Food Sci. Biotechnol. Vol. 15, No. 3, pp. 424 ~ 430 (2006)

Food Science and Biotechnology

Characterization of Low Molecular Weight Polyphenols from Pine (*Pinus radiata*) Bark

Sung Phil Mun* and Chang Sub Ku[1]

Division of Forest Science, College of Agriculture and Life Sciences, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea
[1]Department of Advanced Organic Materials Engineering, Chonbuk National University, Jeonju, Jeonbuk 561-756, Korea

Abstract Low molecular weight polyphenols were isolated from hot water extracts of radiata pine (*Pinus radiata*) bark using a Sephadex LH-20 column and characterized by ^{1}H and ^{13}C NMR, UV, FT-IR, and GC-MS analyses. Major compounds isolated and identified were protocatechuic acid, *trans*-taxifolin, and quercetin. *Trans*-taxifolin, an important intermediate in biosynthetic route of proanthocyanidin (PA), was isolated in large quantities and indicates that PA is a major component of radiata pine bark. Small amounts of polyphenols were identified by GC-MS analysis. The presence of *p*-hydroxybenzoic acid, vanillic acid, protocatechuic acid, *cis*- and *trans*-feruic acid, *p*-coumaric acid, *trans*-caffeic acid, (-)-epicatechin, (+)-catechin, *trans*- and *cis*-taxifolin, (+)-gallocatechin, and quercetin was confirmed by comparison of mass fragmentation patterns and retention times (RT) with authentic samples. In addition, the presence of astringenin, astringenin glycoside, *trans*- and *cis*-leucodelphinidin was strongly assumed from characteristic mass fragment ions due to their conjugated structure and retro Diels-Alder reaction, and also from biosynthetic route of PA. GC-MS analysis allowed us to detect small amounts of phenolic acids and flavonoids and eventually discriminate *trans*- and *cis*-configuration in the identified polyphenols.

Keywords: *Pinus radiata* bark, polyphenols, *trans*-taxifolin, quercetin, protocatechuic acid.

Introduction

Pine (*Pinus radiata*) bark extract has been used as traditional medicines for inflammatory diseases and wound healing in Europe and North America (1). It also has high inhibitory activity against several carbohydrate-hydrolyzing enzymes, indicating its potential as an antihyperglycemic drug (2). Pine phloem extract was recognized as one of the most active extracts in antimicrobial activity (3). Pine bark extract has been ranked among the most potent plant sources for natural phenolic antioxidants (4). Although pine wood is commonly used for producing pulp and board, its bark is completely removed prior to the chipping process due to the high content of lignin/polyphenol that interferes with the manufacturing process. The bark removed from logs is mostly used as boiler fuel, but a huge surplus of the bark is still discarded as a waste residue. In the last few years, the awareness for effective and value-added utilization of these bark wastes has increased (5). The increasing attention on pine bark wastes may be based on its potential as a rich source for polyphenol compounds.

While the chemical composition of pine bark extract is still not completely elucidated, to date, its main constituents are known to be phenolic compounds, broadly divided into monomers (catechin, epicatechin, and taxifolin) and condensed flavonoids classified as proanthocyanidins (PA) (6). Recently, we investigated antioxidant activity of hot water extracts (HWEs) from the barks of 11 *Pinus* species (7). HWE from radiata pine bark showed the higher yield and potent antioxidant activity as compared to the other species studied. The potent antioxidant activity of the HWEs was predominantly dependent on PA content in each bark, indicating the close correlation ($R^2 = 0.97$). In a previous study (8), the PA isolated from radiata pine bark was characterized due to its potent antioxidant activity. Identification of the compounds contained in the HWE of radiata pine bark is therefore important in understanding its further beneficial effects, bioactivity capacity, and the accurate reaction mechanism with enzymes or reactive oxygen species (ROS). The purpose of this study is to isolate and characterize low molecular weight polyphenols in HWE from radiata pine bark.

*Corresponding author: Tel: 82-63-270-2624; Fax: 82-63-270-2631
E-mail: msp@chonbuk.ac.kr
Received January 24, 2006; accepted March 30, 2006

Materials and Methods

Preparative scale extraction of HWE Radiata pine bark obtained from the Sawmilling Co., Ltd. (Christchurch, New Zealand) was dried at 60°C for 48 hr and ground in a Wiley mill. The bark powder (oven dried wt 600 g, 20-80 mesh) was extracted with 6 L of deionized water for 1 hr at 100°C. The extracted bark was transferred into a cotton cloth bag and strongly squeezed by hands, followed by washing with 16 L of deionized water. The extract was filtrated by a 3 μm-filter cartridge. The filtered was evaporated using a large-scale rotary evaporator (N-12; Eyela, Japan) under reduced pressure at 65°C. The concentrate was freeze-dried for 2 days and then vacuum-dried for 2 days under P_2O_5.

Extraction and fractionation methods Separation of monomeric polyphenols from radiata pine bark was conducted as shown in Fig. 1. About 5 g of HWE was dissolved in 50 mL of 70%(v/v) aqueous acetone and filtered followed by evaporation to remove acetone. Lipids were removed from HWE by extracting two times with 100 mL of *n*-hexane. The aqueous layer was extracted five times with 100 mL of ethyl acetate to obtain low

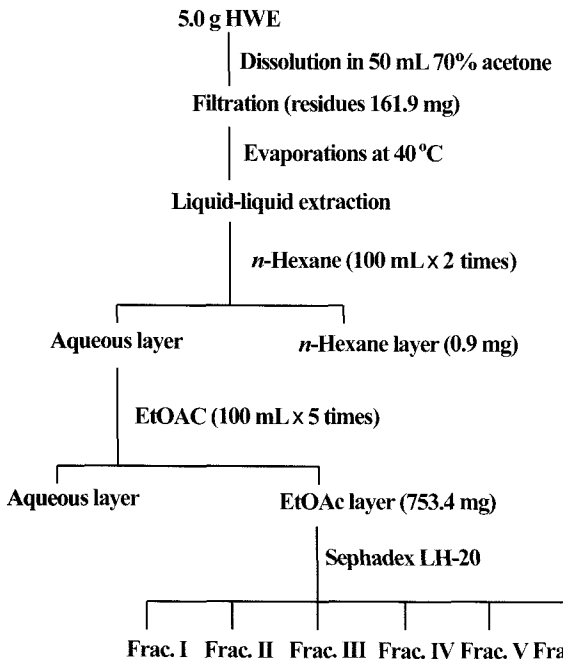

Fig. 1. Schematic flow diagram for fractionation of monomeric polyphenols of HWE from radiata pine bark.

molecular weight polyphenols. Subsequently, the ethyl acetate layer was evaporated under reduced pressure at 50 °C and vacuum-dried to yield 753 mg of reddish brown powder. About 600 mg of the powder was dissolved in ethanol and loaded onto a Sephadex LH-20 column (300× 32 mm i.d.) preconditioned with ethanol. The flow-rate was 1.5 mL/min and fractions were collected using a fraction collector and monitored at 254 nm by a UV detector (UV-D2; Eyela) and TLC development. The combined fractions were again monitored using a diode array spectrophotometer (8452A; Hewlett Packard, USA) in the range from 200 to 400 nm.

Analysis of monomeric flavonoids Fractions I-VI and standard flavonoids [(-)-epicatechin, (+)-catechin, (+)-gallocatechin, (-)-epigallocatechin, *trans*-taxifolin, and quercetin] were dissolved in acetonitrile or acetonitrile containing anhydrous pyridine and silylated with N,O-bis(trimethylsilyl)-triflouroacetamide (BSTFA) as a TMSi reagent at 70°C for 15 min. The TMSi-derivatives were analyzed by gas chromatography coupled with mass spectrometry (GC-MS) and gas chromatography (GC).

GC-MS The fractions I-VI of ethyl acetate soluble extracts were derivatized by addition of BSTFA (100 μL) at 70°C for 15 min. One microliter of sample was injected into a gas chromatograph (GC17A; Shimadzu, Japan) equipped with a flame ionization detector (FID). The peak identification was performed using a GC-MS (QP5050; Shimadzu). Separation of the compounds was achieved using a SPB 5 (30 m × 0.25 mm i.d., 0.25 μm film thickness). The oven temperature was elevated from 200 to 300°C at the rate of 5°C/min and afterward, the final temperature at 300°C was maintained for 20 min. Flow rate of helium gas was held at 1.0 mL/min. Split ratio was adjusted to 10. The MS was operated in electron ionization (EI) mode at 70 eV. Interface temperature was kept at 230 °C. The compounds were identified by comparison with commercially available standards, data reported in the publications (9-18), and in the Wiley 139 computer library.

Spectroscopic procedures The UV spectra of polyphenols isolated were recorded in the range from 200 to 400 nm using the above diode array spectrophotometer with cells of 1 cm path length. The bathochromic shift was observed after addition of shift reagents (NaOH, $AlCl_3$, and NaOAc). FT-IR spectra were recorded in the transmission mode using a FT-IR spectrophotometer (8201PC; Shimadzu). The FT-IR spectra of the pelletized KBr sample were scanned between 4000 and 400/cm. ^1H and ^{13}C NMR spectra were obtained in methanol-d_4 using a NMR spectrometer (JNM-EX 400; Jeol, Japan).

Compound 1 (protocatechuic acid) ^1H NMR (400 MHz, CD_3OD): δ 6.79 (1H, *d*, *J*=7.8 Hz, H-5), 7.41 (1H, *dd*, *J*=1.9 and 7.7 Hz, H-6), 7.43 (1H, *d*, *J*=1.9 Hz, H-2). ^{13}C NMR (100 MHz, CD_3OD): δ 115.7 (C-5), 117.7 (C-2), 123.1 (C-6), 123.8 (C-1), 146.0 (C-3), 151.4 (C-4), 170.1 (C-7). GC-MS 70 eV, *m/z* (TMSi-derivative): 193 (base peak), 370 [M^+].

Compound 2 (*trans*-taxifolin) λ_{max}^{EtOH} 290, 326 nm (sh); (+2N NaOH) 290 → 326 nm; (+$AlCl_3$) 310, 376 nm (sh); (+NaOAc) 290 → 326 nm. ν_{max}/cm: 3437, 3186, 1616, 1477, 1086. ^1H NMR (400 MHz, CD_3OD): δ 4.49 (1H, *d*, *J*=11.5 Hz, H-2), 4.90 (1H, *d*, *J*=11.9 Hz, H-3), 5.87 (1H, *d*, *J*=2.0 Hz, H-6), 5.91 (1H, *d*, *J*=2.0 Hz, H-8), 6.79 (1H, *d*, *J*=7.9 Hz, H-5′), 6.83 (1H, *dd*, *J*=1.9 and 9.8 Hz, H-6′), 6.85 (1H, *d*, *J*=1.4 Hz, H-2′). ^{13}C NMR (100 MHz, CD_3OD): δ 73.7 (C-3), 85.1 (C-2), 96.2 (C-8), 97.3 (C-6), 101.8 (C-10), 115.8 (C-2′), 116.0 (C-5′), 120.8 (C-6′), 129.8 (C-1′), 146.2 (C-4′), 147.0 (C-3′), 164.4 (C-5), 165.2 (C-9), 168.6 (C-7), 198.2 (C-4). GC-MS 70 eV, *m/z* (TMSi-derivative): 368 (base peak), 665 [M^+].

Compound 3 (quercetin) λ_{max}^{EtOH} 258, 374 nm. ν_{max}/cm: 3412, 1665, 1612, 1524, 1450, 1016. ^1H NMR (400 MHz, CD_3OD) δ: 6.18 (1H, *d*, *J*=1.9 Hz, H-6), 6.38 (1H, *d*, *J*=2.0 Hz, H-8), 6.88 (1H, *d*, *J*=8.1 Hz, H-5′), 7.63 (1H, *dd*, *J*=2.0 and 8.4 Hz, H-6′), 7.73 (1H, *d*, *J*=1.9 Hz, H-2′). ^{13}C NMR (100 MHz, CD_3OD): δ 94.4 (C-8), 99.2 (C-6), 104.5 (C-10), 115.9 (C-2′), 116.2 (C-5′), 121.6 (C-6′), 124.1 (C-1′), 137.2 (C-3), 146.1 (C-3′), 147.9 (C-2), 148.7 (C-4′), 158.1 (C-9), 162.4 (C-5), 165.5 (C-7), 177.2 (C-4). GC-MS 70 eV, *m/z* (TMSi-derivative): 647 (base peak), 661 [M^+].

Results and Discussion

The ethyl acetate soluble fraction of HWE from radiata pine bark was eluted with ethanol through a Sephadex LH-20 column (Fig. 2) and monitored using a UV detector and TLC development. Fractions I-VI were obtained, but fractions I and II were mainly containing fatty substances and neutral sugars. Therefore, fractions III-VI, which were assumed to contain the desirable polyphenols, were analyzed by UV spectroscopy (Fig. 3), GC and GC-MS

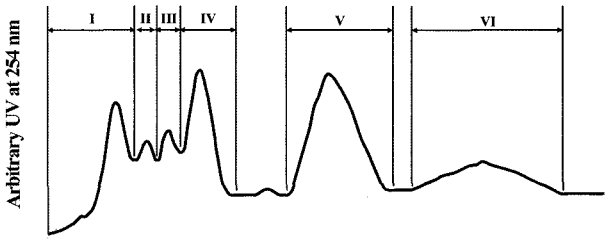

Fig. 2. Elution chromatogram of monomeric polyphenols of ethyl acetate soluble fraction on a Sephadex LH 20 column eluted with ethanol.

(Fig. 4 and Table 1).

Fraction III (10 mg) contained various phenolic acids such as *p*-hydroxybenzoic acid (**1**), vanillic acid (**2**), protocatechuic acid (**3**), *cis*-ferulic acid (**4**), *p*-coumaric acid (**5**), and *trans*-ferulic acid (**6**) (Fig. 4). These phenolic acids were identified by comparison with authentic samples and previous characterizations in the published literatures (9, 10). Although *p*-hydroxybenzoic acid is commonly present in plants, this is the first report of its presence in radiata pine bark. The structural configuration of ferulic acid was evidently discriminated by GC and GC-MS analyses.

The UV spectrum of fraction IV (9 mg) was almost similar to that of protocatechuic acid reported in the literature (19). The presence of a single absorption band in the 240-260 nm region is indicative of a single group in the *para* position, whereas the two absorption bands at 250-260 and 290-300 nm indicate a group in the *para* position as well as one other group in the *ortho* or *meta* positions (20). The two absorption bands at 262 and 292 nm may approximately support the presence of *para* position and either *ortho* or *meta* position in compound 1 obtained from fraction IV. Fraction IV was subjected to GC and GC-MS analyses and the chromatogram was shown in Fig. 4. Compound 1 (**3**) at RT 4.43 min was detected as a major peak with *m/z* 370 (M^+). Compound 1 represented more than 90% of fraction IV. Therefore, without recrystallization, fraction IV was further analyzed by ^1H and ^{13}C NMR. The ^1H NMR spectrum indicated the presence of aromatic signals of an ABX system at 6.79 (*d*, *J*=7.8 Hz), 7.41 (*dd*, *J*=1.9 and 7.7 Hz), and 7.43 (*d*, *J*=1.9 Hz) ppm, respectively assignable to H-5, H-6, and H-2. The ^{13}C NMR spectrum also showed the signals of two oxygen-bearing aromatic ring at 151.4 and 146.0 ppm, and a ketone group at 170.1 ppm. These data indicate that compound 1 is protocatechuic acid.

In Fig. 4, a small peak at RT 8.05 min represented the characteristic fragment ions at *m/z* 396 (M^+) and *m/z* 73 (base peak), corresponding to *trans*-caffeic acid. The mass fragment peaks were identical with those reported in the literatures (10, 11).

The UV spectrum of fraction V (158 mg) showed maximum absorption at 290 nm, characteristics of typical flavanones and dihydroflavonols. A white crystalline compound was precipitated in fraction V and then recrystallized in pure water. Chemical structure of compound 2 (14 mg) was elucidated by the following spectral studies. The UV spectrum of compound 2 was observed at 290 and 326 (sh) nm in ethanol, indicating a typical dihydroflavonol structure with a pyrone C ring. The UV absorption bands were also identical to those reported for taxifolin (21-23). Aluminum chloride is well known to form chelates with flavonoid sites containing 3-

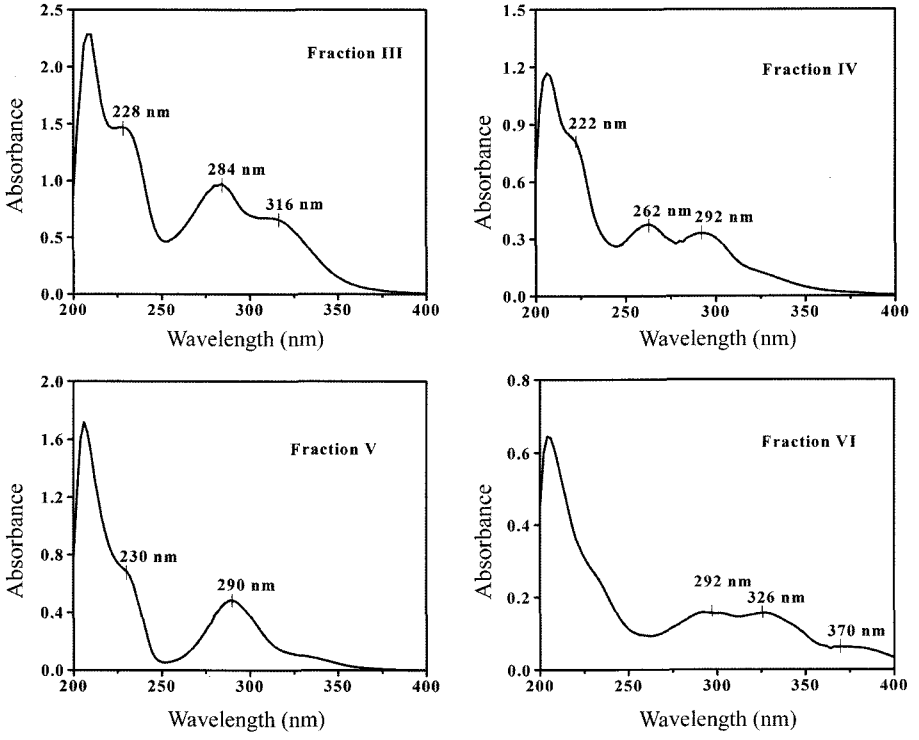

Fig. 3. UV spectra of fractions III-VI obtained from ethyl acetate soluble fraction of HWE from radiata pine bark.

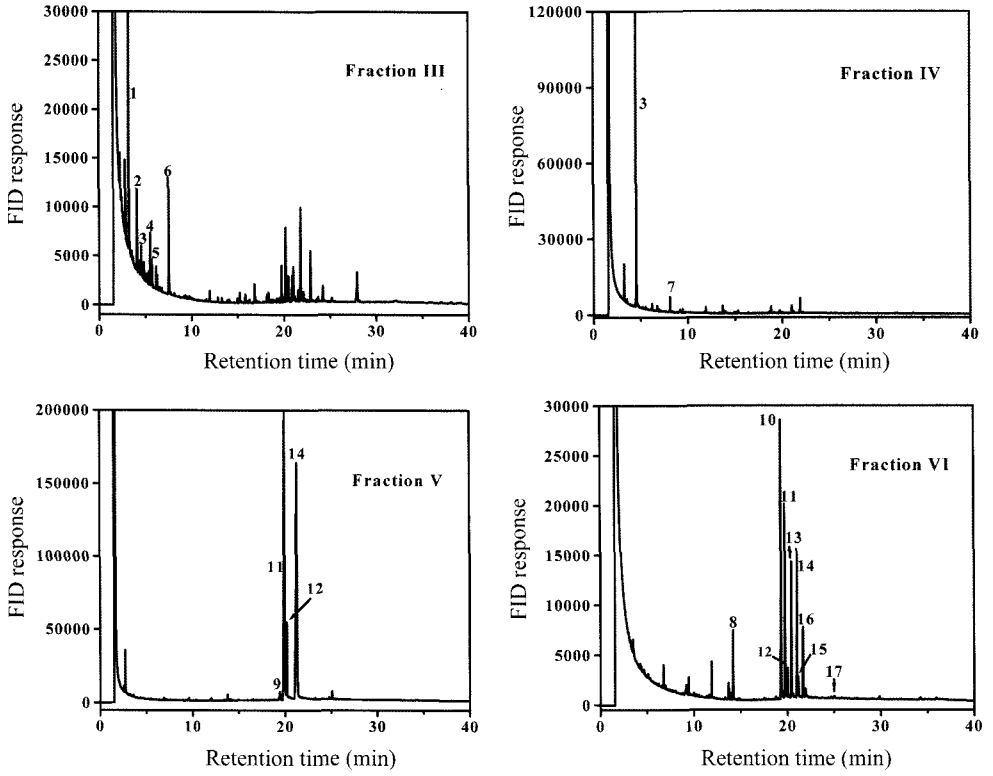

Fig. 4. GC chromatogram of fractions III-VI obtained from ethyl acetate soluble fraction of HWE from radiata pine bark.

hydroxy-4-keto group, 5-hydroxy-4-keto group, and 3',4'-*O*-diphenolic group (24, 25). In particular, the presence of a 3-hydroxyl group or a 5-hydroxyl group further selectively allows the complex formation involving the carbonyl function. Compound 2 formed a complex with aluminum chloride in ethanol and resulted in a characteristic large bathochromic shift of band I (50 nm) and band II (20 nm), suggesting the presence of 5-hydroxy-4-keto group (26, 27). The bathochromic shift (36 nm) after addition of sodium acetate indicated the presence of a free hydroxyl group at C-7 (28). The ^{13}C NMR spectrum of compound 2 showed dihydroflavonol structure from three carbon signals assignable to 85.1 (C-2), 73.7 (C-3), and 198.2 (non-conjugated keto group, C-4) ppm. The ^{1}H NMR spectrum showed the two signals at 4.49 and 4.90 ppm, corresponding to methylene protons of H-2 and H-3, respectively. The high H-2/H-3 coupling constant (J=11.5 Hz) of compound 2 indicated a *trans*-configuration in the C-ring (29, 30). The two doublet signals observed at 5.87 and 5.91 ppm were assigned as *meta*-coupled aromatic protons from the low coupling constant (J=2 Hz) in the A-ring. The proton signals at 6.79 (*d*, J=7.9 Hz), 6.83 (*dd*, J=1.9 and 9.8 Hz), and 6.85 (*d*, J= 1.4 Hz) ppm were assigned to a 1,2,4-trisubstituted benzene ring. Compound 2 was identified as *trans*-taxifolin by comparison of all the spectral data. *Trans*-taxifolin was readily detectable due to its intermediary contribution to biosynthetic route of PA in pine bark (27). The residue after isolation of *trans*-taxifolin in fraction IV was silylated with a TMSi reagent and analyzed using GC and GC-MS (Fig. 4 and Table 1). The residual compounds were identified by comparison of retention time and fragment mass peaks with authentic samples. The two compounds resolved at RT 19.33 and 19.85 min showed the identical M^+ (*m/z* 650) and base peak (*m/z* 368) ions, suggesting that these are related isomers. The compounds were identified as (-)-epicatechin (**9**) and (+)-catechin (**10**), respectively, by comparison of retention time with authentic samples. GC-MS analysis revealed two compounds at RT 20.13 and 21.13 min that were related isomers and indicated the main mass peaks at *m/z* 368 (base peak) and *m/z* 665 (M^+). The two compounds were identified as *cis*-taxifolin (**12**) and *trans*-taxifolin (**14**) by comparison with the isolated *trans*-taxifolin. The coincidence of the base peak (*m/z* 368) between catechins and taxifolins leaded us to hypothesize that the mass fragmentation is followed by a retro Diels-Alder fission (Fig. 5). The main molecular ions, formed by the retro Diels-Alder fission, of these flavonoids gave us information on chemical structure of the B-ring. In fraction V, *trans*-taxifolin may be isolated, to a relatively large extent, due to higher stability of *trans*-taxifolin than its *cis*-isomer (31).

The absorption band at 370 nm of fraction VI (29 mg) was attributed to the presence of flavonols. A greenish yellow crystalline compound was formed in fraction VI. This compound was recrystallized in ethanol and the chemical structure was studied using various spectral analyses. The IR spectrum of compound 3 (3 mg) showed a strong absorption band of hydroxyl groups at 3412/cm and the informative bands at 1665 and 1612/cm, which correspond to the conjugated carbonyl group and aromatic double bond, respectively. The other absorption band observed at 1016/cm is due to aryl ethers. Most flavonols exhibit two major absorption bands in the UV region: band I in the 320-385 nm range representing the B-C ring absorptions and band II in the 240-280 nm range due to

Table 1. GC-MS result for monomeric polyphenols in HWE from radiata pine bark

No.	RT (min)	Compound	M^+, *m/z*	Base peak, *m/z*	Main fragment ions, *m/z*	Fraction
1	3.24	*p*-Hydroxybenzoic acid	282	73	282, 267, 193, 73	III
2	4.13	Vanillic acid	312	297	312, 297, 282, 267, 253, 223, 126, 73	III
3	4.62	Protocatechuic acid	370	193	370, 355, 311, 193, 73	III, IV
4	5.63	*cis*-Ferulic acid	338	338	338, 323, 308, 293, 249, 219, 73	III
5	5.83	*p*-Coumaric acid	308	73	308, 293, 249, 219, 179, 73	III
6	7.68	*trans*-Ferulic acid	338	338	338, 323, 308, 293, 249, 219, 73	III
7	8.05	*trans*-Caffeic acid	396	73	396, 381, 219, 73	III, IV
8	14.31	Astringenin (?)	532	532	532	VI
9	19.33	(-)-Epicatechin	650	368	650, 368, 355	V
10	19.43	Astringenin glycoside (?)	?	532	532	VI
11	19.85	(+)-Catechin	650	368	650, 368, 355	V, VI
12	20.13	*cis*-Taxifolin	665	368	665, 368, 267, 179, 147, 75	V, VI
13	20.53	(+)-Gallocatechin	738	456	738, 648, 456, 355, 281, 267	VI
14	21.13	*trans*-Taxifolin	665	368	665, 368, 267, 179, 147, 75	V, VI
15	21.28	*cis*-Leucodelphinidin (?)	826 (?)	456	753, 739, 456, 369, 267	VI
16	21.79	*trans*-Leucodelphindin (?)	826 (?)	456	752, 737, 456, 369, 267	VI
17	25.12	Quercetin	661	647	661, 647, 575, 559, 207	VI

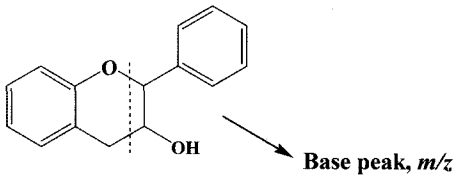

Fig. 5. Retro Diels-Alder fission of flavonoid structure.

the A-C ring absorptions (32). For flavonols, it has been observed that an increase in the number of B-ring hydroxyl groups induces a shift from 3 to 10 nm in band I (33). Therefore, band I is used for structural identification of flavonols. The band I at 374 nm of compound 3 has its characteristic maximal absorption due to the 3',4'-dihydroxyl group in the B-ring of flavonols. The UV spectrum for compound 3 was identical to that of quercetin reported in the literatures (34-36). The observation of mass fragment ions at *m/z* 661 (M^+) and *m/z* 647 (base peak) were also in agreement with the literatures (9, 10). The ^{13}C NMR spectrum displayed the characteristic signals of flavonol moiety at 147.9 (C-2), 137.2 (C-3), and 177.2 (conjugated keto group, C-4) ppm. The two doublet signals observed at 6.18 and 6.38 ppm were assigned as *meta*-coupled aromatic protons from the low coupling constant (J=2 Hz) (37). The proton signals at 6.88 (*d*, J=8.1 Hz), 7.63 (*dd*, J=2.0 and 8.4 Hz), and 7.73 (*d*, J=1.9 Hz) ppm were assigned to be due to 1,2,4-trisubstituted benzene ring. Taken together, these results show that compound 3 is quercetin.

The residue after isolation of compound 3 in fraction VI was silylated with a TMSi reagent and analyzed by GC and GC-MS. The GC-MS results revealed that fraction VI contains (+)-catechin, *cis*-taxifolin, (+)-gallocatechin, *trans*-taxifolin, and quercetin. The mass fragmentation pattern revealed the presence of astringenin, astringenin glycoside, *trans*- and *cis*-leucodelphinidin probably due to their conjugated double bond linkages and retro Diels-Alder fission.

Figure 6 shows chemical structure of the identified polyphenols and Trolox equivalent antioxidant capacity (TEAC). Structure-antioxidant activity relationship of polyphenols has been widely reported in the literatures (38-41). The TEAC assay standardized as Trolox equivalent can be used to compare the structure-antioxidant activity relationship between the well-known polyphenols. Therefore, the previously reported TEAC assays demonstrated the potential antioxidant activity of the identified polyphenols. This antioxidant activity allowed us to speculate about the potential health benefits of pine bark extracts, particularly regarding those conditions caused by free radical-induced disorders. In addition, these monomeric polyphenols identified from radiata pine bark extracts have been found in a number of diets and also have shown remarkable effects *in vivo* such as antioxidant activity (42), protection against cardio-vascular disease, anticarcinogenic activity, and modulation of gene expression. The phenolic acids have also been shown to inhibit lipid peroxidation that causes food spoilage, and preclinical and clinical research has shown that the phenolic acids modulate the immune/inflammatory response and prevent UVR-induced skin cancer in several animal models (43). From our characterization of low molecular weight polyphenols from pine bark HWE, we have shown that radiata pine bark may indeed provide a rich source of these beneficial compounds.

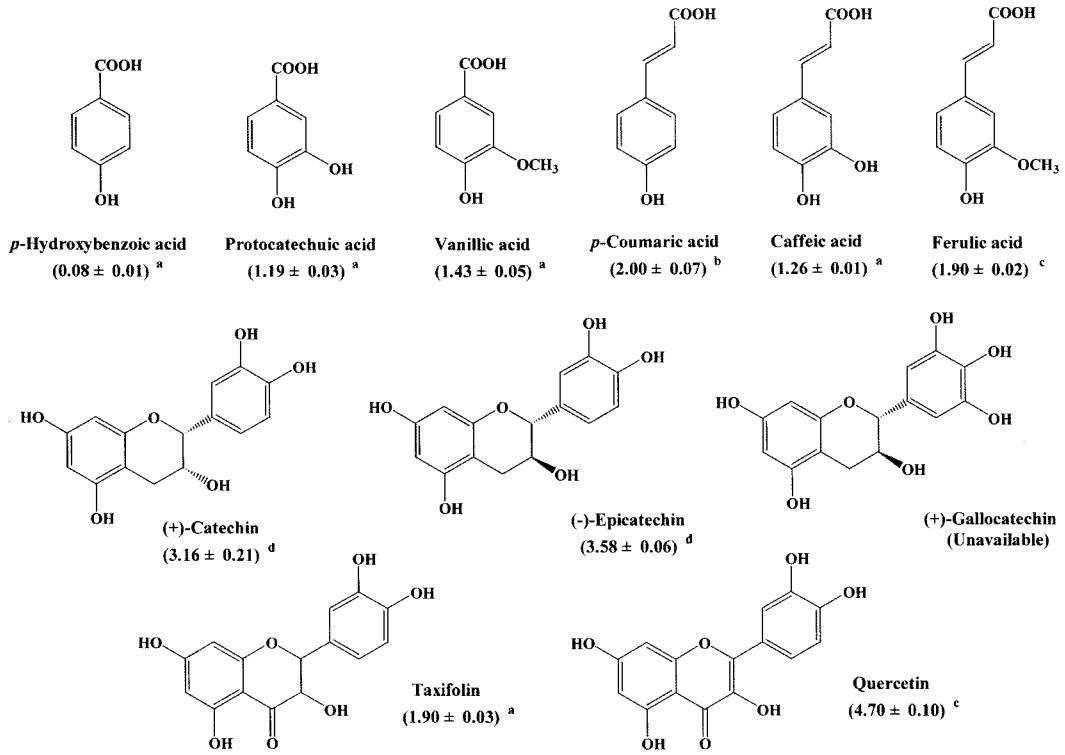

Fig. 6. TEAC (mM) of monomeric polyphenols in HWE from radiata pine bark. [a]Rice-Evans *et al.* (38), [b]Luximon-Ramma *et al.* (39), [c]van den Berg *et al.* (40), [d]Re *et al.* (41).

Acknowledgments

This study was supported by Technology Development Program for Agricultural and Forestry, Ministry of Agriculture and Forestry, Republic of Korea. The authors are grateful to Prof. Bae Young Soo (Department of Wood Science and Technology, Kangwon National University, Korea) for donation of authentic compounds of (-)-epicatechin, (-)-epigallocatechin, and (+)-gallocatechin.

References

1. Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a proanthocyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. Free Radical Bio. Med. 27: 704-724 (1999)
2. Kim YM, Wang MH, Rhee HI. A novel á-glucosidase inhibitor from pine bark. Carbohyd. Res. 339: 715-717 (2004)
3. Rauha JP, Remes S, Heinonen M, Hopia A, Kähkönen M, Kujala T, Pihlaja K, Vuorela H, Vuorela P. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Int. J. Food Microbiol. 56: 3-12 (2000)
4. Virgili F, Pagana G, Bourne L, Rimbach G, Natella F, Rice-Evans C, Packer L. Ferulic acid excretion as a marker of consumption of a French maritina pine (*Pinus Maritima*) bark extract. Free Radical Bio. Med. 28: 1249-1256 (2000)
5. Hassan EBM, Mun SP. Liquefaction of pine bark using phenol and lower alcohols with methanesulfonic acid catalyst. J. Ind. Eng. Chem. 8: 359-364 (2002)
6. Karonen M, Loponen J, Ossipov V, Pihlaja K. Analysis of procyanidins in pine bark with reversed-phase and normal-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. Anal. Chim. Acta 522: 105-112 (2004)
7. Ku CS, Mun SP. Anti-oxidative potential of hot water extracts obtained from different species of pine bark (abstract no PP004). In: Abstracts: 54th Annual Meeting of the Japan Wood Research Society. August 3-5, Sapporo convention center, Sapporo, Japan. Japan Wood Research Society, Sapporo, Japan (2004)
8. Ku CS, Mun SP. Identification and characterization of hot water extracts from *Pinus radiata* bark and their potential bioactivity (abstract no P61530). In: Abstracts: 55th Annual Meeting of the Japan Wood Research Society. March 16-18, Kyoto University Yoshida-south Campus Clock Tower Centennial Hall, Kyoto, Japan. Japan Wood Research Society, Kyoto, Japan (2005)
9. Owen RW, Haubner R, Hull WE, Erben G, Spiegelhalder B, Bartsch H, Haber B. Isolation and structure elucidation of the major individual polyphenols in carob fibre. Food Chem. Toxicol. 41: 1727-1738 (2003)
10. Zhang K, Zuo Y. GC-MS determination of flavonoids and phenolic and benzoic acids in human plasma after consumption of cranberry juice. J. Agric. Food Chem. 52: 222-227 (2004)
11. Franke R, Humphreys JM, Hemm MR, Denault JW, Ruegger MO, Cusumano JC, Chapple C. The Arabidopsis REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism. Plant J. 30: 33-45 (2002)
12. Tokuşoğlu MK, Yıldırum Z. HPLC-UV and GC-MS characterization of the flavonol aglycones quercetin, kaempferol, and myrcetin in tomato pastes and other tomato-based products. Acta Chromatogr. 13: 196-207 (2003)
13. Stevens JF, Hart H, Elema ET, Bolck A. Flavonoid variation in Eurasian *Sedum* and *Sempervivum*. Phytochemistry 41: 503-512 (1996)
14. Watson DG, Pitt AR. Analysis of flavonoids in tablets and urine by gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry. Rapid Commun. Mass Sp. 12: 153-156 (1998)
15. Peng X, Misawa N, Harayama S. Isolation and characterization of *thermophilic bacilli* degrading cinnamic, 4-coumaric, and ferulic acids. Appl. Environ. Microbiol. 69: 1417-1427 (2003)
16. Gonthier MP, Cheynier V, Donovan JL, Manach C, Morand G, Mila I, Lapierre C, Rémésy C. Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. Nutr. Metabol.

461-467 (2003)
17. Kennedy JF, Methacanon P, Lloyd LL. The identification and quantitation of the hydroxycinnamic acid substituents of a polysaccharide extracted from maize bran. J. Sci. Food Agric. 79: 464-470 (1999)
18. Martens DA. Division S-3 - Soil biology & biochemistry: identification of phenolic acid composition of alkali-extracted plants and soils. Soil Sci. Soc. Am. J. 66: 1240-1248 (2002)
19. Dueñas M, Estrella I, Hernández T. Occurrence of phenolic compounds in the seed coat and the cotyledon of peas (*Pisum sativum* L.). Eur. Food Res. Technol. 219: 116-123 (2004)
20. Moran JF, Klucas RV, Grayer RJ, Abian J, Harborne JB, Becana M. Characterization of phenolic glucosides from soybean root nodules by ion-exchange high performance liquid chromatography, ultraviolet spectroscopy and electrospray mass spectrometry. Phytochem. Anal. 9: 171-176 (1998)
21. Le Nest G, Caille O, Woudstra M, Roche S, Guerlesquin F, Lexa D. Zn-polyphenol chelation: complexes with quercetin, (+)-catechin, and derivatives: I optical and NMR studies. Inorgan. Chim. Acta 357: 775-784 (2004)
22. Dellus V, Mila I, Scalbert A, Menard C, Michon V, Herve du Penhoat CLM. Douglas-fir polyphenols and heartwood formation. Phytochemitry 45: 1573-1578 (1997)
23. Mabry TJ, Markham KR, Thomas MB. The systematic identification of flavonoids. Springer, New York, NY, USA. pp. 35-61 (1970)
24. Boudet AC, Cornard JP, Merlin JC. Conformational and spectroscopic investigation of 3-hydroxyflavone-aluminium chelates. Spectrochim. Acta Part A 56: 829-839 (2000)
25. Cornard JP, Boudet AC, Merlin JC. Complexes of Al(III) with 3′4′-dihydroxy-flavone: characterization, theoretical and spectroscopic study. Spectrochim. Acta Part A 57: 591-602 (2001)
26. Bergeron C, Marston A, Antus S, Gauthier R, Hostettmann K. Flavonoids from *Pyrola elliptica*. Phytochemistry 49: 233-236 (1998)
27. Harborne JB, Mabry TJ. The flavonoids: advances in research. Chapman and Hall, New York, NY, USA. pp. 417-446 (1982)
28. Li TM, Li WK, Yu JG. Flavonoids from *Artabotrys hexapetalus*. Phytochemistry 45: 831-833 (1997)
29. Lu Y, Sun Y, Foo LY, McNabb WC, Molan AL. Phenolic glycosides of forage legume *Onobrychis viciifolia*. Phytochemistry 55: 67-75 (2000)
30. Dübeler A, Voltmer G, Gora V, Lunderstädt J, Zeeck A. Phenols from *Fagus sylvatica* and their role in defence against *Cryptococcus fagisuga*. Phytochemistry 45: 51-57 (1997)
31. Trouillas P, Fagnére C, Lazzaroni R, Calliste C, Marfak A, Duroux JL. A theoretical study of the conformational behavior and electronic structure of taxifolin correlated with the free radical-scavenging activity. Food Chem. 88: 571-582 (2004)
32. Markham KR. Flavones, flavonols and their glycosides. In: Methods in Plant Biochemistry, Plant Phenolics. Harborne JB (ed). Academic Press, New York, NY, USA. pp. 197-235 (1989)
33. Marfak A, Trouillas P, Allais DP, Calliste CA, Cook-Moreau J, Duroux JL. Reactivity of flavonoids with 1-hydroxyethyl radical: a γ-radiolysis study. Biochim. Biophy. Acta 1670: 28-39 (2004)
34. Couladis M, Baziou P, Verykokidou E, Loukis A. Antioxidant activity of polyphenols from *Hypericum triquetrifolium* Turra. Phytother. Res. 16: 769-770 (2002)
35. Lewis CE, Walker JRL, Lancaster JE, Sutton KH. Determination of anthocyanins, flavonoids and phenolic acids in potatoes. I: Coloured cultivars of *Solanum tuberosum* L. J. Sci. Food Agric. 77: 45-57 (1998)
36. Peng ZF, Strack D, Baumert A, Subramaniam R, Goh NK, Chia TF, Tan SN, Chia LS. Antioxidant flavonoids from leaves of *Polygonum hydropiper* L. Phytochemistry 62: 219-228 (2003)
37. Lee JY, Moon SO, Kwon YJ, Rhee SJ, Park HR, Choi SW. Identification and quantification of anthocyanins and flavonoids in mulberry (Morus sp.) cultivars. Food Sci. Biotechnol. 13:176-184 (2004)
38. Rice-Evans CA, Miller NJ, Paganaga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Bio. Med. 20: 933-956 (1996)
39. Luximon-Ramma A, Bahorun T, Crozier A, Zbarsky V, Datla KP, Dexter DT, Aruoma OI. Characterization of the antioxidant functions of flavonoids and proanthocyanidins in Mauritian black teas. Food Res. Int. 38: 357-367 (2005)
40. van den Berg R, Haenen GRMM, van den Berg H, Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chem. 66: 511-517 (1999)
41. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio. Med. 26: 1231-1237 (1999)
42. Seog HM, Jung CH, Kim YS, Park HS. Phenolic acids and antioxidant activities of wild ginseng (Panax ginseng C. A. Meyer) leaves. Food Sci. Biotechnol. 14: 371-374 (2005)
43. Gombau L, García F, Lahoz A, Fabre M, Roda-Navarro P, Majano P, Alonso-Lebrero JL, Pivel JP, Castell JV, Gómez-Lechon MJ, González S. *Polypodium leucotomos* extract: antioxidant activity and disposition. Toxicol. In Vitro 20: 464-471 (2006)