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Caffeoyl Triterpenes from Pear (*Pyrus pyrifolia* Nakai) Fruit Peels and Their Antioxidative Activities against Oxidation of Rat Blood Plasma

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ABSTRACT: Six triterpenes, including three caffeoyl triterpenes, were purified and isolated from pear fruit (*Pyrus pyrifolia* Nakai cv. Chuwhangbae) peel extracts using various column chromatography techniques with a guided 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay. The isolated compounds were identified as betulinic aldehyde (**1**), lupeol (**2**), betulinic acid (**3**), 3-*O*-*cis*-caffeoylbetulinic acid (**4**), 3-*O*-*trans*-caffeoylbetulinic acid (**5**), and 3-*O*-*trans*-caffeoyloleanolic acid (**6**) on the basis of nuclear magnetic resonance spectroscopy and electrospray ionization mass spectrometry. Four compounds (**1**, **4**–**6**) were identified from Asian pear fruit for the first time. In addition, compounds **4**–**6**, containing a caffeic acid moiety, showed higher DPPH radical-scavenging and suppression effects against copper ion-induced oxidation of rat blood plasma than other compounds without a caffeic acid moiety.

KEYWORDS: *Pyrus pyrifolia Nakai, pear fruit peel, caffeoyl triterpenes, 1,1-diphenyl-2-picrylhydrazyl, cholesteryl ester hydroperoxide*

INTRODUCTION

Pear (*Pyrus* spp.) belongs to the Rosaceae family and is widely distributed throughout the world.[1] The fruit is commonly eaten fresh and in processed foods such as juice, puree, jellies, and jams.[2,3] Many studies have reported on the chemical constituents and composition in different pear cultivars, including sugars, organic and fatty acids, amino acids, phenolics, vitamins, volatiles, and minerals.[4−10] In particular, phenolic compounds, including arbutin, chlorogenic acid, hydroxycinnamoyl malates, catechins, and procyanidins, have been identified in pear fruit.[10−14] Most studies on the phenolic constituents in pear fruits have been almost exclusively performed on European pears. However, a few phenolic compounds, including arbutin and chlorogenic acid, have also been found in Asian pears.[15,16]

In Asian countries, including Korea and China, pear fruit has been used as a traditional medicine remedy to treat constipation, diuresis, cough, and flu.[16] Several studies have reported that Asian pear fruit exerts biological effects, including antimicrobial, anti-inflammatory, and antioxidative activities.[17−19] In addition, steroids (β-sitosterol, daucosterol, α-amyrin), quercitrin, and triterpenoids (oleanolic acid, ursolic acid, 2β,19α-hydroxyurosolic acid) have been isolated and identified as anti-inflammatory and/or antimicrobial compounds in *Pyrus bretschneideri* R. (Chinese pear).[18,19] We have also isolated various phenolic compounds, including seven phenylpropanoid malate derivatives, coumaroylquinic acid derivatives, and flavonoids from *Pyrus pyrifolia* Nakai cv. Chuhwangbae pear fruit, which is one of the most highly consumed pear fruits in Korea.[20−22] In the course of investigating antioxidative compounds from *P. pyrifolia* fruit peel, we isolated six triterpenes, including three caffeoyl triterpenes, as antioxidative compounds with a guided 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay.

In the present paper we describe the isolation and identification of six triterpenes, including three caffeoyl triterpenes, from the EtOAc-soluble phenolic layer of *P. pyrifolia* fruit peel MeOH extracts. We also report the antioxidative activities of the isolated compounds by measuring the DPPH radical-scavenging and inhibition ability of cholesteryl ester hydroperoxide (CE-OOH) formation in copper ion-induced lipid peroxidation in rat blood plasma.

MATERIALS AND METHODS

Materials and Chemicals. Fresh pear fruit (*P. pyrifolia* Nakai cv. Chuwhangbae) was harvested in Naju City, South Korea, in September 2008. This pear was identified by Prof. Wol-Soo Kim, Laboratory of Pomology, College of Agriculture and Life Science, Chonnam National University. A voucher sample (no. JNU PE 20050831-2) was deposited in the laboratory herbarium. The fresh fruits were hand-peeled at about 3 mm thickness. The fruit peels (15 kg fresh weight) were immediately stored at −70 °C until use. Methanol-d_4 (CD_3OD) was obtained from Merck (Darmstadt, Germany). Solvents used for analyses were of high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific Korea (Seoul, Korea). DPPH, caffeic acid (CA), and spectrophotometric grade trifluoroacetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Methanol (MeOH), acetonitrile (MeCN), and ethyl acetate (EtOAc), which were used for extraction and solvent fractionation, were of extrapure quality and were obtained from Duksan (Ansan, Korea). All other chemicals used were of reagent grade and were obtained from commercial sources.

Extraction and Solvent Fractionation. Fresh pear peels (15 kg fresh weight) were homogenized using a homogenizer (BM-2 Nissei biomixer; Nihonseiki, Osaka, Japan) with MeOH (24 L). After a 3 day extraction at room temperature, the mixture was filtered under vacuum

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through no. 2 filter paper (Whatman, Maidstone, U.K.), and the residue was repeatedly extracted with MeOH (11 L). The solutions extracted with MeOH were combined and concentrated in a vacuum at 38 °C. The MeOH extracts (3708.67 g) were suspended in acidic buffer (0.2 M glycine−0.2 M HCl, pH 3.0, 6 L) and partitioned with EtOAc (6 L, three times). The EtOAc layer was partitioned with phosphate buffer (0.2 M NaH_2PO_4−0.2 M Na_2HPO_4, pH 8.0, 6 L, three times) to produce the EtOAc-soluble neutral layer. The aqueous layer was adjusted to pH 3.0 using 1.0 M HCl and then partitioned with EtOAc (6 L, three times) to yield the EtOAc-soluble acidic layer.

Isolation. The EtOAc-soluble phenolic layer (2.38 g) obtained after solvent fractionation of pear fruit peel MeOH extract was charged on a silica gel column (2.8 × 38 cm, 70−230 mesh; Merck, Darmstadt, Germany) and eluted with *n*-hexane/EtOAc/MeOH (6:4:0, 4:6:0, 2:8:0, 0:10:0, 0:9:1, 0:8:2, 0:7:3, 0:6:4, 0:5:5, 0:0:10, v/v/v; stepwise system, each 200 mL). Fraction C (218.7 mg) obtained after silica gel column chromatography of the EtOAc-soluble phenolic layer was separated on a Sephadex LH-20 column (2.0 × 40 cm) eluted with MeOH/BuOH = 50:50 (v/v; 134.5 mL) as a mobile phase. Fraction C4 obtained after preparative HPLC purification of fraction C was further purified on an ODS column (1.8 × 56 cm, 70−230 mesh, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using H_2O/MeOH as the mobile phase (8:2, 9:1, 9.5:0.5, 0:10, v/v; stepwise system, each 200 mL). Fraction C5 was purified by HPLC on an instrument equipped with a Shim-pack Prep-ODS (H) kit (5 μm, 20 × 250 mm; Shimadzu, Kyoto, Japan) using a linear gradient of 65% MeCN (eluent A) to 95% MeCN (eluent B), starting with 100% A, increasing to 100% B for 30 min, and then holding at 100% B for 50 min. Fraction C6 was injected into an HPLC instrument equipped with a prep-ODS column using an isocratic system of 85% MeCN. HPLC analysis was carried out using a Shim-pack Prep-ODS (H) kit (Shimadzu). The flow rate was 9.9 mL/min, and eluents were monitored at 254 nm.

Structural Analysis. Nuclear magnetic resonance (NMR) spectra were obtained with ^{unit}INOVA 500 and 600 spectrometers (Varian, Walnut Creek, CA) using tetramethylsilane (TMS) as an internal standard in CD_3OD and pyridine-d_5. All mass spectra were acquired on a hybrid ion-trap time-of-flight mass spectrometer (Shimadzu) that was equipped with an electrospray ionization (ESI) source (ESI-MS). Accurate masses were corrected by calibration using sodium trifluoroacetate clusters as internal references. Sample solutions were prepared by dissolving each sample in a solution of methanol to a final concentration of 50 μg/μL. All ions produced were introduced into the instrument for accurate mass determination. Data acquisition and analysis was performed using LC Solution 3.0 software (Shimadzu).

Compound ***1*** *(White Powder).* ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 9.68 (1H, s, H-28), 4.63 (1H, s, H-29a), 4.76 (1H, s, H-29b), 3.19 (1H, dd, *J* = 11.5, 4.5 Hz, H-3), 2.87 (1H, m, H-19), 1.70 (3H, s, H-30), 0.98 (3H, s, H-26), 0.97 (3H, s, H-27), 0.92 (3H, s, H-23), 0.82 (3H, s, H-25), 0.76 (3H, s, H-24). ^{13}C NMR (125 MHz, $CDCl_3$, TMS): for data see Table 1. ESI-MS (positive): *m*/*z* 441.3 $[M + H]^+$.

Compound ***2*** *(White Powder).* ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ 4.69 (1H, s, H-29a), 4.56 (1H, s, H-29b), 3.18 (1H, dd, *J* = 11.5, 5.0 Hz, H-3), 1.68 (3H, s, H-30), 1.02 (3H, s, H-26), 0.96 (3H, s, H-23), 0.94 (3H, s, H-27), 0.82 (3H, s, H-25), 0.78 (1H, s, H-28), 0.76 (3H, s, H-24). ^{13}C NMR (125 MHz, $CDCl_3$, TMS): for data see Table 1. ESI-MS (positive): *m*/*z* 449.4 $[M + Na]^+$.

Compound ***3*** *(White Powder).* ^{1}H NMR spectrum (600 MHz, pyridine-d_5, TMS): δ 4.97 (1H, s, H-29a), 4.79 (1H, s, H-29b), 3.47 (1H, t, *J* = 7.2 Hz, H-3), 1.81 (3H, s, H-30), 1.78 (1H, t, *J* = 11.4 Hz, H-18), 1.24 (3H, s, H-23), 1.08 (3H, s, H-27), 1.07 (3H, s, H-26), 1.00 (3H, s, H-24), 0.84 (3H, s, H-25). ^{13}C NMR (150 MHz, pyridine-d_5, TMS): for data see Table 1. ESI-MS (positive): *m*/*z* 479.3 $[M + Na]^+$.

Compound ***4*** *(White Powder).* ^{1}H NMR (600 MHz, CD_3OD, TMS): δ 7.28 (1H, d, *J* = 1.8 Hz, H-2′), 7.00 (1H, dd, *J* = 8.4, 1.8 Hz, H-6′), 6.80 (1H, d, *J* = 13.2 Hz, H-7′), 6.72 (1H, d, *J* = 8.4 Hz, H-5′), 5.74 (1H, d, *J* = 13.2 Hz, H-8′), 4.71 (1H, s, H-29a), 4.59 (1H, s, H-29b), 4.49 (1H, dd, *J* = 12.0, 4.2 Hz, H-3), 1.70 (3H, s, H-30), 1.02 (3H, s, H-23), 0.98 (3H, s, H-27), 0.89 (3H, s, H-26), 0.85 (3H, s, H-24), 0.78 (3H, s, H-25). ^{13}C NMR (150 MHz, CD_3OD, TMS): for data see Table 1. ESI-MS (positive): *m*/*z* 641.4 $[M + Na]^+$.

Compound ***5*** *(White Powder).* ^{1}H NMR (600 MHz, pyridine-d_5, TMS): δ 8.05 (1H, d, *J* = 16.2 Hz, H-7′), 7.68 (1H, br s, H-2′), 7.24 (1H, br s, H-6′), 7.20 (1H, br s, H-5′), 6.71 (1H, d, *J* = 16.2 Hz, H-8′), 4.97 (1H, s, H-29a), 4.87 (1H, m, H-3), 4.77 (1H, s, H-29b), 1.79 (3H, s, H-30), 1.09 (3H, s, H-27), 1.04 (3H, s, H-26), 0.94 (6H, s, H-23, H-24), 0.78 (3H, s, H-25). ^{13}C NMR (150 MHz, pyridine-d_5, TMS): for data see Table 1. ESI-MS (positive): *m*/*z* 641.4 $[M + Na]^+$.

Compound ***6*** *(White Powder).* ^{1}H NMR (600 MHz, CD_3OD, TMS): δ 7.52 (1H, d, *J* = 16.2 Hz, H-7′), 7.03 (1H, d, *J* = 2.1 Hz, H-2′), 6.94 (1H, dd, *J* = 8.4, 2.1 Hz, H-6′), 6.78 (1H, d, *J* = 8.4 Hz, H-5′), 6.24 (1H, d, *J* = 16.2 Hz, H-8′), 5.25 (1H, t, *J* = 3.3 Hz, H-12), 4.57 (1H, dd, *J* = 12.0, 4.2 Hz, H-3), 2.86 (2H, dd, *J* = 13.8, 3.6 Hz, H-2), 1.28 (3H, s, H-23), 1.18 (3H, s, H-27), 1.00 (3H, s, H-25), 0.94 (3H, s, H-29), 0.97 (3H, s, H-26), 0.91 (3H, s, H-24), 0.83 (3H, s, H-30).

Table 1. ^{13}C NMR Spectroscopic Data for the Isolated Compounds from Pear Fruit Peels

position	1[a]	2[a]	3[b]	4[c]	5[b]	6[c]
1	38.8	38.7	39.6	39.8	38.6	39.5
2	27.4	27.4	28.7	24.8	24.3	24.8
3	79.1	79.0	78.5	82.4	80.4	82.4
4	38.9	38.8	39.9	39.1	38.3	39.1
5	55.3	55.2	56.3	57.1	55.7	57.0
6	18.3	18.3	19.1	19.4	18.5	19.5
7	34.4	34.2	35.2	35.6	34.6	34.1
8	40.9	40.8	41.5	42.1	41.1	40.7
9	50.5	50.4	51.3	52.0	50.7	48.8
10	37.2	37.1	37.9	38.3	37.3	38.3
11	20.8	20.9	21.5	22.3	21.2	24.7
12	25.6	25.1	26.5	27.0	26.0	123.6
13	38.7	38.0	39.0	39.8	38.6	145.4
14	42.6	42.8	43.2	43.7	42.9	43.1
15	29.3	27.3	30.6	30.9	30.3	29.0
16	28.9	35.5	33.2	33.5	32.8	24.2
17	59.4	43.0	57.0	57.7	56.6	47.8
18	48.1	48.2	50.1	50.6	49.7	43.0
19	47.6	48.0	48.1	48.4	47.8	47.4
20	149.8	151.0	151.7	152.2	151.3	31.8
21	29.8	29.8	31.6	31.8	31.2	35.1
22	33.3	40.0	37.9	38.4	37.6	34.0
23	28.0	28.0	29.0	28.7	28.1	28.8
24	15.4	15.4	16.7[d]	16.8	16.3	17.5
25	15.9	16.1	16.7[d]	17.1	16.9	16.1
26	16.2	16.0	16.7[d]	16.9	16.3	17.8
27	14.2	14.5	15.2	15.3	14.9	26.6
28	206.8	18.0	179.3	180.3	178.9	182.2
29	110.2	109.3	110.3	110.3	110.0	33.7
30	19.1	19.3	19.8	19.7	19.5	24.1
1′				117.6	115.9	127.8
2′				144.9	147.7	115.2
3′				148.2	150.5	146.9
4′				115.8	116.8	149.7
5′				124.7	122.1	116.7
6′				128.5	127.0	123.1
7′				145.9	145.7	146.8
8′				118.6	115.7	115.7
9′				168.6	167.4	169.3

[a]The frequency was 125 MHz for ^{13}C NMR in $CDCl_3$. [b]The frequency was 150 MHz for ^{13}C NMR in pyridine-d_5. [c]The frequency was 150 MHz for ^{13}C NMR in CD_3OD. [d]The C-24, C-25, and C-26 carbon signals overlapped.

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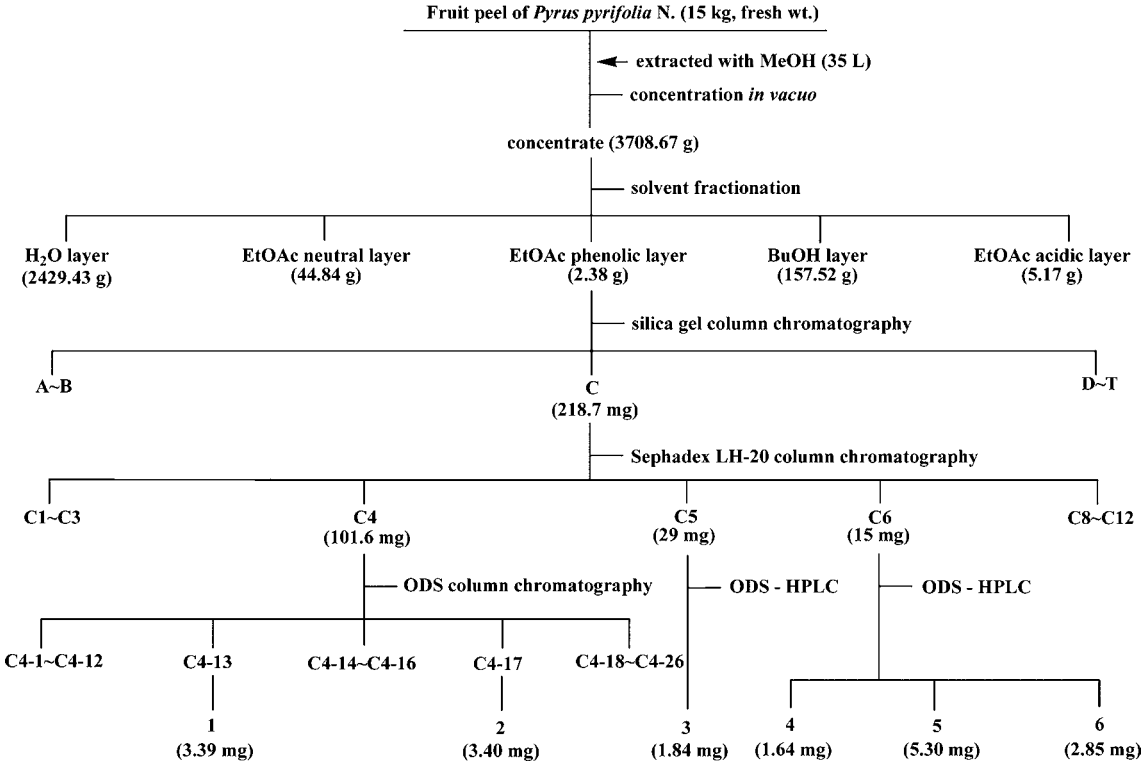

Figure 1. Purification procedure for the isolated compounds from the EtOAc-soluble phenolic layer of the pear fruit peel MeOH extract.

^{13}C NMR (150 MHz, CD_3OD, TMS): for data see Table 1. ESI-MS (positive): m/z 641.4 $[M + Na]^+$.

DPPH Radical-Scavenging Assay. The DPPH radical-scavenging activity was conducted using three different methods. Free radical-scavenging activities of the EtOAc-soluble layers were evaluated according to the method described by Abe et al.,[23] with slight modifications. Briefly, a methanol solution (200 μL) of each sample at different concentrations (10−300 μg) was mixed with a DPPH radical/ethanol solution (1800 μL, final concentration 250 μM). This solution was allowed to sit for 30 min in the dark. The free radical-scavenging activity of each sample was quantified by decolorization of DPPH at 517 nm. The DPPH radical-scavenging activities of the EtOAc-soluble layers were determined as the percentage decrease in the absorbance compared to a blank test.

An assay to purify the antioxidative compounds was conducted by spraying DPPH reagent on a thin-layer chromatography (TLC) plate (silica gel 60 F254, 0.25 mm thickness, Merck).[24] All fractions were purified by various column chromatography techniques, and ODS-HPLC was spotted on a TLC plate and developed using a $CHCl_3$/EtOAc = 9:1 (v/v) mixture. Each fraction was spotted onto the plate. After spraying 100 μM DPPH/EtOH solution onto the plate, the fractions that decolorized the spots were considered reflective of antioxidative activity. In addition, the purity of the fractionated compounds was confirmed by UV and a 1% cerium sulfate solution spray.

The free radical-scavenging activities of the isolated compounds and CA were evaluated by a DPPH radical according to the method described by Abe et al.,[23] with slight modifications. An ethanol solution (100 μL, final concentration 32 μM) of the isolated compounds and CA was added to the DPPH radical/ethanol solution (100 μL, final concentration 250 μM). The solution was then mixed and permitted to stand for 30 min in the dark. The free radical-scavenging activity of each sample was measured at 517 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). The DPPH radical-scavenging activities of the isolated compounds and other samples were determined as the percentage decrease in absorbance as shown by the blank test. The experiments were conducted in triplicate.

Determination of the Inhibitory Effect of the Isolated Compounds against Copper Ion-Induced Oxidation in Rat Blood Plasma. The antioxidative activities of the isolated compounds and CA were evaluated by measuring their inhibitory effects against CE-OOH formation during copper ion-induced oxidation of diluted rat blood plasma according to the method reported by Kim et al.[25] Sprague−Dawley rats (males, 6 weeks old, 180−200 g) were obtained from Samtako Bio Korea (Osan, Korea). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (no. CNU IACUC-YB-R-2013-4). The rats were kept at a temperature of 20 ± 2 °C with a 12 h light/dark cycle and fasted for 15 h prior to blood collection. After anesthesia with diethyl ether, blood was collected from the abdominal aorta into heparinized tubes. Rat plasma was isolated by centrifugation (3000g) at 4 °C for 20 min and stored at −40 °C prior to use. Blood plasma was diluted 4-fold with PBS (pH 7.4). The diluted plasma (640 μL) was added to the isolated compounds and CA (16.7 μM) and then oxidized by adding 100 μL a $CuSO_4$ PBS solution (final concentration 100 μM). The mixture was incubated at 37 °C for 5 h with continuous shaking. The CE-OOH concentration was determined according to the method described by Arai et al.[26] Briefly, aliquots (100 μL) were withdrawn from the incubation solutions and mixed with 3 mL of MeOH containing 2.5 mM butylated hydroxytoluene (BHT). The mixture was sonicated for 1 min and then partitioned with 3 mL of n-hexane by vortexing vigorously for 1 min. The upper layer (n-hexane) was collected, and extraction of the lower layer with 3 mL of n-hexane was repeated. The combined n-hexane phases were evaporated in a rotary evaporator at room temperature. The remaining lipids were dissolved in 100 μL of MeOH/$CHCl_3$ (95:5, v/v), and aliquots were subjected to CE-OOH analysis by reversed-phase HPLC using a TSK-gel Octyl-80TS column (Tosoh, Tokyo, Japan). The effluent was monitored by UV at 235 nm (Shimadzu SPD-10A). The solution of MeOH/H_2O (97:3, v/v) served as a mobile phase, and the flow rate was constant at 1.0 mL/min. The CE-OOH concentration was calculated from a standard curve of cholesteryl linoleate hydroperoxide. Detailed procedures for preparing the cholesteryl linoleate hydroperoxide standard have been published.[26]

Statistical Analysis. All data are expressed as the mean ± standard deviation using the Statistical Package for Social Sciences (SPSS; Chicago, IL) 17.0 programs. Statistical differences in DPPH radical-scavenging activity of the isolated compounds and CA were analyzed by the Tukey–Kramer test ($p < 0.05$).

RESULTS AND DISCUSSION

Isolation of Compounds from the EtOAc-Soluble Phenolic Fraction. The peels of pear fruit have a much higher and more variable phenolic content than the pulp.[10,16] Therefore, the peel was used to investigate the constituents contained in the pear fruit. The MeOH extract (3708.67 g) was obtained from the fresh pear peels (15 kg fresh weight) and was fractionated into five layers: an EtOAc-soluble acidic layer (5.17 g), an EtOAc-soluble phenolic layer (2.38 g), a BuOH-soluble layer (157.52 g), an EtOAc-soluble neutral layer (44.84 g), and a H_2O-soluble layer (2429.43 g).[20] The antioxidative activities of these layers were evaluated using the DPPH (final concentration 250 μM) radical. The EtOAc-soluble acidic and phenolic layers showed higher antioxidative activity than the other fractions (data not shown). Seven (hydroxycinnamoyl)-malic acids and their methyl esters and eight phenolic compounds were already identified from the EtOAc-soluble acidic layer by our previous study.[20,21] In the present study, the purification and isolation of antioxidative active compounds contained in the EtOAc-soluble phenolic layer was performed.

The EtOAc-soluble phenolic layer (2.38 g) was fractionated by silica gel column chromatography using a stepwise system of *n*-hexane/EtOAc/MeOH. Each fraction was developed on silica gel TLC using a $CHCl_3$:EtOAc = 9:1 (v/v) solvent and then sprayed with a DPPH radical (200 μM)/EtOH solution. Twenty fractions (A–T) were obtained on the basis of the detection pattern of the antioxidative active compounds on TLC. In particular, fraction C (*n*-hexane/EtOAc, 218.7 mg) showed higher DPPH radical-scavenging activity and simple patterns on a TLC plate when compared to the other fractions. Therefore, fraction C (218.7 mg) was further fractionated by Sephadex LH-20 column chromatography (MeOH/BuOH, 5:5, v/v) to give 12 active fractions (C1–C12). Of them, the purity of fractions C4, C5, and C6 was relatively higher than that of the other fractions. Fraction C4 [elution volume/total volume (V_e/V_t) = 0.94–1.02, 101.6 mg] was purified by ODS column chromatography using a MeOH/H_2O solvent to give two compounds (**1**, 3.39 mg; **2**, 3.40 mg). Fraction C5 (29 mg, V_e/V_t = 1.06–1.14) was purified by ODS-HPLC using a MeCN/H_2O gradient system to obtain compound **3** (t_R = 32.8 min, 1.84 mg). Fraction C6 (15 mg, V_e/V_t = 1.18–1.30) was purified by ODS-HPLC (isocratic system, 85% MeCN) to afford three compounds [**4**, t_R = 19.2 min (1.64 mg); **5**, t_R = 20.9 min (5.30 mg); **6**, t_R = 24.4 min (2.85 mg)]. The purification and isolation procedures of six compounds from the EtOAc-soluble phenolic layer are indicated in Figure 1. The structures of the isolated compounds were determined on the basis of NMR spectroscopic and MS data.

Structural Elucidation of the Isolated Compounds. Compound **1** was obtained as a white powder. The ^{1}H NMR (500 MHz, $CDCl_3$) spectrum of **1** showed the presence of two geminal olefinic proton signals at δ 4.76 (1H, s, H-29b) and 4.63 (1H, s, H-29a), an aldehyde proton signal at δ 9.60 (1H, s, H-28), an oxygenated methine proton signal at δ 3.19 (1H, dd, J = 11.5, 4.5 Hz, H-3), and six methyl proton signals [δ 0.91 (H-23), 0.75 (H-24), 0.82 (H-25), 0.97 (H-26), 0.96 (H-27), 1.70 (H-30)]. The ^{13}C NMR (125 MHz, $CDCl_3$) spectrum revealed the presence of 30 carbon signals, including an aldehyde carbon signal at δ 206.8 (C-28), a methylene double bond carbon signal at δ 110.2 (C-29), and an oxygenated methine carbon signal at δ 79.1 (C-3) (Table 1). The structure of **1** was suggested to be betulinic aldehyde from the ^{1}H and ^{13}C NMR spectroscopic data. A pseudomolecular ion peak was observed at m/z 441.3 $[M + H]^+$ in the ESI-MS (positive) spectrum, which agreed with the molecular weight (MW 440) of **1**. Therefore, compound **1** was identified as betulinic aldehyde (Figure 2) on the basis of a comparison of its NMR spectroscopic data obtained from the bark of *Alnus japonica*.[27]

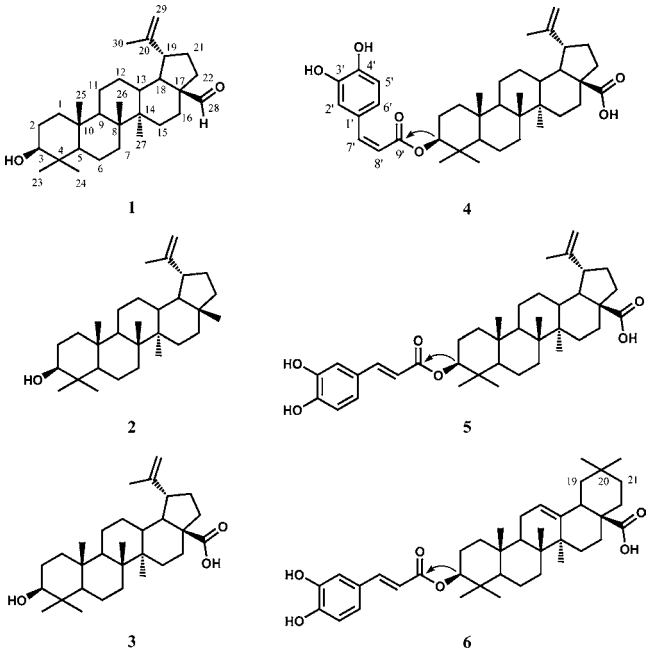

Figure 2. Structure of the isolated compounds and the important heteronuclear multiple bond correlations (arrow).

Compound **2** was obtained as a white powder. A pseudomolecular ion peak was observed at m/z 449.4 $[M + Na]^+$ in the ESI-MS (positive) spectrum, indicating that the molecular weight of **2** was 426. The ^{1}H and ^{13}C NMR spectra of **2** were very closely related to those of **1** (Table 1). Its spectra indicated the presence of a methyl group instead of an aldehyde group in the structure of **1**. The structure of **2** was suggested to be lupeol on the basis of the ESI-MS and 1D NMR spectroscopic data. The ^{13}C NMR spectrum of **2** also agreed with that of lupeol found in the bark of *Hymenocardia acida*.[28] Therefore, compound **2** was unambiguously identified as lupeol (Figure 2).

Compound **3** was obtained as a white powder. A pseudomolecular ion peak was observed at m/z 479.3 $[M + Na]^+$ in the ESI-MS (positive) spectrum, indicating that the molecular weight of **3** was 456. The ^{1}H and ^{13}C NMR spectra of **3** were very closely related to those of **1** (Table 1). Its spectra indicated the presence of a carboxyl group instead of an aldehyde group in the structure of **1**. The structure of **3** was suggested to be betulinic acid from the ESI-MS and 1D NMR spectroscopic data. In addition, the ^{13}C NMR spectrum of **3** agreed with that of betulinic acid reported previously.[29] Therefore, compound **3** was unambiguously identified as betulinic acid (Figure 2).

Compound **4** was obtained as a white powder. The ^{1}H and ^{13}C NMR spectra of **4** were very closely related to those of **1** except for the proton and carbon signals of the phenylpropanoid moiety. The ^{1}H NMR spectrum of **4** showed a CA moiety including trisubstituted benzene ring proton signals at δ 6.72 (1H, d, J = 8.4 Hz, H-5′), 7.00 (1H, dd, J = 8.4 Hz, 1.8 Hz, H-6′), and 7.28 (1H, d, J = 1.8 Hz, H-2′) and two olefinic double bond proton signals at δ 5.74 (1H, d, J = 13.2 Hz, H-8′) and 6.80 (1H, d, J = 13.2 Hz, H-7′)]. In particular, the olefinic double bond was assigned to the *cis* form from the coupling constant value (J = 13.2 Hz) of δ 5.74 (H-8′) and 6.80 (H-7′). These data were supported by the ^{13}C NMR spectrum (Table 1), together with the observation of a carbonyl carbon signal at δ 168.6 (C-9′). Compound **4** was suggested to be *cis*-caffeoylbetulinic acid. The cross-peak of the oxygenated methine proton signal at δ 4.49 (H-3) and the carbonyl carbon signal at δ 168.6 (C-9′) in the heteronuclear multiple bond correlation (HMBC) spectrum indicated that CA was etherified with the C-3 position of betulinic acid (Figure 2). In addition, a pseudomolecular ion peak was observed at m/z 641.4 $[M + Na]^+$ in the ESI-MS (positive) spectrum, which agreed with the molecular weight (MW 618) of **4**. Therefore, compound **4** was determined to be 3-*O*-*cis*-caffeoylbetulinic acid (Figure 2) on the basis of a comparison of its NMR spectroscopic data reported previously.[30]

Compound **5** was obtained as a white powder. The ESI-MS and 1D NMR spectra of **5** were very closely related to those of **4**. That is, the signals assignable to caffeoyl-betulinic acid were detected in almost the same pattern as those of **4**. In addition, the ^{1}H NMR spectrum showed the presence of CA. However, the coupling constant value of δ 6.71 (H-8′) and 8.05 (H-7′) was 16.2 Hz, indicating that the olefinic double bond in the CA moiety was in the *trans* form. Therefore, compound **5** was suggested to be *trans*-caffeoylbetulinic acid. In addition, the cross peak between the oxygenated methine proton signal (δ 4.87, H-3) and the carbonyl carbon signal (δ 167.4, C-9′) in the HMBC spectrum confirmed that CA was etherified with the C-3 position of betulinic acid (Figure 2). Therefore, compound **5** was determined to be 3-*O*-*trans*-caffeoylbetulinic acid (Figure 2) on the basis of a comparison of its NMR spectroscopic data reported previously.[30]

Compound **6** was obtained as a white powder. The molecular weight of **6** was determined to be 618 by the pseudomolecular ion peak detected at m/z 641.4 $[M + Na]^+$ in the ESI-MS (positive) spectrum. When the ^{1}H and ^{13}C NMR spectra of **6** were compared to those of **5**, the caffeoyl moiety was the same but the triterpene moiety differed. The ^{1}H NMR spectrum of **6** showed the presence of seven methyl proton signals [δ 0.83 (H-30), 0.91 (H-24), 0.94 (H-29), 0.97 (H-26), 1.00 (H-25), 1.18 (H-27), and 1.28 (H-23)], an oxygenated methine proton signal at δ 4.57 (1H, dd, J = 12.0, 4.2 Hz, H-3), and an olefinic proton signal at δ 5.25 (1H, t, J = 3.3 Hz, H-12), suggesting an olea-12-en-3-ol skeleton. The ^{1}H NMR spectrum of **6** was supported by its ^{13}C NMR spectrum. The carbonyl carbon signal at δ 182.2 (C-28) suggested that the triterpene structure of **6** was assignable to oleanolic acid. In addition, the HMBC spectrum revealed the cross-peak of the oxygenated methine proton signal at δ 4.57 (H-3) and a carbonyl carbon signal at δ 169.3 (C-9′), indicating that CA was etherified with the C-3 position of oleanolic acid (Figure 2). Therefore, **6** was determined to be 3-*O*-*trans*-caffeoyloleanolic acid (Figure 2) on the basis of a comparison of its NMR spectroscopic data reported previously.[31]

DPPH Radical-Scavenging Activity of the Isolated Compounds. The radical-scavenging activities of six compounds isolated from pear fruit peels were determined with the DPPH radical at the same concentration (32 μM) (Figure 3).

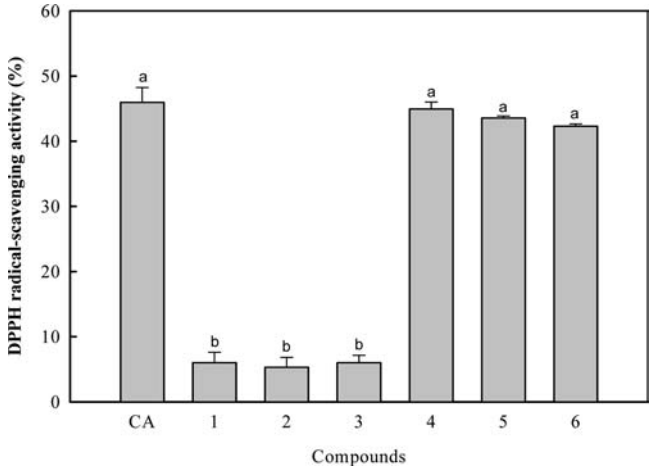

Figure 3. DPPH radical-scavenging activity of the compounds isolated from pear fruit peels. Compounds **1**–**6** were evaluated at a concentration of 32 μM in a DPPH radical (final concentration 250 μM)/ethanol solution. CA (caffeic acid; 32 μM) was used as a positive control. Data are the mean ± standard deviation (n = 3). Different letters "a" and "b" indicate a significant difference ($p < 0.05$).

Caffeoyl triterpenes **4**–**6** showed significantly higher DPPH radical-scavenging activity than the other triterpenes (**1**–**3**), which did not contain a caffeoyl moiety. The DPPH radical-scavenging activities of caffeoyl triterpenes **4**–**6** were also very similar to that of CA in the free form used as a positive control. No significant difference was observed between the antioxidative activities of caffeoyl triterpenes **4**–**6**. However, other triterpenes (**1**–**3**) that did not contain a caffeoyl moiety showed low DPPH radical-scavenging activity in this assay, and their activities did not significantly differ ($p < 0.05$). Many studies have reported that CA exerts high free radical-scavenging activity (Figure 3).[25,32] These results indicate that the catechol group of the caffeoyl moiety is an important active site for radical-scavenging activity of caffeoyl triterpenes **4**–**6**.

Determination of the Inhibitory Effect of the Isolated Compounds against Copper Ion-Induced Oxidation in Rat Blood Plasma. The antioxidative activities of the isolated compounds **1**–**6** and CA as a positive control were determined by the copper ion-induced oxidation system in rat blood plasma (Figure 4). Interestingly, the results showed a pattern very similar to that of the DPPH radical-scavenging experiment (Figure 3). That is, caffeoyltriterpenes **4**–**6** more effectively inhibited CE-OOH formation during oxidation of rat plasma when compared to **1**–**3** (Figure 4). In particular, the inhibition activities against CE-OOH formation of caffeoyl triterpenes **4**–**6** were comparable to that of CA in the free form, indicating that their antioxidant activities were probably contributed mainly by the caffeoyl groups rather than any other partial structure of the triterpene moiety.

Six triterpenes, including three caffeoyl triterpenes, isolated from pear fruit peels were identified as betulin aldehyde (**1**), lupeol (**2**), betulinic acid (**3**), 3-*O*-*cis*-caffeoylbetulinic acid (**4**), 3-*O*-*trans*-caffeoylbetulinic acid (**5**), and 3-*O*-*trans*-caffeoyloleanolic acid (**6**) (Figure 2). Compounds **2** and **3** have been previously isolated as anti-inflammatory compounds from pear

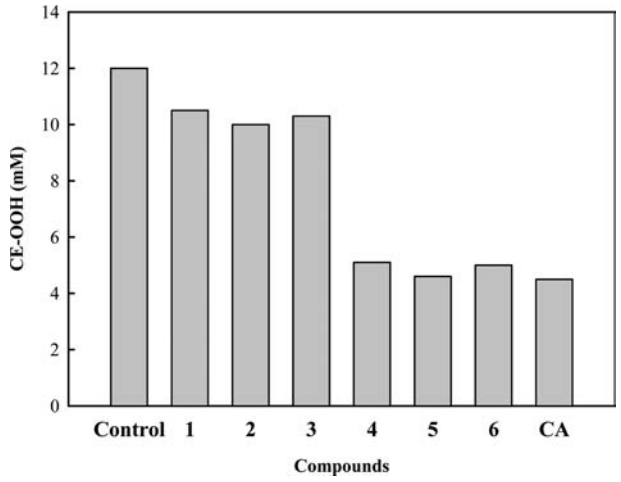

Figure 4. Inhibitory effect of compounds (16.7 μM) isolated from pear fruit peels against copper ion-induced oxidation in rat plasma. Four-fold dilutions of plasma with PBS (pH 7.4) were mixed with an EtOH solution of **1**–**6** (final concentration 16.7 μM) and then oxidized by adding $CuSO_4$ (final concentration 100 μM). The reaction mixture was incubated at 37 °C for 5 h with continuous shaking. CA (caffeic acid; 16.7 μM) was used as a positive control. Data are representative of two experiments.

fruit,[19,20] and caffeoyl triterpenes **4**–**6** have been found in some plants. That is, **4** and **5** in *Salacia cordata*,[33] *Celastrus stephanotifolius*,[30] and *Callistemon lanceolatus*[34] and **6** in *Betula pubescens*[35] and *C. stephanotifolius*[30] have been identified. In addition, compounds **4**–**6** were identified here for the first time in Asian pear fruits[36] and very recently in European pear fruits.[37] However, one triterpene (**1**) has not been previously reported in pear fruits.

Caffeoyl triterpenes exert various biological effects, including inhibition of elastase[34] and anticancer,[30] antimalarial,[38] and anti-inflammatory[39] activities. The results of the antioxidative evaluation indicated that caffeoyl triterpenes **4**–**6** significantly scavenged DPPH radicals and inhibited CE-OOH formation during rat blood plasma oxidation induced by copper ions. It is well-known that the caffeoyl group has high free radical-scavenging and metal-chelating effects.[25,32] These results indicate that caffeoyl triterpenes **4**–**6** are potential radical scavengers and metal-chelating agents. Therefore, these findings warrant further studies to understand the chemical constituents and biological effects of various pear cultivars.

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Notes

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