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Studies on Biological Activity of Wood Extractives (XVII)*1
- Components and Antioxidant activity of *Alnus firma* -

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ABSTRACT

This study is to isolate bio-active compounds from *Alnus firma* and evaluate their antioxidant activity. Dried wood powder of *A. firma* was extracted by organic solvents and fractionated in the sequential extraction steps. The isolated compounds were characterized by EI-MS, ^{13}C- and ^{1}H-NMR including COSY, DEFT, HMQC, and HMBC. Antioxidant activities of the isolated compounds were evaluated by DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging effect. From the wood of *A. firma*, three kinds of diarylheptanoids, alnusodiol (1), alnusonol (2) and alnusone (3), and gallic acid (4) were isolated. Among these four compounds, compound 1, 2, and 3 are isolated from *A. firma* for the first time. The antioxidant activity of gallic acid was 93.5% at the concentration of 100 ppm. This compound showed stronger antioxidant activity than those of other isolated compounds and the reference BHT (butylated hydroxytoluene).

Keywords : *Alnus firma*, diarylheptanoid, alnusodiol, alnusonol, alnusone, antioxidant activity

1. INTRODUCTION

Alnus firma (Betulaceae) is a deciduous tree which is distributed through Korea and Japan. Its bark is somewhat grayish brown in color. The fruits of this plant were used as dye.

Species in the family of Betulaceae are widely studied in various fields. In the case of *Alnus japonica*, a number of diarylheptanoids as well as other phenolics, including secoisolariciresinol diferulate were isolated by Nomura *et al.* (1981). An extracts of *A. japonica* cortex has used for the purpose of purifying blood and curing diarrhea. According to Jeong (2003), the extracts from the cortex of *A. japonica* significantly inhibited carrageenan-induced paw edema at 1, 2, and 3 hours after oral administration.

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Although many species in the family of Betulaceae have studied, phytochemical analysis of the constituents in *A. firma* has not been investigated in detail. This study was carried out to isolate and identify the chemical components from the wood extractives of *A. firma*. The antioxidant activities of the isolated compounds from *A. firma* were also evaluated using DPPH assay method.

2. MATERIALS and METHODS

2.1. General Procedures

EI-MS was performed at 70 eV ionization energy by direct inlet probe method, using a JEOL JMS-600W mass spectrometer (Japan). NMR spectra were obtained using a Varian UI 500 spectrometer (U.S.A) at the operating frequency of 500 MHz (^{1}H) and 125 MHz (^{13}C) at Korea Basic Science Institute in Seoul.

2.2. Plant Materials

The wood of *A. firma* (18-years old) was collected and identified by Dr. Kwon Y. H. (National Arboretum, Korea) at the experimental forest of Korea Forest Research Institute in Jinju, Kyungnam Province, Korea in July 2002. This material was dried at room temperature under shade. Voucher specimens were deposited at the Korea Forest Research Institute, Seoul, Korea.

2.3. Extraction and Fractionation

Air dried wood of *A. firma* was powdered and extracted twice with 95% ethanol (EtOH), and then evaporated to give the crude extracts. The crude extracts were successively partitioned with organic solvents, such as diethyl ether (Et_2O), dichrolomethane (CH_2Cl_2), and ethyl acetate (EtOAc).

2.4. Isolation Procedures

A CH_2Cl_2 soluble fraction (58.4 g) of the wood from *A. firma* was passed through a Sephadex LH-20 and eluted with *n*-hexane-EtOH (1:4, v/v) to yield 10 sets of fraction (AFWD 1～10).

Fraction AFWD 5 was re-chromatographed on silica gel column chromatography with $CHCl_3$-MeOH (10:1, v/v) to give 3 subfractions (AFWD 5-1～5-3). Among these fractions, the second fraction (AFWD 5-2) was further subjected to the repeated column chromatography on silica gel eluted with *n*-hexane-EtOAc-MeOH (5:2:1, v/v /v) to give 4 sets of fractions (AFWD 5-2-1～5-2-4) and compound 1 (35.4 mg) was isolated from AFWD 5-2-3 fraction.

Fraction AFWD 5-1 was further subjected to the repeated column chromatography on Sephadex LH-20 eluted with MeOH-EtOH (5:3, v/v) to give 8 sets of fractions (AFWD 5-1-1～AFWD 5-1-8) and AFWD 5-1-4 was rechromatographed on the silica gel columns with $CHCl_3$-MeOH (20:1, v/v) and *n*-hexane-EtOAc-acetone (3:2:1, v/v), successively, to afford compound 2 (21.4 mg).

Fraction AFWD 5-1-5 was further subjected to the repeated column chromatography on silica gel eluted with benzene-CH_2Cl_2-acetone (10:2:1, v/v/v) to give 12 sets of fractions (AFWD 5-1-5-1～AFWD 5-1-5-12). Compound 3 (28.9 mg) was obtained from the fourth fraction (AFWD 5-1-5-4) of AFWD 5-1-5.

The EtOAc soluble fraction (67.96 g) was subjected to the column chromatography on Sephadex LH-20 eluted with acetone-MeOH (5:1, v/v) to yield 4 sets of fraction (AFWE 1～AFWE 4). Fraction AFWE 1 (47.63 g) was re-chromatographed on silica gel column chromatography with $CHCl_3$-MeOH (5:1, v/v) to

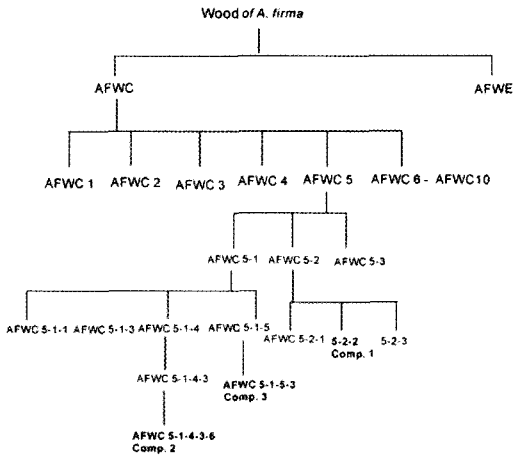


Fig. 1. The scheme of isolation procedure of compound 1, 2, and 3 from the wood of *A. firma*.

give 6 subfractions (AFWE 1-1～AFWE 1-6). Fraction AFWE 1-4 was further chromatographed on silica gel column chromatography with CH_2Cl_2-EtOAc-MeOH (5:2:1, v/v/v) to give 6 subfractions (AFWE 1-4-1～AFWE 1-4-6). AFWE 1-4-3 was purified by the column chromatography on Shephadex LH-20, and eluted with a solvent system of acetone- MeOH (3:1, v/v) to give 3 fractions. Compound 4 (28.3 mg) was finally isolated from AFWE 1-4-3-2 on the silica gel column chromatography with CH_2Cl_2-MeOH (8:1, v/v).

2.5. Spectral Data of Compounds

Compound 1 : Colorless needle: EI-MS *m/z*: 314 ($[M]^+$, base ion), 296, 278, 255, 225, 211, 181, 152, 128, 106, 77. ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) data, see Table 1: ^{1}H-^{1}H COSY correlations: H-4↔H-5, H-19↔H-15, H-8a↔H-8b, H-8a↔H-7, H-13↔H-11. HMBC correlations: H-1→C-3/C-5, H-5→C-3/C-4, H-15→C-17/C-19, H-8a/H-8b→C-6, H-12a/H-12b→C-14.

Compound 2 : Colorless needle: EI-MS *m/z*: 312 ($[M]^+$, base ion), 294, 258, 236, 211, 197, 165, 153, 128, 106, 77, 55. ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) data, see Table 1: ^{1}H-^{1}H COSY correlations: H-8a↔H-8b/H-7/H-9, H-10a↔H-9, H-10b↔H-9, H-1↔H-5, H-8↔H9. HMBC correlations: H-7→C-5, H8→C-6, H12→C-14, H-4→C-6, H-19→C-15/C-13, H-5→C-1/C-7, H-10a/H-10b/H-12a/H-12b/H-13→C-11.

Compound 3 : Colorless needle: EI-MS *m/z*: 294 ($[M]^+$, base ion), 225, 211, 207, 165, 129, 98, 85. ^{1}H-NMR (500 MHz, CD_3OD) and ^{13}C-NMR (125 MHz, CD_3OD) data, see Table 1: ^{1}H-^{1}H COSY correlations: H-1↔H-4, H-4↔H-5, H-9↔H-10, H-15↔H-18↔H-19, H-8↔H-7, H-12↔H-13. HMBC correlation: H-1→C-7, H-4→C-6, H-10→C-8/C-11, H-9→C-11, H-19→C-15, H-15→C-16/C19, H-18→C-16.

Compound 4 : Yellow powder: EI-MS *m/z*: 170 ($[M]^+$, base ion), 153, 135, 126. ^{1}H-NMR (500 MHz. CD_3OD): δ 7.06 (2H, *s*, H-2, -6). ^{13}C-NMR (125 MHz, CD_3OD): 110.3 (*d*, C-2, -6), 122.0 (*s*, C-1), 139.5 (*s*, C-4), 146.3 (*s*, C-3, -5), 170.3 (*s*, C-7). HMBC correlation: H-2/H-6→C-2/C-6/C-1/C-4/C-3/C-5/C-7.

2.6. Antioxidant Activity Test

The antioxidant activity of compounds was assessed on the basis of the scavenging activity of the DPPH free radical method. The sample solutions dissolved in MeOH (4 mL) were added to the solutions of DPPH in MeOH (4.5×10^{-4} M, 1 mL) and the reaction mixture were shaken thoroughly. After 30 minutes of incubation at room temperature, the remaining amounts of DPPH were determined by colorimetry (8452A Diode Array Spectrophotometer, Hewlett Packard Co.) at 520 nm (Blois, 1958). The mixture of 4 mL MeOH with a solution of 1 mL DPPH was used as the control. Butylated hydroxylanisole (BHA) and α-tocopherol are used for the comparison. The mean values were

Table 1. ^{1}H- and ^{13}C-NMR data (δ) of compound 1, 2, and 3[a]

	1		2		3	
Position	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	7.04 (*d*, 2.0)	134.3	6.57 (*d*, 2.0)	134.6	6.87 (*d*, 2.0)	135.4
2	-	128.2	-	127.2	-	126.4
3	-	154.6	-	152.6	-	151.9
4	6.73 (*d*, 8.0)	117.7	6.76 (*d*, 8.0)	117.0	6.74 (*m*)	115.6
5	6.96 (*dd*, 2.0, 8.0)	130.0	7.01 (*dd*, 2.0, 8.0)	130.7	6.97 (*m*)	128.9
6	-	130.5	-	132.5	-	132.8
7	2.80 (*m*)	27.5	2.82 (*m*)	29.1	2.86 (*d*, 5.5)	32.8
8a	1.73 (*m*)	36.0	1.75 (*m*)	35.6	2.50 (*m*)	35.2
8b	2.23 (*m*)		2.39 (*m*)		-	
9	4.03 (*m*)	67.4	4.16 (*m*)	67.4	6.97 (*m*)	149.5
10a	1.91 (dd, 4.5, 7.5)	52.0	2.64 (*dd*, 5.0, 8.5)	54.1	6.53 (d, 15.5)	133.7
10b	-		2.91 (*m*)		-	
11	4.03 (*m*)	67.4	-	212.2	-	202.6
12a	1.73 (*m*)	36.0	2.82 (*m*)	43.0	2.66 (*m*)	39.6
12b	2.23 (*m*)		3.09 (*m*)		-	
13	2.80 (*m*)	27.5	2.91 (*m*)	27.3	3.12 (*s*)	29.5
14	-	130.5	-	132.3	-	131.8
15	7.04 (*d*, 2.0)	134.2	6.76 (*d*, 2.0)	134.6	6.97 (*m*)	133.4
16	-	128.2	-	127.2	-	126.3
17	-	154.6	-	152.3	-	150.9
18	6.73 (*d*, 8.0)	117.7	6.76 (*d*, 8.0)	117.1	6.74 (*m*)	116.3
19	6.96 (*dd*, 2.0, 8.0)	130.0	7.01 (*dd*, 2.0, 8.0)	129.6	6.97 (*m*)	128.2

[a]Values in parentheses are coupling constants (in Hz). Spectra were measured at 125 and 500 MHz.

obtained from triplicate experiments. The following formula was used for the determination of antioxidant activity of each compound:

$$\text{Percentage of Free Radical Scavenging (\%)} = \{1 - (A/B)\} \times 100$$

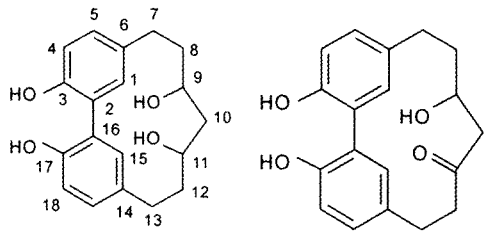
"A" and "B" indicates the absorbance of sample and control solution at 520 nm, respectively.

3. RESULTS and DISCUSSION

3.1. Identification of Compounds

Four compounds were isolated from the wood extracts of *A. firma* through the repeated column chromatography with SiO_2 and Sephadex LH-20.

The molecular formula $C_{19}H_{22}O_4$ of compound 1 was established on the basis of ^{1}H- and ^{13}C-NMR spectra and EI-MS (*m/z* 314 $[M]^{+}$). In the aromatic region of the ^{1}H-NMR spectra of compound 1, the coupling constant between H-1 and H-5 and the coupling constant between H-5 and H-4 indicated a 2, 3, 6-trisubstituted benzene ring (Li *et al.*, 2004). From the ^{13}C-NMR and HMQC spectra showed the presence of five methylenes, eight methines, and six quaternary carbons. In ^{13}C-NMR spectrum, each carbon was assigned as follows; C-8a and -12a at δ 36.0, C-9 and -11 at δ 67.4, C-4 and -18 at δ 117.7, C-5 and -19 at δ 130.0, C-2 and -16 at δ 128.2 and C-3 and -17 at δ 154.6,



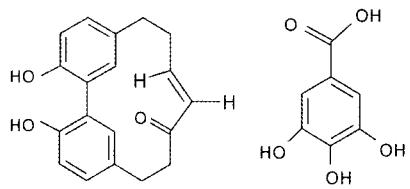


Fig. 2. Compounds isolated from the wood of *A. firma*.

respectively. These chemical shifts of carbon signals were assigned by comparison with the values in the literature (Nomura *et al.*, 1981). Consequently, the structure of compound 1 was concluded to be alnusodiol (Fig. 2).

Compound 2 with the molecular formula $C_{19}H_{20}O_4$ was established from EI-MS, ^{13}C-NMR and ^{1}H-NMR. The ^{13}C-NMR and DEFT spectra showed the signals of five methylenes, seven mechines, and seven quaternary carbons. In the ^{1}H-NMR spectrum, signals on the aromatic region indicated the coupling patterns originated from the two sets of 1, 3, 4-trisubstituted benzene rings: H-4/H-5 (J = 8.0), H-5/H-1 (J = 2.0), H-18 /H-19 (J = 8.0), and H-18/H-15 (J = 2.0), respectively (Li *et al.*, 2003). The correlations in the ^{1}H-^{1}H correlation spectroscopy (COSY) spectrum displayed connectivities between H-8a/H-8b, H-7, H-9, H-10a/H-9, H-10b/H-9, and H-1/H-5. The assigned proton and carbon chemical signals were compared with the values in the literature (Nomura *et al.*, 1981). Consequently, the structure of compound 2 was concluded to be alnusonol (Fig. 2).

Compound 3 was isolated as yellow powder and the EI-MS presented a signal at *m/z* 294 $[M]^+$. The ^{1}H-NMR spectrum of compound 3 shown a doublet (J = 15.5 Hz) at δ 6.53 suggested that the double bond conjugated to the ketone group is *trans*-distributed. In the HMBC spectrum, H-1, H-4, and H-10 were correlated with C-7, C-6, and C-8 and C-11, respectively. The assigned proton and carbon chemical signals were compared with the values in the literature (Nomura *et al.*, 1981). Based on these results and the values previously reported in the literature, this compound was identified as alnusone (Fig. 2).

The compound 4 was obtained as a yellow powder. The EI-MS presented a signal at *m/z* 170, corresponding to molecular formula $C_7H_6O_5$. In ^{1}H-NMR, one set of singlet at δ 7.06 (2H, *s*) which suggests the presence of a phenolic derivatives was assigned to H-2 and H-6. A ^{13}C-NMR signal at δ 170.3 was assigned to carboxyl carbon (C=O, C-7). The ^{13}C-NMR spectrum exhibited typical signals for a galloyl moiety at δ 146.3 (C-3 and C-5), δ 139.5 (C-4), δ 122.0 (C-1), and δ 110.36 (C-2 and C-6). The assigned carbon chemical shifts were compared with the values in the literature (Chevalley *et al.*, 1999 : Kim *et al.*, 1997). Consequently, the structure of compound 4 was concluded to be gallic acid (Fig. 2). Among these four compounds, compound 1, 2, and 3 are isolated from *A. firma* for the first time.

3.2. Antioxidant Activity

The antioxidant activities of four compounds obtained from the wood of *A. firma* were shown in Fig. 3. As shown in Fig. 3, the calculated values of the radical scavenging activity test on the compound 4 were 93.5 and 91.5% at the concentration of 100 and 10 ppm, respectively. Compound 1, 2, and 3 showed a moderate

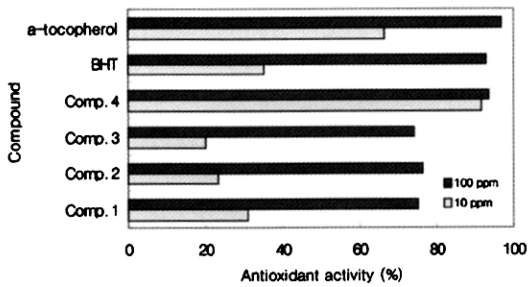


Fig. 3. Free radical scavenging activities of the isolated compounds from the wood of *A. firma*.

antioxidant activities (75.3, 76.5 and 74.3% at 100 ppm, respectively). Among four compounds, compound 4 exhibited the highest radical scavenging activity toward DPPH radical. According to the reports of Cooper-Drive *et al.* (1998), Park *et al.* (2003) and Park *et al.* (2004), the strength of antioxidant activities for the phenolic compounds depends on the number and position of hydroxyl groups.

Recently, natural antioxidants are receiving much attention, therefore, this study indicates that these isolated compounds may be useful for the treatment of oxidative damage and have potential possibility to be natural antioxidants.

4. CONCLUSIONS

From the wood of *A. firma*, four compounds were isolated by column chromatography using Sephadex LH-20 and/or silica gel and identified as follows: alnusodiol (1), alnusonol (2), alnusone (3), and gallic acid (4). Antioxidant activity of four compounds was evaluated by the DPPH free radical scavenging method. From the results, gallic acid showed the strongest antioxidant activity, while other compounds had the moderate antioxidant activity.

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