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In Vitro Antioxidative Effects and Tyrosinase Inhibitory Activities of Seven Hydroxycinnamoyl Derivatives in Green Coffee Beans

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Seven kinds of hydroxycinnamic acid derivatives identified as 3-caffeoylquinic acid (3-CQA), 4-caffeolyquinic acid (4-CQA), 5-caffeoylquinic acid (5-CQA), 5-feruloylquinic acid (5-FQA), 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA), and 4,5-dicaffoylquinic acid (4,5-diCQA) by MS, ^{1}H NMR, and HPLC analyses were isolated from low-quality (immature) and commercial quality green coffee beans. The quantity of chlorogenic acid isomers (10.4 g/100 g), especially 5-CQA, in commercial green coffee beans [*West Indische Bereiding* (West India processing beans from Sumatra Island, Indonesia, WIB)] was higher than that in low-quality beans [9.1 g/100 g, *Eerste Kwaliteit* (Export low-quality beans from Java Island, Indonesia, EK-1, grade 4)], whereas little difference in diCQAs was detected between the two kinds of beans. The free radical scavenging activity of these isolates was evaluated in assay systems using DPPH free radicals and superoxide anion radicals generated by xanthine−XOD. The diCQAs showed strong (1.0−1.8-fold) free radical scavenging activity compared to commonly used antioxidants such as α-tocopherol and ascorbic acid. The potency order of superoxide anion radical scavenging activity was diCQAs > caffeic acid, CQAs > 5-FQA. The activities of the diCQAs were twice as effective as those of CQAs and 4 times as effective as that of 5-FQA. The diCQAs also exhibited more potent (2.0−2.2-fold) tyrosinase inhibitory activities compared to CQAs, arbutin, and ascorbic acid. The isolates exhibited antiproliferation activities in four cancer cell lines, U937, KB, MCF7, and WI38-VA. Among these, KB cells were most sensitive (IC_{50} = 0.10−0.56 mM).

KEYWORDS: Green coffee beans; caffeoylquinic acid; feruloylquinic acid; dicaffeoylquinic acid; antioxidants; tyrosinase; antiproliferation activity

INTRODUCTION

Coffee beans contain considerable amounts of hydroxycinnamic acid derivatives, including 4.3−7.2% caffeoylquinic acids (CQAs), 0.3−1.2% feruloylquinic acids (FQAs), and 0.8−2.5% dicaffeoylquinic acids (diCQAs) (**Figure 1**) (*1*). *Coffea canephora* var. *robusta* contains significantly higher amounts (80−100 mg/g) of these compounds compared to *Coffea arabica* (*1*), and the CQA and diCQA content is one of the factors that determines the quality of coffee beans (*2*, *3*).

Most low-quality green coffee beans consist of immature beans. The ratio of CQAs to total chlorogenic acids (CQAs, FQAs, and diCQAs) in green coffee beans increases with maturity, whereas the ratio of diCQAs decreases (*3*). Contamination of immature beans negatively affects coffee beverage flavor because of the high ratio of diCQAs to CQAs. Therefore, these beans are not marketed in coffee drinks or effectively utilized as resources. We are therefore currently investigating the effective utilization of these coffee beans.

Hydroxycinnamic acid compounds have physiological activities such as being potent antioxidants (*4*, *5*), inhibitors of hepatotoxic and proliferative activity (*6*−*8*), inhibitors of HIV-integrase (*9*−*12*), and enhancers of macrophage spreading and mobility (*13*). Chlorogenic acid (5-CQA) inhibits linoleic acid peroxidation, and 1,3-dicaffeoylquinate inhibits the γ-irradiation-induced oxidation of 5-CQA (*4*, *5*). However, the physiological effects of CQAs, FQA, and diCQAs, which are major hydroxycinnamic acid derivatives in green coffee beans, have not been compared in vitro under the same assay conditions. We compared the following physiological activities of these compounds in four cancer cell lines under the same conditions: DPPH free radical and superoxide anion radical scavenging activities, tyrosinase inhibitory activities, and antiproliferation activities.

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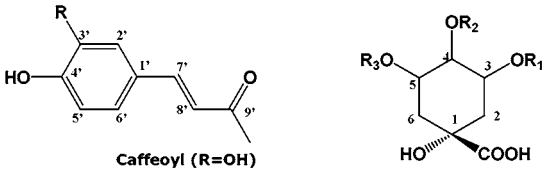

Compound	R_1	R_2	R_3
3-Caffeoylquinic acid (3-CQA)	Caffeoyl	H	H
4-Caffeoylquinic acid (4-CQA)	H	Caffeoyl	H
5-Caffeoylquinic acid (5-CQA)	H	H	Caffeoyl
5-Feruloylquinic acid (5-FQA)	H	H	Feruloyl
3,4-Dicaffeoylquinic acid (3,4-diCQA)	Caffeoyl	Caffeoyl	H
3,5-Dicaffeoylquinic acid (3,5-diCQA)	Caffeoyl	H	Caffeoyl
4,5-Dicaffeoylquinic acid (4,5-diCQA)	H	Caffeoyl	Caffeoyl

Figure 1. Chemical structures of the major component of hydroxycinnamic acid derivatives in coffee beans.

MATERIALS AND METHODS

Sample and Reagents. Low-quality (EK-1) and commercial quality (WIB) coffee beans (*C. canephora* var. *robusta*) obtained from Java and Sumatra Islands, Indonesia, in 2002, respectively, were used. Caffeic acid, quinic acid, and 3,4-dihydroxyhydrocinnamic acid (3,4-diOH HCA) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Sephadex LH-20 was purchased from Amersham Biosciences AB (Uppsala, Sweden), and other chemicals of analytical grade were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Sample Preparation. Two kinds of green coffee beans (100 g) were ground and subjected to extraction three times with 1 L of 70% (v/v) aqueous methanol under reflux at 80 °C for 20 min. The extracts were concentrated to 50 mL and stored at 4 °C for 48 h to form a yellow hydroxycinnamic acid derivatives−K−caffeine complex precipitate (*14*, *15*). The precipitate was dissolved in tartaric acid solution, and the resulting crystalline potassium tartarate was removed. Then, the solution was subjected to extraction with $CHCl_3$ to remove caffeine. The aqueous layer was run through a Sephadex LH-20 column (450 × 26 mm i.d.) and scanned for absorbance at 325 nm. Absorbant fractions were collected by linear gradient methanol (0−70%) in 35 mM acetic acid aqueous solution at 60 mL/h. Each fraction concentrate was subjected to reversed-phase column chromatography in a preparative HPLC PLC-561 system (GL Sciences Inc., Tokyo, Japan), where the conditions were as follows: column, Inertosil ODS-3 250 × 19 mm i.d. (GL Sciences Inc.); column temperature, 40 °C; mobile phase, solvents A (20% CH_3OH in 35 mM acetic acid) and B (methanol), gradient, 0.0 min, A = 100%, 60.0 min, A:B = 1:1, 70.0 min, B = 100%, 80.0 min, A = 100%; detection, UV 326 nm. All samples were eluted at 15 mL/min, and the collected fractions were once again subjected to a Sephadex LH-20 column and eluted with methanol. The purified fractions were then freeze-dried and stored at −20 °C.

Structural Analysis. Fast atom bombardment mass spectra (FAB-MS) were measured with a JEOL Tandem MStation JMS-700TKM mass spectrometer (JEOL Ltd., Tokyo, Japan) using a direct inlet system with Xe for the FAB gas, FAB energy at 6 keV, an emission current of 3 mA, accelerating voltage of 10 kV, scan range with *m*/*z* 50−1000, and glycerol matrices. ^1H NMR spectra were measured with JEOL GSX-500 (500 MHz) instruments in CD_3OD with tetramethylsilane as an internal standard. UV absorption spectra were recorded using a U3310 spectrometer (Hitachi, Tokyo, Japan).

DPPH Radical Scavenging Activity. The DPPH radical scavenging activity of the samples was analyzed according to a modified procedure of Yoshikawa et al. (*16*). Samples in ethanol (EtOH) solution (0.2 mL) were added to a mixture of 100 mM acetate buffer (pH 5.5, 2.0 mL) and 0.1 mM DPPH in EtOH (1.0 mL) in a test tube and left to stand at room temperature in the dark for 30 min. The absorbances of the resulting solutions were measured at 517 nm. Ascorbic acid and α-tocopherol were used as a standard, and the control was prepared without the samples. The activity was expressed as the concentration of sample necessary to give a 50% reduction in the sample absorbance (IC_{50}).

Superoxide Anion Scavenging Activity. Superoxide anion scavenging activity was determined according to a modified method of Matsushige et al. (*17*). Reaction mixtures containing 1.4 mL of 50 mM Na_2CO_3 (pH 10.2), 100 μL of 3 mM xanthine, 100 μL of 3 mM EDTA, 100 μL of BSA (1.5 mg/mL), 100 μL of 75 mM nitro blue tetrazonium, and 50 μL of the samples were preincubated at 30 °C for 10 min, and 50 μL of xanthine oxidase (0.3 unit/mL) was added. After incubation at 30 °C for 20 min, 200 μL of 6 mM $CuCl_2$ was added to stop the reactions and the absorbances were measured at 560 nm. The activity was expressed as the sample concentration necessary to give a 50% reduction in the sample absorbance (IC_{50}).

Tyrosinase Inhibitory Activity. The activity of mushroom tyrosinase (EC 1.14.18.1) was determined using L-tyrosine and L-DOPA as substrates according to a modified method of Tada et al. (*18*). The activity was expressed as the sample concentration that gave a 50% inhibition in the enzyme activity (IC_{50}).

L-Tyrosine as Substrate. Reaction mixtures (1 mL) containing 0.55 mM L-tyrosine in 10 mM phosphate buffer (pH 6.8), tyrosinase (60 units), and the samples were each incubated at 37 °C for 10 min, and the absorbances were measured at 475 nm.

L-DOPA as Substrate. Reaction mixtures (2 mL) containing 0.85 mM L-DOPA in 19 mM phosphate buffer (pH 6.8), tyrosinase (30 units), and the samples were each incubated at 25 °C, and the absorbances at 475 nm were continuously measured from 0.5 to 2 min.

Antiproliferation Activity. Three cell cultures containing human histiocytic lymphoma U937, normal human diploid lung fibroblast WI38, and SV40 virally transformed WI38 (WI38VA) were obtained from the Health Service Research Resources Bank, Osaka, Japan. Human breast carcinoma MCF-7 (ATCC HTB22) and human oral carcinoma KB cells were obtained from the American Type Culture Collection (Rockville, MD) and Tottori University (Yonago, Japan), respectively. The U937 cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum (Flow Laboratories, Irvine, U.K.). The KB cells were cultured in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% calf serum (Flow Laboratories), and the WI38, WI38VA, and MCF-7 cells were cultured in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum. All media were supplemented with 100 units of penicillin/mL, 100 μg of streptomycin/mL, and 60 μg of kanamycin/mL. The cells were cultured at 37 °C in a humidified atmosphere in the presence of 5% CO_2.

The antiproliferation activities of the samples were examined in four human cancer cell lines (U937, KB, MCF-7, and WI38VA) and one normal cell line (WI38), and viable cells were enumerated after incubation with the isolated hydroxycinnamic acid derivatives by WST-1 assay (*19*). The 50% inhibitory concentration (IC_{50}) was determined from two independent experiments ($n = 4$). U937 cells (50 μL) were plated at 5×10^4 cells/well in 96-well tissue culture plates, and then 50 μL of sample solution adjusted to each concentration was added to the wells. After incubation at 37 °C for 1 day, 10 μL of WST-1 reagent (Dojindo Laboratory, Kumamoto, Japan) was added to each well, and incubation was continued for another 3 h. The difference in absorbance between 450 and 650 nm was measured using a plate analyzer (Toyo-sokki Co., Yokohama, Japan). The remaining five kinds of cells were plated at 5×10^3 cells/well in 96-well tissue culture plates and incubated at 37 °C for 1 day. After removal of the culture broth, 100 μL of sample adjusted to each concentration in the medium was added to the wells and incubated for 1 day. WST-1 reagent was added, and the difference in absorbance was measured.

Antiproliferation activity was calculated using the following formula:

$$\text{antiproliferation activity (\%)} = \{[(A_{450}\text{ of the cell culture without sample}) - (A_{450}\text{ of the medium})] - [(A_{450}\text{ of the cell culture with sample}) - (A_{450}\text{ of the medium containing sample})]/[(A_{450}\text{ of the cell culture without sample}) - (A_{450}\text{ of the medium})]\} \times 100$$

Nuclear morphological changes in U937 cells treated by the samples

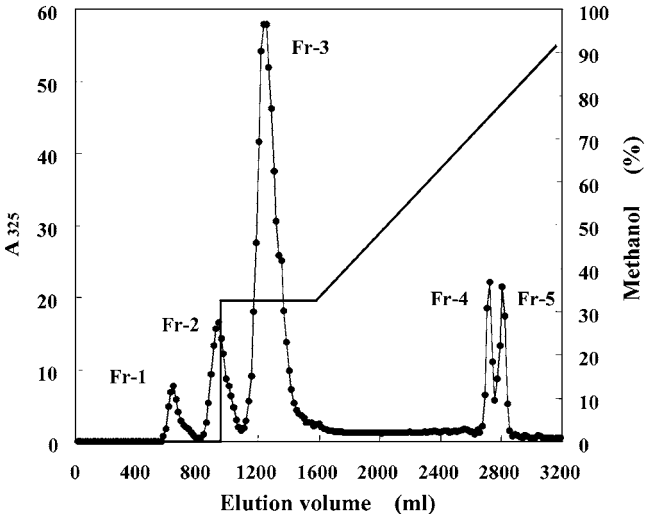

Figure 2. Chromatogram of 70% MeOH extract from green coffee beans obtained by Sephadex LH-20 column chromatography. Fractions containing hydroxycinnamic acid derivatives were eluted with a linear gradient of methanol (0–70%) in 35 mM acetic acid at 60 mL/h.

were observed under a fluorescence microscope after staining by Hoechst 33258 (Sigma-Aldrich).

Statistical Analysis. All values were expressed as the mean ± standard deviation (SD). Data analysis was carried out with Statcel software (OMS Publishing Inc., Tokyo, Japan). Student's *t* test for unpaired observations was used for the statistical evaluation of differences, and a *p* value below 0.05 or 0.01 was considered to be statistically significant.

RESULTS AND DISCUSSION

Isolation of Compounds That Absorbed at 326 nm from Green Coffee Beans. Five separate peaks at 326 nm were obtained from extracts of low-quality and commercial quality green coffee beans by Sephadex LH-20 column chromatography (**Figure 2**). Peaks 2 and 5 were further separated into two peaks by preparative HPLC purification, respectively. Purification combined with Sephadex LH-20 column chromatography and preparative HPLC was effective in isolating seven kinds of compounds at 326 nm from the extracts.

The HPLC profiles of the peaks at 326 nm from the two kinds of green coffee beans were identical to those previously reported (*20*, *21*). HPLC analysis with mobile phase containing 35 mM acetic acid instead of 2 mM phosphoric acid (*22*) made it possible to clearly separate seven peaks from the extracts.

Identification of Seven Compounds Isolated from Green Coffee Beans by Instrumental Analysis. HPLC retention times, FAB-MS and MS-MS data, and ^{1}H NMR data on the isolated seven compounds are listed in **Tables 1** and **2**, respectively. All isolated compounds had a UV absorption maximum at 325 nm. FAB-MS data on compounds **1**, **2**, and **4** gave a molecular ion at 354. For MS-MS with positive ion mode, $[M + 1]^+$ gave an ion at *m*/*z* 163 as a base peak resulting from positive ionization of the carbonyl oxygen of the caffeoyl moiety reported by Fang et al. (*23*). Three compounds also produced an ion at *m*/*z* 191 corresponding to the quinic moiety fragment in MS-MS of $[M - 1]^-$. These MS data indicated that three compounds were chlorogenic acid isomers. The ^{1}H NMR spectra of compounds **1**, **2**, and **4** were the sum of the spectra of caffeic acid and quinic acid (**Table 2**). The chemical shifts of the C-3, C-4, and C-5 protons in the three compounds are shifted downfield from those of quinic acid. Corse et al. (*24*) reported that the chemical shifts are directly related to substitution of the hydroxyl groups of quinic acid. Morishita et al. (*20*) and Islam et al. (*25*) determined the position of the ester bond in the quinic acid moiety of the chlorogenic acid derivatives on the basis of their chemical shifts. On the basis of the chemical shifts of the C-3, C-4, and C-5 protons, compounds **1**, **2**, and **4** were identified as 3-CQA, 4-CQA, and 5-CQA, respectively. The ^{1}H NMR spectrum of 5-CQA was identical to that of commercial 5-CQA, and the NMR data of these compounds were similar to those reported by Morishita et al. (*20*). The HPLC retention times of compounds **1**, **2**, and **4** also matched those of 3-CQA, 4-CQA, and 5-CQA reported by Ky et al. (*22*), respectively. From these results, compounds **1**, **2**, and **4** were identified as 3-CQA, 4-CQA, and 5-CQA, respectively.

Compound **3** in FAB-MS had a molecular ion at 368. For MS-MS analysis, $[M + 1]^+$ indicated an ion at *m*/*z* 177 corresponding to the positive ionization fragment of the carbonyl oxygen of the feruloyl moieties, and $[M - 1]^-$ gave an ion at *m*/*z* 191 corresponding to the fragments of quinic acid. The ^{1}H NMR spectrum of compound **3** was similar to that of 5-FQA reported by Morishita et al. (*20*). From these results and a comparison of the HPLC retention times, compound **3** was identified as 5-FQA.

FAB-MS of compounds **5**−**7** gave a molecular ion at 516. For MS-MS analysis, $[M + 1]^-$ gave two ions at *m*/*z* 355 and 163 corresponding to the chlorogenic acid fragment derived from the loss of a caffeoyl group and the positive ionization fragment of the carbonyl oxygen of the caffeoyl moiety, respectively, and $[M - 1]^-$ gave five ions at *m*/*z* 353, 191, 179, 173, and 135. The ion at *m*/*z* 353 indicated a chlorogenic acid fragment derived from the loss of a caffeoyl group. Ions at *m*/*z* 191 and 173 indicated fragments of the quinic moiety, and ions at *m*/*z* 179 and 135 indicated fragments of the caffeoyl moiety (*23*). These MS data indicated that compounds **5**−**7** were dicaffeoylquinic acid isomers. The ^{1}H NMR peaks of C-3, C-4, and C-5 in compounds **5**−**7** were shifted downfield from those of quinic acid, which indicated substitution of the hydroxyl groups of quinic acid (**Table 2**). From these results, compounds **5**−**7** were identified as 3,5-diCQA, 3,4-diCQA, and 4,5-diCQA, respectively. The NMR data were similar to those reported by Morishita et al. (*20*), Basnet et al. (*6*), and Islam et al. (*25*). The HPLC retention times of compounds **5**−**7** also matched those of dicaffeoylquinic acid isomers reported by Ky et al. (*22*), respectively.

The yields of seven isolated compounds from 100 g of low-quality and commercial quality green coffee beans are listed in **Table 3**. Chlorogenic acid monoisomeres, especially 5-CQA, were present in higher amounts in commercial beans (WIB) than in low-quality beans (EK-1), but little change in diCQAs was detected between WIB and EK-1 beans. These results are similar to those of Menezes (*3*); the chlorogenic acid content of the monoisomers increased with the maturity of the coffee beans.

Antioxidative Activity of Hydroxycinnamic Acid Derivatives. DPPH radical and superoxide anion radical scavenging activities (IC_{50}) of the seven hydroxycinnamic acid derivatives ranged from 5.6 to 10 μM and from 4.3 to 36 μM, respectively (**Figures 3** and **4**). Superoxide anion radical scavenging activity exhibited a greater difference among the samples tested compared with DPPH radical scavenging activity. The diCQAs had a higher DPPH radical scavenging activity than α-tocopherol and ascorbic acid, which are commonly used as antioxidants, and CQAs and 5-FQA ($p < 0.01$, **Figure 3**). The diCQAs also showed a greater superoxide anion radical scavenging activity compared with CQAs and caffeic acid ($p < 0.01$, **Figure 4**), whereas 5-FQA and 3,4-diOH HCA showed weak activity. Furthermore, quinic acid had no activity. Saturation at the C7 double-bond position (3,4-diOH HCA) and

Table 1. Identification of Hydroxycinnamic Acid Derivatives Isolated from Green Coffee Beans Using Their Spectral Characteristics in HPLC and Positive and Negative Ions in FAB-MS and MS-MS

compd	HPLC retention time (min)	positive ions		negative ions		estimated MW	identification
		MS $[M+1]^+$	MS-MS (*m/z*)	MS $[M-1]^-$	MS-MS (*m/z*)		
1	1.45	355	163	353	191	354	3-CQA
2	17.7	355	163	353	191	354	4-CQA
3	23.6	369	177	367	191	368	5-FQA
4	14.8	355	163	353	191	354	5-CQA
5	38.6	517	163, 355	515	135, 173, 173, 179, 191, 353	516	3,5-diCQA
6	35.8	517	163,355		135, 173, 173, 179, 191, 353	516	3,4-diCQA
7	44.0	517	163,355		135, 173, 173, 179, 191, 353	516	4,5-diCQA

Table 2. ^{1}H NMR Spectral Data[a] of Compounds **1**–**7**, QA, CA, and FA

carbon	compd						
	1	**2**	**3**	**4**	QA	CA	FA
2	2.18	2.19	2.22	2.18	2.18		
3	5.66	4.32	4.21	4.30	4.01		
4	3.94	5.04	3.82	3.94	3.68		
5	4.32	4.35	5.42	5.41	4.18		
6	2.28, 2.01	2.29, 2.02	2.29, 2.02	2.29, 2.02	2.21, 1.94		
2	6.99	7.00	7.21	7.00		7.02	7.19
5	6.73	6.75	6.81	6.75		6.76	6.79
6	6.88	6.91	7.08	6.91		6.93	7.05
7	6.19	6.21	6.32	6.21		6.18	6.36
8	7.50	7.52	7.58	7.52		7.51	7.55
1-OH	4.82	4.83	4.88	4.81	4.80		
4-OH	4.07 or 4.66	4.07 or 4.65	4.08 or 4.66	4.12 or 4.65	4.01 or 4.61		
5-OH	4.07 or 4.66	4.07 or 4.65	4.08 or 4.66	4.12 or 4.65	4.01 or 4.61		
7-OH	12.10	12.06	12.09	12.04	12.01		
3-OH	9.49	9.46		9.42		9.40	
4-OH	9.11	9.10	9.11	9.06		9.02	9.02
OCH_3			4.07				4.12

carbon	compd		
	5	**6**	**7**
2	2.18	2.20	2.18
3	5.49	5.68	4.33
4	3.95	5.12	5.06
5	5.43	4.35	5.64
6	2.30, 2.12	2.29, 2.03	2.35, 2.14
2,2	7.11, 7.09	7.03, 7.00	7.06, 7.03
5,5	6.80, 6.78	6.74, 6.73	6.76, 6.73
6,6	7.00, 6.98	6.91, 6.88	6.91, 6.88
7,7	6.38, 6.28	6.29, 6.21	6.24, 6.21
8,8	7.63, 7.61	7.62, 7.53	7.59, 7.56
1-OH	5.07	5.04	5.02
4-OH	4.41 or 4.95	4.38 or 4.92	4.36 or 4.89
5-OH	4.41 or 4.95	4.38 or 4.92	4.36 or 4.89
7-OH	12.39	12.39	12.36
3,3-OH	9.79, 9.76	9.81, 9.77	9.78, 9.76
4,4-OH	9.48, 9.42	9.45, 9.42	9.44, 9.41

Table 3. Yield of Hydroxycinnamic Acid Derivatives in WIB and EK-1 Grade 4 (Grams per 100 g of Dry Weight Basis)

compd	WIB	EK-1 grade 4
3-CQA	0.69 ± 0.03	0.85 ± 0.04
4-CQA	1.53 ± 0.13	1.46 ± 0.06
5-CQA	5.55 ± 0.31	4.38 ± 0.19
5-FQA	1.40 ± 0.88	1.12 ± 0.03
3,4-diCQA	0.31 ± 0.01	0.33 ± 0.04
3,5-diCQA	0.52 ± 0.03	0.50 ± 0.04
4,5-diCQA	0.34 ± 0.07	0.44 ± 0.12

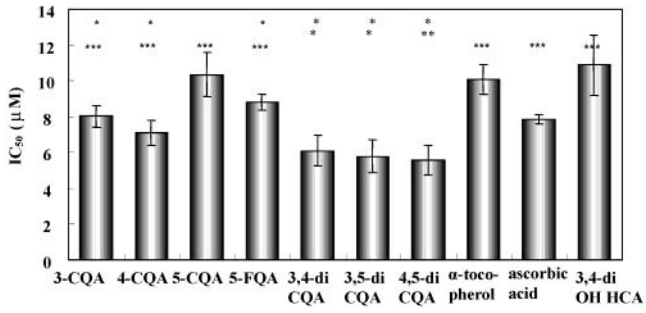

Figure 3. DPPH radical scavenging activity of chlorogenic acid isomers. Results are expressed as the mean ± SD ($n = 5$). ∗, $p < 0.01$ versus α-tocopherol; ∗∗, $p < 0.01$ versus ascorbic acid; ∗∗∗, $p < 0.01$ versus diCQAs.

methoxylation at the C3 position (5-FQA) of the caffeoyl group lowered the scavenging activity of the superoxide anion radical to less than half of those of caffeic acid and 5-CQA, respectively (**Figure 4**). This double bond is able to conjugate with other double bonds and hydroxyl groups in the phenol ring of the caffeoyl group, which may act as an acceptor of free radicals and may easily abstract the hydrogen atom from the caffeoyl group. These results suggest that the antioxidative activity of

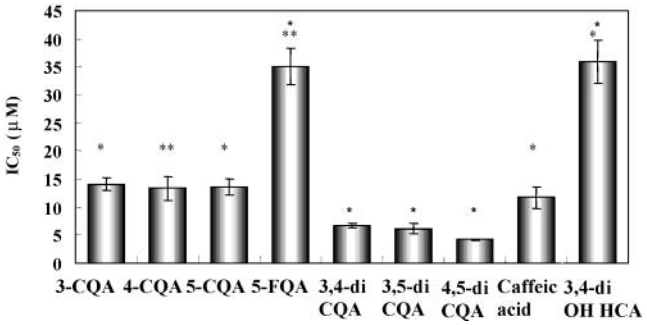

Figure 4. Superoxide anion radical scavenging activity of cinnamoyl acid derivatives isolated from coffee beans. Results are expressed as the mean ± SD ($n = 5$). ∗, $p < 0.01$ versus caffeic acid; ∗∗, $p < 0.01$ versus diCQAs.

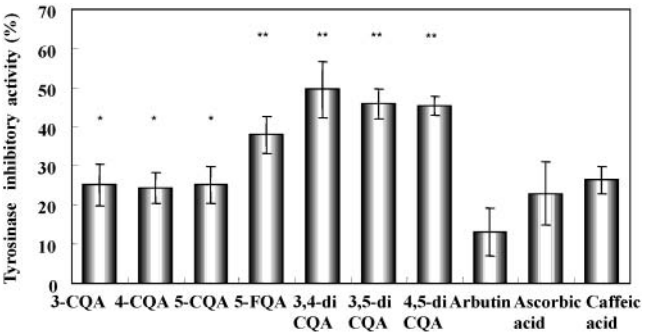

Figure 5. Tyrosinase inhibitory activity of cinnamoyl acid derivatives isolated from coffee beans using L-tyrosine as a substrate. Each sample was tested at 90 μM. Results are expressed as the mean ± SD ($n = 5$). ∗, $p < 0.05$ versus arbutin; ∗∗, $p < 0.01$ versus arbutin, ascorbic acid, and caffeic acid.

hydroxycinnamic acid derivatives depends on the number of caffeoyl groups.

Nakatani et al. (*21*) reported that 3-, 4-, and 5-CQAs found in prunes (*Prunus domestica* L.) have strong antioxidant effects. The DPPH radical scavenging effects of 3- and 4-CQAs and 4,5-diCQAs were stronger than those of α-tocopherol and cysteine (*25*), and the effect of 3,5-diCQA was stronger than that of 5-CQA (*4*). Lee (*26*) reported that the antioxidative activities of polyphenols in general are dependent on the addition of a caffeoyl group to the quinic core. In this experiment, we obtained similar results because the number of caffeoyl groups in the compound contributes to the scavenging activity of DPPH and superoxide radicals rather than the linkage positions of caffeoyl groups on the quinic core.

Tyrosinase Inhibitory Activity of Isolated Hydroxycinnamic Acid Derivatives. Tyrosinase activity was evaluated by measuring the amount of dopachrome produced from L-tyrosine or L-DOPA by tyrosinase. Arbutin and ascorbic acid (typical tyrosinase inhibitors) at 90 μM inhibited the production of dopachrome from L-tyrosine at 13 and 23% and from L-DOPA at 0 and 34%, respectively. The diCQAs most significantly inhibited the formation of dopachrome from L-tyrosine (45−50%) and from L-DOPA (51−59%, $p < 0.01$, **Figures 5** and **6**). No inhibitory activity was detected for 3,4-diOH HCA, which suggests that the double bond at the C7 position in the caffeoyl group might be essential for the expression of tyrosinase inhibitory activity. Kubo and Kinst-Hori (*27*) reported that cinnamic acid and cinnamic aldehyde inhibit the tyrosinase at 0.70 and 0.98 mM, respectively, whereas phenylpropionaldehyde does not express this inhibitory activity. The double bond at the C7 position of caffeoyl group is required to form a stable

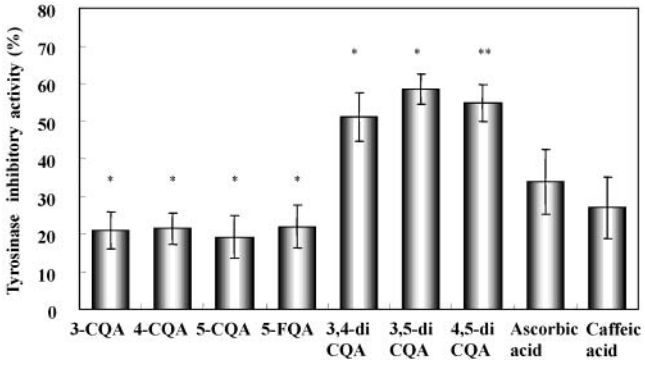

Figure 6. Tyrosinase inhibitory activity of cinnamoyl acid derivatives isolated from coffee beans using L-DOPA as a substrate. Each sample was tested at 90 μM. Results are expressed as the mean ± SD ($n = 5$). ∗, $p < 0.05$ versus ascorbic acid; ∗∗, $p < 0.01$ versus ascorbic acid and caffeic acid.

Table 4. Antiproliferation Activity of Hydroxycinnamic Acid Derivatives Isolated from Green Coffee Beans on Several Cancer Cell Lines[a]

	IC_{50} (mM) for cancer cell line				
compd	U937	KB	MCF-7	WI38	WI38VA
3-CQA	1.20 ± 0.31	0.18 ± 0.02	0.89 ± 0.44	5.87 ± 2.47	4.93 ± 0.84
4-CQA	0.74 ± 0.03	0.16 ± 0.03	0.92 ± 0.48	3.56 ± 0.09	5.99 ± 2.08
5-CQA	1.54 ± 0.27	0.14 ± 0.02	0.74 ± 0.38	5.87 ± 2.47	8.18 ± 3.42
5-FQA	3.07 ± 0.55	0.56 ± 0.03	1.62 ± 0.81	3.46 ± 0.41	4.65 ± 0.48
3,4-diCQA	0.57 ± 0.01	0.16 ± 0.02*	0.31 ± 0.15	0.50 ± 0.03*	0.75 ± 0.05
3,4-diCQA	0.56 ± 0.05	0.18 ± 0.01*	0.32 ± 0.16*	0.52 ± 0.04*	0.47 ± 0.03
4,5-diCQA	0.76 ± 0.06	0.10 ± 0.01*	0.37 ± 0.19*	0.62 ± 0.03*	0.58 ± 0.02

[a] Results are expressed as the mean ± SD ($n = 4$). ∗, $p < 0.01$ vs CQAs (3-CQA, 4-CQA, 5-CQA) and 5-FQA.

Schiff base with a primary amino group in the enzyme, which should show inhibitory activity.

Recently, three kinds of diCQAs in steamed sweet potato were found to suppress melanogenesis in mouse melanoma cells as shown by Shimozono et al. (*28*). Hydroxycinnamic acid derivatives from low-quality green beans are present as antioxidants and skin-whitening agents in cosmetics.

Antiproliferative Activity of Hydroxycinnamic Acid Derivatives Isolated from Green Coffee Beans. The antiproliferative activities of the seven isolated hydroxycinnamic acid derivatives are summarized in **Table 4** on the basis of their IC_{50} values. The proliferation of the five kinds of culture cells tested was inhibited in a dose-dependent manner (data not shown). Among the culture cells tested, KB cells were most sensitive to the derivatives ($p < 0.01$), CQAs ($IC_{50} = 0.14-0.18$ mM), 5FQA ($IC_{50} = 0.56$ mM), and diCQAs ($IC_{50} = 0.1-0.18$ mM). To our knowledge, this is the first report on the antiproliferative activity of CQAs and diCQAs on the KB cancer cell line. The diCQAs showed antiproliferative activities at lower IC_{50} against WI38, WI38VA, and MCF-7 ($p < 0.01$) compared to CQAs and 5FQA. These results suggest that the number of caffeoyl groups might be a dominant factor in determining the potency of inhibitory activity on the growth of adherent cells. Nagaoka et al. (*7*) reported on the antiproliferative activity of methyl esters of diCQAs and triCQA on murine colon 26-L5 carcinoma, human HT-1080 fibrosarcoma, murine B16-BL6 melanoma, and human lung carcinoma A-549 cells at $IC_{50} = 2.94-148$ μM, and their results approximately agreed with ours. WI38 and WI38VA cells expressed lower sensitivity against CQAs and FQA ($p < 0.01$). However, the IC_{50} values of CQAs and diCQAs for the normal cell line WI38 were almost the same as those for the WI38VA cancer cell line, which suggests that these compounds exhibit cytotoxicity toward normal cells.

5-FQA was not effective against the proliferation of the U937, KB, and MCF-7 cancer cell lines compared to CQAs and diCQAs ($p < 0.01$). Methoxylation at the C3 position of the caffeoyl group might result in lower antiproliferative activity against the cancer cell lines other than the scavenging activity of superoxide anion radicals (**Figure 4**). Furthermore, apoptotic nuclear morphological changes were observed in U937 cells treated with 120 μM diCQAs after 1 day of culturing.

Conclusions. We isolated seven kinds of hydroxycinnamic acid derivatives, three diCQAs, three CQAs, and one FQA, from green coffee beans. The amount of diCQAs in low-quality green beans was almost the same as that in commercial quality beans. DiCQAs exhibit more potent antioxidative, tyrosinase inhibitory, and antiproliferation activities compared to FQA and CQAs. Our findings suggest that hydroxycinnamic acid derivatives, especially diCQAs, from low-quality green coffee beans, which are unused agricultural resources, might be useful as protective agents against active oxygen species, as skin-whitening agents in cosmetics, and as chemopreventive agents.

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