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Isolation of Phytochemicals from *Salvia plebeia* Using Countercurrent Chromatography Coupled with Reversed-phase HPLC

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Abstract – *Salvia plebeia* R. Br. is a plant which has been used as an edible crop and traditional medicine in Asian countries. In this study, HPLC-PDA analysis and countercurrent chromatography (CCC) coupled with reversed-phase (RP) HPLC method were applied to isolate ten isolates from 3.3 g of *n*-butanol soluble extract from hot-water extract of *S. plebeia.* The use of CCC enabled us to efficiently fractionate the starting material with less sample loss and facilitate the isolation of compounds from *S. plebeia* extract using RP-HPLC. The isolates were determined to be caffeic acid (**1**), 6-hydroxyluteolin 7-*O*-β-D-glucoside (**2**), eudebeiolide B (**3**), (*R*)-rosmarinic acid (**4**), homoplantaginin (**5**), eudebeiolide D (**6**), plebeiolide C (**7**), salpleflavone (**8**), eupafolin (**9**) and hispidulin (**10**) based on the spectroscopic evidence.
Keywords – *Salvia plebeia,* Lamiaceae, Countercurrent chromatography, Reversed-phase HPLC

Introduction

Salvia plebeia R.Br. is an annual or biennial plant belonging to the Lamiaceae family and distributed in Asian regions including Korea, China, Japan, India and Australia.[1] In Korea, the whole parts of *S. plebeian* are used as an edible crop with the common name of 'Gombo-baechu' because the appearance of this plant is close to that of cabbage. In terms of traditional medicine usage in Korea, *S. plebeian* have been used to treat inflammation-related disease such as common cold, cough, hepatitis.[1] Recent biological studies have revealed that *S. plebeian* possessed anti-asthma, anti-atopic dermatitis, anti-cancer, anti- obesity, antioxidant, and anti-influenza effects.[1,2] Diverse phytochemicals have been identified from *S. plebeian* such as flavonoids, terpenoids and phenolic acids, and flavonoids are regarded as principal biologically active components of this plant.[2] Recently, many efforts have been performed to develop a functional food using *S. plebeian* extract since this plant have shown versatile biological effects. Therefore, phyto-chemical studies are should be accomplished such as identification and isolation of active or marker molecules to meet the compounds needs for chemistry manufacturing controls (CMC), biological assays and further scientific investigation.

In the preliminary HPLC analysis, we found that hot-water extract of *S. plebeia* especially contained a great amount of rosmarinic acid as a main constituent giving rise to suppressing the peak intensity of the other minor compounds (Fig. 1 and 2). Conventionally, silica gel-based column chromatography (CC) is applied for initial fractionation of plant extract because silica gel is a one of inexpensive sorbents. During multiple isolation steps with silica gel, some active molecules with small amounts can be lost due to the significant irreversible absorption to silica gel and chemical degradation by acid-catalyzed rearrangement reaction. Usually, around 30% of sample mass is irreversibly bind to silica gel in a single chromatography step, which can cause complete loss of minor active molecules.[3] Therefore, silica gel-based isolation method should be excluded as much as possible for recovering minor compounds from sample extract, which is particularly emphasized for limited amount of sample mass.

In the current study, countercurrent chromatography (CCC) coupled with reversed-phase (RP) HPLC method was applied to isolate phytochemcals from the hot-water extract of *S. plebeia* to facilitate recovering minor molecules

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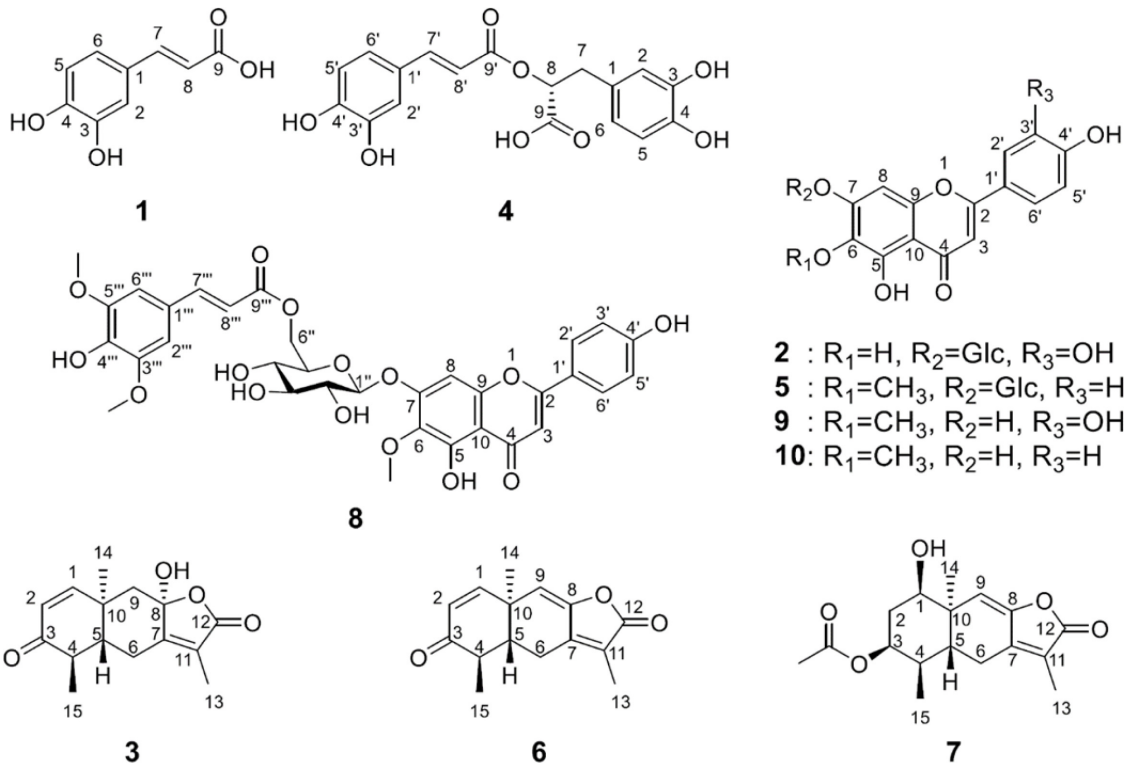

Fig. 1. Chemical structures of compounds **1**–**10** from hot-water extract of *S. plebeia*.

as well as rosmarinic acid. The structures of isolates were determined through spectroscopic evidence such as ^{1}H-, ^{13}C-NMR, ESI-Q-TOF-MS, circular dichroism (CD) and optical rotation (Fig. 1).

Experimental

General experimental procedures – The chemical structures of the isolates were determined by NMR spectrum data using an Avance 500 spectrometer (Bruker, Karlsruhe, Germany), J-815 spectrometer (Jasco, Tokyo, Japan) and P-2000 polarimeter (Jasco, Tokyo, Japan). High-performance counter current chromatography (HPCCC) was performed on a MIDI HPCCC (Dynamic Extraction, Berkshire, UK). The preparative HPLC was performed with a Gilson HPLC system (Middleton, WI, USA) with a Luna C18(2) (21.2 × 250 mm I.D., 5 μm; Phenomenex, Torrance, CA, USA). The analytical HPLC was conducted using a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) and analysis was accomplished by Luna C18 (4.6 × 250 mm I.D., 5 μm; Phenomenex, Torrance, CA, USA). ESI-Q-TOF-MS spectra data were obtained using an Agilent 6530 Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA, USA). Silica gel 60 (40-63 μm, 230-400 mesh; Merck, Kenilworth, NJ, USA), ZEOprep 90 C18 (40-63 μm; ZEOCHEM, Uetikon, Switzerland) and Sephadex LH-20 (Ge healthcare, Uppsala, Sweden) were used in liquid column chromatography. Organic solvents for partition and column chromatography were purchased from Dae-Jung Chemical Co. Ltd. (Seoul, Korea).

Plant material – The hot-water extract of *S. plebeia* was prepared and provided from FromBIO (Suwon, Gyenoggi-do, Korea). The whole part of *S. plebeia* was purchased from the local markets in Deagu-si (Misan Yakcho Nongjang) and extracted using the hot-water at 85 °C for 8 hrs. The extraction yield of hot-water extract was 21%.

HPLC analysis – The samples from *S. plebeia* were analyzed by HPLC-PDA. A gradient elution of water (0.01% TFA, A) and MeOH (0.01% TFA, B) mixture were used; 0–5 min (90% A), 5–40 min (90 to 0% A), 40–50 min (0% A). The flow rate of the mobile phase was 1.0 ml/min and HPLC chromatograms were monitored at 254 and 340 nm.

Extraction and isolation – The hot-water extract of *S. plebeia* (50 g) was suspended in H_2O and sequentially partitioned with *n*-BuOH. The *n*-BuOH layer was concentrated under reduced pressure to give *n*-BuOH soluble extract (3.4 g). The *n*-BuOH soluble extract (1.65

g × 2 times) was subjected to HPCCC using a biphasic solvent system of $CHCl_3$-MeOH-IPA-H_2O (6:6:1:4, v/v/v/v) (rotation speed - 1400 rpm; normal-phase mode; flow-rate: 25.0 ml/min; detection wavelength: 280 nm). After HPCCC experiments, HPLC-PDA analysis was performed to pool the peak fractions showing similar HPLC chromatogram pattern and six peak fractions (Fr. 1–5) were yielded according to the HPLC-PDA analysis and the solution retained in the HPCCC column after run was extruded and pooled to give Fr. 6. Fraction 1 (314 mg) was divided by two subfraction (Fr. 1.1–1.2) using RP-MPLC [CH_3OH/H_2O (20:80 and 100:0, v/v), flow rate: 6.0 ml/min]. Fraction 1.1 was purified by 45% CH_3OH using RP-HPLC to give compound **3** (6.1 mg). Compounds **6** (4.1 mg) and **7** (5.0 mg) were isolated by RP-HPLC [MeCN-H_2O, 46:54 (v/v), flow rate: 5.0 ml/min] from Fr. 1.2. Compound **10** (36.9 mg) was isolated by RP-MPLC [CH_3OH-H_2O, 15:85→100:0 (v/v), flow rate: 5.0 ml/min] from Fr. 2 (269 mg). Fraction 3 (231 mg) was subjected to RP-MPLC [CH_3OH-H_2O,15:85→100:0 (v/v), flow rate: 5 ml/min] to divide two subfraction (Fr. 3.1–3.2.). Fraction 3.1 was separated by RP-HPLC with 55% CH_3OH as a mobile phase to afford compound **8** (7.3 mg). Fraction 3.2 was purified by RP-HPLC using CH_3OH-H_2O mixture (62:38, flow rate: 5.0 ml/min) to yield compound **9** (12.6 mg). Fraction 4 (190.7 mg) was subjected to a RP-HPLC using a gradient elution of CH_3OH-H_2O mixture [15:85→100:0 (v/v), flow rate: 5.0 ml/min] to afford compounds **1** (12.1 mg) and **5** (42.9 mg). Compounds **2** (13.1 mg) and **4** (36.0 mg) were isolated from Fr. 6 (548 mg) by RP-HPLC [CH_3OH-H_2O, 20:80→81:19 (v/v), flow rate: 5.0 ml/min]. Fraction 5 (1.35 g) was purified by RP-MPLC using a gradient elution of CH_3OH-H_2O mixture [15:85→80:20 (v/v), flow rate: 10 ml/min] to give 971 mg of compound **4**.

Caffeic acid (1) – white amorphous powder; $C_9H_8O_4$; ESI-Q-TOF-MS: *m/z* 179.0347 $[M-H]^-$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 7.38 (1H, d, *J* = 15.8 Hz, H-7), 7.01 (1H, d, *J* = 2.1 Hz, H-2), 6.94 (1H, dd, *J* = 8.1, 2.1 Hz, H-6), 6.75 (1H, d, *J* = 8.1 Hz, H-5), 6.16 (1H, d, *J* = 15.8 Hz, H-8); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 168.30 (C-9), 148.05 (C-4), 145.57 (C-3), 144.17 (C-7), 125.80 (C-1), 121.02 (C-6), 115.74 (C-5), 115.74 (C-8), 114.56 (C-2).

6-Hydroxyluteolin 7-*O*-β-D-glucoside (2) – pale yellow powder; $C_{21}H_{20}O_{12}$; ESI-Q-TOF-MS: *m/z* 463.0876 $[M-H]^-$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 7.42 (1H, dd, *J* = 8.2, 2.3 Hz, H-6'), 7.40 (1H, d, *J* = 2.3 Hz, H-2'), 6.96 (1H, s, H-8), 6.90 (1H, d, *J* = 8.2 Hz, H-5'), 6.70 (1H, s, H-3), 5.02 (1H, d, *J* = 7.2 Hz, H-1"), 3.75 (1H, dd, *J* = 11.2, 5.2 Hz, H-6"a), 3.16-3.54 (5H, o, H-2", 3", 4", 5", 6"b) (o: peak overlapped); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 182.26 (C-4), 164.26 (C-2), 151.30 (C-7), 149.71 (C-4'), 149.01 (C-9), 146.59 (C-5), 145.75 (C-3'), 130.43 (C-6), 121.65 (C-1'), 118.95 (C-6'), 115.93 (C-5'), 113.46 (C-2'), 105.81 (C-10), 102.53 (C-3), 100.93 (C-1"), 93.96 (C-8), 77.27 (C-3"), 75.79 (C-5"), 73.19 (C-2"), 69.64 (C-4"), 60.64 (C-6").

Eudebeiolide B (3) – colorless crystal; $C_{15}H_{18}O_4$; ESI-Q-TOF-MS: *m/z* 261.1130 $[M-H]^-$; CD(MeOH) Δε -30.9 (217 nm), -16.5 (242.5 nm); ^{1}H-NMR (500 MHz, CD_3OD): δ 6.87 (1H, d, *J* = 10.0 Hz, H-1), 5.91 (1H, d, *J* = 10.0 Hz, H-2), 2.86 (1H, dd, *J* = 13.6, 3.4 Hz, H-6a), 2.56 (1H, dq, *J* = 13.3, 6.6 Hz, H-4), 2.37-2.39 (2H, o, H-6b, 9a), 1.82 (3H, d, *J* = 1.4 Hz 13-C$\underline{H}_3$), 1.73 (1H, t, *J* = 12.5 Hz, H-5), 1.66 (1H, d, *J* = 13.4 Hz, H-9b), 1.40 (3H, s, 14-C$\underline{H}_3$), 1.22 (3H, d, *J* = 6.7 Hz, 15-C$\underline{H}_3$) (o: peak overlapped); ^{13}C-NMR (125 MHz, CD_3OD): δ 202.85 (C-3), 174.38 (C-12), 161.04 (C-1, 7), 126.71 (C-2), 123.83 (C-11), 105.21 (C-8), 51.74 (C-5), 49.10 (C-9), 43.67 (C-4), 38.53 (C-10), 25.93 (C-6), 19.06 (C-14), 12.43 (C-15), 8.38 (C-13).

(*R*)-Rosmarinic acid (4) – pale yellow powder; $C_{18}H_{16}O_8$; ESI-Q-TOF-MS: *m/z* 359.0767 $[M-H]^-$; $[\alpha]_D^{25}$= +33.2 (c 0.1, CH_3OH); ^{1}H-NMR (500 MHz, CD_3OD): δ 7.55 (1H, d, *J* = 15.9 Hz, H-7'), 7.04 (1H, d, *J* = 2.0 Hz, H-2'), 6.95 (1H, dd, *J* = 8.2, 2.1 Hz, H-6'), 6.78 (1H, d, *J* = 8.2 Hz, H-5'), 6.75 (1H, d, *J* = 2.1 Hz, H-2), 6.70 (1H, d, *J* = 8.1 Hz, H-5), 6.61 (1H, dd, *J* = 8.1, 2.1 Hz, H-6), 6.26 (1H, d, *J* = 15.9 Hz, H-8'), 5.19 (1H, dd, *J* = 8.4, 4.3 Hz, H-8), 3.10 (1H, dd, *J* = 14.4, 4.3 Hz, H-7a), 3.01 (1H, dd, *J* = 14.3, 8.5 Hz, H-7b); ^{13}C-NMR (125 MHz, CD_3OD): δ 173.65 (C-9), 168.6 (C-9'), 149.89 (C-4), 147.87 (C-3), 146.96 (C-7'), 146.31 (C-3'), 145.43 (C-4'), 129.41 (C-1), 127.81 (C-1'), 123.29 (C-6), 121.93 (C-6'), 117.72 (C-2'), 116.64 (C-5), 116.43 (C-5'), 115.36 (C-8'), 114.56 (C-2), 74.77 (C-8), 38.07 (C-7).

Homoplantaginin (5) – pale yellow powder; $C_{22}H_{22}O_{11}$; ESI-Q-TOF-MS: *m/z* 463.1241 $[M+H]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 12.95 (1H, s, 5-O$\underline{H}$), 7.95 (2H, d, *J* = 8.8 Hz, H-2', 6'), 7.02 (1H, s, H-8), 6.94 (2H, d, *J* = 8.8 Hz, H-3', 5'), 6.86 (1H, s, H-3), 5.11 (1H, d, *J* = 7.3 Hz, H-1"), 3.77 (3H, s, 6-OC$\underline{H}_3$), 3.73 (1H, brd, *J* = 10.2 Hz, H-6"a), 3.21-3.52 (5H, o, H-2", 3", 4", 5", 6b") (o: peak overlapped); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 182.28 (C-4), 164.28 (C-2), 161.45 (C-4'), 156.46 (C-7), 152.46 (C-9), 152.12 (C-5), 132.49 (C-6), 128.56 (C-2', 6'), 120.99 (C-1'), 116.00 (C-3', 5'), 105.71 (C-10), 102.64 (C-3), 100.18 (C-1"), 94.35 (C-8), 77.26 (C-3"), 76.71 (C-5"), 73.15 (C-2"), 69.55 (C-4"), 60.59 (C-6"), 60.29 (6-O$\underline{C}H_3$).

Eudebeiolide D (6) – colorless crystal; $C_{15}H_{16}O_3$; CD

(MeOH) Δε 11.0 (216.6 nm), -5.0 (250.6 nm); ^{1}H-NMR (500 MHz, CD_3OD): δ 7.11 (1H, d, J = 10.0 Hz, H-1), 5.92 (1H, s, H-9), 5.92 (1H, d, J = 10.0 Hz, H-2), 2.99 (1H, dd, J = 17.4, 4.2 Hz, H-6a), 2.51-2.61 (2H, o, H-4, 6b), 2.20 (td, J = 13.1, 4.2 Hz, H-5), 1.91 (3H, d, J = 1.8 Hz, 12-C$\underline{H}_3$), 1.30 (3H, s, 14-C$\underline{H}_3$), 1.23 (3H, d, J = 6.8 Hz, 15-C$\underline{H}_3$) (o: peak overlapped); ^{13}C-NMR (125 MHz, CD_3OD): 202.61 (C-3), 172.83 (C-12), 158.87 (C-1), 150.80 (C-8), 148.72 (C-7), 128.01 (C-2), 122.77 (C-11), 116.91 (C-9), 47.95 (C-5), 43.56 (C-4), 40.55 (C-10), 24.34 (C-6), 21.78 (C-14), 12.10 (C-15), 8.53 (C-13).

Plebeiolide C (7) – colorless crystal; $C_{17}H_{22}O_5$; ESI-Q-TOF-MS: *m/z* 307.1544 $[M+H]^+$; CD(MeOH) Δε -1.3 (250.5 nm), 2.9 (283.7 nm); ^{1}H-NMR (500 MHz, CD_3OD): δ 5.81 (1H, s, H-9), 4.97 (1H, q, J = 3.0 Hz, H-3), 3.74 (1H, t, J = 3.0 Hz, H-1), 2.90 (1H, m H-6a), 2.30-2.40 (2H, o, H-5, 6b), 2.06 (2H, m, H-2), 2.02 (3H, s, OAc-C$\underline{H}_3$), 1.94 (1H, m, H-4), 1.88 (3H, d, J = 1.6 Hz, 13-C$\underline{H}_3$), 1.06 (3H, s, 14-C$\underline{H}_3$), 0.99 (3H, d, J = 6.8 Hz, 15-C$\underline{H}_3$) (o: peak overlapped); ^{13}C-NMR (125 MHz, CD_3OD): δ 173.47 (C-12), 173.08 (-$\underline{C}OCH_3$), 150.20 (C-8), 149.94 (C-7), 121.12 (C-11), 120.32 (C-9), 75.05 (C-3), 74.44 (C-1), 42.94 (C-10), 37.38 (C-5), 35.23 (C-4), 33.25 (C-2), 23.49 (C-6), 21.31 (-CO$\underline{C}H_3$), 19.19 (C-14), 15.56 (C-15), 8.39 (C-13).

Salpleflavone (8) – pale yellow powder; $C_{33}H_{32}O_{15}$; ESI-Q-TOF-MS: *m/z* 669.1820 $[M+H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ 7.61 (2H, d, J = 8.8 Hz, H-2', 6'), 7.35 (1H, d, J = 15.8 Hz, H-7'''), 6.81 (2H, d, J = 8.8 Hz, H-3', 5'), 6.76 (1H, s, H-8), 6.34 (1H, s, H-3), 6.32 (2H, s, H-2''', 6'''), 6.17 (1H, d, J = 15.8 Hz, H-8'''), 5.14 (1H, d, J = 7.6 Hz, H-1''), 4.80 (1H, dd, J = 11.8, 2.7 Hz, H-6''a), 4.22 (1H, dd, J = 11.8, 9.0 Hz, H-6''b), 3.91 (3H, s, 6-OC$\underline{H}_3$), 3.60 (6H, s, 3''', 5'''-OC$\underline{H}_3$), 3.40-3.86 (4H, o, H-2'', 3'', 4'', 5'') (o: peak overlapped); ^{13}C-NMR (125 MHz, CD_3OD): δ 184.26 (C-4), 168.63 (C-9'''), 166.57 (C-2), 162.87 (C-4'), 157.57 (C-7), 154.21 (C-5), 154.05 (C-9), 149.17 (C-3''', 5'''), 147.62 (C-7'''), 139.31 (C-4'''), 134.42 (C-6), 129.41 (C-2', 6'), 126.29 (C-1'''), 122.77 (C-1'), 116.92 (C-3', 5'), 115.51 (C-8'''), 107.66 (C-10), 106.20 (C-2''', 6'''), 103.27 (C-3), 101.18 (C-1''), 95.72 (C-8), 78.07 (C-3''), 75.70 (C-5''), 74.68 (C-2''), 72.79 (C-4''), 64.77 (C-6''), 61.65 (6-O$\underline{C}H_3$), 56.39 (3''', 5'''-O$\underline{C}H_3$).

Eupafolin (9) – pale yellow powder; $C_{16}H_{12}O_7$; ESI-Q-TOF-MS: *m/z* 317.0661 $[M+H]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 13.08 (1H, s, 5-O$\underline{H}$), 7.41 (1H, dd, J = 8.2, 2.2 Hz, H-6'), 7.39 (1H, d, J = 2.2 Hz, H-2'), 6.88 (1H, d, J = 8.2 Hz, H-5'), 6.66 (1H, s, H-3), 6.55 (1H, s, H-8), 3.75 (3H, s, 6-OC$\underline{H}_3$); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 182.00 (C-4), 163.92 (C-2), 157.40 (C-7), 152.78 (C-5), 152.37 (C-9), 149.71 (C-4'), 145.74 (C-3'), 131.36 (C-6), 121.51 (C-1'), 118.96 (C-6'), 116.00 (C-5'), 113.32 (C-2'), 103.99 (C-10), 102.37 (C-3), 94.12 (C-8), 59.92 (6-O$\underline{C}H_3$).

Hispidulin (10) – pale yellow powder; $C_{16}H_{12}O_6$; ESI-Q-TOF-MS: *m/z* 299.0556 $[M-H]^-$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 13.07 (1H, s, 5-O$\underline{H}$), 7.95 (2H, d, J = 8.8 Hz, H-2', 6'), 6.94 (2H, d, J = 8.8 Hz, H-3', 5'), 6.77 (1H, s, H-3), 6.58 (1H, s, H-8) 3.75 (3H, s, 6-OC$\underline{H}_3$); ^{13}C-NMR (125 MHz, DMSO-d_6): δ 182.08 (C-4), 163.74 (C-2), 161.17 (C-4'), 157.52 (C-7), 152.75 (C-9), 152.41 (C-5), 131.40 (C-6), 128.44 (C-2', 6'), 121.20 (C-1'), 115.95 (C-3', 5'), 103.95 (C-10), 102.35 (C-3), 94.26 (C-8), 59.91 (6-O$\underline{C}H_3$).

Results and Discussion

As described in Introduction part, the hot-water extract of *S. plebeia* contained large amount of rosmarinic acid and its peak area occupied around 70% of total peak area in the HPLC chromatogram (Fig. 2A). When the hot-water extract was partitioned with *n*-BuOH, the minor peaks were more clearly detected than those of hot-water extract (Fig. 2B). The yield of *n*-BuOH soluble extract from the hot-water extract was low (6–7%), which indicated that the most mass of hot-water extract was composed of water-soluble metabolites which were not detected in this HPLC-PDA analysis. It is reasonable that the higher the amount of starting material, the greater the possibility for separating trace minor compounds. However, non-absorptive chromatographic method should be applied to isolate minor constituents since the yield of *n*-BuOH soluble extract is very low and rosmarinic acid still detected as the main compound. To meet the goal, CCC was employed in this study since CCC uses only liquids as a stationary and a mobile phase showing no irreversible absorption and chemical reaction of target compound during fractionation and isolation process.[4] Therefore, CCC can be the best choice to fractionate and isolate trace compounds of limited starting extract.

The *n*-BuOH soluble extract *S. plebeia* was fractionated by CCC using the biphasic solvent system of $CHCl_3$-MeOH-IPA-H_2O (6:6:1:4, v/v/v/v). As shown in Fig. 3, CCC produced five fractions (Fr. 1–5) and stationary phase extruded after CCC run (Fr. 6). The HPLC-PDA analysis revealed that each fraction contained one ~ three compounds with high purity and trace minor compounds were more clearly detected in the HPLC chromatograms. Even, compounds **4** and **10** were isolated as a single compound in this one-step CCC run. These results showed the strong point of CCC in that CCC method is

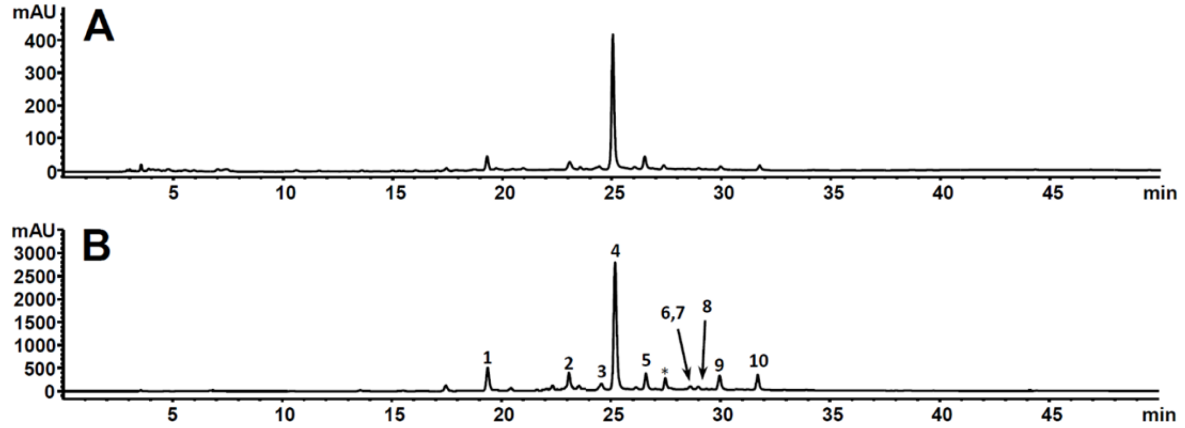

Fig. 2. HPLC chromatograms of hot-water extract of *S. plebeia* (**A**), and its *n*-BuOH soluble extract (**B**). Peaks: caffeic acid (**1**), 6-hydroxyluteolin 7-*O*-β-D-glucoside (**2**), eudebeiolide B (**3**), (*R*)-rosmarinic acid (**4**), homoplantaginin (**5**), eudebeiolide D (**6**), plebeiolide C (**7**), salpleflavone (**8**), eupafolin (**9**), hispidulin (**10**); * unidentified peak.

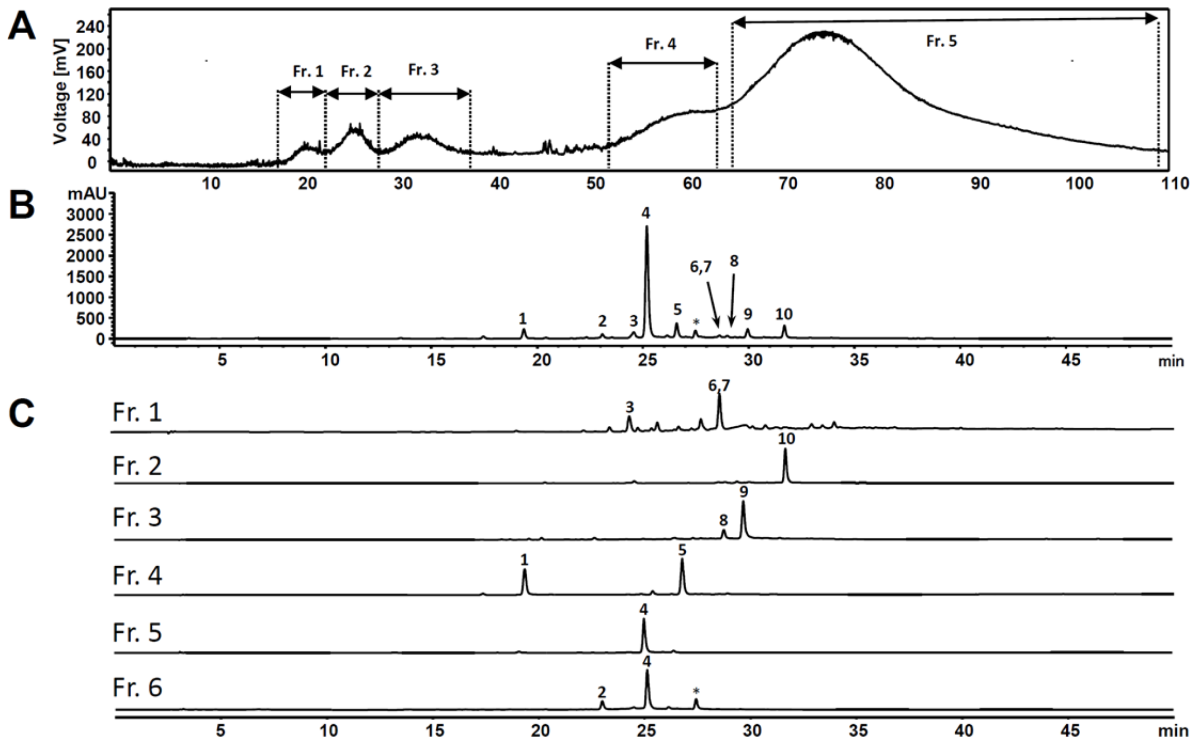

Fig. 3. HPCCC chromatogram of *n*-BuOH soluble extract from *S. plebeian* (**A**), HPLC chromatogram of *n*-BuOH soluble extract from *S. plebeian* (**B**) and HPLC chromatogram of HPCCC peak fractions (**C**). Peaks: caffeic acid (**1**), 6-hydroxyluteolin 7-*O*-β-D-glucoside (**2**), eudebeiolide B (**3**), (*R*)-rosmarinic acid (**4**), homoplantaginin (**5**), eudebeiolide D (**6**), plebeiolide C (**7**), salpleflavone (**8**), eupafolin (**9**), hispidulin (**10**); * unidentified peak.

excellent to fractionate extract and isolate a single compound at the same time. Further preparative RP-HPLC was utilized to isolate and increase the purities of compounds.

HPCCC followed by preparative RP-HPLC afforded ten compounds and chemical structures of isolates were determined through spectroscopic data. The compound marked star (*) was not identified because the purity of compound was not satisfactory enough to determine the chemical structure. The HPLC-PDA chromatograms of isolates are shown in Fig. 4.

The molecular formula of compound **1** was deduced to be $C_9H_8O_4$ according to the $[M-H]^-$ peak in the ESI-Q-TOF-MS at *m/z* 179.0347. The ^{1}H-NMR spectrum indicated

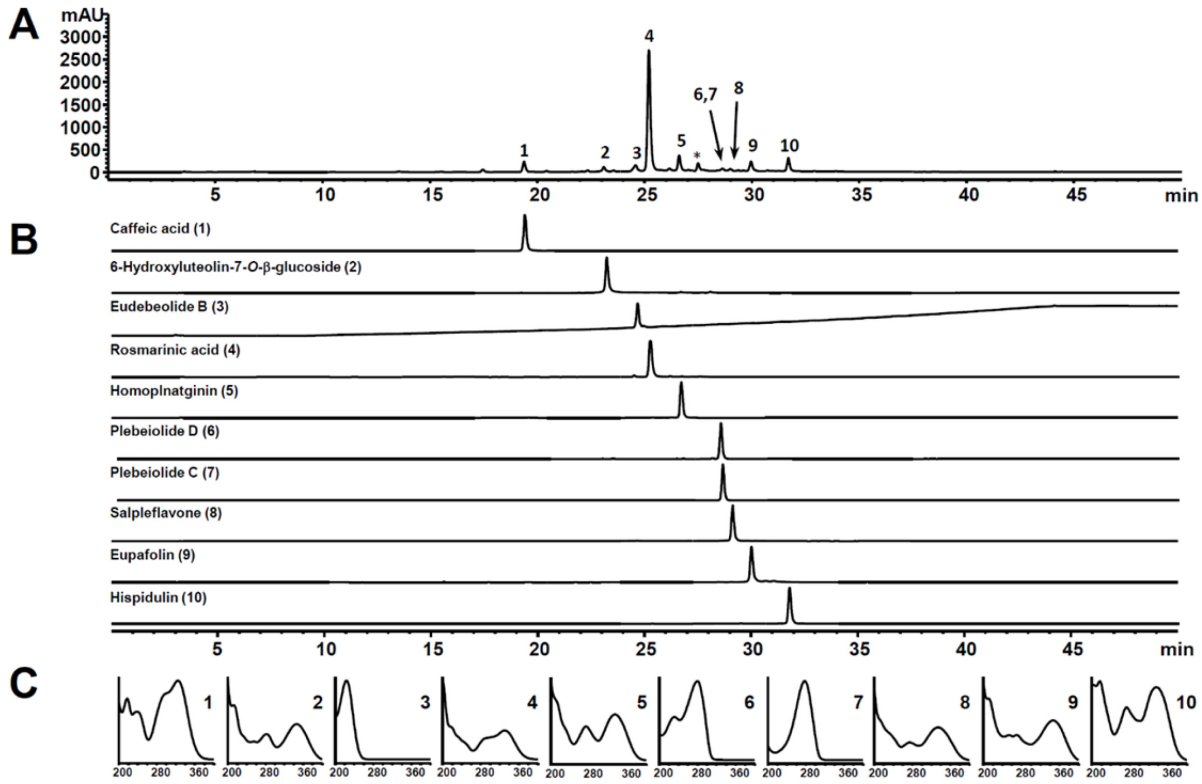

Fig. 4. HPLC chromatograms of *n*-BuOH soluble extract from *S. plebeian* (**A**), ten isolates (**B**) and UV spectra of compounds **1**–**10** generated by HPLC-PDA analysis (**C**).

an 1,3,4-trisubstitued benzene ring [δ_H 7.01 (1H, d, J = 2.1 Hz, H-2), 6.94 (1H, dd, J = 8.1, 2.1 Hz, H-6), 6.75 (1H, d, J = 8.1 Hz, H-5)] and signals of *trans* olefinic protons at [δ_H 7.38 (1H, d, J = 15.8 Hz, H-7), 6.16 (1H, d, J = 15.8 Hz, H-8)]. In the ^{13}C-NMR spectrum, 9 carbon signals were observed including a carboxyl group at δ_C 168.30. Therefore, the structure of compound **1** was determined to be caffeic acid.[5]

The ^{1}H-NMR spectrum of **2** showed an 1,3,4-trisubstitued benzene ring [δ_H 7.42 (1H, dd, J = 8.2, 2.3 Hz, H-6'), 7.40 (1H, d, J = 2.3 Hz, H-2'), 6.90 (1H, d, J = 8.2 Hz, H-5')], two singlets at δ_H 6.70 (1H, s, H-3), and 6.96 (1H, s, H-8). The ^{1}H-NMR spectrum indicated the presence of 6- or 8-hydroxyluteolin skeleton. Furthermore, an anomeric proton signal of sugar moiety was observed at δ_H 5.11 (1H, d, J = 7.3 Hz, H-1"). The sugar moiety was identified to be glucose from the ^{13}C-NMR spectrum, and it was connected to C-7 position of aglycone *via* the HMBC cross peak at δ_H 5.02 (H-1")/δ_C 151.30 (C-7). The singlet at δ_H 6.96 was determined to be H-8, because it was correlated with carbon signals at δ_C 151.30 (C-7), 149.01 (C-9), 130.43 (C-6) and 105.81 (C-10) in the HMBC experiment. Therefore, chemical structure of compound **2** was established to be 6-hydroxyluteolin 7-*O*-β-D-glucoside.[6]

The molecular formula of compound **3** ($C_{15}H_{18}O_4$) was determined from a molecular ion peak at *m/z* 261.1130 $[M-H]^-$ in the ESI-Q-TOF-MS. The ^{1}H-NMR showed the resonances of *cis*-olefinic group at [δ_H 6.87 (1H, d, J = 10.0 Hz, H-1), 5.91 (1H, d, J = 10.0 Hz, H-2)], two methine resonances at δ_H 2.56, 1.73, two methylene groups [δ_H 2.86 (dd, J = 13.6, 3.4 Hz, H-6a), 2.37-2.39 (2H, o, H-6b, 9a), 1.73 (1H, t, J = 12.5 Hz, H-5)] and three methyl groups (δ_H 1.82, 1.40, 1.22). The ^{13}C-NMR spectrum detected 15 carbon signals, including a carbonyl group at δ_C 202.85 (C-7) and an α,β-unsaturated-γ-lactone moiety at [δ_C 174.38 (C-12), 161.04 (C-7), 123.83 (C-11)], which indicated the presence of a sesquiterpene lactone skeleton. The complete assignments of methyl groups were determined by HMBC experiment which showed correlation peaks at δ_H 1.22 (15-CH_3)/δ_C 202.85 (C-3), 43.67 (C-4) and 51.74 (C-5); δ_H 1.40 (14-CH_3)/δ_C 161.04 (C-1), 51.74 (C-5), 49.1 (C-9) and 38.53 (C-10) and δ_H 1.82 (13-CH_3)/δ_C 161.04 (C-7), 123.83 (C-11) and 174.38 (C-12). These results demonstrated that compound **3** possessed a 3-oxo-8-hydroxyeudesman-1,7-dien-8,12-olide skeleton. The relative and absolute stereochemistry was deduced by NOESY and CD spectra, respectively. The NOESY spectrum showed correlations peaks δ_H 2.56

(H-4)/δ_H 1.40 (14-CH_3) and δ_H 1.73 (H-5)/δ_H 1.22 (15-CH_3) and the CD spectrum displayed negative Cotton effects at 217 nm (Δε -30.9) and 242.5 nm (Δε -16.5). From the above results, the structure of compound **3** was determined as (4*R*,5*R*,8*R*,10*R*)-3-oxo-8-hydroxyeudesman-1,7-dien-8,12-olide (eudebeiolide B).[7]

The molecular formula of compound **4** was deduced to be $C_{18}H_{16}O_8$ by ESI-Q-TOF-MS, showing a molecular ion peak $[M-H]^-$ at *m/z* 359.0767. The ^{1}H-NMR spectrum displayed the partial structure of a caffeic acid resonance close to compound **1**. The other signals were observed to be an 1,3,4-trisubstitued aromatic ring at δ_H 6.75 (1H, d, *J* = 2.1 Hz, H-2), 6.70 (1H, d, *J* = 8.1 Hz, H-5) and 6.61 (1H, dd, *J* = 8.1, 2.1 Hz, H-6), methine signal at δ_H 5.19 (1H, dd, *J* = 8.4, 4.3 Hz, H-8) and methylene group [δ_H 3.10 (1H, dd, *J* = 14.4, 4.3 Hz, H-7a), 3.01 (1H, dd, *J* = 14.3, 8.5 Hz, H-7b)], which was corresponding to a dihydroxycaffeic acid moiety. In the ^{13}C-NMR spectrum, 18 carbon resonances were observed corresponding to rosmarinic acid.[ref)] The stereochemistry of C-8 position was determined as *R*-form by an optical rotation value at $[\alpha]_D^{25}$= +33.2. Consequently, compound **4** was established to be (*R*)-rosmarinic acid.[8]

The ^{1}H-NMR spectrum of **5** showed resonances characteristic for hispidulin skeleton including an 1,4-disubstitued benzene ring [δ_H 7.95 (2H, d, *J* = 8.8 Hz, H-2', 6'), 6.94 (2H, d, *J* = 8.8 Hz, H-3', 5')], two singlets at δ_H 6.86 (1H, s, H-3), and 7.02 (H-8), and a methoxy signal [δ_H 3.77 (3H, s, 6-O$\underline{CH_3}$)]. In addition, a doublet derived from a sugar unit were found at δ_H 5.11 (1H, d, *J* = 7.3 Hz, H-1"). The ^{13}C-NMR detected 22 carbon resonances corresponding to a hispidulin and a glucose moieties. The connectivity of glucose confirmed by the HMBC correlation of δ_H 5.11 (H-1")/δ_C 156.46 (C-7). Therefore compound **5** was identified to be homoplantaginin (hispidulin 7-glucoside).[9]

The ^{1}H- and ^{13}C-NMR spectra of **6** were similar to those of **3** with differences in that carbon signal at δ_C 49.10 showed downfield shift to δ_C 116.91 and H-9 signals at δ_H 2.37-2.39 (1H, o, H-9a), 1.66 (1H, d, *J* = 13.4 Hz, H-9b) was changed to δ_H 5.92 (1H, s, H-9). In the same manner, the relative and absolute configuration of **6** was determined by NOESY and CD spectra, respectively. Collectively, compound **6** was (4*R*,5*R*,10*R*)-3-oxoeudesman-1,7,8-trien-8,12-olide (eudebeiolide D).[7]

The molecular formula of compound **7** was established to be $C_{17}H_{22}O_5$ on the basis of its ESI-Q-TOF-MS spectrum (*m/z* 307.1544 $[M+H]^+$). The ^{1}H and ^{13}C-NMR spectra showed the similar signals to those of **6** except for the absence of olefinic resonances and a ketone signal. In addition, two carbon signals characteristic for an acetoxyl group were observed at δ_C 173.08 and 21.31. In the HMBC experiment, long range correlation was observed between δ_H 4.97 (H-3) and δ_C 173.08 (C-3), which indicated that acetoxyl group was connected to C-3 position. Likewise, the stereochemistry of compound **7** was determined by NOESY and CD spectra. Therefore, compound **7** was identified as (1*R*,3*S*,4*R*,5*R*,10*R*)-3-acetyloxy 1-hydroxyeudesman-7,8-dien-8,12-olide (plebeiolide C).[10]

The ^{1}H-NMR spectrum of **8** was similar to that of compound **5**, except for an additional sinapic acid moiety including *trans*-olefinic signals at δ_H 7.35 (1H, d, *J* = 15.8 Hz, H-7'"), 6.17 (1H, d, *J* = 15.8 Hz, H-8'") and two methoxy groups [δ_H 3.60 (6H, s, 3'", 5'"-OC$\underline{H_3}$)] and a singlet at δ_H 6.32 (2H, s, H-2'", 6'"). The HMBC correlation between δ_H 4.80 (H-6") and δ_C 168.63 (C-9'") confirmed that sinapic acid was connected to C-6" position. Consequently, structure of compound **8** was determined to be salpleflavone.[11]

The molecular formula of compounds **9** and **10** was established to be $C_{16}H_{12}O_7$ and $C_{16}H_{12}O_6$ from molecular ion peaks at *m/z* 317.0661 $[M+H]^+$ and 299.0556 $[M-H]^-$, respectively. The ^{1}H-NMR spectra of compounds **9** and **10** were almost close to each other including two singlets [δ_H 6.88 (1H, d, *J* = 8.2 Hz, H-5'), 6.66 (1H, s, H-3) for **9**; 6.77 (1H, s, H-3), 6.58 (1H, s, H-8) for **10**] and a methoxy resonance [3.75 (3H, s, 6-OC$\underline{H_3}$) for **9** and **10**]. The difference was that compound **9** possessed a 1,3,4-trisubstitued benzene ring [δ_H 7.41 (1H, dd, *J* = 8.2, 2.2 Hz, H-6'), 7.39 (1H, d, *J* = 2.2 Hz, H-2'), 6.88 (1H, d, *J* = 8.2 Hz, H-5')], while a 1,4-disubstitued benzene ring [δ_H 7.95 (2H, d, *J* = 8.8 Hz, H-2', 6') and 6.94 (2H, d, *J* = 8.8 Hz, H-3', 5')] was observed in compound **10**. From the spectroscopic evidence and literature values, compounds **9** and **10** were elucidated to be eupafolin[12] and hispidulin.[13]

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