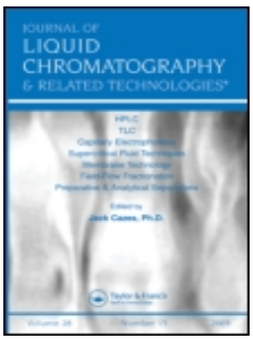

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Preparative Isolation of Polar Antioxidant Constituents from *Abies koreana* Using Centrifugal Partition Chromatography Guided by DPPH•-HPLC Experiment

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Preparative separation of antioxidant constituents from the leaves of *Abies koreana* Wilson (Pinaceae) was performed by centrifugal partition chromatography (CPC) with a two-phase solvent system of ethyl acetate-isopropanol-water (9:1:10, *v/v*) target-guided by DPPH•-HPLC experiment. In DPPH•-HPLC experiment, *trans*-piceid exerted antioxidant activity, which was purified by Diaion-HP 20 macroporous resin chromatography and subsequent CPC. Maltol (98.3 mg), dihydrokaempferol 7-*O*-*β*-D-glucopyranoside (230.4 mg), and *trans*-piceid (165.9 mg) were purified from 2.19 g of crude extract with the purity over 95% by HPLC analysis. The chemical structures of isolated compounds were determined by ^{1}H, ^{13}C NMR, and ESI-MS.

Keywords: *Abies koreana* Wilson, centrifugal partition chromatography, DPPH•-HPLC experiment, dihydrokaempferol 7-O-*β*-D-glucopyranoside, maltol, *trans*-piceid

Introduction

Abies koreana Wilson (Pinaceae) is an evergreen conifer tree that indigenously inhabits the high mountains in Korea. *A. koreana* showed various biological activities such as a cytotoxic effect,[1,2] an anti-acne effect caused by drug-resistant skin pathogen,[3] memory-enhancing effects on a scopolamine induced amnesia mouse model,[4] and an inhibitory effect on nitric oxide against lipopolysaccharide induced in BV-2 microglia cells.[5]

The phytochemical studies on *A. koreana* have isolated and reported a number of mono-, sesqui-, and triterpenoids; lignans; and flavonoids.[1–6] However, only a few biological activities of secondary metabolites from *A. koreana* were performed.

Studies on biological activities often require a certain amount of purified chemicals. Hence, economical and effective techniques for the isolation of phytochemicals were required. To that purpose, DPPH•-HPLC experiment guided antioxidant isolation methods by centrifugal partition chromatography were developed to obtain purified active phytochemicals.[7,8] CPC is a hydrostatic solid support free, liquid–liquid chromatography technique. This solid free sorbent system allows benefits such as no-peak tailing, irreparable sample absorption, sample loss, or variety of biphasic solvent systems.[9,10]

In this article, CPC method guided by a DPPH•-HPLC experiment was developed to purify antioxidant compounds from *A. koreana*. Two-phase solvent systems, composed of ethyl acetate–isopropanol–water, were evaluated as compounds in *A. koreana* and were polar. The CPC operation with solvent system of ethyl acetate–sopropanol–water (9:1:10, *v/v/v*) led to the successful isolation of maltol (98.3 mg), dihydrokaempferol 7-*O*-*β*-D-glucopyranoside (230.4 mg), and *trans*-piceid (165.9 mg) from 2.19 g of crude extract with a purity over 95% by HPLC analysis.

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Experimental

Apparatus and Reagents

A SCPC-100 + 1000 (Armen instrument, France) apparatus and a Spot prep II HPLC instrument (an automated HPLC system: an injector, a pump, a UV/Vis detector, a fraction collector, a digital screen, Armen Instrument, France) were combined to form a CPC system. In this study, 1000 mL rotor was applied. HPLC analyses were carried out using an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) controlled by Chemstation software. It was equipped with a G1322A 1260 Degasser, a G1312C 1260 Binary Pump, a G1329B autosampler, a G1316A column oven, and a G1315D DAD detector. Structural assignments were based on a Bruker model digital AVANCE III 400 (Bruker, Germany) with an Advion Expression CMS system (New York, NY, USA). The optical rotation were measured on a Jasco-P-2000 polarimeter at the sodium D line (589 nm), at 25°C using a 50 mm path length cell. HPLC grade acetonitrile and water and analytical grade organic solvents were purchased from Daejung Chemical (Gyonggi-do, Korea).

Preparation of A. koreana *Sample for CPC*

The leaves and stems of *A. koreana* Wilson were collected from a Hanyang University Herbarium (Hanyang University, Ansan, Gyeonggi-do 426-791, Republic of Korea). Voucher specimen was deposited in the laboratory. Dried leaves and stems (3.9 kg) were extracted three times with 7.5 L of methanol for 90 min under ultra-sonication. The solution was filtered and evaporated by rotary evaporation *in vacuo*, and then, freeze-dried to obtain 564 g residue (yield, 14.4%). Afterward, a 20 g of methanolic extract was suspended in 1-L distilled water and was subjected to Diaion HP-20 column chromatography (Mitsubishi, Japan) to afford four fractions. Subsequently, the column was eluted with 2-L deionized water, 10% ethanol in water, 50% ethanol in water, and 100% ethanol. These four fractions, A–Ds (8.26 g, 1.03 g, 4.27 g, and 3.01 g, respectively) were concentrated by a rotary evaporator and stored in refrigerator (−20°C)

DPPH•-HPLC Experiment

Fraction C (50% ethanol fraction) was dissolved in methanol at a concentration of 10 mg/mL. Five hundred μL of fraction C solution reacted with same volume of DPPH solution (500 μL, 3.8 mg/mL in methanol), and, then, the mixture was incubated at 37°C in a water bath for 30 min and passed through a 0.45-μm filter prior to HPLC analysis. And, diluted fraction C solution with methanol (5 mg/mL) was used as a control. These two solutions were analyzed by an Agilent 1260 HPLC system with an INNO C18 column (4.6 × 250 mm, 5 μm, YoungJin Biochrom, Korea). The mobile phase consisted of acetonitrile (1% formic acid, solvent A) and water (1% formic acid, solvent B) in a gradient mode: 0 min–2 min, 10% A; 2 min–17 min, 10–60% A; 17.01 min–25 min, 100% A. The flow rate was 1 mL/min, whereas the injection volume was 10 μL. The DAD detector measured UV spectrum over a range of 210 to 400 nm and the chromatogram of the effluents was recorded at 280 nm.

Selection of Solvent System

Using analytical HPLC, the *K* values of targeted peaks were determined. Several compositions of the two-phase solvent systems consisting of *n*-hexane–ethyl acetate–isopropanol–water or ethyl acetate–isopropanol–water were tested on the bases of the partition coefficient of the target compounds. Concisely, 15–20 mg of fraction C was transferred to a 2-mL tube and percolated with 1.8 mL of respective phase (1:1, *v/v*) of two-phased immiscible solvent systems. The tube was then shaken, followed by thorough equilibration with centrifuge. After one minute centrifugation, the equal volume of the upper and lower phases were analyzed separately by analytical HPLC to examine the applicability of the two-phase solvent system. The *K* values of target compounds were calculated by following equation:

$$K = \text{the peak areas of upper phase in HPLC} / \text{the peak areas of lower phase in HPLC}$$

CPC Separation Procedure

The CPC separation was performed with a two-phase solvent system composed of ethyl acetate–isopropanol–water with a volume ratio 9:1:10 and operated as follows: upper and lower phase solvents were prepared when the aforementioned solvents were torrentially mixed and completely equilibrated. The 1000-mL volume of CPC rotor was filled with upper organic phase as stationary phase at a flow rate of 50 mL/min in ascending mode at a speed of 800 rpm rotation. Then, the rotation speed of rotor was accelerated to 1200 rpm, and the lower aqueous phase as the mobile phase was carried into the rotor in descending mode at a 10 mL/min flow rate. When the CPC rotor reached hydrostatic equilibrium, indicated by the outlet exuding a clear mobile phase (300 mL of stationary phase out of 1000 mL rotor volume, 38 bar), the sample solution (2.19 g of *A. koreana* extract dissolved in 12 mL of mixed upper and lower phase) was subjected to the Armen CPC system. The effluent from the outlet of the CPC rotor was continuously monitored at the wavelength of 280 nm and scan mode (230–400 nm). Each peak fraction was collected and separated on the bases of the elution profile to afford three CPC peak fractions **I–III**. The fractions were concentrated and analyzed by analytical HPLC.

HPLC Analysis and Identification of the CPC Peak Fractions

The crude extract Diaion HP-20, separated fractions A–D, and CPC purified peak fractions **I–III** were analyzed by HPLC. HPLC conditions are as previously mentioned. Identification of the CPC purified compounds were carried out by ESI-MS and NMR. The ESI-MS spectra conditions were as follows: positive and negative ion mode; mass range, *m/z* 100–1200; capillary temperature, 200°C; capillary voltage, 150 V; source voltage offset, 30; Source voltage span, 10; Source gas temperature, 150°C; ESI voltage, 3,500 V. ^{1}H (400 MHz), ^{13}C (100 MHz) together with 2D NMR including ^{1}H-^{1}H COSY, HSQC, and HMBC were measured on a Bruker model digital AVANCE III 400 NMR. The NMR spectrum were processed by the MestReNova software (Mestrelab Research, Santiago de Compostela, Spain).

Results and Discussion

Fractionation with Diaion HP-20 Resin

Diaion HP-20 column chromatography was mainly used to isolate the phenolic compounds from natural products.[11] Considering the major compounds in *A. koreana*, including flavonoids and phenolic compounds, the removal of nonpolar impurity such as chlorophylls and lipids are required during the column chromatography. The HPLC analysis of *A. koreana* crude extracts indicated from the UV spectrum in each peak before 5 min or after 15 min showed that most of organic acids were eluted before 5 min and other nonpolar constituents after 15 min (Fig. 1). Table 1 revealed that major constituents were concentrated 1.8, 3.8, and 3.2 fold, respectively, in 50% ethanol eluted fraction.

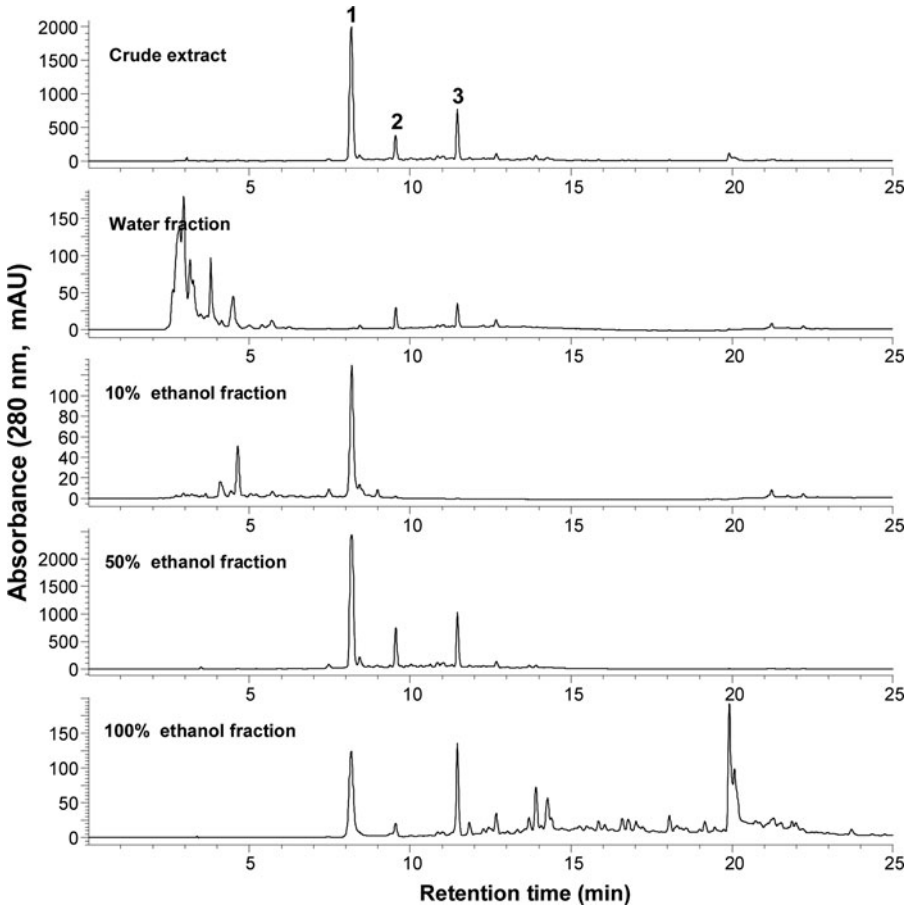

Fig. 1. HPLC chromatograms of Diaion HP-20 macroporous resin chromatography. Major components of *A. koreana* were eluted at 50% ethanol fraction. HPLC conditions: Column, Inno Column C18 (4.6 × 250 mm, 5 µm, YoungJin Biochrom, Korea); mobile phase, acetonitrile (1% formic acid, solvent A)-water (1% formic acid, solvent B) in a gradient mode as follows: 0 min–2 min, 10% A; 2 min–17 min, 10–60% A; 17.01 min–25 min, 100% A; flow rate, 1 mL/min; UV wavelength, 280 nm; injection volume, 10 µL.

Screening Antioxidants by DPPH–HPLC Experiment

The DPPH–HPLC method was applied to detect antioxidants in *A. koreana* extract, which was able to screen antioxidants from complex mixtures, particularly for natural products without sample pretreatment. The peak area of compound **3** decreased in HPLC chromatogram after spiking with DPPH solution, whereas there were no changes in other peak areas of compounds **1** and **2** in DPPH–HPLC analysis (Fig. 2). In order to determine the chemical structures of compounds, CPC was used to isolate and purify major compounds from the 50% ethanol fraction obtained by Diaion HP-20 column chromatography.

Table 1. Contents of Maltol (**1**), Dihydrokaempferol-7-*O*-*β*-D-glucopyranoside (**2**), and *Trans*-piceid (**3**) in *A. koreana* according to the purification processes; crude extract, Diaion HP-20 macroporous resin, and CPC operation

	Contents of compounds (% of dry weight)		
Samples	Maltol (**1**)	Dihydrokaempferol-7-*O*-*β*-D-glucopyranoside (**2**)	*Trans*-piceid (**3**)
Crude extract	4.27	0.92	1.40
Diaion HP-20 fraction	7.71	3.52	4.43
CPC peak fractions	>99	95	97

Content of each compound was calculated by calibration curve. Standard compounds were isolated by preparative HPLC and purity was confirmed by NMR.

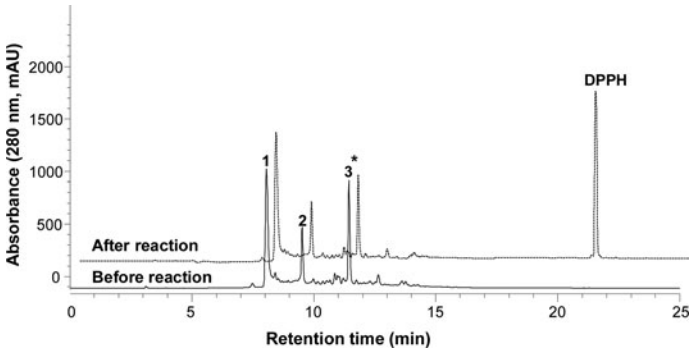

Fig. 2. HPLC-UV and DPPH-HPLC-UV chromatograms of *A. koreana* extract. HPLC conditions were the same as described in Fig. 1.

Table 2. Partition coefficients (K) of target compounds of *A. koreana* extract in different solvent systems

No	Solvent system	Volume ratio	Setting time	*K* Values				
				Maltol (**1**)	α*	Dihydrokaempferol-7-*O*-β-D-glucopyranoside (**2**)	α**	*Trans*-piceid (**3**)
1	E:I:W	9:1:10	23 s	**1.99**	3.2	**0.62**	1.5	**2.96**
2	E:I:W	8:2:10	24 s	2.02	1.9	1.07	2.0	4.11
3	E:I:W	7:3:10	9 s	2.00	1.3	1.52	2.3	4.52
4	E:I:W	6:4:10	1 m 20 s	1.80	3.0	1.64	2.0	3.54
5	H:E:I:W	0.5:5:10	15 s	1.19	3.1	0.38	0.8	0.96
6	H:E:I:W	0.5:5:7:10	1 m 10 s	1.12	1.1	1.06	1.1	1.20
7	H:E:I:W	0.5:5:5:11	1 m 18 s	1.53	1.5	1.02	1.4	2.20
8	H:E:I:W	0.5:5:5:12	2 m	1.68	1.3	1.15	1.4	2.07
9	H:E:I:W	1:5:5:11	59 s	1.42	2.1	0.67	1.2	1.66

E: ethyl acetate; I: isopropanol; W: water; H: *n*-hexane.
*Separation factor between compounds **1** and **2**.
Separation factor between compounds **2 and **3**.

Optimization of Two-Phase Solvent System

Finding out an appropriate two-phase solvent system accounts for the most crucial part in CPC separation. The selection of the finest two-phase solvent system, among various solvent systems in different ratios and variety solvents, entails the consideration of the chemical's familiarity with one of the phases in an immiscible two-phase solvent. In a bid to satisfy a two-phase solvent system, there is a set of requirements that are universally accepted for CPC separation such as: i) sufficient retaining of the stationary phase; ii) a short set-up time of solvent preparation (less than 25 second); iii) ideal partition coefficient (K value) ($0.5 \leq K \leq 2.0$), iv) adequate sample solubility; and v) adequate separation factor (greater than 1.5 between compounds) ($\alpha = K_2 / K_1$, $K_2 > K_1$).[12,13] In order to find the two-phase solvent system that is appropriate for CPC isolation of three major compounds in *A. koreana*, we examined K values in various solvent systems consisting of ethyl acetate–isopropanol–water or *n*-hexane–ethyl acetate–isopropanol–water in different ratios (Table 2). To begin with, the partition coefficient that far exceeded the aforementioned rule iii), for instance, solvent system No. **2, 3**, and **4**, which were far beyond K value 3, were excluded. Afterward, solvent system No. **5, 6**, and **9** were also ruled out because separation factors were too small to separate target compounds. In addition, solvent system No. **7** and **8** should be excluded as well because these solvent systems were not able to retain the stationary phase in the CPC column in our preliminary experiment as they have a long setting time, breaking rule ii). Therefore, solvent system No. 1 (ethyl acetate–isopropanol–water, 9:1:10, *v/v/v*) was chosen for CPC operation.

Preparative Separation Target Compounds by CPC

The two-phase solvent system of ethyl acetate–isopropanol–water (9:1:10, *v/v/v*) was used for the CPC separation procedure. As shown in Fig. 3, *A. koreana* fraction C (2.19 g) was separated excellently into three response major CPC peak fractions; **I** (82.5–110 min), **II** (165–230 min), and **III** (255–350 min) in a descending mode. Each CPC peak fraction from **I** to **III** was concentrated to yield 98.3 mg of peak fraction **I**, 230.4 mg of peak fraction **II**, and 165.9 mg of peak fraction **III**. Afterward, those fractions obtained from the CPC chromatogram were analyzed via analytical HPLC. Fraction peaks **I–III** from the CPC with a descending mode corresponded to the peak order **2, 1**, and **3** in the HPLC chromatogram in Fig. 3. The purities of maltol (**1**), dihydrokaempferol-7-*O*-β-D-glucopyranoside (**2**), and *trans*-piceid (**3**) were demonstrated to be >99, 95, and 97%, respectably, according to HPLC peaks area at 280 nm.

Structural Identification of CPC Peak Fractions

The chemical structures of three major compounds isolated from *A. koreana* were elucidated by interpretation of ^{1}H, ^{13}C NMR including 2D NMR (COSY, HSQC, and HMBC), and ESI-MS together with the comparisons from existing literature.[5,14–16]

Peak Fraction I: Yellowish amorphous powder; $[\alpha]_D^{25}$ 32.9° (*c* 0.2, methanol); UV λmax (nm): 225.8, 285.4; ^{1}H NMR (400 MHz, methanol-d_4) $\delta = 7.36$ (2H, bd, $J = 6.0$, H-2′, 6′), 6.83 (2H, bd, $J = 7.1$, H-3′, 5′), 6.22 (1H, d, $J = 10.1$, H-8), 6.19 (1H, d, $J = 10.1$, H-6), 5.01 (1H, H-2), 4.96 (1H, H-1″), 4.59 (1H, H-3), 3.86 (1H, H-6″a), 3.67 (1H, H-6″b), 3.44 (1H, H-2″), 3.44 (1H, H-5″), 3.40 (1H, H-3″), 3.37 (1H, H-4″); ^{13}C NMR (100 MHz, methanol-d_4) $\delta = 199.4$ (C-4), 167.2 (C-7), 164.7 (C-5), 164.2 (C-9), 159.4 (C-4′), 130.5 (C-2′, 6′), 129.0 (C-1′), 116.2 (C-3′, 5′), 103.6 (C-10), 101.2 (C-1″), 98.3 (C-6), 97.0 (C-8), 85.2 (C-2), 78.2 (C-3″), 77.8 (C-5″), 74.6 (C-2″), 73.8 (C-3), 71.1 (C-4″), 62.2 (C-6″); ESIMS *m/z* 472.9 $[M + Na]^+$. Compared with the data given in literature,[14] peak fraction I was identified as dihydrokaempferol 7-*O*-β-D-glucopyranoside (**2**)

Peak Fraction II: Reddish amorphous powder; UV λmax (nm): 213.3, 274.2; ^{1}H NMR (400 MHz, methanol-d_4) $\delta = 7.94$ (1H, d, $J = 5.5$, H-6), 6.39 (1H, d, $J = 5.5$, H-5), 2.34 (3H, s, H-CH3); ^{13}C NMR (100 MHz, methanol-d_4) $\delta = 175.2$ (C-4), 156.3 (C-6), 152.3 (C-2), 144.6 (C-3), 114.5 (C-5), 14.2

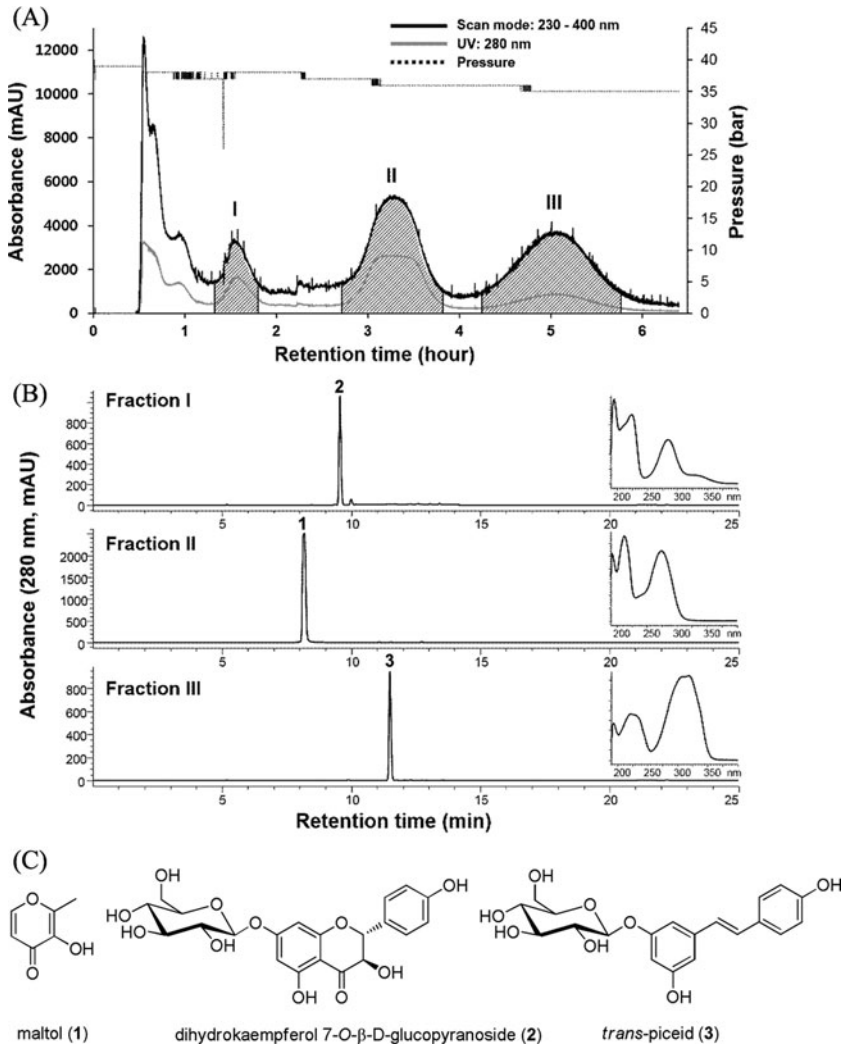

Fig. 3. CPC chromatogram of *A. koreana* extract (A), HPLC analysis of isolated compounds (B), and chemical structures of isolated compounds (C). CPC operation conditions: two-phase solvent system, ethyl acetate–isopropanol–water with a volume ratio 9:1:10; descending mode, mobile phase: lower aqueous phase; flow-rate, 10 mL/min; rotation speed, 1200 rpm; monitored at 280 nm and scan mode (230–400 nm). HPLC conditions were the same as described in Fig. 1.

(C-CH3); ESIMS *m/z* 126.9 $[M+H]^+$. Compared with the data given in literature,[5,15] peak fraction II was identified as maltol (**1**)

Peak Fraction III: White solid; $[\alpha]_D^{25}$ the 25 should be over the D $-63.2°$ (*c* 0.3, methanol); UV λmax (nm): 226.9, 319.0; ^{1}H NMR (400 MHz, methanol-d_4) $\delta = 7.37$ (2H, d, $J = 8.6$, H-2′, 6′), 7.02 (1H, d, $J = 16.3$, H-8), 6.85 (1H, d, $J = 16.3$, H-7), 6.79 (1H, t, $J = 1.6$, H-2), 6.77 (2H, d, $J = 8.6$, H-3′, 5′), 6.62 (1H, t, $J = 1.6$, H-6), 6.45 (1H, t, $J = 2.1$ H-4), 4.90 (1H, d, $J = 7.4$, H-1″), 3.93 (1H, H-6″a), 3.72 (1H, H-6″b), 3.46 (1H, H-2″), 3.47 (1H, H-5″), 3.43 (1H, H-3″), 3.39 (1H, H-4″); ^{13}C NMR (100 MHz, methanol-d_4) $\delta = 160.5$ (C-3), 159.6 (C-5), 158.5 (C-4′), 141.5 (C-1), 130.3 (C-1′), 130.0 (C-8), 128.9 (C-2′, 6′), 126.7 (C-7), 116.5 (C-3′, 5′), 108.3 (C-6), 107.0 (C-2), 104.0 (C-4), 102.4 (C-1″), 78.2 (C-5″), 78.0 (C-3″), 74.9 (C-2″), 71.4 (C-4″), 62.6 (C-6″): ESIMS *m/z* 412.8 $[M+Na]^+$. Compared with the data given in literature,[16] peak fraction III was identified as *trans*-piceid (**3**).

Conclusions

A labor saving and an economical preparative isolation technique for antioxidants of *A. koreana* was developed by a DPPH$^\bullet$–HPLC experiment and CPC with a solvent system of ethyl acetate–isopropanol–water (9:1:10, *v/v/v*). This system allows the excellent separation of target components with using just about 5 L of mobile phase in 360 min without an extra purification process. The present study will support further biological studies in *A. koreana* and an easy access to its secondary metabolites.

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