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Stilbenoids of Korean Pine (*Pinus koraiensis*) Inner Bark*[1]

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ABSTRACT

Pinus koraiensis inner bark was collected and extracted with 95% ethanol. The extracts were concentrated and then sequentially fractionated using *n*-hexane, CH_2Cl_2, EtOAc, and H_2O to be freeze dried. A portion of EtOAc fraction (6.6 g) was chromatographed on a Sephadex LH-20 column using aqueous methanol to isolate (+)-catechin (**1**), (-)-epicatechin (**2**), and *trans*-pinostilbenoside (**3**). Resveratrol (**4**) and *trans*-pinostilbene (**5**) were isolated by column chromatography using EtOH-hexane mixture after purification with aqueous methanol. The structures of these stilbenosides and flavans were characterized by spectroscopic tools using NMR and MS.

Keywords : *Pinus koraiensis*, inner bark, column chromatography, stilbene derivatives

1. INTRODUCTION

Korean pine (*P. koraiensis*) distributes in the Amur and maritime provinces of Russia, in Korea and China, and in a few spots of Japan (Mirov, 1967; Vidakovic, 1991). The species has been introduced into Europe in 1846, where its altitudinal distribution generally ranges from 600 to 1,000 m (Vidakovic, 1991). The tree is a prominent reforestation species in Korea and has been planted in 86,000 ha. accounting for about 30% of Korean artificial forests (Kim and Mishiro, 1998).

The chemical compositions of the species have been reported on wood and bark. Stilbene glycosides, *Z*-pinostilbenoside, *E*-desoxyrhaponticin and *E*-resveratroloside were isolated from the bark (Song, 2001). Two stilbenoids and five flavonoids were isolated from the wood (Lee *et al*., 2003). The bark of Korean pine has an antibacterial activity (Kim *et al*., 2001) and stilbenoids, which are major components of the chemical constituents of the species, also have various biological activities such as antioxidation, antiasthma and antiallergy (Singh *et al*., 1997; Miura *et al*., 2000; Shimizu *et al*., 2000; Song *et al*., 2001).

Recently there are many trials to use the bark of *Pinus* species for manufacturing functional cosmetics or supplementary health foods. This study was carried out to investigate the chemical constituents of Korean pine inner bark as a

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part of the purpose for the development of bioactive products.

2. MATERIALS and METHODS

2.1. Plant Material

26 years old of *P. koraiensis* tree was collected in the experimental forest of Kangwon National University, Bong-Myung Ri, Gangwon Province, Korea in April 2009. A voucher specimen (WSE 0904-2) has been deposited at the herbarium in the Department of Wood Science and Engineering. The inner bark was carefully stripped and then air-dried at room temperature for 2 weeks before grinding.

2.2. General Experiment

NMR spectra were recorded on a Bruker Avance DPX 400 and 600 spectrometers using tetramethylsilane (TMS) as an internal standard and chemical shifts are given in δ (ppm). MALDI-TOF-MS was performed with a Voyager-DE STR spectrometer. FAB-MS were recorded on a Micromass Autospec M363 spectrometer using *m*-nitro benzyl alcohol (NBA) as a matrix. Column chromatography was done on a lipophilic Sephadex LH-20 (25～100 μm, Sigma) column. Eluents were collected using a fraction collector (Gilson FC 204). Thin layer chromatography (TLC) was performed on DC-Plastikfolien Cellulose F (Merck) plates and developed with TBAW (*t*-BuOH-HOAC-H_2O (3:1:1, v/v/v)) and 6% aqueous HOAc. Visualization was done by UV light (254 and 365 nm) or by spray with vanillin (vanillin : EtOH : H_2SO_4, 15 : 250 : 2.5, w/v/v) solution followed by heating.

2.3. Extraction and Isolation

The air-dried, ground inner bark of *P. koraiensis* (1.87 kg) was immersed in 95% ethanol at room temperature for 3 days. After filtration (Advantec No. 2), the bark sample was extracted two more times. The filtrates were combined and evaporated on a rotary evaporator under the reduced pressure at 40°C. A part of the aqueous residue (57.5 g) was successively fractionated on a separatory funnel and freeze-dried to give *n*-hexane (0.97 g), CH_2Cl_2 (1.03 g), EtOAc (12.7 g), and H_2O (40.3 g) soluble fractions.

A portion of EtOAc soluble fraction (6.3 g) was chromatographed on a Sephadex LH-20 column using MeOH-H_2O (2 : 1, v/v) to afford 5 fractions: F1 (1.0 g), F2 (0.55 g), F3 (1.80 g), F4 (1.2 g), F5 (0.16 g), and F6 (1.2 g). F2 (0.55 g) was retreated on a Sephadex column with MeOH-H_2O (1 : 3, v/v) to give compounds **1** (8 mg) and **3** (0.4 g). F3 (1.8 g) was also washed again with MeOH-H_2O (1 : 2, v/v) to afford compounds **1** (72 mg), **2** (8 mg), and **3** (0.62 g). F5 (0.16 g) was rechromatographed on a Sephadex column with MeOH-H_2O (1 : 3, v/v) to separate 6 subfractions. F5-6 (50 mg) was retreated on a Sephadex column with EtOH-hexane (3 : 1, v/v) to give compounds **4** (15 mg) and **5** (12 mg).

2.3.1. (+)-catechin (1)

Yellow amorphous powder. R_f : 0.53 (TBAW) and 0.33 (6% HOAc). ^{1}H-NMR (400 MHz, δ, CD_3OD) : 2.50 (1H, *dd*, *J* = 8.1 Hz and 16.1 Hz, H-4ax), 2.84 (1H, *dd*, *J* = 5.4 Hz and 16.1 Hz, H-4eq), 3.97 (1H, *m*, H-3), 4.57 (1H, *d*, *J* = 7.5 Hz, H-2), 5.86 (1H, *d*, *J* = 2.4 Hz, H-6), 5.93 (1H, *d*, *J* = 2.3 Hz, H-8), 6.72 (1H, *dd*, *J* = 1.7 Hz and 8.1 Hz, H-6′), 6.76 (1H, *d*, *J* = 8.1 Hz, H-5′), 6.84 (1H, *d*, *J* = 1.8 Hz, H-2′). ^{13}C-NMR (100 MHz, δ, CD_3OD) : 28.55 (C-4), 68.84 (C-3), 82.88 (C-2), 95.53 (C-8), 96.32 (C-6), 100.85 (C-10), 115.28 (C-2′), 116.12 (C-5′), 120.08 (C-6′), 132.24 (C-1′), 146.26 (C-3′), 146.28 (C-4′), 156.95 (C-9), 157.61 (C-5),

Table 1. ^{1}H and ^{13}C-NMR chemical shift (ppm) of compounds **3-5**

	3[a]		**4**[b]		**5**[b]	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		139.1		139.9		139.9
2	6.61 *br s*	102.6	6.45 *d* (*J* = 2.0)	104.4	6.56 *br s*	104.7
3		160.5		158.2		161.1
4	6.25 *t* (*J* = 2.0)	100.5	6.16 *t* (*J* = 2.0)	101.3	6.24 *br s*	103.0
5		158.5		158.2		159.1
6	6.57 *br s*	105.9	6.45 *d* (*J* = 2.0)	104.4	6.53 *br s*	106.3
7	6.99 *d* (*J* = 16.4)	126.8	6.80 *d* (*J* = 16.3)	125.6	6.87 *d* (*J* = 16.3)	125.8
8	7.11 *d* (*J* = 16.4)	127.8	6.95 *d* (*J* = 16.3)	128.0	7.03 *d* (*J* = 16.3)	128.9
1′		130.6		129.0		130.4
2′	7.53 *d* (*J* = 8.8)	127.5	7.35 *d* (*J* = 8.5)	127.4	7.40 *d* (*J* = 8.7)	128.4
3′	7.03 *d* (*J* = 8.7)	116.3	6.76 *d* (*J* = 8.4)	115.1	6.77 *d* (*J* = 8.4)	116.0
4′		156.9		157.0		157.8
5′	7.03 *d* (*J* = 8.7)	116.3	6.76 *d* (*J* = 8.4)	115.1	6.77 *d* (*J* = 8.4)	116.0
6′	7.53 *d* (*J* = 8.8)	127.5	7.35 *d* (*J* = 8.5)	127.4	7.40 *d* (*J* = 8.7)	128.4
OCH_3	3.74 s	54.9			3.72 *s*	55.4
Glc						
1	4.90 *d* (*J* = 7.3)	100.1				
2	3.27 *m*	73.1				
3	3.27 *m*	76.5				
4	3.19 *m*	69.6				
5	3.36 *m*	76.9				
6	3.48, 3.70 *m*	60.6				

a) Spectra measured at 400 MHz (^{1}H) and 100 MHz (^{13}C).
b) Spectra measured at 600 MHz (^{1}H) and 125 MHz (^{13}C).

157.86 (C-7).

2.3.2. (-)-epicatechin (**2**)

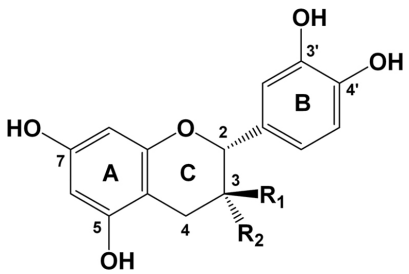
Brown amorphous powder. R_f : 0.37 (TBAW) and 0.31 (6% HOAc). ^{1}H-NMR (400 MHz, δ, CD_3OD) : 2.73 (1H, *dd*, *J* = 2.8 Hz and 16.8 Hz, H-4ax), 2.86 (1H, *dd*, *J* = 4.5 Hz and 16.8 Hz, H-4eq), 4.17 (1H, *m*, H-3), 4.81 (1H, *s*, H-2), 5.91 (1H, *d*, *J* = 2.3 Hz, H-6), 5.94 (1H, *d*, *J* = 2.3 Hz, H-8), 6.75 (1H, *d*, *J* = 8.1 Hz, H-5′), 6.80 (1H, *dd*, *J* = 1.8 Hz and 8.3 Hz, H-6′), 6.97 (1H, *d*, *J* = 1.7 Hz, H-2′). ^{13}C-NMR (100 MHz, δ, CD_3OD) : 29.31 (C-4), 67.54 (C-3), 79.92 (C-2), 95.93 (C-8), 96.43 (C-6), 100.11 (C-10), 115.37 (C-2′), 115.93 (C-5′), 119.44 (C-6′), 132.34 (C-1′), 145.83 (C-3′), 145.99 (C-4′), 157.42 (C-9), 157.73 (C-5), 158.05 (C-7).

2.3.3. *trans*-pinostilbenoside (**3**)

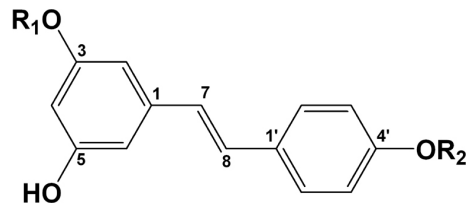
White amorphous powder. R_f : 0.72 (TBAW) and 0.34 (6% HOAc). MALDI-TOF-MS : Calculated for $C_{21}H_{24}O_8$ 404, Found *m/z* 405 $[M + H]^+$, 427 $[M + Na]^+$. ^{1}H and ^{13}C-NMR (CD_3 OD) see Table 1.

2.3.4. Resveratrol (**4**)

Brown amorphous powder. R_f : 0.68 (TBAW) and 0.00 (6% HOAc). MALDI-TOF-MS : Cal-



R_1 = OH, R_2 = H : (+)-catechin **(1)**
R_1 = H, R_2 = OH : (-)-epicatechin **(2)**



R_1 = CH_3, R_2 = β-D-glucose : *trans*-pinostilbenoside **(3)**
R_1 = R_2 = H : resveratrol **(4)**
R_1 = CH_3, R_2 = H : *trans*-pinostilben **(5)**

Fig. 1. The chemical structures of compounds 1-5 isolated from *P. koraiensis* inner bark.

culated for $C_{14}H_{12}O_3$ 228, Found *m/z* 229 $[M + H]^+$. ^1H and ^{13}C-NMR $(CD_3)_2SO$) see Table 1.

2.3.5. *trans*-pinostilben (**5**)

Brown amorphous powder. R_f : 0.76 (TBAW) and 0.00 (6% HOAc). FAB-MS : Calculated for $C_{15}H_{14}O_3$ 242, Found *m/z* 243 $[M + H]^+$. ^1H and ^{13}C-NMR (CD_3OD) see Table 1.

3. RESULTS and DISCUSSION

Three stilbene derivatives, *trans*-pinostilbenoside (**3**), resveratrol (**4**), and *trans*-pinostilben (**5**) were isolated by a Sephadex LH-20 column using aqueous methanol and EtOH-hexane mixture from EtOAc soluble fraction of the inner bark of *P. koraiensis*. (+)-catechin (**1**) and (-)-epicatechin (**2**) were also purified using a Sephadex column and aqueous methanol. Their structures were characterized by NMR and MS spectroscopy.

Compounds **1** and **2** are very common in many plant sources and characterized as (+)-catechin and (-)-epicatechin using authentic literature data (Harbone and Mabry, 1982).

Compound **3** was purified as white amorphous powder and the molecular formular was deduced to be $C_{21}H_{24}O_8$ on the basis of the peak at *m/z* 427 $[M + Na]^+$ in the positive MALDI-TOF MS. In the ^1H-NMR spectrum, aromatic H-2, H-4 and H-6 appeared at δ 6.61, 6.25 and 6.57, respectively. Two *trans* olefinic protons (H-7 and H-8) showed at δ 6.99 and 7.11 (J = 16.4 Hz), respectively. Two sets of aromatic symmetric protons (H-2′,6′ and H-3′,5′) gave two signals at δ 7.53 (J = 8.8 Hz) and 7.03 (J = 8.7 Hz), respectively (Song *et al*., 2001). The anomeric proton (H-1″) showed a doublet signal at δ 4.90 and its coupling constant was 7.3 Hz indicating β-glucose. The other proton signals appeared at δ 3.19～3.70 representing the typical glucose molecule. The methoxyl protons gave a typical signal at δ 3.74. In the ^{13}C-NMR spectrum, C-3 containing a methoxyl group gave a signal at δ 160.5 and the methoxyl carbon appeared at δ 54.9. The other hydroxyl containing C-5 showed a signal at δ 158.5. C-4′ connecting to the glucose unit gave a signal at δ 156.9. Two sets of symmetrical carbons, C-2′,6′ and C-3′,5′, appeared at δ 127.5 and 116.3, respectively. C-1 of the glucose appeared at δ 100.1 suggesting β-form and the other carbons gave typical signals among δ 60.6~76.9. HMBC spectrum showed a correlation between C-3 and methoxy protons and also gave another correlation between C-4′ and glucose H-1 which confirm the proper structure

linkages of compound **3**. According to the combination of NMR and literature data (Gromova *et al*., 1975; Song, 2001; Rimando and Suh, 2008), the compound was characterized as *trans*-pinostilbenoside.

Compounds **4** and **5** were obtained as brown amorphous powder. In the ^{1}H-NMR spectrum of compound **4**, the symmetrical H-2 and H-6 gave one double signal at δ 6.45 (J = 2.0 Hz) and H-4 showed a triplet at δ 6.16. The other two sets of symmetrical protons, H-2′,6′ and H-3′,5′, showed two doublet signals at δ 7.35 and 6.76, respectively. H-7 and H-8, *trans*-olefinic protons, gave doublet signals at δ 6.80 and 6.95, respectively, with 16.3 Hz of coupling constant. In the ^{13}C-NMR spectrum of compound **4**, the hydroxyl containing symmetrical C-3 and C-5 gave a signal at δ 158.2 and another hydroxyl containing C-4′ showed a signal at δ 157.0. Two *trans*-olefinic C-7 and C-8 gave signals at δ 125.6 and 128.0, respectively. Three pairs of symmetrical carbons, C-2 and C-6, C-2′ and C-6′, and C-3′ and C-5′, appeared at δ 104.4, 127.4, and 115.1, respectively. On the basis of NMR data, compound **4** was determined as resveratrol (Koh *et al*., 2001; Commodari *et al*., 2005).

Compound **5**, *trans*-pinostilben, showed almost same NMR spectrum as those of compound **4**, besides the substituted methoxyl group at C-3 that confirmed a correlation between C-3 and methoxy proton by HMBC spectrum. Thus compound **5** was identified as *trans*-pinostilben (Regev-Shoshani *et al*., 2003).

4. CONCLUSIONS

Three stilbene derivatives, *trans*-pinostilbenoside, resveratrol, and *trans*-pinostilben, includeing (+)-catechin and (-)-epicatechin, were isolated from EtOAc fraction of *Pinus koraiensis* inner bark by Sephadex LH-20 column chromatography using aqueous methanol and EtOH-hexane mixture as eluting solvents. Their structures were characterized by NMR, includeing two dimensional HMBC, and MALDI-TOF MS. To our best knowledge, resveratrol and *trans*-pinostilben were first isolated from the inner bark of *Pinus koraiensis*.

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