Preparative isolation of monoterpenes from a gamma-irradiated mutant of *Perilla frutescens* var. c*rispa* by centrifugal partition chromatography

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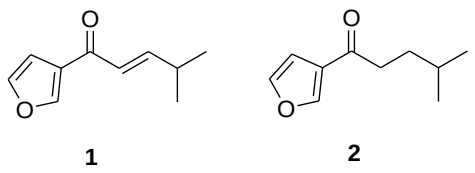
1. Introduction

Perilla frutescens var. *crispa* (Lamiaceae) is widely distributed in Asia, its leaves are used as food and traditional medicine [1]. *P. frutescens* var. *crispa* has diverse components, such as monoterpenes, flavonoids, and phenolic acids, which have several beneficial activities including antioxidant [2, 3], anti-inflammatory [4], and anticancer [5] effects. Our research group has developed a new cultivar of *P. frutescens crispa* (cv. Antisperill), using a gamma-irradiated mutant of the original plant *P. frutescens* var. *crispa*. with a high content of monoterpenes compared to the original cultivar [6]. The supercritical carbon dioxide (SC-CO_2) extract of this new cultivar, compared with that of the original cultivar, possesses higher content of monoterpenes, isoegomaketone (**1**) and perilla ketone (**2**) (Fig. 1), and higher anti-inflammatory activity [7,8].

Centrifugal partition chromatography (CPC) was a liquid–liquid separation system that partitions solutes between two immiscible liquid phases [9]. As CPC has advantages of no irreversible adsorption allowing for high product recovery and enhancement of sample-loading capacity, process flexibility [10, 11]. Thus, CPC has been used extensively for preparative separation and purification of natural products [12].

For developing health functional food and/or botanical drug product using the SC-CO_2 extract of the leaves of *P. frutescens* var. *crispa* (cv. Antisperill), large amounts of purified compounds are required to evaluate the *in vivo* and *in vitro* anti-inflammatory potential of this plant. For this purpose, we established an efficient CPC method for the separation of two monoterpenes from the SC-CO_2 extract of the leaves of *P. frutescens* var. *crispa* (cv. Antisperill).



1 **2**

Fig. 1. Chemical structures of compounds **1** and **2** isolated from the leaves of *P. frutescens* var. *crispa* (cv. Antisperill).

2. Methods and Results

2.1 General

CPC was performed on the Gilson CPC 250 system (Gilson Inc., Middleton, WI, USA) equipped with a 250 mL rotor, a 10 mL sample loop, a Shimadzu LC-8A pump (Shimadzu, Kyoto, Japan), and a Shimadzu SPD-10A UV/Vis detector. The equipment used for HPLC analysis was the Agilent 1200 system (Agilent Technologies Co., Santa Clara, CA, USA) equipped with a YMC-Triart C18 column (5 μm, 250 mm × 4.6 mm; YMC Co.) and the ChemStation software. The NMR experiment was performed on the JNM-ECA 500 MHz NMR instrument (JEOL Ltd., Tokyo, Japan) with tetramethylsilane as an internal standard. All other chemicals and solvents used in this study were of analytical grade.

2.2 Plant material

P. frutescens var. *crispa* (cv. A*ntisperill*) was developed by gamma irradiating (200 Gy) the seeds of the original plant *P. frutescens* var. *crispa* using a labeled Cobalt (^{60}Co) source, followed by selection based on the screening of anti-inflammatory activity and active compound concentrations as well as the examination of stable inheritance of phenotype for 3 years (1995–1998) at the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute (Jeongeup-si, Jeollabuk-do, Korea).

2.3 Preparation of crude sample

The dried leaves of *P. frutescens* var. *crispa* (cv. Antisperill) (45 kg) were pulverized and then prepared by the SC-CO_2 extraction method using the supercritical fluid extraction system (SCFE-P100; Ilshin Autoclave Co., Daejeon, Korea). The powdered sample was placed into the extraction column of the SC-CO2 extractor. The predetermined conditions for the procedure were as follows: pressure, 400 bar; temperature, 50°C, CO2 flow rate (99.9%), constant at 3 L/min; and extraction time, 4 h. The resultant oil was collected (480 g; 1.92% w/w) and stored in a refrigerator at 4°C.

2.4 Selection of the two-phase solvent system

The *K* values for the lower aqueous mobile phase were instantly applied as the $K_{U/L}$ value, and its proper range was $0.5 \leq K \leq 1.0$; the *K* values for the upper organic mobile phase were calculated as the reciprocal of the $K_{U/L}$ value, and its suitable range was $1.0 \leq K \leq 2.0$. The separation factor (α) value was the ratio of the two K values and was obtained by dividing the K values of the two compounds ($\alpha = K_1/K_2$, where $K_1 > K_2$). The values were recommended to be >1.5 [11]. In brief, 1 mg crude sample was added to a 1.5 mL tube, and then, 500 μL of each phase of the pre-equilibrated two-phase solvent system was added and vigorously shaken. After the two-phase sample were thoroughly equilibrated, 200 μL of each phase was collected and subjected to HPLC analysis (Table 1).

Table 1. The partition coefficient (*K*) and separation factor (α) of compounds **1** and **2** in different solvent systems.

Solvent system (*n*-Hexane-EtOAc-EtOH-Water)	*K* values		α value
	1	**2**	α_{23}
5 : 5 : 5 : 5	0.21	0.14	1.5
6 : 4 : 5 : 5	0.22	0.14	1.6
7 : 3 : 5 : 5	0.25	0.14	1.7
6 : 4 : 6 : 4	0.47	0.31	1.5
7 : 3 : 6 : 4	0.52	0.31	1.7
7 : 3 : 7 : 3	0.97	0.61	1.6
7 : 3 : 8 : 2	1.49	1.04	1.4
8 : 2 : 7 : 3	0.23	0.14	1.7
8 : 2 : 8 : 2	1.59	1.04	1.5
9 : 1 : 9 : 1	2.22	1.67	1.3

2.5 CPC separation

The SC-CO_2 extract (500 mg) of the leaves of *P. frutescens* var. *crispa* (cv. Antisperill) was dissolved in a 1:1 (*v*/*v*) mixture (5 mL each) of the two-phase solvent system (*n*-hexane–ethyl acetate–ethanol–water = 8:2:8:2). CPC separation of compounds **1** and **2** was performed using the two-phase solvent system of n-hexane–ethyl acetate–ethanol–water (8:2:8:2) in the ascending mode (the upper organic mobile phase). In this solvent condition, the stationary phase retained in the column was 76%. After elution of the peaks II and I, extrusion of the stationary phase was performed at 42 min. Compounds **1** (56.12 mg yield) and **2** (78.60 mg yield) corresponding to the peak fractions II and III, respectively, were successfully isolated from the SC-CO2 extract (Fig. 2).

2.5 HPLC-DAD analysis of identification of compounds

The SC-CO_2 extract and each peak fraction from CPC were weighed accurately and dissolved in MeOH at 1.0 and 0.5 mg/mL, respectively, and filtered through a syringe filter (0.45 μm) for HPLC analysis. The HPLC analysis was performed using an YMC-Triart C18 column (5 μm, 250 × 4.6 mm; YMC Co., Kyoto, Japan) with a gradient solvent system of acetonitrile and water (45:55–55:45). The flow rate was maintained at 0.8 mL/min, and the injection volume was set to 10 μL. Chromatograms were acquired at 254 nm using the DAD detector. Compounds **2** (98.9% purity) and **3** (97.3% purity) corresponding peak fractions II and III, respectively, were successfully purified from the SC-CO_2 extract. HPLC chromatogram of peaks I and II are shown in Fig. 3A and 3B.

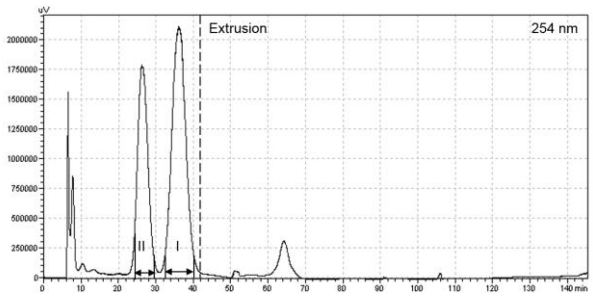


Fig. 2. CPC separation of the SC-CO2 extract of the leaves of *P. frutescens* var. *crispa* (cv. Antisperill) using n-hexane/ethyl acetate/ethanol/water (8:2:8:2, *v*/*v*) in an ascending mode (for CPC conditions, see Section 2.). The extrusion was performed after 42 min.

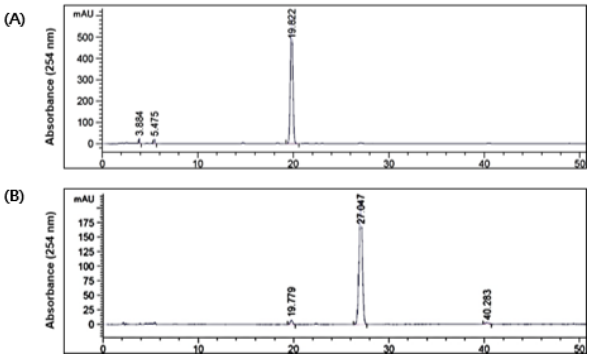


Fig. 3. HPLC chromatograms of CPC peak fractions II (A) and II (B).

2.6 Structural identification

The chemical structures of the target compounds were determined by ^{1}H and ^{13}C NMR spectroscopy and the NMR data are follows.

NMR data of peak I: positive ESI-MS *m/z* 165.0 $[M+H]^+$. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.03 (1H, s, H-5), 7.44 (1H, d, *J* = 1.5, H-2), 7.00 (1H, dd, *J* = 15.3, 1.5 Hz, H-8), 6.81 (1H, d, *J* = 1.5, H-4), 6.47 (1H, dd, *J* = 15.3, 1.5 Hz, H-7), 2.52 (1H, m, H-9), 1.10 (6H, s, 10 and H-11); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 184.2 (C-6), 154.6 (C-8), 147.2 (C-5), 147.2 (C-2), 129.3 (C-3), 124.1 (C-7), 109.2 (C-4), 31.3 (C-9), and 21.4 (C-10 and C-11). Peak I was identified as isoegomaketone (**1**) on comparing its data with the data provided elsewhere [8].

NMR data of peak II: positive ESI-MS *m/z* 167.1 $[M+H]^+$. ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 8.00 (1H, s, H-5), 7.40 (1H, d, *J* = 1.5, H-2), 6.73 (1H, d, *J* = 1.5, H-4), 2.70 (2H, t, *J* = 7.0 Hz, H-7), 1.58 (3H, m, H-8 and H-9), 0.89 (6H, d, *J* = 6.5, H-10 and H-11); ^{13}C-NMR

($CDCl_3$, 125 MHz): δ 195.7 (C-6), 147.7 (C-5), 144.3 (C-2), 128.2 (C-3), 109.1 (C-4), 38.9 (C-7), 33.6 (C-8), 28.2 (C-9), and 22.7 (C-10 and C-11). Peak II was identified as perilla ketone (**2**) on comparing its data with the data described elsewhere [8].

3. Conclusions

CPC was successfully applied to separate monoterpenes, the anti-inflammatory component of *P. frutescens* var. *crispa* (cv. Antisperill). Isoegomaketone (**1**) and the major compound, perilla ketone (**2**) were purified using the n-hexane–ethyl acetate–ethanol–water (8:2:8:2, *v*/*v*) solvent system in an ascending mode. Our results indicate that CPC is a useful method in separating and purifying two major compounds from the SC-CO_2 extract of *P. frutescens* var. *crispa* (cv. Antisperill).

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