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Identification of Afzelin and Quercitrin from *Pinus koraiensis* and Their Contents in Genus *Pinus* Using HPLC/UV Analysis

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Abstract – Phytochemical constituents were isolated from *Pinus koraiensis* needles by open column chromatography. The structures isolated from the ethyl acetate fraction of *P. koraiensis* needles were elucidated as afzelin (**1**) and quercitrin (**2**) on the basis of spectroscopic analyses including ^{1}H-NMR and MS. The amounts of afzelin (**1**) and quercitrin (**2**) in genus *Pinus* were determined by HPLC/UV analysis. Afzelin (**1**) and quercitrin (**2**) contents were highest in *P. thunbergii* needles. Because *P. thunbergii* needles contain high amounts of afzelin (**1**) and quercitrin (**2**), they have promising potential as new additives to natural medicinal products, health supplements, and beverages.
Keywords – Afzelin, Analysis, Flavonoid, HPLC, *Pinus koraiensis*, Quercitrin

Introduction

Pinus koraiensis is found in the Amor and maritime provinces of Russia, throughout the mountains of Korea, on Changbai Mountain of China, and on the Japanese islands of Honshu and Shikoku.[1-3] *P. koraiensis* is planted on roadsides in cities to provide shade and is used to rehabilitate degraded regions in Korea. *P. koraiensis* is a hardy ornamental tree, although it is slow growing. It begins producing seed after 15 years, but abundant seed crops are not produced for 30 or more years. In addition to the high economic value of its seeds, wood from this tree has long been used for fine quality furniture in northeastern Asia.[4] *P. koraiensis* is one of the most important sources for timber and for use as a traditional medicinal plant.[5]

Genus *Pinus* have been reported to possess anti-fatigue, anti-aging, and anti-inflammatory properties.[6] Genus *Pinus* contains various pharmaceutical components such as 5-hydroxy-7-methoxyflavone, chrysin, pinocembrin, galangin, 3-hydroxy-5-methoxystilbene, pinosylvin, gallic acid, protocatechuic acid, vanillic acid, syringic acid, *p*-coumaric acid, scopoletin, and (+)-catechin.[7,8] *P. koraiensis* seeds are a nutritional food rich in phenolic compounds. The anti-oxidant activity of phenolic compounds has been widely accepted by the scientific community and is believed to prevent diseases.[9,10]

Drugs derived from plants are major importance to the health of human-being. Although many of these useful drugs have been synthesized, very few are produced commercially by synthesis. The value of genus *Pinus* is being rediscovered as a new approach to material resources. With this proposition, the major objective of this paper is to develop health food containing genus *Pinus* by analysis of phytochemical constituents from *P. koraiensis*. And optimum HPLC/UV analysis conditions were developed to analyze the most relevant phytochemical constituents in genus *Pinus*.

Experimental

Plant materials – *Pinus koraiensis* needles were collected in Pocheon, Korea in March, 2012. A voucher specimen was deposited at the Herbarium of the Department of Integrative Plant Science, Chung-Ang University, Korea. Other genus *Pinus*, including *P. banksiana*, *P. densiflora*, *P. densiflora* for. *multicaulis*, *P. koraiensis*, *P. parviflora, P. rigida*, *P. strobus,* and *P. thunbergii*, were purchased from the Plant Extract Bank of KRIBB in Deajeon, Korea.

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Instruments and reagents – Ethanol (EtOH), methanol (MeOH), *n*-hexane, chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) (SamChun Pure Chemical Co., Pyeongtaek, Korea) were used as the mobile phase in medium-pressure liquid chromatography (MPLC) (Biotage, Uppsala, Sweden). DMSO (Sigma-Aldrich, St. Louis, MO) was used as the NMR solution. Electron ionization-mass spectrometry (EI-MS) was conducted with a Jeol JMS-600W (Tokyo, Japan) mass spectrometer. ^{1}H-nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AVANCE 600 NMR (Bremen, Germany) spectrometer using TMS as the internal standard. Chemical shifts are reported in parts per million (δ), and coupling constants (*J*) are expressed in Hertz. An Eyela rotary evaporator system (Tokyo, Japan) under reflux *in vacuo* was used for evaporation. Thin-layer chromatography was conducted with Kiesel gel 60 F_{254} (Art. 5715, Merck Co., Darmstadt, Germany) plates (silica gel, 0.25 mm layer thickness), and compounds were visualized by spraying with 10% H_2SO_4 in MeOH, followed by heating to 100 °C. Column chromatography was conducted with a Sephadex LH-20 column (bead size: 25 - 100 μ; Sigma-Aldrich Co., USA). MPLC was equipped with cartridges (KP-SIL, 39 × 225 mm). HPLC chromatograms were recorded with a Waters Breeze system (MA, USA) equipped with a Waters 1525 binary HPLC pump and 2489 system UV/VIS detector. The water and MeOH used in this research were of HPLC grade, and all other reagents were of analytical grade.

Extraction and isolation – *P. koraiensis* needles (2 kg) were dried, finely powdered, and then extracted with 70% EtOH for 3 h (6 L × 5) under reflux at 65 - 75 °C. After filtration and removal of solvent *in vacuo*, the EtOH extract (70.8 g) was collected. This extract was suspended in distilled water and successively partitioned with *n*-hexane (11.9 g), CH_2Cl_2 (8.7 g), EtOAc (8.5 g), and *n*-BuOH (16.4 g). A portion of the EtOAc fraction (2 g) was used for MPLC with a stepwise gradient of $CHCl_3$/MeOH solvent systems to yield eight subfractions. Subfractions 4 - 6 were repeatedly chromatographed on a Sephadex LH-20 column eluted with MeOH/water (1 : 3 → 1 : 0) in order to yield compounds **1** (5.3 mg) and **2** (6.1 mg).

Compound **1**: Brown powder; $C_{21}H_{20}O_{10}$; EI-MS *m/z*: 432 $[M]^+$ (21.5), 404 (5.3), 389 (7.1), 302 (13.6), 286 (100.0), 258 (8.8), 147 (16.7); ^{1}H-NMR (600 MHz, DMSO-d_6): Table 1

Compound **2**: Yellow powder; $C_{21}H_{20}O_{11}$; EI-MS *m/z*: 432 $[M-O]^+$ (11.5), 302 (100.0), 286 (70.1), 257 (8.9), 147 (19.8); ^{1}H-NMR (600 MHz, DMSO-d_6): Table 1

Sample preparation – To analyze of compounds **1** and **2** from the needles and stems of genus *Pinus* (*P. banksiana*, *P. densiflora*, *P. densiflora* for. *multicaulis*, *P. koraiensis*, *P. parviflora, P. rigida*, *P. strobus,* and *P. thunbergii*), the residue was dissolved in 1 ml of MeOH and then sonicated for 10 min. The resultant solution was filtered with a Whatman 0.2 μm nylon syringe filter. The resulting solution was used for HPLC analysis.

Table 1. ^{1}H-NMR spectral data for compounds **1** and **2**

No.	**1**	**2**
6	6.18 br d	6.20 d (*J* = 1.9 Hz)
8	6.40 br d	6.42 d (*J* = 1.9 Hz)
2'	7.97 d (*J* = 7.2 Hz)	7.53 br d
3'	6.89 d (*J* = 7.2 Hz)	–
5'	6.89 d (*J* = 7.2 Hz)	6.88 d (*J* = 7.3 Hz)
6'	7.97 d (*J* = 7.2 Hz)	7.49 br d
1"	5.57 br d	5.52 br d
2" - 5"	4.63 - 3.54	4.23 - 3.66
6"	1.20 br d	1.20 br d
5-OH	12.63 s	12.65 s

Chemical shifts are reported in parts per million (δ), and coupling constants (*J*) are expressed in Hertz.

HPLC condition – A Discovery® C18 (4.6 × 250 mm, 5 μm) column was used for simultaneous determination of compounds **1** and **2**. The mobile phase was 0.2% acetic acid in water (solvent A) and MeOH (solvent B). The gradient solvent system was solvents A and B (60 : 40), and increased in linear gradients to solvents A and B (40 : 60) for 40 min, then to solvents A and B (0 : 100) for 5 min, and finally to solvents A and B (60 : 40) for 20 min. The injection volume was 10 μl and flow rate was 1 ml/min. The UV spectra were recorded at 330 nm for quantification of flavonoids. All injections were performed in triplicate.

Limit of detection (LOD) and limit of quantification (LOQ) – Validation of the HPLC method for compounds **1** and **2** as standard compounds was determined by LOD and LOQ. Method linearity was established by triplicate injections in the range of 0.125 - 10.0 mg/ml. Seven calibration solutions were injected in triplicate, and five replicate analyses of the calibration solutions were performed. Calibration curves were constructed by linear regression of the peak area-ratios (Y) of compounds **1** and **2** versus concentration (X) in mg/ml. The relative standard deviation was used as a measure of repeatability. The percent recoveries were evaluated by calculating the ratio of amount detected versus amount added. LOD and LOQ values were determined separately at signal to noise ratios (S/N) of 3 and 10, respectively.

Calibration curve – Stock solutions (1 mg/ml) of compounds **1** and **2** were prepared in MeOH, and the solution content was successively reduced to 50% in

order to create different concentrations. Analyte contents were determined from the corresponding calibration curves. The calibration functions of compounds **1** and **2** were calculated using peak area (Y), concentration (X, μg/10 μl), and mean value (n = 5) ± standard deviation.

Results and Discussion

Confirmation of the chemical structures of compounds **1** and **2** was carried out by a combination of ^{1}H-NMR and EI-MS. Typical flavonoid signals were observed in the ^{1}H-NMR spectra of compounds **1** and **2**. Singlet signals at δ 12.63 - 12.64 indicate the presence of 5-OH in flavonoids. The ^{1}H-NMR spectrum of compound **1** indicate a flavonoid with A_2B_2 splitting at δ 7.97 (2H, d, J = 9.0 Hz) and 6.89 (2H, d, J = 9.0 Hz) in the B-ring. The existence of H-6 and -8 were confirmed by two broad singlet signals (δ 6.18 and 6.40, respectively). The ^{1}H-NMR spectrum also supported that this skeleton has a rhamnoside on the basis of distinguishing peaks of rhamnoside (δ 1.20 and 5.57). The ^{1}H-NMR spectrum of compound **2** indicate a flavonoid with ABX splitting at δ 6.88 (1H, d, J = 7.3 Hz), 7.49 (1H, br d), and 7.53 (1H, br d) in the B-ring. The existence of H-6 and -8 were confirmed by two broad singlet signals (δ 6.20 and 6.42, respectively). The ^{1}H-NMR spectrum also supported that this skeleton has a rhamnoside on the basis of distinguishing peaks of rhamnoside (δ 1.20 and 5.52). Based on the obtained spectroscopic data, the purified compounds were identified as afzelin (**1**) and quercitrin (**2**).[11-18] The structures of afzelin (**1**) and quercitrin (**2**) are shown in Fig. 1. Greater diversity in flavonol structures was found in genus *Pinus.*[19] Afzelin (**1**) and quercitrin (**2**) have previously been isolated from various parts of *Rhododendron* plants.[20,21] Afzelin (**1**) have been shown to exhibit high aldose reductase inhibitory activity.[22] Quercitrin (**2**) have been shown to exert intestinal anti-inflammatory effects in experimental models of rat colitis[23,24] and releases quercetin to implement its anti-inflammatory effect by inhibiting the NF-κB pathway.[25]

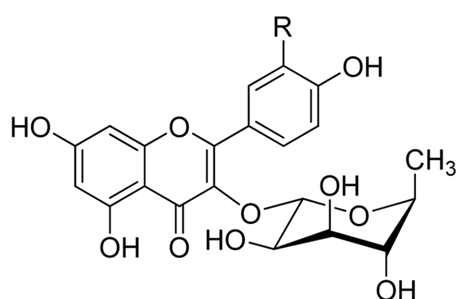


Fig. 1. Structures of compounds **1** and **2**.

HPLC separation of afzelin (**1**) and quercitrin (**2**) was conducted for qualitative and quantitative analyses using a reverse phase system with a mobile phase consisting of 0.2% acetic acid in water and MeOH. The standard calibration curves for afzelin (**1**) and quercitrin (**2**) are shown in Table 2. Using optimized analytical methods, the amounts of afzelin (**1**) and quercitrin (**2**) in genus *Pinus* were simultaneously determined (Fig. 2). The contents of afzelin (**1**) and quercitrin (**2**) were highest in *P. thunbergii* needles (108.456 and 1.380 μg/mg ext., respectively) and *P. rigida* stems (0.062 and 0.206 μg/mg ext., respectively).

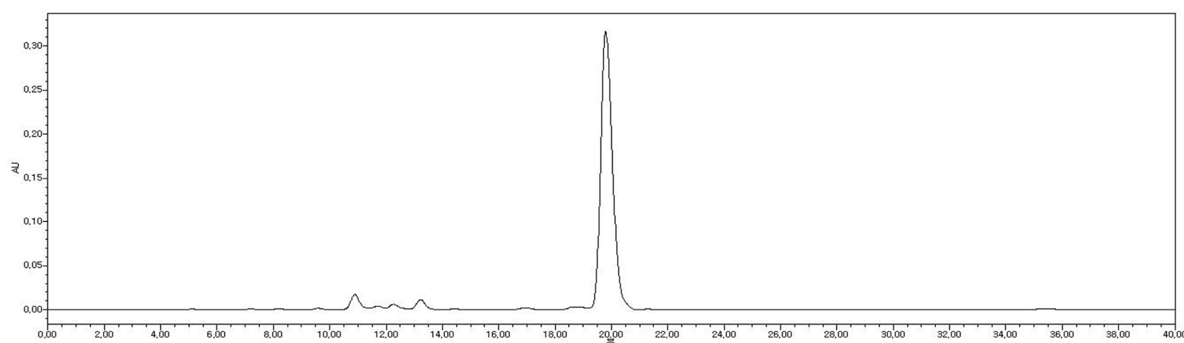
The LOD and LOQ under the present chromatographic conditions were determined at signal to-noise ratios (S/N) of 3 and 10, respectively. The LOD and LOQ of afzelin (**1**) and quercitrin (**2**) were 0.141 - 0.471 and 0.122 - 0.364 mg/ml, respectively (Table 3).

These results demonstrate that *P. thunbergii* contains large amounts of afzelin (**1**) and quercitrin (**2**) and has promising potential as a new additive for natural medicinal products, health supplements, and beverages. Afzelin (**1**) and quercitrin (**2**) are potential efficacy or index components for data analysis. KFDA approval will soon be conducted for this procedure.

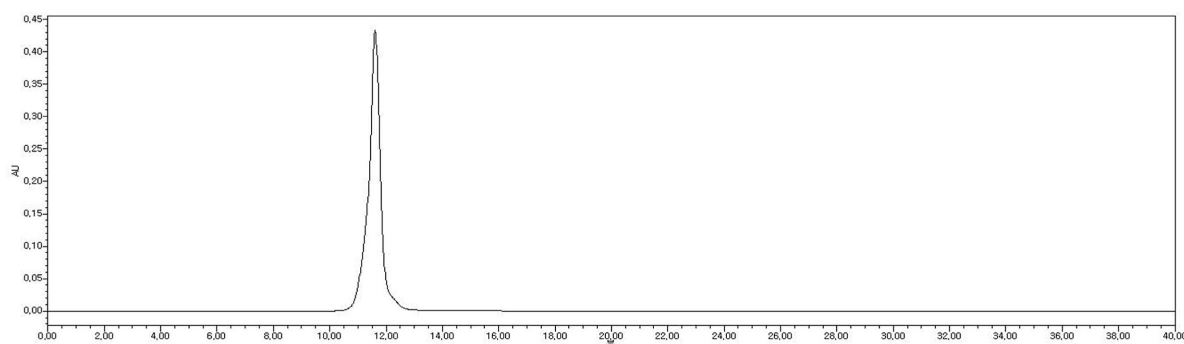
Table 2. Calibration curves of compounds **1** and **2**

Compound	Linear range (μg/ml)	Linear regression equation Y = aX + b		Correlation coefficient (r^2)
		Slope (a)	Intercept (b)	
1	0.5 - 15	936.06	0.3994	1
2	0.5 - 15	1280.5	0.4851	1

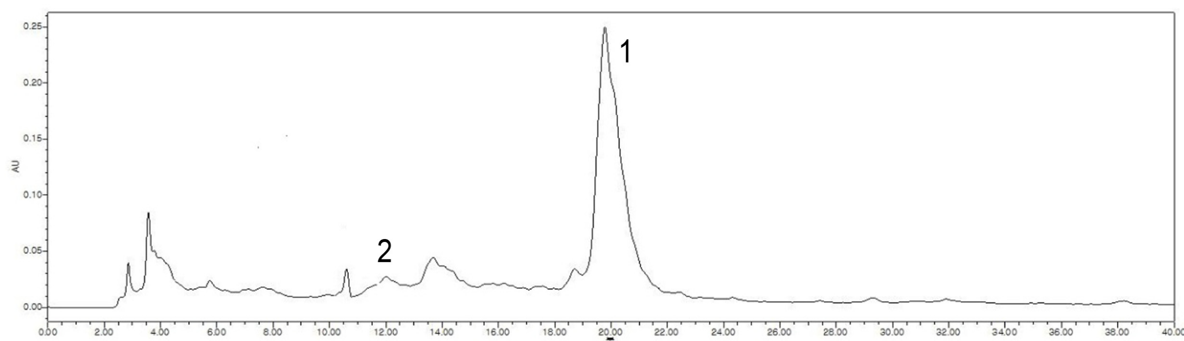
Y = Peak area, X = Concentration of standard (mg/ml)
r^2 = Correlation coefficient for three data points from calibration curve



A



B



C

Fig. 2. HPLC chromatograms of compounds **1** (A, 19.6 min) and **2** (B, 11.6 min), and the MeOH extract of *P. thunbergii* needles (C).

Table 3. LOD and LOQ values of compounds **1** and **2**

Compound	Regression equation	r^2	Linear range (mg/ml)	LOD (mg/ml)	LOQ (mg/ml)
1	Y = 351.04X – 0.2337	0.9996	0.015-0.5	0.141	0.471
2	Y = 439.32X – 3.8365	0.9985	0.015-0.5	0.122	0.364

Y = Peak area, X = Concentration of standard (mg/ml)
r^2 = Correlation coefficient for three data points from calibration curve

Table 4. Contents of compounds **1** and **2** in the MeOH extracts of genus *Pinus* needles and stems

Sample	Content (μg/mg ext.)			
	Needles		Stems	
	1	**2**	**1**	**2**
P. banksiana	8.763 ± 0.008	0.0535 ± 0.001	–	–
P. densiflora	27.197 ± 0.120	0.4398 ± 0.001	–	–
P. densiflora for. *multicaulis*	0.041 ± 0.001	0.0615 ± 0.001	–	–
P. koraiensis	31.724 ± 0.064	0.4929 ± 0.001	–	–
P. parviflora	22.205 ± 0.087	0.0881 ± 0.001	–	–
P. rigida	14.047 ± 0.021	0.6669 ± 0.001	0.062 ± 0.001	0.206 ± 0.001
P. strobus	20.327 ± 0.012	0.4551 ± 0.001	–	–
P. thunbergii	108.456 ± 0.146	1.3796 ± 0.001	–	–

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