Phenolics and eudesmanolide from aged common sage exudate with sugar

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Abstract Nineteen compounds including one new flavanone were isolated from the juice of aged common sage exudate with sugar (ACSE). The isolated compounds were identified by NMR and MS analyses as levodopa methyl ester (**1**), 3,4-dihydroxybenzoic acid (**2**), (*S*)-8-hydroxy-4-hydroxy-phenylpropanoic acid (**3**), 4-hydroxybenzoic acid ethyl ester (**4**), *cis*-caffeic acid (**5**), *trans*-caffeic acid (**6**), esculetin (**7**), (*S*)-8-hydroxy-3,4-dihydroxy-phenylpropanoic acid ethyl ester (**8**), *cis*-rosmarinic acid (**9**), *trans*-rosmarinic acid (**10**), *trans*-rosmarinic acid methyl ester (**11**), 6-methoxy-7,8,3',5'-tetrahydroxyflavanone (**12**), nepetin (**13**), *trans*-caffeic acid ethyl ester (**14**), luteolin (**15**), *cis*-caffeic acid ethyl ester (**16**), 6-methoxynaringenin (**17**), 1 α -acetoxy-2-oxo-eudesman-3,7(11)-dien-8 β ,12-olide (**18**), and hispidulin (**19**). Compound **12** was isolated for the first time from nature and seven compounds (**1**, **3**, **4**, **7**, **8**, **14**, and **18**) were newly identified from common sage. Of them, 15 isolated phenolic compounds (**1–3**, **5–8**, **10–15**, **17**, and **19**) were detected in ACSE juice, while only **10** was detected in the fresh common sage.

Keywords *Salvia plebeia* · Common sage · Fermented juice · Flavanone · Eudesmanolide

Introduction

Aged products of plant exudates (APE) are manufactured by aging after food and medicinal materials (vegetables, fruits, herbs, etc.) are mixed and extracted with a high concentration of sugar [1–3]. These products are widely consumed in Korea due to the belief that they provide health benefits [1]. Several APE have been reported to exert beneficial biological properties, such as antioxidant, anti-obesity, and anticancer activities [3–5]. Nevertheless, information on the chemical constituents and biological effects of APE is much more limited than that of the original plant materials. In addition, scientists question whether APE are fermented by microorganisms at such high sugar concentrations. In fact, it has been reported that various microorganisms including yeasts are involved in the fermentation of plant exudates by sugar [6]. In particular, osmophilic yeasts, *Candida* sp., *Zygosaccharomyces* sp., and *Saccharomyces* sp. were dominant at high sugar concentrations during natural fermentation of plant materials, such as aloe, onion, *Prunus mume*, kale, and sea tangle [6]. Therefore, it is likely that microorganisms produce bioactive compounds and metabolize native compounds contained in raw materials by fermentation during the aging of APE.

Salvia plebeia R. Br. (Lamiaceae, common sage) is widely distributed in the Asian countries, of Korea, China, and Japan. This plant has been used in folk medicine for healing hepatitis, inflammation, and hemorrhoids [7]. Previous studies on the chemical constituents of common sage established the presence of flavonoids, terpenes, lignans, and caffeic acid derivatives [7–10]. Common sage is especially rich in flavonoids and phenolic acids. Several recent studies have reported that common sage exerts certain biological activities including liver and gastric

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protection, as well as antioxidant, antibacterial, anticancer, anti-inflammatory, anti-allergic, and anti-anaphylactic effects [11–14]. Therefore, the ingestion of common sage for the prevention of various diseases has recently received increased attention.

The aged common sage exudate by sugar (ACSE) is commonly consumed in Korea. However, the fermentation characteristics, as well as the chemical constituents and biological effects of ACSE have not yet been established. We hypothesized that fermentation during the aging process of ACSE would produce metabolites absent in fresh common sage. In our preliminary study [15], we confirmed that ACSE contained diverse compounds different from those found in fresh common sage through a chemical profiling study using high-pressure liquid chromatography (HPLC) analysis. The chemical analyses of the constituents of ACSE would provide important information concerning quality control and evaluation of the biological effects of ACSE. Therefore, we carried out the isolation and structural elucidation of the chemical constituents of ACSE.

In this study, 19 compounds [two *p*-hydroxybenzoic acid derivatives, eight phenylpropanoid derivatives, three rosmarinic acid derivatives, five flavonoids (one new compound), and one eudesmanolide] were isolated and structural-determined from ACSE. In addition, the presence of 15 isolated compounds in ACSE was confirmed by HPLC analysis.

Materials and methods

Materials and chemicals

Fermented common sage juice was provided from Saeselwon Company in Damyang County, South Korea. Briefly, fresh common sage was collected in June of 2013 in Damyang County, and the fresh leaf (8.5 kg) was mixed with sucrose (5.7 kg). The leaf was removed after 3 months and the ACSE juice was naturally aged in a sealed jar at room temperature for 9 months. Deuterated methanol (CD_3OD) and acetone- d_6 , with tetramethylsilane as an internal standard, were purchased from Merck Co. (Darmstadt, Germany). Methanol (MeOH), acetonitrile (MeCN), *n*-butanol (*n*-BuOH), and ethyl acetate (EtOAc) were obtained from Duksan (Ansan, Korea).

HPLC analysis of fresh common sage and its ACSE juice

ACSE juice (100 mL) was added to distilled water (250 mL) and successively partitioned with *n*-hexane (350 mL, 3 times), EtOAc (350 mL, 3 times), and water-saturated *n*-BuOH (BuOH, 350 mL, 3 times). Each layer

was concentrated with rotary evaporation. Meanwhile, a mixture of fresh common sage and sucrose (6:4, w/w) was homogenized and extracted with MeOH (1.2 L). The MeOH extract obtained after rotary evaporation was partitioned using the same method as for the ACSE juice. The layers obtained after the solvent fractionation of both samples were analyzed by octadecylsilane (ODS)-HPLC using an ODS-80Ts (TSK-gel, 5 μm , 4.6 mm $\times$ 250 mm, Tosoh, Tokyo, Japan). The compounds were eluted with a gradient system of 5% MeOH containing 2% acetic acid in $\text{H}_2\text{O} \rightarrow 100\%$ MeOH for 50 min. The flow rate was 1.0 mL/min, and the detection wavelength was 254 nm.

Extraction and isolation

ACSE juice (4 L) was added to distilled water (6 L) and partitioned with *n*-hexane (10 L, 3 times). The aqueous layer was successively partitioned with EtOAc (10 L, 3 times) and BuOH (10 L, 3 times). After concentration, the EtOAc layer (11.135 g) was loaded onto a Diaion HP-20 column (5.0 cm $\times$ 83 cm; Mitsubishi Chemical Industrial, Tokyo, Japan) and eluted by a step-wise system of 10% MeOH in $\text{H}_2\text{O} \rightarrow 100\%$ MeOH (each step, 3.5 L). Twenty fractions (A–T) were obtained by the analysis of ODS-HPLC (column: ODS-80Ts, 4.6 mm $\times$ 250 mm Tosoh; gradient: 30% MeOH containing 2% acetic acid in $\text{H}_2\text{O} \rightarrow 70\%$ MeOH in H_2O for 50 min; flow rate: 1.0 mL/min; wavelength: 254 nm). Fractions C + D, F, L, M, P, and S were purified by ODS-HPLC using a Shim-pack Prep-ODS (H) Kit (10 μm , 20 cm $\times$ 250 mm; Shimadzu, Kyoto, Japan; flow rate: 9.9 mL/min; wavelength: 280 nm). Fraction C + D (223.2 mg) was subjected to ODS-HPLC (gradient: 10% MeOH in H_2O with pH adjusted to 2.65 by TFA $\rightarrow$ 25% MeOH in H_2O for 40 min) to obtain **1** (t_R 30.6 min, 4.0 mg). Compounds **2** (t_R 19.0 min, 2.8 mg) and **3** (t_R 23.0 min, 3.4 mg) were isolated from fraction F (108.1 mg) by ODS-HPLC (gradient: 15% MeOH in H_2O with pH adjusted to 2.65 by TFA $\rightarrow$ 40% MeOH in H_2O for 40 min). Compounds **4** (t_R 3.2 min, 2.6 mg) and **5** (t_R 6.0 min, 4.2 mg) were isolated from fraction L (207.9 mg) by ODS-HPLC (gradient: 20% MeOH in H_2O with pH adjusted to 2.65 by TFA $\rightarrow$ 60% MeOH in H_2O for 25 min). Compounds **6** (t_R 16.2 min, 30.8 mg), **9** (t_R 27.9 min, 3.67 mg), **10** (t_R 29.5 min, 5.16 mg), and **11** (t_R 29.8 min, 5.06 mg) with subfraction M2 (t_R 18.6 min, 5.6 mg) were isolated from fraction M (2113.2 mg) by ODS-HPLC (gradient: 25% MeOH in H_2O with pH adjusted to 2.65 by TFA $\rightarrow$ 65% MeOH in H_2O for 40 min). Compounds **7** (t_R 14.3 min, 1.1 mg) and **8** (t_R 19.9 min, 1.2 mg) were isolated from subfraction M2 (5.6 mg) by ODS-HPLC using an ODS-80Ts column (TSK-gel, 4.6 mm $\times$ 250 mm, Tosoh; gradient: 25% MeOH in H_2O with pH adjusted to 2.65 by TFA $\rightarrow$ 65%

MeOH in H₂O for 40 min; flow rate: 1.0 mL/min; wavelength: 254 nm). Compounds **12** (t_R 12.6 min, 52.1 mg), **13** (t_R 14.7 min, 1.0 mg), and **14** (t_R 15.2 min, 50.4 mg), among with subfraction P4 (t_R 18.1 min, 30.6 mg) from fraction P (359.8 mg) and **17** (t_R 16.7 min, 3.0 mg) and **18** (t_R 23.5 min, 3.0 mg) from fraction Q (156.0 mg) were purified by ODS-HPLC (gradient: 50% MeOH in H₂O with pH adjusted to 2.65 by TFA → 80% MeOH for 40 min). Compounds **15** (t_R 16.3 min, 3.0 mg) and **16** (t_R 18.4 min, 0.9 mg) was isolated from subfraction P4 (30.6 mg) by ODS-HPLC (column: μ Bondapak C18, 10 μ m, 7.8 mm $\times$ 300 mm, Waters, USA; gradient: 100% H₂O with pH adjusted to 2.65 by TFA → 60% MeOH for 40 min; flow rate, 5.0 mL/min; wavelength: 254 nm). Compound **19** (t_R 14.2 min, 2.6 mg) was isolated from fraction Q (97.7 mg) by ODS-HPLC (gradient: 30% MeOH in H₂O with pH adjusted to 2.65 by TFA → 80% MeOH in H₂O for 25 min).

Structural analysis

The nuclear magnetic resonance (NMR) spectra of **1–19** were recorded using ^{unity}INOVA 500 and 600 spectrometers (Varian, Walnut Creek, CA, USA). All mass spectra were recorded on a hybrid ion-trap time-of-flight mass spectrometer (LCMS-IT-TOF, Shimadzu) equipped with an electrospray ionization (ESI) source (ESIMS).

Compound 1 ¹H-NMR (500 MHz, CD₃OD) δ 46.66 (1H, br. s, H-2), 6.67 (1H, d, J = 7.0 Hz, H-5), 6.52 (1H, dd, J = 7.0, 2.0 Hz, H-6), 2.77 (1H, dd, J = 11.5, 4.0 Hz, H-7a), 2.89 (1H, dd, J = 11.5, 6.0 Hz, H-7b), 4.29 (1H, dd, J = 6.0, 4.0 Hz, H-8), 3.65 (3H, s, -OCH₃); ESIMS (negative) m/z 153.05 [M-H]⁻.

Compound 2 ¹H-NMR (500 MHz, CD₃OD) δ 7.43 (1H, br. s, H-2), 6.79 (1H, d, J = 7.5 Hz, H-5), 7.41 (1H, dd, J = 7.5, 2.0 Hz, H-6); ESIMS (negative) m/z 153.05 [M-H]⁻.

Compound 3 ¹H-NMR (500 MHz, CD₃OD) δ 7.07 (2H, d, J = 8.0 Hz, H-2 and H-6), 6.70 (2H, d, J = 8.0 Hz, H-3 and H-5), 2.81 (1H, dd, J = 14.0, 4.5 Hz, H-7a), 3.01 (1H, dd, J = 14.0, 7.5 Hz, H-7a), 4.27 (1H, dd, J = 7.5, 4.5 Hz, H-8); ESIMS (negative) m/z 181.0 [M-H]⁻.

Compound 4 ¹H-NMR (500 MHz, CD₃OD) δ 7.87 (2H, d, J = 8.5 Hz, H-2 and H-6), 6.81 (2H, d, J = 8.5 Hz, H-3 and H-5), 4.13 (2H, m, H-1'), 1.25 (3H, J = 7.0 Hz, H-2'); ESIMS (negative) m/z 165.05 [M-H]⁻.

Compound 5 ¹H-NMR (500 MHz, CD₃OD) δ 7.03 (1H, br. s, H-2), 6.76 (1H, d, J = 8.5 Hz, H-5), 6.93 (1H, br. d, J = 8.5 Hz, H-6), 7.75 (1H, d, J = 9.5 Hz, H-7), 6.16 (1H, d, J = 9.5 Hz, H-8); ESIMS (negative) m/z 179.0 [M-H]⁻.

Compound 6 ¹H-NMR (500 MHz, CD₃OD) δ 7.03 (1H, d, J = 2.0 Hz, H-2), 6.78 (1H, d, J = 8.0 Hz, H-5), 6.94 (1H, dd, J = 8.0, 2.0 Hz, H-6), 7.53 (1H, d, J = 16.0 Hz, H-7), 6.22 (1H, d, J = 16.0 Hz, H-8); ESIMS (negative) m/z 179.0 [M-H]⁻.

Compound 7 ¹H-NMR (500 MHz, CD₃OD) δ 6.17 (1H, d, J = 9.0 Hz, H-3), 7.78 (1H, d, J = 9.5 Hz, H-4), 6.94 (1H, s, H-5), 6.75 (1H, s, H-8); ESIMS (negative) m/z 176.85 [M-H]⁻.

Compound 8 ¹H-NMR (500 MHz, CD₃OD) δ 6.67 (1H, d, J = 2.4 Hz, H-2), 6.66 (1H, dd, J = 7.8 Hz, H-5), 6.53 (1H, dd, J = 7.8, 2.4 Hz, H-6), 2.88 (1H, dd, J = 13.8, 5.4 Hz, H-7a), 2.77 (1H, dd, J = 13.8, 7.2 Hz, H-7b), 4.26 (1H, dd, J = 7.2, 5.4 Hz, H-8), 4.13 (2H, m, H-1'), 1.22 (3H, t, J = 7.2 Hz, H-2'); ¹³C-NMR (125 MHz, CD₃OD) δ 130.0 (C-1), 117.8 (C-2), 146.3 (C-3), 145.2 (C-4), 116.3 (C-5), 122.0 (C-6), 41.3 (C-7), 73.5 (C-8), 175.7 (C-9), 62.1 (C-1'), 14.6 (C-2'); ESIMS (negative) m/z 225.05 [M-H]⁻.

Compound 9 ¹H-NMR (600 MHz, CD₃OD) δ 6.70 (1H, d, J = 2.0 Hz, H-2), 6.68 (1H, d, J = 8.0 Hz, H-5), 6.53 (1H, dd, J = 8.0, 2.0 Hz, H-6), 2.96 (1H, d, J = 14.5, 7.0 Hz, H-7a), 3.05 (1H, d, J = 14.5, 4.3 Hz, H-7b), 5.12 (1H, dd, J = 8.5, 4.3 Hz, H-8), 7.38 (1H, d, J = 2.0 Hz, H-2'), 6.77 (1H, d, J = 8.0 Hz, H-5'), 7.04 (1H, dd, J = 8.0, 2.0 Hz, H-6'), 6.82 (1H, d, J = 13.0 Hz, H-7'), 5.78 (1H, d, J = 13.0 Hz, H-8'); ESIMS (negative) m/z 359.05 [M-H]⁻.

Compound 10 ¹H-NMR (600 MHz, CD₃OD) δ 6.74 (1H, d, J = 2.0 Hz, H-2), 6.69 (1H, d, J = 8.0 Hz, H-5), 6.61 (1H, dd, J = 8.0, 2.0 Hz, H-6), 3.00 (1H, dd, J = 14.0, 8.5 Hz, H-7a), 3.10 (1H, dd, J = 14.0, 4.3 Hz, H-7b), 5.18 (1H, dd, J = 8.3, 4.3 Hz, H-8), 7.04 (1H, d, J = 2.0 Hz, H-2'), 6.77 (1H, d, J = 8.0 Hz, H-5'), 6.95 (1H, dd, J = 8.0, 2.0 Hz, H-6'), 7.54 (1H, d, J = 16.0 Hz, H-7'), 6.26 (1H, d, J = 16.0 Hz, H-8'); ESIMS (negative) m/z 359.05 [M-H]⁻.

Compound 11 ¹H-NMR (600 MHz, CD₃OD) δ 6.76 (1H, d, J = 2.0 Hz, H-2), 6.72 (1H, d, J = 8.0 Hz, H-5), 6.56 (1H, dd, J = 8.0, 2.0 Hz, H-6), 2.96 (1H, d, J = 14.5, 7.0 Hz, H-7a), 2.73 (1H, d, J = 14.5, 4.3 Hz, H-7b), 5.42 (1H, dd, J = 8.5, 4.3 Hz, H-8), 7.53 (1H, d, J = 2.0 Hz, H-2'), 6.90 (1H, d, J = 8.0 Hz, H-5'), 7.21 (1H, br. d, J = 8.0, 2.0 Hz, H-6'), 7.83 (1H, d, J = 16.0 Hz, H-7'), 6.34 (1H, d, J = 16.0 Hz, H-8'), 3.67 (3H, s, -OCH₃); ESIMS (negative) m/z 373.1 [M-H]⁻.

Compound 12 ¹H- and ¹³C-NMR data are shown in Table 1; HR-ESIMS (negative) m/z 317.0660 [M-H]⁻ (calculated for C₁₆H₁₂O₇, m/z 317.0661, +0.1 mDa).

Table 1 ^1H - (500 MHz) and ^{13}C - (125 MHz) NMR data of **12** in acetone- d_6

Position	δ_{H} (int., mult., J in Hz)	δ_{C}
2	5.38 (1H, dd, 13.0, 3.0)	80.4
3a	3.14 (1H, dd, 17.0, 13.0)	43.7
3b	2.73 (1H, dd, 17.0, 3.0)	
4	—	198.2
5	6.00 (1H, s)	95.7
6	—	129.9
7	—	156.4
8	—	156.1
9	—	159.6
10	—	103.4
1'	—	131.6
2'	6.86 (1H, br. s)	116.1
3'	—	146.1
4'	7.03 (1H, s)	114.8
5'	—	146.4
6'	6.86 (1H, br. s)	119.2
-OCH ₃	3.77 (3H, s)	60.8

Compound 13 ^1H -NMR (500 MHz, CD_3OD) δ 6.55 (2H, s, H-3 and H-8 overlapping signals), 7.38 (1H, br. s, H-2'), 6.90 (1H, d, J = 8.4 Hz, H-5'), 7.39 (1H, dd, J = 8.4, 1.8 Hz, H-6'), 3.88 (3H, s, -OCH₃); ^{13}C -NMR (125 MHz, CD_3OD) δ 166.6 (C-2), 103.6 (C-3), 184.4 (C-4), 154.8 (C-5), 133.0 (C-6), 158.9 (C-7), 95.4 (C-8), 154.2 (C-9), 105.9 (C-10), 123.8 (C-1'), 114.2 (C-2'), 147.2 (C-3'), 151.2 (C-4'), 116.9 (C-5'), 120.4 (C-6'), 61.1 (-OCH₃); ESIMS (negative) m/z 315.0 $[\text{M}-\text{H}]^-$.

Compound 14 ^1H -NMR (500 MHz, CD_3OD) δ 7.03 (1H, br. s, H-2), 6.77 (1H, d, J = 8.0 Hz, H-5), 6.91 (1H, br. d, J = 8.0 Hz, H-6), 7.51 (1H, d, J = 16.0 Hz, H-7), 6.22 (1H, d, J = 16.0 Hz, H-8), 4.17 (2H, m, H-1'), 1.27 (3H, t, J = 7.0 Hz, H-2'); ESIMS (negative) m/z 206.9 $[\text{M}-\text{H}]^-$.

Compound 15 ^1H -NMR (500 MHz, CD_3OD) δ 6.54 (1H, s, H-3), 6.20 (1H, br. s, H-6), 6.44 (1H, br. s, H-8), 7.38 (1H, br. s, H-2'), 6.89 (1H, d, J = 8.4 Hz, H-5'), 7.39 (1H, dd, J = 8.4, 1.8 Hz, H-6'); ^{13}C -NMR (125 MHz, CD_3OD) δ 166.6 (C-2), 103.6 (C-3), 184.4 (C-4), 154.8 (C-5), 100.2 (C-6), 166.1 (C-7), 95.1 (C-8), 163.3 (C-9), 104.0 (C-10), 123.8 (C-1'), 115.2 (C-2'), 146.9 (C-3'), 149.6 (C-4'), 115.4 (C-5'), 120.4 (C-6'); ESIMS (negative) m/z 286.9 $[\text{M}-\text{H}]^-$.

Compound 16 ^1H -NMR (600 MHz, CD_3OD) δ 7.03 (1H, d, J = 2.0 Hz, H-2), 6.73 (1H, d, J = 8.4 Hz, H-5), 7.01 (1H, dd, J = 8.4, 2.0 Hz, H-6), 7.53 (1H, d,

J = 13.0 Hz, H-7), 6.25 (1H, d, J = 13.0 Hz, H-8), 4.16 (2H, dd, J = 14.4, 7.2 Hz, H-1'), 1.31 (3H, t, J = 7.2 Hz, H-2'); ^{13}C -NMR (150 MHz, CD_3OD) δ 127.8 (C-1), 115.2 (C-2), 145.8 (C-3), 148.3 (C-4), 116.9 (C-5), 123.0 (C-6), 144.8 (C-7), 115.9 (C-8), 168.6 (C-9), 61.3 (C-1'), 14.6 (C-2'); ESIMS (negative) m/z 206.9 $[\text{M}-\text{H}]^-$.

Compound 17 ^1H -NMR (500 MHz, CD_3OD) δ 5.32 (1H, br. d, J = 13.2 Hz, H-2), 2.70 (1H, dd, J = 17.3, 13.2 Hz, H-3a), 3.11 (1H, dd, J = 17.3, 2.8 Hz, H-3b), 5.97 (1H, s, H-8), 7.31 (2H, d, J = 8.5 Hz, H-2' and H-6'), 6.82 (2H, d, J = 8.5 Hz, H-3' and H-5'), 3.79 (3H, s, -OCH₃); ESIMS (negative) m/z 301.05 $[\text{M}-\text{H}]^-$.

Compound 18 ^1H - and ^{13}C -NMR data are shown in Table 2; ESIMS (negative) m/z 302.95 $[\text{M}-\text{H}]^-$.

Compound 19 ^1H -NMR (600 MHz, CD_3OD) δ 6.95 (1H, s, H-3), 6.93 (1H, s, H-8), 7.96 (2H, d, J = 8.4 Hz, H-2' and H-6'), 7.26 (2H, d, J = 8.4 Hz, H-3' and H-5'), 3.98 (3H, s, -OCH₃); ^{13}C -NMR (125 MHz, CD_3OD) δ 164.9 (C-2), 103.8 (C-3), 183.5 (C-4), 154.5 (C-5), 132.9 (C-6), 159.2 (C-7), 95.5 (C-8), 154.0 (C-9), 105.7 (C-10), 122.6 (C-1'), 129.3 (C-2' and C-6'), 117.2 (C-3' and C-5'), 163.0 (C-4'), 60.6 (-OCH₃); ESIMS (negative) m/z 299.05 $[\text{M}-\text{H}]^-$.

Table 2 ^1H - (600 MHz) and ^{13}C - (150 MHz) NMR data of compound **18** in CD_3OD

Position	δ_{H} (int., mult., J in Hz)	δ_{C}
1	6.00 (1H, s)	80.4
2	—	194.6
3	5.27 (1H, s)	126.5
4	—	164.0
5	3.33 (1H, m)	49.7
6a	3.24 (1H, dd, 12.9, 3.6)	25.6
6b	2.50 (1H, dd, 12.9, 6.6)	
7	—	122.4
8	5.00 (1H, m)	79.0
9a	2.52 (1H, m)	43.1
9b	0.90 (1H, t, 7.2)	
10	—	44.2
11	—	163.0
12	—	172.0
13	1.87 (3H, s)	8.4
14	1.09 (3H, s)	12.3
15	2.09 (3H, s)	22.0
1'	—	176.8
2'	2.19 (3H, s)	20.6

HPLC analysis of the isolated compounds in MeOH extract of fresh common sage and ACSE juice

The fresh common sage (15.4 g fresh wt.) was homogenized with 100% MeOH (150 mL), extracted at room temperature for 24 h, and vacuum filtered through No. 2 filter paper (Whatman). The remaining residue was extracted again with 80% MeOH (150 mL) using the same extraction procedure described above. The 100 and 80% MeOH solutions were combined and concentrated with rotary evaporation. The concentrates (3.2 g) were suspended in distilled water (70 mL) and partitioned with *n*-hexane (70 mL, 3 times) and EtOAc (70 mL, 3 times), successively. After concentration, the EtOAc layer (2 mg/mL) was dissolved in MeOH. The ACSE juice (7 mL, 15.4 g fresh common sage eq. wt.) was added to distilled water (63 mL). The solvent fractionation of the ACSE juice was performed under the same conditions used for that of the fresh common sage MeOH extract. The EtOAc layers were injected to ODS-HPLC using Shiseido C18 (UG 120, 5 μ m, 4.6 mm $\times$ 250 mm, Tokyo, Japan). The compounds were eluted using a gradient system of 10% MeOH containing 2% acetic acid in H₂O to 70% MeOH in H₂O over 40 min and holding at 70% MeOH for 45 min. The flow rate was 1.0 mL/min, and the detection wavelength was 254 nm. The 15 phenolic compounds isolated in this study were used as external standards.

Results and discussion

Isolation and identification of compounds from the EtOAc layer of ACSE juice

To compare the chemical constituents of fresh common sage and ACSE juice, the MeOH extract of fresh common sage + sucrose and ACSE juice were successively solvent fractionated with *n*-hexane, EtOAc, and BuOH. These layers were analyzed by analytical scale ODS-HPLC using an ODS-80Ts column and a gradient system of 5% MeOH containing 2% acetic acid in H₂O $\rightarrow$ 100% MeOH. The HPLC results showed that the compounds contained in ACSE differed significantly from those of fresh common sage + sucrose (data not shown). In particular, new peaks were observed on the HPLC chromatograms of the *n*-hexane and EtOAc layers of ACSE. The HPLC chromatogram of the BuOH layer of ACSE was very similar to that of fresh common sage + sucrose. These results indicate that various compounds are likely being produced during the aging process of ACSE, and that diverse compounds produced during aging are mainly present in the EtOAc layer of the ACSE juice. Therefore, we purified and

isolated the chemical constituents from the EtOAc layer of ACSE juice.

The ACSE juice (4 L) was successively solvent fractionated with *n*-hexane and EtOAc to afford the EtOAc layer. The concentrated EtOAc layer (5.17 g) was chromatographed on a Diaion HP-20 column (step-wise system: 10% MeOH $\rightarrow$ 100% MeOH). The resulting 20 fractions (A-T) were grouped by chemical profiles using ODS-HPLC. Nineteen compounds (**1**–**19**) were isolated from fractions C + D, F, L, M, P, Q, and S by ODS-HPLC purification under various HPLC conditions. The isolation and purification procedures for **1**–**19** are summarized in Fig. 1. Of them, 17 compounds were identified as 3,4-dihydroxybenzoic acid (**2**) [16], (*S*)-8-hydroxy-4-hydroxyphenylpropanoic acid (**3**) [17], *cis*-caffeic acid (**5**) [18], *trans*-caffeic acid (**6**) [18], esculetin (**7**) [19], *cis*-rosmarinic acid (**9**) [19], *trans*-rosmarinic acid (**10**) [19], *trans*-rosmarinic acid methyl ester (**11**) [19], nepetin (**13**) [10], *trans*-caffeic acid ethyl ester (**14**) [20], luteolin (**15**) [21], *cis*-caffeic acid ethyl ester (**16**) [22], 6-methoxynaringenin (**17**) [10], and hispidulin (**19**) [10] (Fig. 2) by comparison of their NMR and MS data to those previously reported. Seven compounds (**9**–**11**, **13**, **14**, **16** and **17**) were also confirmed by 2D-NMR experiments. Three compounds were determined by MS and 1D- and 2D-NMR experiments to be levodopa methyl ester (**1**), 4-hydroxybenzoic acid ethyl ester (**4**), and (*S*)-8-hydroxy-3,4-dihydroxyphenylpropanoic acid ethyl ester (**8**).

Structural-elucidation of isolated compounds **12** and **18**

The ESI–MS (negative) spectrum of **12** revealed a deprotonated ion peak at m/z 317.06 [M–H][–], which was indicative of a molecular weight of 318. In addition, the molecular formula (C₁₆H₁₂O₇) of **12** was determined by the deprotonated peak [M–H][–] at m/z 317.0660 (calculated m/z 317.0661) in the HR–ESI–MS (negative) spectrum. The ¹H- and ¹³C-NMR spectra of **12** indicated the presence of a typical flavanone. The ¹H-NMR spectrum of **12** showed four singlet proton signals assigned to the benzene ring at δ 6.00 (1H, s, H-5), 6.86 (2H, br. s, H-2' and H-6') and 6.86 (1H, br. s, H-4'), one *sp*³ methine proton signal at δ 5.38 (1H, dd, J = 13.0, 3.0 Hz, H-2), and methylene proton signals at δ 3.14 (1H, dd, J = 17.0, 13.0 Hz, H-3a) and 2.73 (1H, dd, J = 17.0, 3.0 Hz, H-3b). A methoxy proton signal at δ 3.77 (3H, s, –OCH₃) was also observed (Table 1). The ¹H-NMR structural assignment of **12** was supported by the presence of 16 carbon signals, including one carbonyl carbon at δ 198.2 (C-4) in the ¹³C-NMR spectrum (Table 1). The MS and 1D-NMR results suggested that **12** was a tetrahydroxyflavanone possessing a methoxy group. Furthermore, the complete structure of **12**

Fig. 1 Procedure used to isolate compounds 1–19 from the fermented common sage juice

Fig. 2 Chemical structures of the isolated compounds 1–19

was determined by ^1H - ^1H COSY, HSQC, NOE, and HMBC experiments. The correlations of H-2' to C-4' and C-6', H-4' to C-2' and C-6', and H-6' to C-2' and C-4' in the HMBC spectrum confirmed the presence of three B ring protons of a flavanone skeleton (Fig. 3). Therefore, the

remaining methoxy and two hydroxyl groups are located on the A-ring, which was confirmed by HMBC and NOE experiments. The observation of the HMBC correlations of H-2 and H-5 to C-4 was indicative of the C-5 proton in a flavanone structure. The enhancement of H-5 by irradiation

Fig. 3 HMBC (arrows) and NOE/NOESY (dotted lines) correlations of the isolated compounds **12** and **18**

of the methoxy proton at δ 3.77 (3H, s, $-\text{OCH}_3$) in the NOE experiment indicated that the methoxy group was connected to the C-6 of 7,8,3',5'-tetrahydroxyflavanone (Fig. 2). Therefore, the planar structure of compound **12** was unambiguously determined to be 6-methoxy-7,8,3',5'-tetrahydroxyflavanone (Fig. 2). The absolute stereochemistry for 3,5-dihydroxybenzene group in C-2 of **12** should be further determined by analysis of circular dichroism or X-ray crystallography.

The ESI-MS (negative) spectrum of **18** showed a pseudomolecular ion peak at m/z 302.95 $[\text{M}-\text{H}]^-$, indicating a molecular weight of 304. The ^1H -NMR spectrum of **18** exhibited the presence of one double bond proton signal at δ 5.27 (H-3), and two methylene proton signals at δ 3.24 (H-6a), 2.50 (H-6b), 2.52 (H-9a), and 0.90 (H-9b) (Table 2). Additionally, three methine proton signals at δ 6.00 (H-1), 3.33 (H-5), and 5.00 (H-8) and four methyl proton signals at δ 1.87 (H-13), 1.09 (H-14), 2.09 (H-15), and 2.19 (H-1') were observed. The ^1H -NMR structural assignment of **18** was supported by the presence of 17 carbon signals, including three carbonyl carbons at δ 194.6 (C-2), 172.0 (C-12), and 176.8 (C-1'), in the ^{13}C -NMR spectrum (Table 2). The presence of three quaternary carbons of double bond at δ 164.0 (C-4), 122.4 (C-7), and 163.0 (C-11), and one quaternary sp^3 carbon at δ 44.2 (C-10), was also confirmed by the HSQC experiment. Among with the molecular formula of **18**, the chemical shifts of four sp^2 quaternary carbons indicated the presence of one ketone carbon at δ 194.6 (C-2) and two carboxylic groups with the signals at δ 172.0 (C-12) and 176.8 (C-1'). The MS and 1D-NMR results suggested that **18** was eudesmanolide with an acetyl group. The complete structure of **18** was determined by ^1H - ^1H COSY, HSQC, NOESY, and HMBC experiments. The HMBC correlations were indicative of eudesma-3(4),7(11)-dien-8,12-olide and an acetyl group. In particular, a correlation of H-1 at δ 6.00 to C-1' at δ 176.8

was observed in the HMBC spectrum, indicating that the acetyl group was esterified with C-1 of eudesma-3(4),7(11)-dien-8,12-olide (Fig. 3). The observation of NOESY correlations of H-1 at δ 6.00 and H-5 at δ 3.33 as well as H-8 at δ 5.00 and H-14 at δ 1.09 indicated that H-1 and H-5 had the same configuration, and those of H-8 and H-14 configuration were different (Fig. 3). Therefore, the structure of compound **18** was unambiguously determined to be 1 α -acetoxy-2-oxo-eudesman-3,7(11)-dien-8 β ,12-olide (Fig. 2).

Occurrence of the isolated phenolic compounds in fresh common sage and ACSE juice by HPLC analysis

To confirm the occurrence of the isolated compounds in fresh common sage + sucrose and ACSE juice, the EtOAc layers were analyzed by an analytical scale ODS-HPLC using a Shiseido C18 column and a gradient system of 10% MeOH containing 2% acetic acid in $\text{H}_2\text{O} \rightarrow 70\%$ MeOH in H_2O . For the caffeic acids (**5** and **6**), rosmarinic acids (**9** and **10**), and caffeic acid ethyl esters (**14** and **16**), both the *trans* and *cis* isomers were present. The *cis* forms (**5**, **9**, and **16**) might be respectively produced as artifact from the respective *trans* forms (**6**, **10**, and **14**) during the purification and isolation procedures. Therefore, 15 isolated phenolic compounds, namely **1**–**3**, **5**–**8**, **10**–**15**, **17**, and **19** (excluding **5**, **9**, **16**, and **18**) were used as external standards. Detection of these 15 compounds on the HPLC chromatogram of the ACSE juice indicated they were also present in ACSE juice [Fig. 4(B)]. Of the 15 isolated compounds, only compound **10** was detected on the HPLC chromatogram of the fresh common sage + sucrose [Fig. 4(A)]. Caffeic acid (**6**), esculetin (**7**), rosmarinic acid (**10**), nepetin (**13**), luteolin (**15**), 6-methoxynaringenin (**17**), and hispidulin (**19**) have been previously found in common

Fig. 4 HPLC chromatograms for the EtOAc layers of (A) fresh common sage + sucrose MeOH extract and (B) the ACSE juice and (C) external standards of phenolic compounds. **1:** levodopa methyl ester; **2:** 3,4-dihydroxybenzoic acid; **3:** (*S*)-8-hydroxy-4-hydroxy-phenylpropanoic acid; **4:** 4-hydroxybenzoic acid ethyl ester; **6:** *trans*-caffeic acid; **7:** esculetin; **8:** (*S*)-8-hydroxy-3,4-dihydroxy-phenylpropanoic acid ethyl ester; **10:** *trans*-rosmarinic acid; **11:** *trans*-rosmarinic acid methyl ester; **12:** 6-methoxy-7,8,3',5'-tetrahydroxyflavanone; **13:** nepetin; **14:** *trans*-caffeic acid ethyl ester; **15:** luteolin; **17:** 6-methoxynaringenin; **19:** hispidulin

sage [8–10]. Therefore, most of the isolated compounds might be present in small amounts in common sage, and/or produced during the aging of the common sage exudate with sugar.

Of the compounds isolated from the ACSE juice, five compounds (**1**, **4**, **8**, **11**, and **14**) were in the methyl- or ethyl-esterified forms. Such compounds are widely present in foods [23]. The carboxylic acids of these compounds could be esterified with MeOH, EtOH, or EtOAc under acidic conditions [24, 25]. Therefore, these compounds could likely be produced as artifacts during the extraction and purification steps. Methyl-esterified forms (**11**), as well as ethyl-esterified forms (**1**, **4**, **8**, and **14**), were detected in the ACSE juice [Fig. 4(B)], whereas they were not detected in the fresh common sage [Fig. 4(A)]. Previous studies have reported that phenolic acids are esterified by microorganisms during fermentation [23, 26]. Therefore, the presence of **1**, **4**, **8**, **11**, and **15** in the ACSE juice was definitively verified and might be produced by fermentation during its aging.

Flavonoids are widely distributed as both free and glycoside forms in the plant kingdom, and their glycosides are hydrolyzed during fermentation [27, 28]. Flavonoids **13**, **15**, **17**, and **19** identified in this study and their glycosides have already been found in common sage [8–10]. Here we confirmed the presence of the flavonoid aglycones **13**, **15**, **17**, and **19** in the ACSE juice [Fig. 4(B)]. Their presence could not be confirmed in fresh common sage in this study, though their aglycone and glycoside forms are known to be in fresh common sage [8–10]. It is suggested that flavonoid

aglycones **13**, **15**, **17**, and **19** may be present in common sage at concentrations near the limit of detection of the HPLC method. In addition, these flavonoid aglycones might be produced from their glycosides by enzymatic hydrolysis from fermentation during ACSE manufacturing. A new flavonoid **12** isolated in this study was detected in the ACSE juice [Fig. 4(B)], but not in fresh common sage [Fig. 4(A)]. Therefore, compound **12** might exist as a glycoside in common sage.

In this study, 19 compounds, including two *p*-hydroxybenzoic acid derivatives (**2** and **4**), eight phenylpropanoid derivatives (**1**, **3**, **5–8**, **14**, and **16**), three rosmarinic acid derivatives (**9–11**), five flavonoids (**12**, **13**, **15**, **17**, and **19**), and one eudesmanolide (**18**), were isolated and identified from the ACSE (Fig. 2). Previous studies have reported the presence of phenylpropanoid derivatives, rosmarinic acid derivatives, flavonoids and their glycosides, and eudesmanolide in common sage [8–10]. However, to the best of our knowledge, compounds **1**, **3**, **4**, **7**, **8**, **14**, **18**, and **19** were newly identified from common sage and compound **12** was isolated for the first time from nature. In addition, we confirmed the occurrence of 15 phenolic compounds (**1–4**, **6–8**, **10–15**, **17**, and **19**) in the ACSE juice. To understand what occurs in common sage exudate during the ACSE manufacturing, further chemical profiling will be conducted using the 15 phenolic compounds isolated in this study as external standards.

Diverse previous studies have reported that phenolic compounds, including flavonoids, exhibit various biological effects, such as antioxidant, anti-inflammatory, and

anticancer activities [29–31]. Additionally, several eudesmanolides, including compounds **18** identified in this study, have anti-inflammatory, anti-tumor, and anticancer properties [32–34]. Therefore, phenolics and eudesmanolides could partially contribute to the beneficial health effects of ACSE juice. Therefore, this investigation may provide important information in understanding the biological effects of ACSE juice. However, high sugar intake can cause the health risks, such as obesity and diabetes [35]. Because the ACSE juice is comprised of a high sugar concentration, it is very important to find out optimum intake of ACSE juice to maintain and enhance our health. Therefore, further studies on the relationship between health and the optimum intake of ACSE juice are necessary.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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