Isolation and Structure Determination of New Antioxidative Ferulic Acid Glucoside Esters from the Rhizome of *Alpinia speciosa*, a Zingiberaceae Plant Used in Okinawan Food Culture

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An assay-guided isolation gave three antioxidants including two newly identified compounds from the rhizomes of *Alpinia speciosa*, which is used as an important plant in the food culture of the Okinawa area of Japan. Spectroscopic analysis of the two new compounds revealed them to be new glucoside esters of ferulic acid. The antioxidant activity of the esters was measured using two different methods. Both compounds showed greater activity than that of Trolox in the TLC method; however, one of the compounds showed weaker inhibitory activity than that of Trolox and epicatechin against AMVN-induced methyl linoleate oxidation.

Keywords: *Antioxidant; ferulic acid ester; glucoside; Zingiberaceae; rhizome; Alpinia speciosa; epicatechin*

INTRODUCTION

Natural antioxidants are important materials for the prevention of the oxidative deterioration of foods. Some antioxidants are also expected to have preventative activity against oxidation-related diseases by their ingestion. Many researchers have sought new antioxidants from natural sources, especially edible or medicinal plants. The plant family Zingiberaceae has about 1400 species and most of them grow or are cultivated in tropical and subtropical Asia (Hegnauer, 1963). The plants typically have large rhizomes and people in the area have used them for food, spice, or traditional medicine in their culture. In the rhizomes, various secondary metabolites are accumulated, which show useful biological activities. We have screened the presence of antioxidants in the rhizomes of the Zingiberaceae plants, which were collected in South-East Asia (Jitoe et al., 1992) and found new curcuminoid antioxidants (Masuda et al., 1992, 1993, 1994, 1995). *Alpinia speciosa* belongs to the Zingiberaceae and is widely cultivated in the Okinawa area of Japan. The Okinawan people use this plant extensively in their food culture: for example, the leaves for food wrapping and the seeds for health maintenance (Yoshikawa, 1976). The plant also has large rhizomes; however, the useful constituents of the rhizomes have not yet been sufficiently investigated (Kimura et al., 1966; Itokawa et al., 1981; Pooter et al., 1995). During the course of our studies to find structurally new antioxidants from Zingiberaceae plants, we have investigated the rhizomes using our TLC antioxidant assay (Masuda and Jitoe, 1994). In this paper, we report in detail the isolation and structural elucidation of two new feruloyl esters, together with epicatechin from an ethyl acetate-soluble extract of *Alpinia speciosa* rhizomes.

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MATERIALS AND METHODS

Materials. Dry rhizomes of *Alpinia speciosa* were kindly provided by Nakazen (Nishihara, Okinawa, Japan). Methyl linoleate was purchased from Tokyo Kasei (Tokyo, Japan) and used after purification by silica gel column chromatography (ethyl acetate:hexane = 1:40). Linoleic acid was purchased from Kishida Chemicals (Osaka, Japan) and used without purification. Sodium dodecyl sulfate (SDS) and 2,2′-azobis(2,4-dimethylvaleronitrile)(AMVN) were obtained from Wako Chemical Industries (Osaka, Japan). Trolox was purchased from Tokyo Kasei (Tokyo, Japan). Silica gel for column chromatography (silica gel 60, No. 9385) and silica gel TLC plates (Kieselgel 60 F254) were purchased from Merck (Darmstadt, Germany). Other reagents and solvents were purchased from Nakalai Tesque (Kyoto, Japan).

Extraction and Fractionation. (Figure 2). The dry rhizomes of *Alpinia speciosa* (5.8 kg) were soaked in methanol (26 L) for 1 week at room temperature. After filtration, the filtrate was evaporated under reduced pressure to give a methanol extract (706 g). The extract was mixed with hexane-saturated methanol (3.5 L) and the precipitate produced was filtered off. The filtrate was partitioned with methanol-saturated hexane (3.5 L) to give a hexane fraction (H) and a methanol fraction. The methanol fraction was suspended in water (2 L) and sequentially extracted with ethyl acetate (2 L) and *n*-butanol (2 L) to give an ethyl acetate fraction (E) and a butanol fraction (B), respectively. After evaporation, the yields of these fraction were 25 g for (H), 144 g for (E), and 29 g for (B). One hundred milliliters of the water layer was evaporated to give a water fraction (W) (7.5 g), which was used for the antioxidant assay. The ethyl acetate fraction (144 g) was loaded on a silica gel column (silica gel 1.5 kg) and eluted with 6 L each of 50% acetone in hexane, 80% acetone in hexane, 100% acetone, 5% methanol in acetone, 10% methanol in acetone, 20% methanol in acetone, 30% methanol in acetone, 50% methanol in acetone, and 100% methanol. The eluate was collected in two 3 L fractions each for 50% acetone in hexane

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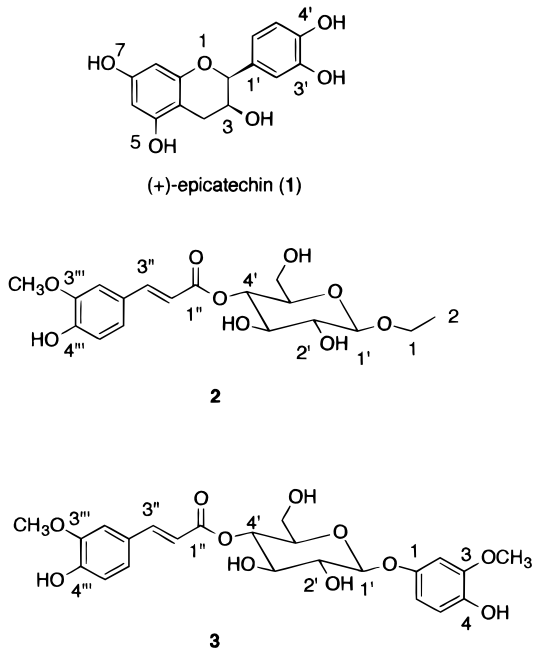

Figure 1. Chemical structures of (+)-epicatechin (**1**) and newly identified antioxidants (**2** and **3**).

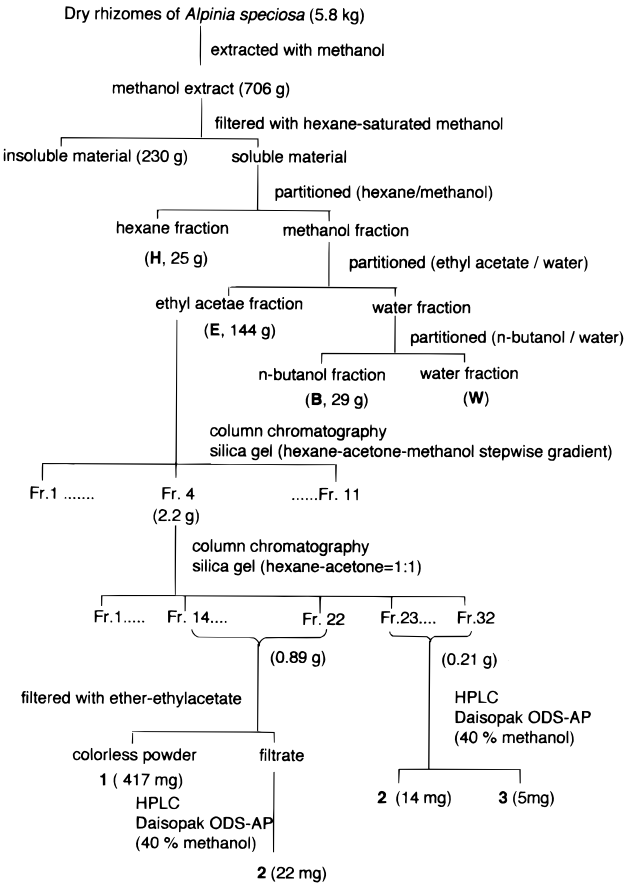

Figure 2. Isolation procedure of antioxidants (**1**, **2**, and **3**) from *Alpinia speciosa* rhizomes.

elution and 80% acetone in hexane elution, and 6 L each for the other solvent elutions, affording 11 fractions. The antioxidant activity of these fractions is shown in Figure 3.

Isolation of Antioxidants 1, 2, and 3. As one of the most active fractions, fraction 4 was chosen for the new antioxidant isolation. fraction 4 (2.2 g) was again subjected to silica gel column chromatography and eluted with acetone−hexane (1:1). Each 12 mL of the eluent was collected, which afforded 32

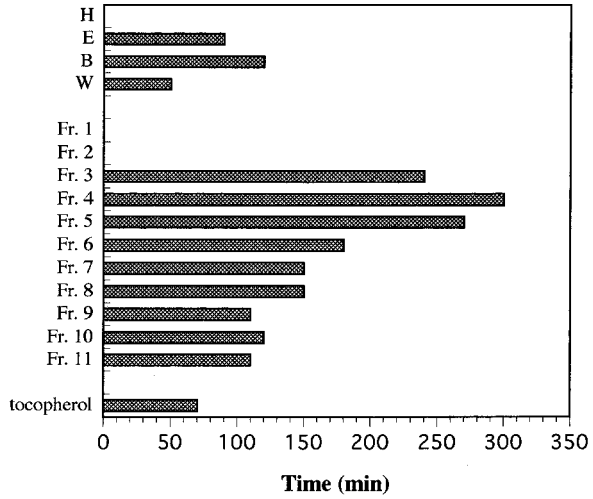

Figure 3. Antioxidant activity of the fractions from rhizomes of *Alpinia speciosa*. Bar shows induction period of each sample (10 μg/spot on TLC), which is the time for disappearance of a clear fluorescent spot in the TLC assay method. Five micrograms of α-tocopherol was used as the reference. H (hexane), E (ethyl acetate), B (butanol), and W (water) indicate the fraction names of the solvent partition and fraction numbers indicate the names of collected fractions in the first column chromatography as illustrated in Figure 2. Data were obtained in duplicate and averaged.

fractions. Fractions 14−22 were combined and evaporated, giving 0.89 g of solid. The solid was crystallized from ether−ethyl acetate to afford (+)-epicatechin (**1**, 417 mg). The mother liquid was evaporated to give a residue (0.36 g). One hundred milligrams of the residue was separated by HPLC using a preparative ODS column with 40% methanol in water (Daisopak SP-120-5-ODS-AP (20 × 250 mm), flow rate: 8 mL/min), affording 22 mg of new compound **2** from the peak at 23 min retention time (Figure 4A). Fractions 23−32 of the previous silica gel column chromatography were also combined and evaporated (0.21 g). Ninetyseven milligrams of the residue was separated by HPLC under the same conditions to give **2** (14 mg) and **3** (5 mg), at 23 and 25 min retention times, respectively (Figure 4B).

Instruments. HPLC was carried out using a Gulliver HPLC system (JASCO, Tokyo). NMR spectra were recorded by a EX-400 spectrometer (JEOL, Tokyo) using the standard ^{1}H and ^{13}C software supplied by the manufacturer. MS were recorded by an SX-102A spectrometer (JEOL, Tokyo) with EI and FAB modes. Optical rotation was measured by a DIP-360 polarimeter (JASCO, Japan) using a 10 cm cell.

Antioxidant Assays. *1. TLC Method*. This assay was performed as previously described (Masuda and Jitoe, 1994) with slight modification. Five microliters of each sample solution containing the appropriate amount of test sample was charged on a silica gel TLC plate (Kieselgel 60 F254) using a 5 μL disposable micropipet (Drummond, Broomall) to make a ca. 6 mm diameter spot. Acetone was employed as a vehicle to make the spot of the fraction samples. To obtain the best result for pure samples, appropriate solvent systems, which have the developing ability to result in a 0.3 R_f value for each sample, were chosen, (10% methanol in CH_2Cl_2 for Trolox, 20% methanol in CH_2Cl_2 for **2** and **3**, 25% methanol in CH_2Cl_2 for **1**). After the solvent was completely removed from the plate in vacuo, a 3% solution of linoleic acid in hexane (Kishida, Osaka) was sprayed. The plate was continuously irradiated by UV (254 nm) in a Fluorescence Analysis Cabinet (CM-10, Spectroline, Westbury) using a UV light (ENF-340C/J, Spectroline, Westbury). Fluorescent-emission light from each sample spot was observed every 15 min.

2. AMVN-Induced Method. To 33 μL of methyl linoleate in a test tube, a 25 mM acetone solution of the test sample (40 μL), 1 M acetonitrile solution of AMVN (30 μL), and 100 μL of acetone were sequentially added. After the solution was well mixed by a Vortex (Scientific Industry, Bohemia, NY), the

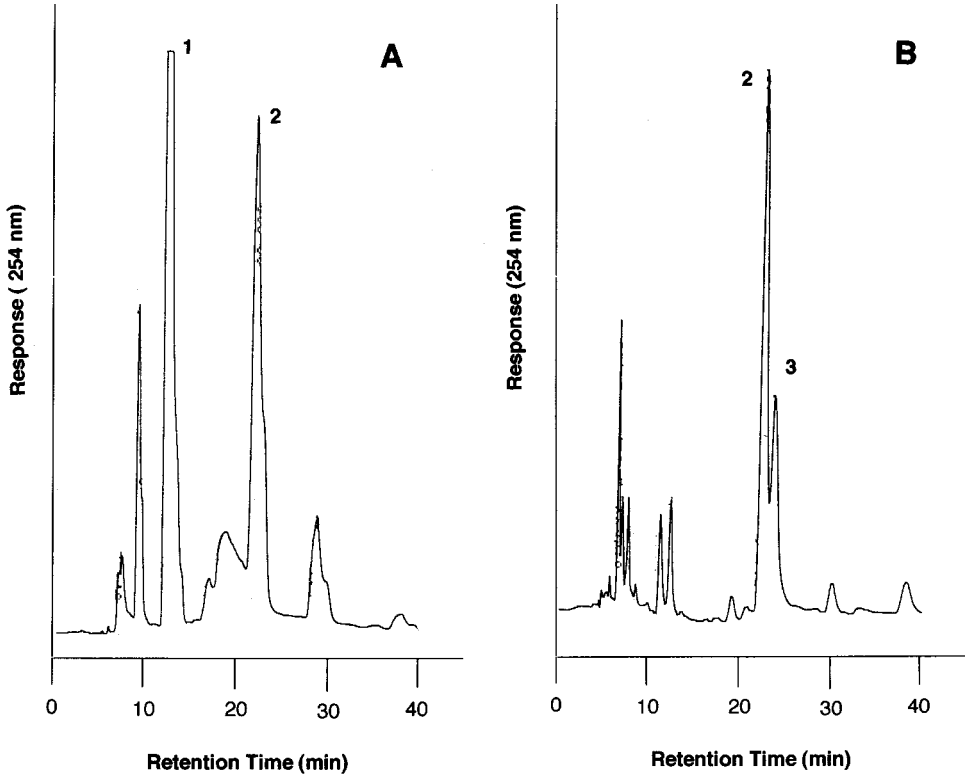

Figure 4. Preparative HPLC patterns of antioxidant-active fractions 14−22 (mother liquid of epicatechin) (A) and fraction 23−32 (B) of the second column chromatography. Column, Daisopak SP-120-5-ODS-AP (20 × 250 mm); flow rate, 8 mL/min; detection, 254 nm, elution solvent, 40% CH_3OH in H_2O.

acetone and acetonitrile were completely evaporated in vacuo. To the mixture 2 mL of 0.5 M sodium dodecyl sulfate (SDS)−0.05 M phosphate buffer (pH 7.4) was added, and well stirred by a Vortex for 2 min, and subsequently by a sonicator (Bransonic 2210, Emerson-Japan, Atsugi, Japan) for 3 min. The mixture was then incubated at 37 °C with shaking. At 30 min intervals, 20 μL of the mixture was taken up and diluted with 380 μL of methanol. Ten microliters of the diluted solution was injected into the HPLC system under the following conditions: column,YMCPak ODS-A-S-5 (4.6 × 150 mm, YMC, Kyoto); solvent, 90% CH_3CN in H_2O; flow rate, 1 mL/min; detection, 234 nm. All stereoisomers of methyl linoleate hydroperoxide (MeLOOH) were observed at 4.6 min. The amount of the methyl linoleate hydroperoxide was calculated by a calibration curve obtained using pure MeLOOH (Terao and Matsushita, 1977).

Antioxidant 1. Colorless powder, mp 235−236 °C (dec) (lit. 235−237 °C, Cadogan et al. 1996); EI-MS, *m/z* (relative intensity) 290 $[M]^+$ (50%), 230 (30%), 152 (50%) and 139 (100%); $[\alpha]_D^{22}$ +52° (C 0.50, H_2O−acetone = 1:1) [lit. $[\alpha]_D^{20}$ +59° (aq acetone), Cadogan et al. 1996]. ^{1}H NMR (acetone-d_6) δ 4.85 (s, H2), 4.18 (brs, H3), 2.70 (brd, J = 17.0 and 3.2 Hz, H4), 2.83 (dd, J = 17.0 and 4.5 Hz, H4), 5.88 (d, J = 1.8 Hz, H6), 5.99 (d, J = 1.8 Hz, H8), 7.01 (d, J = 1.8, H2′), 6.75 (d, J = 8.0 Hz, H5′), 6.80 (dd, J = 8.0 and 1.8 Hz). ^{13}C NMR (acetone-d_6) δ 79.6 (C2), 67.1 (C3), 29.2 (C4), 157.4 (C5[a]), 95.9 (C6[b]), 157.8 (C7), 96.3 (C8[b]), 157.8 (C9[a]), 100.0 (C10), 132.5 (C1′), 115.5 (C2′[c]), 145.5 (C3′[d]), 145.6 (C4′[d]), 115.7 (C5′[c]), 119.6 (C6′) [a−d assignments may be interchangeable.] (Markham et al., 1982).

Antioxidant 2. Colorless solid. Positive FABMS (magic bullet) *m/z* 407.1332 $[M + Na]^+$, calculated for $C_{18}H_{24}O_9Na$: 407.1318. $[\alpha]_D^{27}$ −34° (c 0.59, acetone). ^{1}H and ^{13}C NMR, see Table 1.

Antioxidant 3. Colorless solid. Negative FABMS (magic bullet) *m/z* 477.1423 $[M-H]^-$, calculated for $C_{23}H_{25}O_{11}$: 477.1397. $[\alpha]_D^{27}$ −27° (c 0.15, acetone). ^{1}H NMR, see Table 2.

Tetraacetate of Antioxidant 2. Two milligrams of **2** was dissolved in pyridine (0.5 mL) and acetic anhydride (0.5 mL) at room temperature. After standing for 2 h, the mixture was evaporated in vacuo. The residue was purified by silica gel TLC (2.5% methanol in CH_2Cl_2) to give a tetraacetate (2 mg). FABMS *m/z* 575 $[M + Na]^+$, ^{1}H NMR, see Table 1.

Table 1. ^{1}H and ^{13}C NMR Data of 2 and Tetraacetate of 2 (400 MHz for ^{1}H, 100 MHz for ^{13}C)

position	2 (acetone-d_6) ^{1}H[a]	2 (acetone-d_6) ^{13}C[b]	tetraacetate of **2** ($CDCl_3$) ^{1}H[c]
1	3.40−3.65 (m)	65.3 (t)	3.60 (dq, 10.0, 6.8)
			3.92 (dq, 10.0, 6.8)
2	1.14 (t, 6.3)	15.5 (q)	1.11 (t, 6.8)
1′	4.31 (d, 7.3)	103.8 (d)	4.56 (d, 7.8)
2′	3.26 (t, 7.3)	75.9 (d)[d]	5.01 (dd, 9.8, 7.8)
3′	3.65 (dd, 9.0,7.3)	75.7 (d)[d]	5.23 (t, 9.8)[g]
4′	4.82 (brt, 9.0)	72.2 (d)	5.31 (t, 9.8)[g]
5′	3.40−3.65 (m)	75.5 (d)[d]	3.78 (m)
6′	3.40−3.65 (m)	62.4 (t)	4.19 (dd, 12.5, 3.0)
			4.25 (dd, 12.5, 4.8)
1″		167.3(s)	
2″	6.38 (d, 15.6)	115.4 (d)[e]	6.31 (d, 15.5)
3″	7.61 (d, 15.6)	146.4 (d)	7.63 (d, 15.5)
1‴		127.3 (s)	
2‴	7.33 (d, 1.8)	111.2 (d)	7.09 (brs)
3‴		148.8 (s)[f]	
4‴		150.2 (s)[f]	
5‴	6.85 (d, 7.8)	116.0 (d)[e]	7.06 (d, 8.0)
6‴	7.12 (dd, 7.8, 1.8)	124.1 (d)	7.13 (brd, 8.0)
3‴-OCH_3	3.90 (s)	56.3 (q)	3.88 (s)
4‴-$OCOCH_3$			2.16 (s)
CH_3CO			1.99 (s), 2.02 (s), 2.04 (s)

[a,c] Coupling pattern and coupling constants (J in hertz) are in parentheses. [b] Coupling pattern, which are obtained by DEPT spectra, is in parentheses. [d−g] Assignments may be interchangeable.

RESULTS AND DISCUSSION

Fractionation of the Extract of *Alpinia speciosa* and Isolation of Antioxidants 1, 2, and 3. Methanol was chosen for extraction in this project, because it has a wide solubility for low molecular and moderate polar

Table 2. ^{1}H NMR Data of Compound 3 (400 MHz)

position	**3** (acetone-d_6)[a]	**3** (pyridine-d_5)[b]
2	6.77 (d, 2.6)	*c*
5	6.68 (d, 8.6)	*c*
6	6.54 (dd, 8.6, 2.6)	*c*
1′	4.85 (d, 8.0)	5.59 (d, 7.0)
2′	3.5−3.8 (overlapped)	4.41 (dd, 10.0, 7.0)
3′	3.5−3.8 (overlapped)	4.53 (t, 10.0)
4′	4.89 (t, 9.6)	5.86 (t, 10.0)
5′	3.5−3.8 (overlapped)	4.05−4.40
6′	3.5−3.8 (overlapped)	4.05−4.40
2″	6.40 (d, 16.2)	6.62 (d, 16.0)
3″	7.63 (d, 16.2)	7.96 (d, 16.0)
2‴	7.34 (d, 2.0)	*c*
5‴	6.85 (d, 8.4)	*c*
6‴	7.13 (dd, 8.4, 2.0)	*c*
3-OCH_3	3.78 (s)	3.70 (s)
3‴-OCH_3	3.90 (s)	3.80 (s)

[a,c] Coupling pattern and coupling constants (J in hertz) are in parentheses. [c] Overlapped with large solvent signals.

substances including the antioxidant-active phenolic compounds. A methanolic extract from the dry rhizomes of *A. speciosa* was separated into four fractions (H, E, B, and W) by a solvent partition method. The partition method and the yields of each fraction are shown in Figure 2. The antioxidant activities of the fractions were measured by the TLC method and the data is summarized in Figure 3. Although the hexane fraction (H) showed no activity under the conditions used, the other three fractions showed antioxidant activity. The ethyl acetate (E) and the butanol fractions (B) showed much stronger activity than that of the α-tocopherol (5 μg/spot). The efficiency of the ethyl acetate fraction was less than that of the butanol fraction; however, the yield was higher, which made us choose this fraction for further separation. The ethyl acetate fraction (E) was further fractionated using silica gel column chromatography with stepwise gradient elution, affording 11 fractions (fractions 1−11). The yields of these fractions were 3.4 g for fraction number 1 (fraction 1), 51.0 g for 2 (fraction 2), 2.9 g for 3 (fraction 3), 2.2 g for 4 (fraction 4), 6.0 g for 5 (fraction 5), 6.3 g for 6 (fraction 6), 12.0 g for 7 (fraction 7), 7.6 g for 8 (fraction 8), 8.5 g for 9 (fraction 9), 10.0 g for 10 (fraction 10), and 3.2 g for 11 (fraction 11). These fractions were assayed by the TLC method and the data are shown in Figure 3. Strong antioxidant activity was observed from the third fraction (fraction 3) to the final fraction (fraction 11), with fraction 4 being highest. Thus, the fourth fraction was chosen for purification of the antioxidants present. This fraction was loaded on a silica gel column and eluted with acetone−hexane (1:1). The eluate was collected in 12 mL aliquots and the elution of active compounds was observed starting from the 14th collected fraction (data not shown). From the results of the constituent analysis by TLC, fractions 14−22 and fractions 23−32 were combined and evaporated. Crystallization of the residue from the fractions 14−22 gave compound **1** as the main constituent of the fractions, and then compound **2** was isolated as the next main compound by preparative HPLC. From fractions 23−32, compounds **2** and **3** were also isolated by HPLC as the two main compounds in these fractions (Figure 4).

Structure Determination of Compounds 1, 2, and 3. Compound **1** was identical to (+)-epicatechin by comparison of its physicochemical and spectroscopic data with reported data (Cadogan et al., 1996; Markham et al., 1982). Compound **2** has the molecular formula $C_{18}H_{24}O_9$ based on high-resolution FABMS. The ^{1}H NMR spectrum of **2** showed typical signals due to a trisubstituted benzene ring [δ 7.33 (1H, d, J = 1.8 Hz), 7.12 (1H, dd, J = 7.8 and 1.8 Hz), 6.85 (1H, d, J = 7.8 Hz)], downfield-shifted trans olefin [δ 7.61 (1H, d, J = 15.6 Hz), 6.38 (1H, d, J = 15.6 Hz)], and a phenolic methoxyl group [δ 3.90 (3H, s)]. These data indicated the presence of a ferulic acid structure, which was confirmed by an NOE observation between the methoxyl signal at 3.90 ppm and the aromatic signal at 7.33 ppm. Many other proton signals were observed around 3.5−5 ppm, indicating the presence of a sugar moiety. To identify the sugar, **2** was acetylated with acetic anhydride in pyridine and then analyzed by ^{1}H NMR. In the spectrum of the tetraacetate of **2**, signals due to the protons at the 1-, 2-, 3-, and 4-positions of the acetylated sugar moiety all showed diaxial couplings [δ 4.56 (d, J = 7.8 Hz), 5.01 (dd, J = 9.8 and 7.8 Hz), 5.23 (t, J = 9.8 Hz), 5.31 (t, J = 9.8 Hz), respectively], which revealed that the sugar was β-glucopyranose. The position of attachment of the feruloyl group was determined to be at the 4-position of the sugar, because the proton signal of the 4-position, which was assigned based on a decoupling experiment, was typically downfield-shifted to 4.82 ppm. The attached group at the anomeric position of the sugar was determined to be an ethyl group by the proton signals [δ 1.11 (3H, t, J = 6.8 Hz), 3.60 (1H, dq, J = 10.0 and 6.8 Hz), 3.92 (dq, J = 10.0 and 6.8 Hz)], which were clearly observed in the tetraacetate of **2**. Thus, the structure of compound **2** was determined to be ethyl 4-*O*-feruloyl-β-glucopyranoside as shown in Figure 1. Compound **3** has the molecular formula $C_{23}H_{26}O_{11}$ based on negative FABMS data. Although the ^{1}H NMR data of **3** was very similar to that of **2**, signals due to an additional trisubstituted benzene [δ 6.77 (d, 2.6 Hz), 6.68 (d, J = 8.6 Hz), 6.54 (dd, J = 8.6 and 2.6 Hz)] were observed instead of the signals due to the ethyl group in **2**. The position of attachment of the benzene ring was confirmed as the 1-position of the sugar moiety by the NOE observations between H1′ and H2, and between H1′ and H6 in the NOE differential spectra of **3**. The spectra also revealed that a methoxyl group [δ 3.78 (3H, s)] was attached to the 3-position of the benzene ring from the NOE observation between H2 and the methoxyl signal. The axial coupling constants in H1′, H2′, H3′, and H4′ [δ 5.59 (d, J = 7.0 Hz), 4.41 (dd, J = 10.0 and 7.0 Hz), 4.53 (t, J = 10.0 Hz), 5.86 (t, J = 10.0 Hz), respectively] on the glycosyl portion of **3**, which were observed in pyridine-d_5, identified that the sugar as β-glucopyranose. The downfield shifted value of the chemical shift of H4′ [δ 4.89 ppm] indicated a ferulic acid moiety [δ 7.34 (d, J = 2.0 Hz), 6.85 (d, J = 8.4 Hz), 7.13 (dd, J = 8.4 and 2.0 Hz), 6.40 (d, J = 16.2 Hz), 7.63 (d, J = 16.2 Hz)] that was esterified to the 4-hydroxyl group of the sugar. Thus, compound **3** was determined to be 4-hydroxy-3-methoxyphenyl 4-*O*-feruloyl-β-glucopyranoside, as illustrated in Figure 1.

Antioxidant Activity of 1, 2, and 3. We employed the TLC method as a simple antioxidant assay for isolation work (Masuda and Jitoe, 1994). The antioxidant activity of the pure isolated compounds (**1**, **2**, and **3**) was measured by this method and compared with Trolox as the reference antioxidant. These results are shown in Figure 5. This figure shows that all isolated compounds have a higher antioxidant activity than that of Trolox, which can be determined from the fluorescence-persisting time of spot of each compound (Chang et al.,

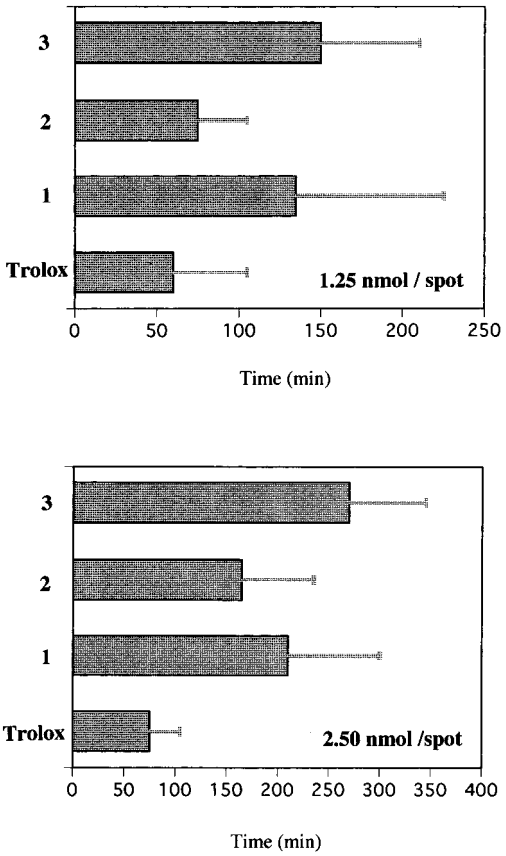

Figure 5. TLC assay results for the isolated compounds (**1**, **2**, and **3**) and Trolox. Each large bar shows the period of clear fluorescent spot and each small bar shows the period during which the fluorescent spot disappears. Data were obtained in duplicate and averaged.

1983). Epicatechin has recently attracted much attention as a potent antioxidant from tea (Ho et al., 1997). One of the newly identified compounds, **3**, has more potent activity than that of epicatechin in this TLC method under both 1.25 nmol/spot and 2.5 nmol/spot conditions. Ferulic acid is an antioxidant-active phenolic acid and widely distributed in plants (Graf, 1992). The feruloyl moiety observed in structures **2** and **3** should contribute to their antioxidant activity. Moreover, **3** has a hydroquinone substituent at the 1-position of the glucose moiety. Although hydroquinone and related compounds have a strong antioxidant activity (Mukai et al., 1992), the glucoside derivative of hydroquinone is less active than the original hydroquinone (Ioku et al., 1992). It is well-known that a methoxyl group at the ortho-position of the phenol group enhances the activity (Cuvelier et al., 1992). This *o*-methoxyl effect may contribute to the strong activity of **3**. Another antioxidant assay, which can establish a rate factor of antioxidation, was carried out using compounds **1** and **2**, of which sufficient was isolated, and Trolox as a reference. The method is due to HPLC analysis of the time course accumulation of methyl linoleate hydroperoxide, which was formed by the AMVN-induced oxidation of methyl linoleate. These results are shown in Figure 6. The data show that Trolox is the most efficient, followed by epicatechin. Aruoma et al. (1997) reported the rate constants of various phenolics for the reaction with a peroxyl radical and showed that Trolox had a 2-fold order of magnitude higher rate constant (2.2×10^8 M^{-1} s^{-1}) than those of ferulic acid (7.5×10^6 M^{-1} s^{-1}) and epicatechin (7.3×10^6 M^{-1} s^{-1}). This may

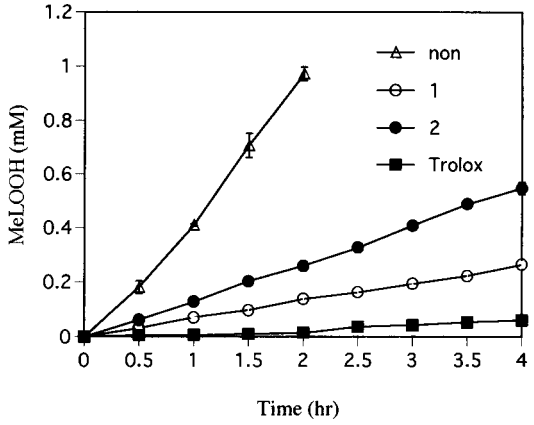

Figure 6. Inhibitory activity of **1**, **2,** and Trolox (0.5 mM each) against AMVN-induced oxidation of methyl linoleate (MeLO). "Non" means a control experiment result, which was performed without antioxidant. Data were obtained in triplicate and averaged. Each SD was expressed by an error bar when it was larger than the used symbol.

be one of the reasons for the more efficient activity of Trolox in this assay system. Compound **3** showed a stronger activity than those of epicatechin and Trolox in the TLC method. Thus, its efficient activity was expected for other antioxidant assays including this AMVN-induced method. Unfortunately, this compound could not be isolated in sufficient amount for the AMVN-induced assay at this stage. For further investigation, preparation by synthesis need to be developed.

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