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Diarylheptanoid and Flavonoid with Antioxidant Activity from *Alnus japonica* Steud on DPPH Free Radical Scavenging Assay

- Research Note -

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Abstract

***Alnus japonica* Steud (Betulaceae) has long been used as a Korean traditional medicine for gastric disorders, hepatitis, and fatty liver. As a part of our study on the identification of secondary metabolites of naturally occurring bioactive compounds, we isolated 1,7-bis(4-hydroxyphenyl)-3,5-heptanediol (1), 5-hydroxy-1,7-bis(4-hydroxyphenyl)-3-heptanone (2), 5,3′-dihydroxy-7,4′-dimethoxyflavone (3) and 3,5,7,3′,4′-pentahydroxyflavone (4) from the dichloromethane and ethylacetate-soluble fractions of *Alnus japonica* Steud. These compounds showed significant antioxidant activity in a concentration-dependent manner. The IC_{50} values of compounds 1, 2, 3 and 4 were 30.1, 37.4, 20.2 and 13.7 μg/mL, respectively, through the scavenging capacity of 1,1-diphenyl-2-picrylhydrazyl radical assay.**

Key words: *Alnus japonica*, Betulaceae, diarylheptanoid, flavonoid, antioxidant activity, DPPH assay

INTRODUCTION

Plants are a proven source of numerous phytochemical agents and secondary metabolites, and it is reasonable to believe that plants contain additional bioactive compounds that remain undiscovered. Bioactive compounds can represent extra-nutritional constituents that are naturally present in small quantities within a food matrix. Development of high-quality plant varieties containing increased levels of bioactive compounds may increase the nutritional value of natural plant sources (1,2).

Alnus japonica Steud (Betulaceae), a fast growing deciduous tree that grows well in heavy clay soils, has long been used as a traditional medicine for gastric disorder, hepatitis and fatty liver in Korea. Recently, bioactive compounds with anti-oxidative activity and human low-density lipoprotein oxidation inhibition were reported from the leaves and fruits of *Alnus japonica* (3,4).

To evaluate the potential of *Alnus japonica* as a functional food with medicinal properties, diarylheptanoid and flavonoids, which have antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, were isolated from dichloromethane (IC_{50}= 38.1 μg/mL) and ethylacetate (IC_{50}=18.0 μg/mL) soluble fractions of methanolic extract of *Alnus japonica* Steud through an activity-monitored column chromatographic isolation method (5).

In this study, we used the DPPH radical, a stable free radical, for the evaluation of the radical-scavenging effects of compounds. Although the reactivity with the DPPH radical may or may not permit a clear-cut definition of their radical-scavenging abilities or antioxidant effects, examination of the scavenging effects on the DPPH radical described here has been useful for providing basic information on the scavenging ability of compounds and their structure-activity relationships (6). Identification of chemical structures was elucidated on the basis of 1D and 2D NMR experiments.

The present study concludes that bark of *Alnus japonica* Steud possesses potent antioxidant potential which may contribute to the prevention and/or delay of degenerative diseases by interfering with pathologies associated with oxidative stress.

MATERIALS AND METHODS

Plant material

Alnus japonica was collected in Korea, in 2003. A sample of the fully ground bark of *Alnus japonica* used in this study was deposited at the Lifetree Biotechnology Co., Ltd. (Suwon, Korea). The dried bark material was stored at ambient temperature and milled just prior to

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the present investigation.

Chemicals

DPPH was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The compounds were dissolved in acetone (Me_2CO-d_6) and dimethylsulfoxide (DMSO-d_6) and stored at -20°C. Other chemicals were purchased from commercial sources and were of the highest purity available.

Instrumental analyses

Melting points (mp) were determined using a Mitamura-Riken melting point apparatus and are uncorrected. The Ultraviolet (UV) and Infrared (IR) spectra were recorded on a Hitachi 3100 UV/Vis and JASCO Fourier transform (FT)-IR-5300 spectrophotometer, respectively. Electron impact mass spectrometry (EI-MS) spectra were obtained on a Hewlett Packard model 5985B gas chromatography (GC)/MS system. A Bruker AMX500 spectrometer was used to record nuclear magnetic resonance (NMR) spectra (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane (TMS), and Me_2CO-d_6 and DMSO-d_6 as an internal standard and NMR solvents, respectively.

General experiment

Thin-layer chromatographic (TLC) analysis was performed on silica gel (Kieselgel 60 F_{254} plates; 0.25 mm layer thickness; Merck, Darmstadt, Germany), with compounds visualized by spraying with 10% $FeCl_3$ in methanol (MeOH) after developing samples. Silica gel (Merck 60 A, 230~400 mesh ASTM) and Sephadex LH-20 (25~100 μm; Pharmacia Fine Chemicals, Piscataway, NJ, USA) were used for open column (3.0×42 cm, 1.5×28 cm) and vacuum column (1.0×15 cm) chromatographic separation.

Extraction and isolation of bioactive compounds

The dried ground bark of *Alnus japonica* Steud (2.0 kg) was extracted with MeOH three times for 3 hr in a hot water bath. The combined methanolic extract was partitioned between dichloromethane (CH_2Cl_2) and water, and with the more polar layer then partitioned with ethylacetate (EtOAc) and *n*-butanol. The fractions were assayed before separating additional chromatographic subfractions. Then, fractions with the desired activity were applied for the isolation of bioactive components. The elutes from the condensation yielded a solid material which was further purified by recrystallization with highly-purified MeOH to give the pure compounds of **1~4**.

DPPH free radical-scavenging activity assay

This assay is based on the capacity of a substance for scavenging stable DPPH free radicals. Reaction mixtures containing test samples (5mL, dissolved in DMSO) and DPPH ethanolic solution (95 mL, final DPPH concentration is 300 mM) in 96-well micro filter plates were incubated at 37°C for 30 min, and absorbances were measured at 515 nm. Percent inhibition by samples treatment was determined by comparison with a DMSO-treated control group. IC_{50} values denote the concentration of samples which is required to scavenge 50% of DPPH free radicals (7). On the basis of reaction conditions, and in order to confirm the usefulness of the assay, commercial antioxidants including ascorbic acid and 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) were also evaluated for their free radical-scavenging activities (Table 1).

Table 1. IC_{50} (μg/mL) of the isolated compounds from *Alnus japonica* Steud determined by DPPH free radical-scavenging assay

Compounds	DPPH[1)]
1	30.1
2	37.4
3	20.2
4	13.7
Ascorbic acid[2)]	22.4
BHA[2)]	20.6

[1)]DPPH free radical scavenging activity (IC_{50}: μg/mL).
[2)]Control compounds.

RESULTS AND DISCUSSION

Spectral data of compounds 1~4

1,7-Bis(4-hydroxyphenyl)-3,5-heptanediol **(1)** : White powder from MeOH; mp 156~158°C; UV λ_{max} (MeOH) (log ε): 265 (4.70), 323 (4.20); λ_{max} (MeOH+NaOH) (log ε) 278 (4.80), 368 (4.57) nm; IR (KBr) ν_{max} 3425 (OH), 1695, 1510 (aromatic C=C) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity %): 316 $[M]^+$ (34.1), 298 $[M-H_2O]^+$ (14.6), 280 $[M-2H_2O]^+$ (42.7); ^1H-NMR (Me_2CO-d_6, 500 MHz): δ 1.64~1.71 (2H, *m*, H-6), 2.47~2.76 (8H, *m*, H-1,2,4,7), 4.05 (2H, *t*, *J*=6.0 Hz, H-3,5), 6.49~6.53 (2H, *m*, H-6′,6″), 6.70~6.74 (6H, *m*, H-2′,2″, 3′,3″,5′,5″); ^{13}C-NMR (Me_2CO-d_6, 125 MHz): δ 26.7 (C-1), 31.2 (C-7), 39.6 (C-6), 45.0 (C-2), 50.5 (C-4), 67.1 (C-3), 67.6 (C-5), 114.8 (C-3′), 115.0 (C-3″), 115.3 (C-2′), 115.5 (C-2″,5″), 115.7 (C-5′), 119.8 (C-6′), 119.6 (C-6″), 133.0 (C-1′), 134.2 (C-1″), 143.3 (C-4′).

5-Hydroxy-1,7-bis(4-hydroxyphenyl)-3-heptanone **(2)** : White plates from MeOH; mp 140~141°C; UV λ_{max} (MeOH) (log ε): 260 (4.68), 320 (4.15) nm; λ_{max} (MeOH+NaOH) (log ε) IR (KBr) ν_{max} 3420 (OH), 1670, 1590 (aromatic C=C), 1276 (C-O) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity %): 314 $[M]^+$ (100.0), 296 $[M-H_2O]^+$

(24.0), 286 $[M-CO]^+$ (35.1), 278 $[M-2H_2O]^+$ (35.8); ^{1}H-NMR (Me_2CO-d_6, 500 MHz): δ 1.64~1.71 (2H, *m*, H-6), 2.47~2.76 (8H, *m*, H-1,2,4,7), 4.05 (1H, *t*, *J*=6.0 Hz, H-5), 6.49~6.53 (2H, *m*, H-6′,6″), 6.70~6.74 (6H, *m*, H-2′,2″,3′,3″,5′,5″); ^{13}C-NMR (Me_2CO-d_6, 125 MHz): δ 28.7 (C-1), 31.0 (C-7), 39.3 (C-6), 45.1 (C-2), 50.0 (C-4), 67.0 (C-5), 115.0 (C-3′), 115.1 (C-3″), 115.3 (C-2′), 115.4 (C-2″), 115.5 (C-5″), 115.7 (C-5′), 119.5 (C-6′), 119.6 (C-6″), 133.0 (C-1′), 134.0 (C-1″), 143.1 (C-4′), 143.3 (C-4′), 210.7 (C-3).

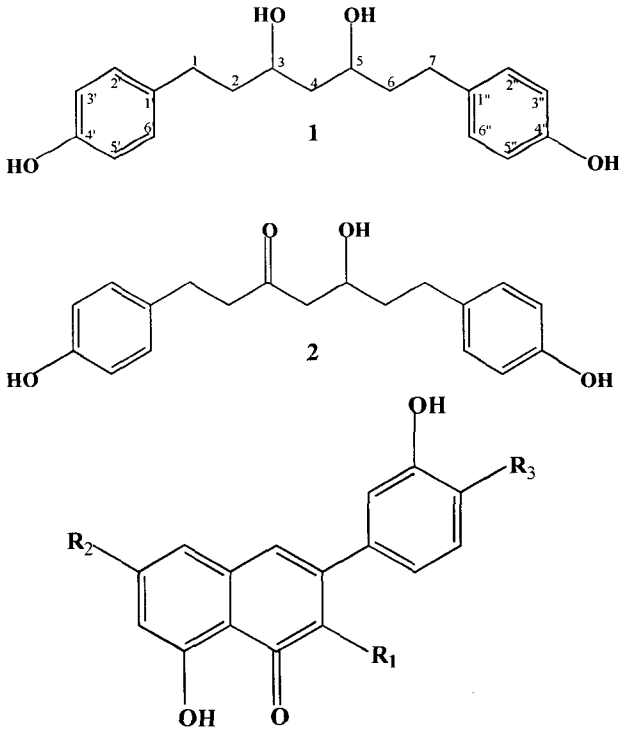
5,3′-Dihydroxy-7,4′-dimethoxyflavone **(3)** : Yellow crystal from MeOH; mp 230~232°C (dec.); UV λ_{max} (MeOH) (log ε): 265 (4.70), 323 (sh, 4.55), 368 (4.69) nm; λ_{max} (MeOH+NaOH) (log ε): 280 (4.82), 315 (sh, 4.67), 420 (4.80) nm; IR (KBr) ν_{max} 3425 (OH), 1655 (α,β-unsaturated C=O), 1610, 1510 (aromatic C=C), 1256 (aromatic C-O) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity %): 315 $[M+1]^+$ (32.0), 314 $[M]^+$ (100.0), 286 $[M-CO]^+$ (32.5); ^{1}H NMR and ^{13}C NMR data were consistent with those in the literature (8).

3,5,7,3′,4′-Pentahydroxyflavone **(4)** : Yellow crystal from MeOH; mp 313~314°C (dec.); UV λ_{max} (MeOH) (log ε): 260 (4.67), 325 (sh, 4.53), 360 (4.76) nm; λ_{max} (MeOH+NaOH) (log ε): 282 (4.85), 318 (sh, 4.69), 418 (4.83) nm; IR (KBr) ν_{max} 3420 (OH), 1645 (α,β-unsaturated C=O), 1610, 1511 (aromatic C=C), 1258 (aromatic C-O) cm^{-1}; EI-MS (70 eV) *m/z* (relative intensity %): 303 $[M+1]^+$ (14.7), 302 $[M]^+$ (100.0), 274 $[M-CO]^+$ (12.9); ^{1}H NMR and ^{13}C NMR data were consistent with those in the literature (9).

Isolation and structure elucidation of compounds 1~4

Methanol extract of *Alnus japonica* cortex powder was evaluated initially for antioxidant activity at a final concentration of 100 μg/mL using the DPPH free radical-scavenging test system. The preliminary distribution pattern of antioxidant activity was categorized as active (>80% inhibition), moderately active (50~80% inhibition), inactive (<50% inhibition). For the active solvent-soluble fractions showing over 80% inhibition in this primary screen, isolation and chemical structure determination of compounds were determined. The CH_2Cl_2 and EtOAc-soluble fractions of *Alnus japonica* Steud bark exhibited significant antioxidant activity with IC_{50} values of 38.1 μg/mL and 18.0 μg/mL, respectively, in the DPPH free radical-scavenging assay. These fractions were then chromatographed over a silica gel and Sephadex LH-20 column using a $CHCl_3$-MeOH gradient and MeOH to isolate pure compounds which were monitored by TLC patterns revealed by a UV lamp and 10% $FeCl_3$-MeOH spray. Complete identification of the isolated compounds made use of various physical and chemical methods, including EI-MS spectrometry, UV/Vis, IR and ^{1}H-NMR and ^{13}C-NMR spectroscopy. The structures of the compounds (Fig. 1) were identified by comparing spectra with published data (8-12). The isolated compounds were determined as 1,7-bis(4-hydroxyphenyl)-3,5-heptanediol (**1**), 5-hydroxy-1,7-bis(4-hydroxyphenyl)-3-heptanone (**2**), 5,3′-dihydroxy-7,4′-dimethoxyflavone (**3**), and 3,5,7,3′,4′-pentahydroxyflavone (**4**).

Compound **1** was formulated as $C_{19}H_{24}O_4$ and obtained as a white powder, with a molecular weight of *m/z* 316 based on EI-MS data, and exhibited UV absorption bands at 265 and 323 nm, which were shifted up-field by the addition of an alkali solution. The ^{1}H-NMR spectrum of **1** showed the presence of five methylenes over δ 1.64~2.76 and two parts of 1,4-two substituted aromatic rings over δ 6.49~6.74. The ^{13}C-NMR spectrum of **1** showed five methylenes, two oxygen-bearing methines and two 4-hydroxyphenyl groups. Assignments of each proton and carbon were confirmed by HMQC and HMBC spectra. Thus, the structure of **1** was identified as 1,7-bis(4-hydroxyphenyl)-3,5-heptanediol.



3 : R_1=H; R_2=OCH_3; R_3=OCH_3
4 : R_1=OH; R_2=OH; R_3=OH

Fig. 1. Chemical structure of compounds 1~4 from *Alnus japonica* Steud.
1: 1,7-Bis(4-hydroxyphenyl)-3,5-heptanediol.
2: 5-Hydroxy-1,7-bis(4-hydroxyphenyl)-3-heptanone.
3: 5,3′-Dihydroxy-7,4′-dimethoxyflavone.
4: 3,5,7,3′,4′-Pentahydroxyflavone.

Compound **2** had a greenish blue coloration with spraying of $FeCl_3$ on a silica TLC plate. Compound **2** was formulated as $C_{19}H_{22}O_4$ and obtained as white plates, with a molecular weight of *m/z* 314, and *m/z* 296 and *m/z* 278, corresponding to loss of one and two water molecules from the parent ion peak. The ^{1}H-NMR and ^{13}C-NMR spectra of **2** were very similar to those of compound **1** except the presence of carbonyl group at 1276 cm^{-1} in its IR spectrum, instead of a hydroxyl group. The ^{1}H-NMR spectrum of **2** also showed the presence of five methylenes over δ 1.64~2.76 and two parts of 1,4-two substituted aromatic rings over δ 6.49~6.74. The ^{13}C-NMR spectrum of **2** showed five methylenes, one oxygen-bearing methane, two 4-hydroxyphenyl groups and one carbonyl signals. Assignments of each proton and carbon were confirmed by HMQC and HMBC spectra. Thus, the structure of compound **2** was identified as 5-hydroxy-1,7-bis(4-hydroxyphenyl)-3-heptanone. These observations showed that the hydroxyl group was attached at the C-5 location. To date, diarylheptanoids from plants were isolated through activity-guided isolation methods and identified as having anti-inflammatory, cytotoxic, anti-leishmanial and chemopreventive potentials (13-17).

Compounds **3** and **4** gave characteristic flavonoid color reactions (purplish brown with $FeCl_3$, yellow with NaOH, yellowish orange with Mg-HCl, pink with Zn-HCl). The UV, EI-MS, ^{1}H NMR and ^{13}C NMR spectral data of the aromatic parts of the compounds were characterized by major bands that resembled those of the flavonoid skeleton. The structures of compounds **3** and **4** were identified by comparing instrumental spectra with published data (8,9). The isolated antioxidant compounds from *Alnus japonica* Steud were identified as 5,3′-dihydroxy-7,4′-dimethoxyflavone (pilloin) (**3**) and 3,5,7,3′,4′-pentahydroxyflavone (quercetin) (**4**). Quercetin had a significant preventive effect on benzo[α]pyrene-induced DNA damage, and had a potential chemopreventive effect on the carcinogenesis of lung cancer induced by benzo[α]pyrene (18).

Scavenging effects of compounds on DPPH free radical assay

The DPPH-scavenging effects of compounds **1**~**4** are presented in Table 1. The concentrations of compounds required for 50% radical scavenging was shown to be in the range of 13.7 to 37.4 μg/mL.

There were significant differences between the scavenging abilities of compounds **1** and **2**, and between compounds **3** and **4**. It was dependent on their structures and the number of hydroxyl group. Compounds **1** and **4** were more effective radical scavengers than compounds **2** and **3**. It is known that many flavonoids are effective antioxidants (19,20). It is also well recognized that the structural features of flavonoids important for radical-scavenging or antioxidant function are the *ortho*-dihydroxyl moiety in the B ring, 2,3-double bond in conjugation with the carbonyl group at C-4 position; and the additional presence of both 3-hydroxyl and 5-hydroxyl groups (21,22).

Free radicals, species with one or more unpaired electrons, are produced in normal or pathological cellular metabolism, from xenobiotics, or through ionizing radiation. Electron acceptors such as molecular oxygen react easily with free radicals, to become radicals themselves (reactive oxygen species; ROS). Oxygen free radicals or reactive oxygen species such as superoxide anion radicals, hydrogen peroxide, hydroxy radicals and singlet oxygen are continuously generated in cells exposed to an aerobic environment, and have been associated with the genesis of tumors, as well as age dependent diseases such as atherosclerosis, arthritis, and neurodegenerative disorders (23-25). It is possible that various diseases associated with aging populations can be reversed by removing avoidable sources of ROS and/or by enhancing antioxidant defense system (26).

The present study concludes that *Alnus japonica* Steud possesses potent antioxidant components which may contribute to the prevention and/or delay of chronic diseases by interfering with disease processes associated with oxidative stress.

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