6%) and HWE (23.4%). These results revealed that the bark components of *P. radiata* can be more favorably extracted with water than those of other pine bark varieties. HWE yields of *P. radiata* bark at liquor ratios of 1:50 (Table 1) and 1:10 (Table 2) were consistent, although the liquor ratio employed affected those of the other varieties. This also showed that extractable components were higher in *P. radiata* bark than in other varieties.

Polyphenol content and antioxidant activity of hot water extracts from pine barks

All the pine bark samples were extracted with hot water at a liquor ratio of 1:10 for 1 h. Table 2 shows the yield, phenolic components, and antioxidant activity of HWE from pine bark. The HWE yields of pine barks varied widely from 2.4% to 23.2% of the dry weight. In particular, the HWE yields from *P. radiata* (23.3%) and *Pinus rigida* (14.4%) barks were relatively higher than those from the other varieties, with those of other pine bark samples ranging from 2.4% to 6.9%. The contents of total phenolics and PA indicated distinct differences among the pine bark varieties. Namely, *Pinus koraiensis* HWE [862 mg (+)-catechin/g HWE] showed the highest total phenolic con-

Table 2. Yield, total phenolic content, proanthocyanidin content, and antioxidant activity of HWEs from various pine bark varieties

Leaf number	Species	Yield[a] (%)	TP[b] (mg/g)	PA[c] (mg/g)	PA/TP	DPPH scavenging activity (%)	
						25.0 μg/ml	12.5 μg/ml
2	*P. densiflora*	5.1	411	384	0.93	91.5	54.8
	P. thunbergii	2.5	541	103	0.19	44.5	20.6
	P. banksiana	4.1	501	27	0.05	25.2	12.4
	P. contorta	6.9	403	102	0.25	36.8	17.8
3	*P. rigida*	14.4	558	489	0.88	93.1	64.6
	P. taeda	2.4	338	51	0.15	26.0	13.9
	P. rigida × *taeda*	2.7	111	41	0.37	29.7	15.3
	P. serotina	2.9	184	37	0.20	30.0	16.7
	P. radiata	23.2	629	404	0.64	92.5	67.9
5	*P. koraiensis*	3.8	862	49	0.06	19.3	12.1
	P. parviflora	4.5	226	21	0.09	3.8	4.8

TP, Total phenolic; PA, proanthocyanidin; DPPH, 1,1-diphenyl-2-picrylhydrazyl
[a] Values determined at a liquor ratio of 1:10
[b] Determined by Folin-Ciocalteu assay, in units of milligrams (+)-catechin equivalent per gram HWE
[c] Determined by vanillin–H_2SO_4 assay, in units of milligrams (+)-catechin equivalent per gram HWE

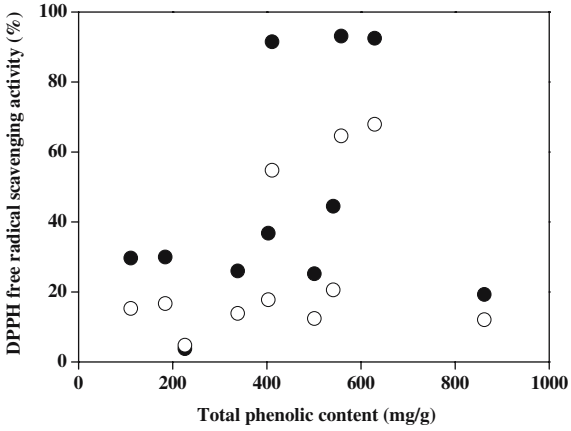

Fig. 1. Correlation between total phenolic content and antioxidant activity. *Filled circles*, 25 μg/ml; *open circles*, 12.5 μg/ml

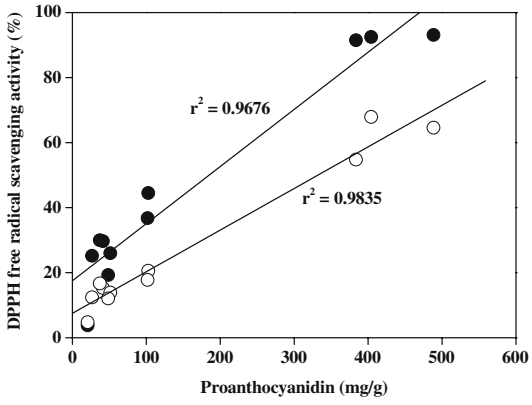

Fig. 2. Correlation between proanthocyanidin content and antioxidant activity

tent, but its antioxidative activity was low at 19.3% at 25.0 μg/ml and 12.1% at 12.5 μg/ml. Figure 1 shows the random plots between total phenolic content determined by Folin-Ciocalteu assay and antioxidant activity. This result showed a low correlation between total phenolic content and antioxidant activity. Although the Folin-Ciocalteu assay has been generally used to determine total phenolic content in crude extracts from plant sources, the assay did not provide a specific result for the crude bark extracts. Many studies have failed to find significant correlation between total phenolic content and antioxidant activity of plant extracts.[15] Our finding may be attributed to the influence of individual antioxidant components and the presence of oxidizable nonphenolic or reducing polar impurities (acids and sugars) in HWEs from pine barks, which is consistent with those factors described in the literature.[16,17] This may be because monophenols are less efficient antioxidants than polyphenols[18] as well as the nonphenolic impurities being able to positively react with the Folin-Ciocalteu phenol reagent, thereby reducing molybdenum with a spectrophotometrically measurable blue color. However, we further considered that lipids containing conjugated diene bonds and free sugars extractable from pine barks under hot water conditions influence the correlation between total phenolic content and antioxidant activity because pine bark generally contains high concentrations of waxes and fatty materials and free sugars. In addition, it may be explained on the basis that the reactions of antioxidants with DPPH free radicals are different from their reactions with the Folin-Ciocalteu reagent in the total phenolic assay. The Folin-Ciocalteu reagent is sensitive to a wide range of substrates, which are easily oxidized, but the DPPH free radical exhibits different sensitivity to various antioxidants depending on their kinetic reactions with the DPPH free radical. Some phenolic antioxidants that react strongly with the Folin-Ciocalteu reagent may not react with DPPH free radicals.[19]

Meanwhile, the antioxidant capacity of PA determined by vanillin–H_2SO_4 assay was estimated using a linear correlation coefficient. It was found that pine bark with high PA content in HWE exhibits potent antioxidant activity by readily donating hydrogen to the DPPH free radical. The extracts from *Pinus densiflora*, *P. radiata*, and *P. rigida* indicated antioxidant activity of greater than 90% at 25.0 μg/ml owing to high PA content. Our results indicated that antioxidant activity correlated well with PA content (Fig. 2). It could be considered that the antioxidant activity of HWE from pine bark was significantly dependent on the content of PA with a flavan-3-ol structure. It may also be explained from the general fact that pine bark extracts are mostly found to contain an entire series of procyanidins from monomers to longer polymers, although monomeric components such as taxifolin, quercetin, protocatechuic acid, ferulic acid, caffeic acid, etc., are present in bark HWEs.[3,20,21] Finally, quantification of PA content using the vanillin–H_2SO_4 assay would be helpful in evaluating antioxidant properties of pine bark extracts. We also suggest that PA plays an important role in antioxidant activities of pine bark extracts and *P. radiata* bark containing high PA content can be considered as a natural resource for biological and pharmaceutical applications. Nowadays, growing interest in the antioxidant and biological activities of bark extracts from *Pinus maritima*, which is patented under the trade name of Pycnogenol, have stimulated the search of various pine barks for potent antioxidants. This effort is expended because the value-added utilization of pine bark extracts with potent antioxidant activities is possible due to similarity in their chemical composition. Although estimating antioxidant activity of pine bark extracts can be considered to be a fundamental study, the results obtained may be used as an important assessment for potential use in industrial applications.

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