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Isolation and Structural Determination of Free Radical Scavenging Compounds from Korean Fermented Red Pepper Paste (*Kochujang*)

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Abstract Sixteen antioxidative active compounds isolated from the EtOAc layer of MeOH extracts of *kochujang*, Korean fermented red pepper paste, were structurally elucidated as fumaric acid, methyl succinate, succinic acid furan-2-yl ester methyl ester (gochujangate, a novel compound), 2-hydroxy-3-phenylpropanoic acid, 3,4-dihydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 6,7-dihydroxy-2H-chromen-2-one (esculetin), caffeic acid, *cis-p*-coumaric acid, *trans-p*-coumaric acid, daidzin, genistin, apigenin 7-*O*-β-D-apiofuranosyl(1→2)-β-D-glucopyranoside, apigenin 7-*O*-β-D-glucopyranoside, and quercetin 3-*O*-α-L-rhamnopyranoside by mass spectrometry (MS) and nuclear magnetic resonance (NMR) experiments. These compounds were analyzed for the first time as antioxidants from *kochujang*.

Keywords: antioxidant, flavonoid glycoside, *kochujang*, succinic acid furan-2-yl ester methyl ester (gochujangate), phenolic compound

Introduction

Kochujang, a traditional Korean hot pepper soybean paste, is a representative seasoning, along with soy sauce and soy paste, in Korean dishes (1,2). It is prepared by fermentation after mixing together red pepper powder, waxy rice flour, salt, and *meju*, which is fermented by natural microflora or by pure strains such as *Aspergillus* and *Bacillus* sp. after cooling steamed soybeans (3,4). The most notable feature of *kochujang* is the hot red pepper that it contains. Red pepper possesses various beneficial compounds such as capsaicinoids, carotenoids, free amino acids, flavonoids, and ascorbic acid (5-10). Particularly, capsaicin and its derivatives have become of interest in recent years with reports of having biological functions such as body-fat suppressive effects through β-adrenergic stimulation (11); lipid lowering effects on triglycerides, with reductions of lipogenesis in rat livers (12), and the acceleration of hepatic enzyme activities, including glucose 6-phosphate dehydrogenase and adipose lipoprotein lipase (13). However, *kochujang* is fermented by the microorganisms in the *meju*. Therefore, it is suggested that the native compounds within the starting materials may be converted by the metabolism of microorganism during fermentation.

Many studies have been carried out on the quality improvement of *koji* (14-16), as well as on the changes of taste components (amino acids, organic acids, free sugars, nucleotides) (17), flavor components (18,19), and microflora and enzyme (amylase and protease) activities (20) during *kochujang* fermentation, and in the development of functionally enriched products (21-25). These studies were principally related to improving the quality. In addition, *kochujang* is reported to have various biological activities; indicating reducing effects on body weight and lipid levels in rats fed a high-fat diet (26-28).

However, the bioactive compounds contained in *kochujang* have not been investigated. Also, studies on the conversion of its bioactive compounds by fermentation have not been carried out. Therefore, we isolated and elucidated the structures of antioxidative active compounds contained in commercially available *kochujang*, as a primary approach to explore the biological functions of *kochujang*.

Materials and Methods

Material and chemicals The commercial *kochujang* was purchased at a Korean market (Gwangju) in November 2006. It was prepared by mass-production in quality controlled factories and the constitution on the label was listed up red pepper flour (11.3%), starch syrup, wheat flour, glutinous rice, *meju* made with soybean, and calcium. Trifluoroacetic acid (TFA) and 2,4-dihydroxybenzoic acid were supplied by Sigma-Aldrich (St. Louis, MO, USA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fumaric acid, 3,4-dihydroxybenzoic acid, caffeic acid, and *trans p*-coumaric acid were obtained from Sigma-Aldrich. A chlorotrimethylsilane solution and *N*,*O*-bis(trimethylsilyl) acetamide were purchased from Fluka Chemika (Buchs, Switzerland). All other chemicals and solvents were of analytical grade.

Assay for purification of antioxidants The isolation of antioxidative active compounds was guided by the thin layer chromatography (TLC)-DPPH assay, as described previously (29). Briefly, each fraction obtained in the process of purification was spotted on a silica gel TLC

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(silica gel 60 F254, 0.25 mm thickness; Merck, Darmstadt, Germany) and developed using a suitable solvent. Then, DPPH ethanol solution (200 μM) was sprayed on the dried TLC plate. The spots that were visualized by decolorization were regarded as active antioxidants.

Extraction and isolation procedures The *kochujang* (3 kg) was extracted with methanol (MeOH, 12 L, 2 times) at room temperature. The combined MeOH extracts were filtered (No. 2, Whatman, International Ltd., Maidstone, England) and concentrated *in vacuo* at 40°C. The MeOH extracts (1,639 g) were then suspended with H_2O (3,020 mL) and successively partitioned with *n*-hexane (3 L, 3 times), chloroform ($CHCl_3$, 3 L, 3 times), ethyl acetate (EtOAc, 3 L, 3 times), and water-saturated *n*-butanol (BuOH, 3 L, 3 times). Each layer was evaporated *in vacuo* at 38°C. The EtOAc fraction (4.92 g) was subjected to column chromatography on a silica gel (Kieselgel 60, 70-230 mesh, 2.8 i.d.×57 cm, Merck). The elution was performed with $CHCl_3$/EtOAc/MeOH (10:0:0, 8:2:0, 6:4:0, 4:6:0, 2:8:0, 0:10:0, 0:8:2, 0:6:4, 0:4:6, 0:2:8, 0:0:10, v/v/v, each 500 mL). Fractions C and F obtained from the silica gel column chromatography were respectively purified by different methods. Fraction C (296.4 mg) was chromatographed on a Sephadex LH-20 column (25-100 mesh, 2.5 i.d.×55 cm, MeOH; Pharmacia Fine Chemicals, Uppsala, Sweden). Two (fr. C2 and C3) of the fractions, from a classified group of 5 (fr. C1-C5), were purified by high performance liquid chromatography (HPLC)-1 and HPLC-5 (Table 1), respectively. The active fractions obtained were then further purified by HPLC under various conditions (HPLC-2, 3, 4, and 6; Table 1). In addition, fraction F, also obtained from silica gel column chromatography, was purified by HPLC-7 (Table 1), and 3 fractions (fr. F2-F4) of 6 fractions (fr. F1-F6) were successively purified by HPLC-8-13 (Table 1). The isolation procedures for the 16 compounds are shown in Fig. 1.

Instrumental analysis of isolated compounds Nuclear magnetic resonance (NMR) spectra were obtained with a Varian ^{unit}INOVA 500 spectrometer (Walnut Creek, CA, USA) using tetramethylsilane as the internal standard in CD_3OD.

Gas chromatography-mass spectrometry (GC-MS) was performed using GC (QP2010; Shimadzu, Kyoto, Japan) under the following GC (GC2010; Shimadzu) conditions: column, VB-1 capillary (0.25 mm×60 m, 10 μm film thickness, Valco Instruments Co. Inc., Huston, TX, USA); carrier gas, helium; injection port temperature, 220°C; split ratio, 20:1; and flow rate, 1.5 mL/min. The column oven temperature was increased from 60 to 220°C at 4°C/min, and then maintained at 220°C for 15 min. Trimethylsilylation was carried out by heating the samples with 20 mL of pyridine/*N*,*O*-bis(trimethylsilyl)acetamide/trimethylchlorosilane (10:5:1, v/v/v) at 50°C for 30 min.

The MS spectra were obtained from electrospray ionization (ESI)-MS spectrometer (API 3200Qtrap; Applied Biosystems, Framingham, MA, USA) and fast bondardment mass spectrometry (FABMS) spectrometer (JMS-700; Jeol Ltd., Tokyo, Japan) with matrix gradient (3-nitrobenzyl alcohol).

Compound 1: White crystal powder (0.4 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 6.75 (2H, s, H-2 and 3). Trimethylsilylated **1**: GC/MS *m/z* (rel. int.) 245 [$(M\text{-}CH_3)^+$, 100], 217 {$[M\text{-}(CH_3)_3]^+$, 5.18}, 143 {$[M\text{-}COOSi(CH_3)_3]^+$, 19.24}, 115 {$[COOSi(CH_3)_3]^+$, 5.96}, 73 {$[Si(CH_3)_3]^+$, 56.69%}, 45 {$[(CH_3)_3]^+$, 12.97}.

Compound 2: Colorless crystal (1.1 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 3.67 (3H, s, $-OCH_3$), 2.59 [4H, s, $-(CH_2)_2-$]. Trimethylsilylated **2**: GC/MS *m/z* (rel. int.) 204 (M^+, 0.47), 173 $[(M\text{-}OCH_3)^+$, 17.15], 157 {$[M\text{-}(CH_3)_3]^+$, 4.48}, 145 {$[M\text{-}(CH_3)_4]^+$, 1.24}, 129 {[M-TMS]$^+$, 16.89}, 114 {$[M\text{-}OSi(CH_3)_3]^+$, 9.55}, 89 {$[OSi(CH_3)_3]^+$, 100}, 73 {$[Si(CH_3)_3]^+$, 73.77}.

Compound 3: Colorless liquid (3.8 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.18 (1H, d, *J*=3.5 Hz, H-3), 6.58

Table 1. HPLC conditions for the isolation and purification of antioxidants from the EtOAc layer of *kochujang* MeOH extracts

Condition No.	System[1)]	Column[2)]	Mobile phase[3)]	Flow rate (mL/min)	Detection (nm)	Column temp. (°C)
HPLC-1	A	D	5% MeCN^I	2.0	254	40
HPLC-2	B	E	H_2O^I	1.0	254	r.t.[4)]
HPLC-3	B	F	H_2O^I	0.9	254	r.t.
HPLC-4	A	E	H_2O^I	1.0	254	40
HPLC-5	A	F	10% MeOH^I	1.0	254	40
HPLC-6	A	G	5% MeCN^I	1.0	254	40
HPLC-7	C	H	J	9.0	280	r.t.
HPLC-8	B	D	40% MeOH	2.0	254	r.t.
HPLC-9	B	G	40% MeOH	1.0	254	r.t.
HPLC-10	B	F	30% MeOH	1.0	254	r.t.
HPLC-11	B	F	40% MeOH	1.0	254	r.t.
HPLC-12	B	D	50% MeOH	2.0	254	r.t.
HPLC-13	B	F	45% MeOH	1.0	254	r.t.

[1)]A: Pump, LC-20AD (Shimadzu, Kyoto, Japan); detector, SPD-20A (Shimadzu). B: Pump, Model 510 Solvent Delivery System (Waters, Milford, MA, USA); detector, Model 486 Tunable Absorbance Detector (Waters). C: Pump, P580 Pump (Dionex, Idstein, Germany); detector, UVD-170S UV/VIS Detector (Dionex).

[2)]D: μBondapak C18, 10 μm, 7.8×300 mm (Waters); E: μBondapak C18, 5 μm, 3.9×300 mm (Waters); F: RP-Amide C16, 5 μm, 4.6×250 mm (Supelco Inc., Bellefonte, PA, USA); G: TSKgel ODS-80Ts, 5 μm, 4.6×250 mm (Tosoh Corp., Kyoto, Japan); H: μBondapak C18, 10 μm, 19×300 mm (Waters).

[3)]I: pH of the mobile phases was adjusted to 2.65 by trifluoroacetic acid; J: 25%-70% MeOH, 30 min gradient elution.

[4)]Room temperature.

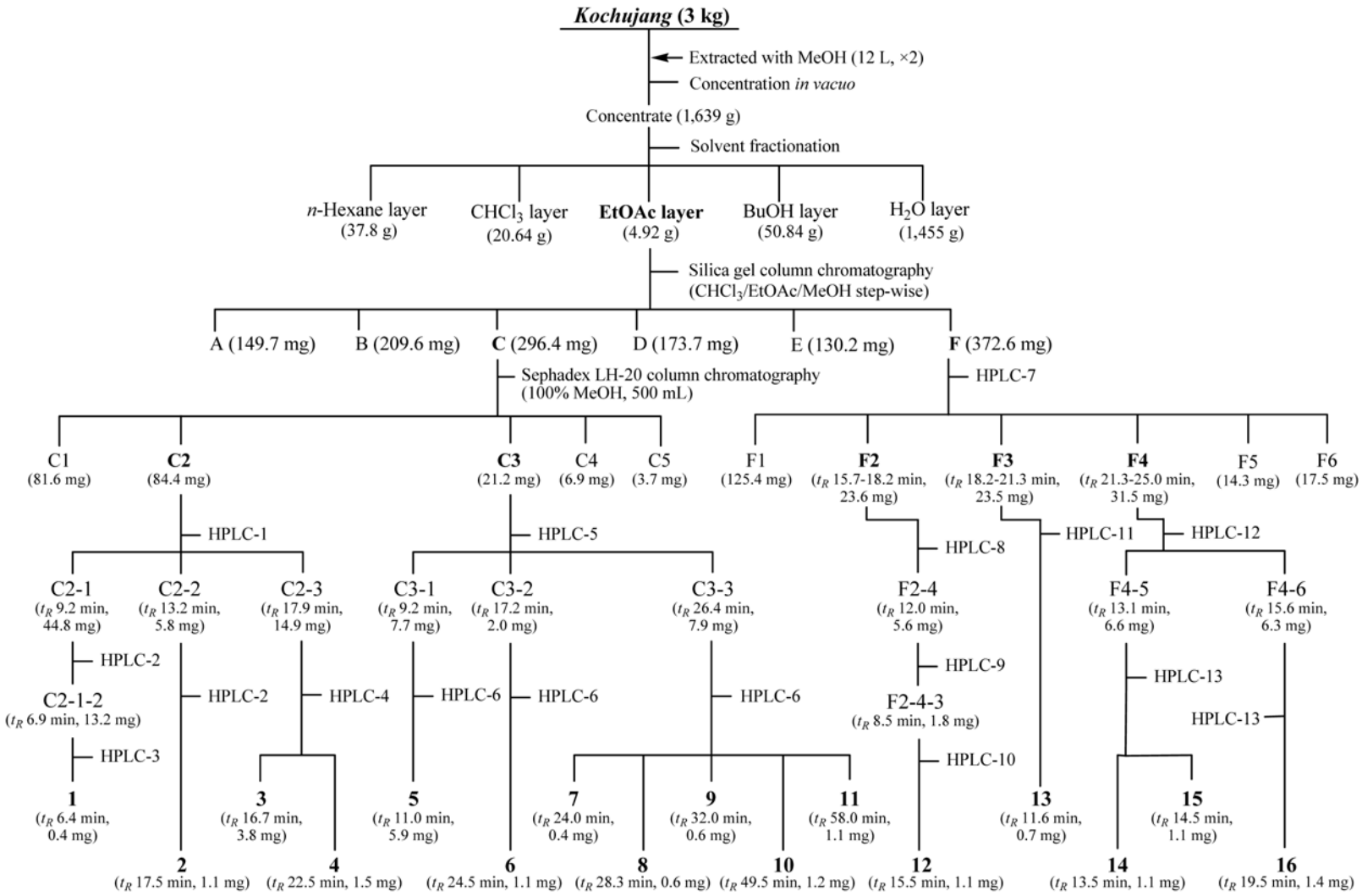

Fig. 1. Isolation procedure for compound 1-16.

(1H, dd, *J*=1.5, 3.5 Hz, H-4), 7.71 (1H, br. s, H-5), 3.67 (3H, s, -OCH_3), 2.59 (4H, s, H-2' and H-3'); ^{13}C-NMR (CD_3OD, 125 MHz) δ 176.12 (C-1'), 174.94 (C-4'), 161.55 (C-2), 147.91 (C-5), 118.83 (C-3), 113.03 (C-4), 52.35 (-OCH_3), 29.95 (C-2' and 3'); FABMS (positive) *m/z* 199.09 $[M+H]^+$.

Compound 4: Colorless crystal (1.5 mg). Trimethylsilylated **4**: GC/MS *m/z* (rel. int.) 309 [$(M-H)^+$, 0.12], 295 [$(M-CH_3)^+$, 4.24], 267 {$[M-(CH_3)_3]^+$, 7.60}, 220 {$[M-OSi(CH_3)_3]^+$, 13.05}, 193 {$[M-COOSi(CH_3)_3]^+$, 56.66}, 147 {$[M-COO(Si(CH_3)_3)_2]^+$, 49.31}, 133 {$[M-(OSi(CH_3)_3)_2]^+$, 6.15}, 103 {$[M-CO(OSi(CH_3)_3)_2]^+$, 3.37}, 91 {$[M-CHCO(OSi(CH_3)_3)_2]^+$, 6.86}, 73 {$[Si(CH_3)_3]^+$, 100}, 45 {$[(CH_3)_3]^+$, 14.49}.

Compound 5: Pale yellow powder (5.9 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.43 (1H, br. s, H-2), 7.42 (1H, dd, *J*=2.0, 8.5 Hz, H-6), 6.80 (1H, d, *J*=8.5 Hz, H-5); ESI-MS (negative) *m/z* 153.0 $[M-H]^+$.

Compound 6: White powder (1.1 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.35 (1H, br. d, *J*=8.0 Hz, H-6), 6.99 (1H, br. d, *J*=7.5 Hz, H-4), 6.73 (1H, br. t, *J*=8.0 Hz, H-5); ^{1}H-NMR (D_2O, 500 MHz) δ 7.48 (1H, br. d, *J*=8.0 Hz, H-6), 7.17 (1H, br. d, *J*=8.0 Hz, H-4), 6.90 (1H, br. t, *J*=8.0 Hz, H-5); ESI-MS (negative) *m/z* 153.0 $[M-H]^+$.

Compound 7: Colorless crystal (0.4 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.68 (1H, d, *J*=8.5 Hz, H-6), 6.31 (1H, dd, *J*=2.5, 8.5 Hz, H-5), 6.28 (1H, d, *J*=2.5 Hz, H-3); ESI-MS (negative) *m/z* 153.1 $[M-H]^+$.

Compound 8: Pale yellow crystal (0.6 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.78 (1H, d, *J*=9.5 Hz, H-4), 6.94 (1H, s, H-5), 6.75 (1H, s, H-8), 6.18 (1H, d, *J*=9.5 Hz, H-3); ESI-MS (negative) *m/z* 177.1 $[M-H]^+$.

Compound 9: Yellow crystal (0.6 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.53 (1H, d, *J*=16.0 Hz, H-1'), 7.03 (1H, d, *J*=2.0 Hz, H-2), 6.93 (1H, dd, *J*=2.0, 8.5 Hz, H-6), 6.77 (1H, d, *J*=8.5 Hz, H-5), 6.22 (1H, d, *J*=16.0 Hz, H-2'); ESI-MS (negative) *m/z* 179.2 $[M-H]^+$.

Compound 10: Colorless crystal (1.2 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.60 (2H, d, *J*=8.5 Hz, H-2 and 6), 6.74 (2H, d, *J*=8.5 Hz, H-3 and 5), 6.81 (1H, d, *J*=13.0 Hz, H-1'), 5.77 (1H, d, *J*=13.0 Hz, H-2'); ESI-MS (negative) *m/z* 163.0 $[M-H]^+$.

Compound 11: Colorless crystal (1.1 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.60 (1H, d, *J*=15.5 Hz, H-1'), 7.45 (2H, d, *J*=8.5 Hz, H-2, 6), 6.80 (2H, d, *J*=8.5 Hz, H-3, 5), 6.28 (1H, d, *J*=15.5 Hz, H-2'); ESI-MS (negative) *m/z* 163.0 $[M-H]^+$.

Compound 12: White powder (1.1 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 8.21 (1H, s, H-2), 8.15 (1H, d, *J*=9.0 Hz, H-5), 7.38 (2H, dd, *J*=2.0, 6.5 Hz, H-2' and 6'), 7.25 (1H, d, *J*=2.0 Hz, H-8), 7.22 (1H, dd, *J*=2.0, 9.0 Hz, H-6), 6.85 (2H, dd, *J*=2.0, 6.5 Hz, H-3' and 5'), 5.11 (1H, d, *J*=8.0 Hz, H-1"), 3.93 (1H, dd, *J*=2.0, 12.0 Hz, H-6"a), 3.71 (1H, dd, *J*=6.0, 12.0 Hz, H-6"b), 3.4-3.6 (4H, m, H-2"-5"); ESI-MS (negative) *m/z* 415.2 $[M-H]^+$.

Compound 13: White powder (0.7 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 8.15 (1H, s, H-2), 7.39 (2H, d, *J*=8.5 Hz, H-2' and 6'), 6.85 (2H, d, *J*=8.5 Hz, H-3' and 5'), 6.71 (1H, d, *J*=2.0 Hz, H-8), 6.52 (1H, d, *J*=2.0 Hz, H-6), 5.05 (1H, d, *J*=7.0 Hz, H-1"), 3.91 (1H, dd, *J*=2.0 Hz, 12.0, H-6"a), 3.71 (1H, dd, *J*=5.5, 12.0 Hz, H-6"b), 3.3-4.0 (4H, m, H-2"-5"); ESI-MS (negative) *m/z* 431.2 $[M-H]^+$.

Compound 14: Yellow crystal (1.1 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.88 (2H, d, *J*=8.5 Hz, H-2' and 6'), 6.93 (2H, d, *J*=8.5 Hz, H-3' and 5'), 6.79 (1H, br. s, H-8), 6.65 (1H, s, H-3), 6.46 (1H, d, *J*=2.0 Hz, H-6), 5.46 (1H, d, *J*=1.5 Hz, H-1'"), 5.15 (1H, d, *J*=7.5 Hz, H-1"), 4.04 (1H, d, *J*=9.5 Hz, H-4'"a), 3.95 (1H, d, *J*=1.5 Hz, H-2'"), 3.93 (1H, dd, *J*=2.0, 12.5 Hz, H-6"a), 3.82 (1H, d, *J*=9.5 Hz, H-4'"b), 3.71 (1H, dd, *J*=6.0, 12.5 Hz, H-6"b), 3.67 (1H, t, *J*=7.5 Hz, H-2"), 3.64 (1H, t, *J*=9.0 Hz, H-3"), 3.54 (1H, br. s, H-5", overlapped with H-5'"), 3.54 (2H, br. s, H-5'", overlapped with H-5"), 3.41 (1H, t, *J*=9.0 Hz, H-4"); ^{13}C-NMR (CD_3OD, 125 MHz) δ 184.22 (C-4), 166.96 (C-2), 164.80 (C-7), 163.21 (C-4'), 163.08 (C-5), 159.14 (C-9), 129.81 (C-2'), 129.81 (C-6'), 123.16 (C-1'), 117.24 (C-3'), 117.24 (C-5'), 111.05 (C-1'"), 107.22 (C-10), 104.24 (C-3), 101.14 (C-6), 100.20 (C-1"), 96.15 (C-8), 80.87 (C-3'"), 75.59 (C-4'"), 78.89 (C-2"), 78.59 (C-3"), 78.44 (C-5"), 78.32 (C-2'"), 71.45 (C-4"), 66.02 (C-5'"), 62.66 (C-6"); ESI-MS (negative) *m/z* 563.2 $[M-H]^+$.

Compound 15: Pale yellow powder (1.1 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.88 (2H, d, *J*=8.5 Hz, H-2' and 6'), 6.93 (2H, d, *J*=8.5 Hz, H-3' and 5'), 6.82 (1H, br. s, H-8), 6.65 (1H, s, H-3), 6.50 (1H, br. s, H-6), 5.06 (1H, d, *J*=7.5 Hz, H-1"), 3.95 (1H, br. d, *J*=12.0 Hz, H-6"a), 3.71 (1H, dd, *J*=5.5, 12.0 Hz, H-6"b), 3.38-3.7 (4H, m, H-2"-5"); ESI-MS (negative) *m/z* 431.1 $[M-H]^+$.

Compound 16: Yellow crystal (1.4 mg); ^{1}H-NMR (CD_3OD, 500 MHz) δ 7.34 (1H, d, *J*=2.0 Hz, H-2'), 7.31 (1H, dd, *J*=2.0, 8.3 Hz, H-6'), 6.91 (1H, d, *J*=8.3 Hz, H-5'), 6.37 (1H, d, *J*=2.3 Hz, H-8), 6.20 (1H, d, *J*=2.3 Hz, H-6), 5.35 (1H, d, *J*=1.5 Hz, H-1"), 4.21 (1H, dd, *J*=1.5, 3.3 Hz, H-2"), 3.74 (1H, dd, *J*=3.3, 9.3 Hz, H-3"), 3.42 (1H, m, H-5"), 3.33 (1H, t, *J*=9.3 Hz, H-4"), 0.94 (3H, d, *J*=6.0 Hz, H-6"); ^{13}C-NMR (CD_3OD, 125 MHz) δ 179.83 (C-4), 166.03 (C-7), 163.40 (C-5), 159.48 (C-2), 158.70 (C-9), 149.96 (C-4'), 146.59 (C-3'), 136.39 (C-3), 123.13 (C-1'), 123.00 (C-6'), 117.09 (C-2'), 116.52 (C-5'), 106.06 (C-10), 103.73 (C-1"), 99.94 (C-6), 94.83 (C-8), 72.29 (C-3"), 72.05 (C-2"), 73.41 (C-4"), 72.19 (C-5"), 17.80 (C-6"); ESI-MS (negative) *m/z* 447.1 $[M-H]^+$.

Results and Discussion

Isolation of antioxidative active compounds A water suspension of the *kochujang* (3 kg) MeOH extracts (1,639 g) was continuously solvent-fractionated with *n*-hexane, $CHCl_3$, EtOAc, and water-saturated *n*-BuOH. The EtOAc layer (4.92 g), which indicated the presence of various antioxidative active compounds in the TLC-DPPH assay, was subjected to silica gel column chromatography ($CHCl_3$/EtOAc/MeOH) using a step-wise procedure. Relatively high DPPH radical scavenging activities were observed for fractions C ($CHCl_3$/EtOAc=4:6, v/v, 296.4 mg) and F (EtOAc/MeOH=8:2, v/v, 372.6 mg). Fraction C was chromatographed on a Sephadex LH-20 column (MeOH) to give 2 active fractions: C2 (Ve/Vt 0.85-1.02, 84.4 mg) and C3 (Ve/Vt 1.03-1.30, 21.2 mg). The fractions C2, C3, and F were repeatedly purified using HPLC under various conditions (Table 1) according to the procedure of Fig. 1, to yield compound **1-16**. The behavior of the antioxidative active compounds during the HPLC purification procedure is also indicated in Fig. 1.

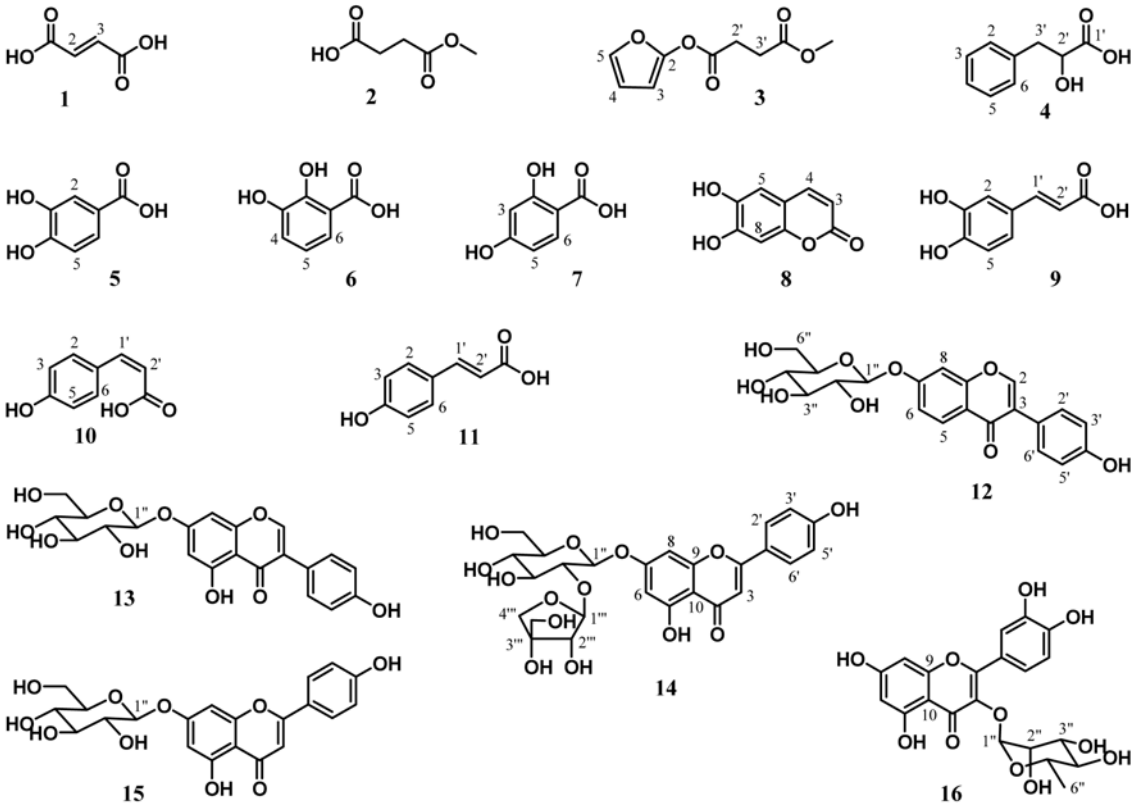

Fig. 2. Structures of antioxidative active compound 1-16 isolated from the EtOAc layer of *kochujang* MeOH extracts.

Structural elucidation of the isolated compounds The ^{1}H-NMR spectrum of **1** showed only one sp^2 carbon proton signal (δ 6.75, H-2 and 3), suggesting the presence of equivalent hydrogens in a low molecular organic acid. Compound **1** was subjected to GC/MS analysis after trimethylsilylation. The MS spectrum of **1** was in accordance with the library (Wiley7) data for trimethylsilylated fumaric acid. In addition, the ^{1}H-NMR spectrum of **1** was consistent with that of standard fumaric acid. Therefore, compound **1** was confirmed as fumaric acid (Fig. 2).

The ^{1}H-NMR spectrum of **2** exhibited a methoxyl carbon (δ 3.67, -OCH_3) and a sp^3 carbon proton signal [δ 2.59, -$(CH_2)_2$-]. The spectral data suggested that **2** was methyl succinate. Therefore, the trimethylsilylated **2** was also analyzed by GC/MS. A molecular ion $[M]^+$ was observed at the mass-to-charge ratio (*m/z*; relative intensity) 204 (0.47%) with other ions formed by the fragmentation: $[M\text{-}CH_3]^+$, $[M\text{-}OCH_3]^+$, $[M\text{-}(CH_3)_3]^+$, and [M-trimethylsilylane]$^+$ at *m/z* 189, 173, 157, and 129, respectively, which were ascribable to the trimethylsilylated methyl succinate. Consequently, compound **2** was identified as methyl succinate (Fig. 2).

The ^{1}H-NMR spectrum of **3** showed a similar pattern to **2** [δ 3.67 (-OCH_3), 2.59 (H-2' and H-3')] as a partial structure. In addition, it displayed 3 sp^2 carbon proton signals [δ 7.18 (H-3), 6.58 (H-4), 7.71 (H-5)], suggesting the presence of furan substituted in the C-2 position. The results suggested that **3** was linked by the esterification of 2-furanol with methyl succinate. A series of linkages among the 3 sp^2 carbon protons and 1 quaternary carbon in the furan ring were confirmed by ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC, Fig. 3) experiments. Moreover, the molecular ion $[M+H]^+$ signal (*m/z* 199.09) was detected from the fast atom bombardment mass spectrometry (FABMS, positive) spectrum. Therefore, the structure of **3** was unambiguously determined to be succinic acid furan-2-yl ester methyl ester (gochujangate, Fig. 2). To our knowledge, this is the first isolation of 2-furanol and methyl succinate as a conjugated form.

The ^{1}H-NMR spectrum of fraction (t_R 22.5 min) contained **4** purified from fraction C2-3 by HPLC-4 (Table 1) showed a mixed pattern of more than 5 compounds. Therefore, a portion of the mixture was subjected to trimethylsilylation, and then analyzed by GC-MS. The GC-MS spectrum of a prominent peak (t_R 31.2 min) on the GC chromatogram showed fragment ion signals (compound **4** in the materials and methods) assignable to trimethylsilylated 2-hydroxy-3-phenylpropanoic acid, and this was in good agreement with the library data (Wiley7). Therefore, the structure of **4** was concluded to be 2-hydroxy-3-phenylpropanoic acid (Fig. 2).

The ^{1}H-NMR spectra of **5** and **7** showed similar mutual patterns except for the chemical shifts [**5**, δ 7.43 (H-2), 7.42 (H-6), 6.80 (H-5); **7**, δ 7.68 (H-6), 6.31 (H-5), 6.28 (H-3)]. It was suggested that these compounds were 3 substituted phenolics. The ESI-MS spectra (negative) displayed peaks at *m/z* 153.0 (**5**) and 153.1 (**7**), which were ascribable to the $[M\text{-}H]^-$ ions, respectively, suggesting they were dihydroxy-benzoic acids. In addition, the ^{1}H-NMR spectra of **5** and **7** were in good agreement with those

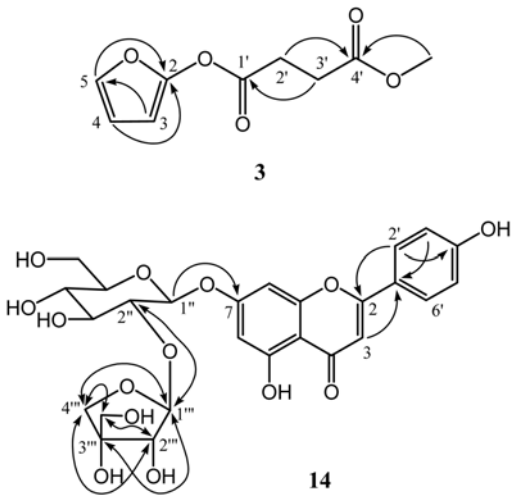

Fig. 3. Important HMBC correlations (arrows) of compound 3 and 14.

obtained from the standards of 3,4-dihydroxybenzoic acid and 2,4-dihydroxybenzoic acid, respectively. Consequently, the compounds were identified as 3,4-dihydroxybenzoic acid (**5**, Fig. 2) and 2,4-dihydroxybenzoic acid (**7**, Fig. 2).

The ESI-MS spectrum (negative) of **6** exhibited a pseudomolecular ion peak $[M-H]^+$ at *m/z* 153.0 with another predominant fragment ion peak $[M-COOH]^+$ at *m/z* 108.9, which were again ascribable to a dihydroxybenzoic acid. The signal splitting patterns and coupling constant values of 3 sp^2 carbon proton signals [D_2O, δ 7.48 (H-6), 7.17 (H-4), 6.90 (H-5)] of **6** in the ^{1}H-NMR spectrum confirmed the presence of a sequential three substituted aromatic benzene ring. Therefore, the ^{1}H-NMR spectral data of **6** were compared with the data of a previous report (30) and superimposed with the data from the reference. As a result, compound **6** was identified as 2,3-dihydroxybenzoic acid (Fig. 2).

The ^{1}H-NMR measurement of **8** showed the typical spectral pattern [δ 7.78 (H-4), 6.94 (H-5), 6.75 (H-8), 6.18 (H-3)] of 6,7-dihydroxychromen-2-one. In addition, the ESI-MS spectrum (negative) also displayed a signal related to a molecular ion [*m/z* 177.1 $(M-H)^+$]. Furthermore, the ^{1}H-NMR spectral data of **8** were in consistent with those reported previously (31), and therefore, the structure of **8** was identified as 6,7-dihydroxy-2H-chromen-2-one (esculetin, Fig. 2).

The structure of **9** and **11** were assigned by a method similar to the one used for **5** and **7**. Specifically, compounds **9** and **11** were identified as caffeic acid (**9**, Fig. 2) and *trans-p*-coumaric acid (**11**, Fig. 2), respectively, by ^{1}H-NMR analyses based on comparisons with the NMR spectral data (data not shown) of standards.

The ^{1}H-NMR spectrum of **10** showed 4 sp^2 carbon proton (6H) signals [δ 7.60 (H-2 and 6), 6.74 (H-3 and 5), 6.81 (H-1'), 5.77 (H-2')] was closely related to those of **11**. The proton signals (δ 6.81 and 5.77) of H-1' and H-2' in **10** showed downfield shifts by 0.79 and 0.51 ppm from those of **11**, respectively. Moreover, the coupling constant values (13.0 Hz) of H-1' and H-2' in **10** were observed to be smaller values than those (15.5 Hz) in **11**. The results revealed that the structure of **10** was *cis-p*-coumaric acid (Fig. 2), with the support of ESI-MS data [negative, *m/z* 163.0 $(M-H)^+$] and the comparison of the ^{1}H-NMR spectroscopic data in the literature (32).

In the ESI-MS spectrum (negative), a pseudomolecular ion peak of **12** was detected at *m/z* 415.2 $[M-H]^+$, together with another fragment ion at *m/z* 253.1 suggesting the presence of a monosaccharide. The ^{1}H-NMR spectrum of **12** showed evidence for glucose [δ 5.11 (H-1"), 3.93 (H-6"a), 3.71 (H-6"b), 3.4-3.6 (4H, H-2"-5")] as well as an aglycone of daidzein [δ 8.21 (H-2), 8.15 (H-5), 7.38 (H-2' and 6'), 7.25 (H-8), 7.22 (H-6), 6.85 (H-3' and 5')]. The coupling constant (J=8.0 Hz) of the anomeric proton signal (δ 5.11, H-1") indicated that the glucose had a β-configuration. When the ^{1}H-NMR spectral data of **12** were compared with those of the literature (33), **12** was unambiguously identified as daidzin (Fig. 2), which was etherified by oxygen between the anomeric carbon of glucose and the C-7 position of daidzein.

The structure of **13** was identified in a similar manner as **12**. The ^{1}H-NMR spectrum of **13** was closely related to that of **12** except for the absence of a proton signal on the C-5 position of **13**, suggesting **13** to be genistin. The ESI-MS spectrum (negative) showed a *m/z* 431.2 $[M-H]^+$ signal, indicating the molecular weight (432) of **13**, which was ascribable to genistin. Therefore, the structure of **13** was identified to be genistin (Fig. 2), based on comparisons with the ^{1}H-NMR data of genistin that was isolated from a fermented soy food (33).

The molecular weight of **14** was analyzed to be 564 from its ESI-MS (negative) spectrum, with *m/z* 563.2 $[M-H]^+$. The ^{13}C-NMR spectrum of **14** exhibited the presence of 26 carbons including 15 carbon signals of an aglycone [δ 184.22, carbonyl carbon signal (C-4); δ 166.96-96.15, 14 sp^2 carbon signals] and 11 carbon signals of 2 monosaccharides [δ 111.5 (C-1'"), 100.20 (C-1"), δ 80.87-62.66 (C-2"-C-6' and C-2'"-C-6'")]. The ^{1}H-NMR spectrum showed evidence for apigenin, including 7 proton signals of an A ring [δ 6.79 (H-8), 6.46 (H-6)]; B ring [δ 7.88 (H-2' and 6'), 6.93 (H-3' and 5')]; and C ring [δ 6.65 (H-3)] as an aglycone. The structure of the sugar moiety was confirmed to be β-D-apiofuranosyl-β-D-glucopyranose based on the 1D- and 2D-NMR experiments. Furthermore, from the HMBC spectrum (Fig. 3), the cross peaks from H-1'" to C-2" and H-1" to C-7 together with other significant cross peaks clearly supported the structure of **14** as apigenin 7-*O*-β-D-apiofuranosyl(1→2)-β-D-glucopyranoside (Fig. 2).

The ^{1}H-NMR spectrum of **15** was very similar to that of **14** except for the lack of signals ascribable to apiose. Therefore, the structure was suggested to be an apigenin glucoside. The ESI-MS spectrum (negative) showed signals of *m/z* 431.1 $[M-H]^+$ and 268.1 $[M-glucose]^+$. Compound **15** was identified to be apigenin 7-*O*-β-D-glucopyranoside (Fig. 2) by the direct comparison of the ^{1}H-NMR data of **15** with the data of apigenin glucoside from the leaves of *Zostera marina* (34).

The ^{1}H- and ^{13}C-NMR data of **16** showed typical spectral patterns of a flavonoid monosaccharide. Especially, in the ^{1}H-NMR spectrum, 5 sp^2 carbon proton signals [δ 7.34 (H-2'), 7.31 (H-6'), 6.91 (H-5'), 6.37 (H-8), 6.20 (H-6)] suggested the presence of quercetin as aglycone. In addition, it showed evidence for the monosaccharide of α-L-rhamnopyranoside, including anomeric proton [δ 5.35 (H-

1")] and methyl proton [δ 0.94 (H-6")] signals. The ESI-MS spectrum (negative) also displayed signals related to the molecular ion [*m/z* 447.1 $(M-H)^+$] and to quercetin rhamnopyranoside [*m/z* 300.1 $(M\text{-rhamnose})^+$]. The ^1H- and ^{13}C-NMR data of **16** corresponded to those of a quercetin 3-*O*-α-L-rhamnopyranoside that had been isolated from *Mammea longifolia* (35). Therefore, the structure of **16** was identified as quercetin 3-*O*-α-L-rhamnopyranoside (Fig. 2).

Sixteen compounds were isolated from the EtOAc layer of *kochujang* MeOH extracts, and were structurally elucidated as fumaric acid (**1**), methyl succinate (**2**), succinic acid furan-2-yl ester methyl ester (gochujangate, **3**), 2-hydroxy-3-phenylpropanoic acid (**4**), 3,4-dihydroxybenzoic acid (**5**), 2,3-dihydroxybenzoic acid (**6**), 2,4-dihydroxybenzoic acid (**7**), 6,7-dihydroxy-2H-chromen-2-one (esculetin, **8**), caffeic acid (**9**), *cis*-*p*-coumaric acid (**10**), *trans*-*p*-coumaric acid (**11**), daidzin (**12**), genistin (**13**), apigenin 7-*O*-β-D-apiofuranosyl(1→2)-β-D-glucopyranoside (**14**), apigenin 7-*O*-β-D-glucopyranoside (**15**), and quercetin 3-*O*-α-L-rhamnopyranoside (**16**) (Fig. 2).

Compound **1-3** consist of an organic acid and its analogues, respectively. Particularly, the partial structure, 2-furanol, of **3** (gochujangate) has been detected as a volatile compound in boiled leeks (*Allium tuberosum*) (36) and fermented aronia-spirit (37). The 2-furanol from *kochujang,* as well as from the leek and aronia-spirit, may be formed through boiling and fermentation. Therefore, it is presumed that gochujangate (**3**) may be produced by the esterification of 2-furanol with methyl succinate (**2**) during fermentation. Compound **4-11** are naturally occurring antioxidants of phenolic acids and their derivatives, which are present in the human diet. Compound **12** and **13** are major isoflavone glucosides contained within soybeans (38), and therefore, it is thought that the compounds in *kochujang* may be derived from soybeans, one of its main ingredients. On the other hand, compound **14-16**, which are apigenin and quercetin glycosides, are assumed to be from the pepper, because their presence in pepper has already been reported (9,10). Interestingly, all of the flavonoids isolated during this study were glycosides, although the presence or absence of the aglycones has not been confirmed.

The microorganisms participating in the fermentation of *kochujang* products prepared by traditional methods, and by mass-production in quality controlled factories, are different from each other (20). Therefore, it is thought that additional investigation is needed on the discrepancy of the bioactive constituents occuring between *kochujang* products made by different preparation methods, in order to standardize and elevate the quality of *kochujang*. Moreover, the origin and content on the antioxidants isolated from *kochujang* are important factors for further investigation, in terms of its functionality and quality control. In conclusion, we revealed at the molecular level, the presence of various antioxidants in the EtOAc layer of *kochujang* extracts. It is presumed that these antioxidative active compounds may be associated with a broad variety of beneficial properties in human health. The results of this study may be useful as a data base, in relation to research on the functionality of *kochujang* and as indicators for quality control.

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