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Content of Antioxidative Caffeoylquinic Acid Derivatives in Field-Grown *Ligularia fischeri* (Ledeb.) Turcz and Responses to Sunlight

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ABSTRACT: *Ligularia fischeri* (Ledeb.) Turcz, a commercial leafy vegetable, contains caffeoylquinic acid derivatives (CQAs) as major phenolic constituents. The HPLC chromatograms of leaf extracts collected from different areas in Korea showed a significant variation in CQA amount, and two tri-*O*-caffeoylquinic acids (triCQAs) were purified and structurally identified by NMR and MS from this plant. Radical scavenging activities among CQAs were found to be increased in proportion to the number of caffeoyl groups. Since this plant prefers damp and shady growth conditions, the effects of sunlight were investigated by growing plantlets in sunlight and shade for four weeks. Greater leaf thickness and higher phenolic contents were found for leaves grown in sunlight than in shade. Four major CQAs—5-mono-*O*-caffeoylquinic acid (5-monoCQA), and 3,4-, 3,5-, and 4,5-di-*O*-caffeoylquinic acid (diCQA)—were induced by solar irradiation, whereas the content of these compounds decreased steadily in shade leaves. The leaves of *L. fischeri* clearly showed adaptation responses to sunlight, and these characteristics can be exploited for cultivation of this plant for potential use as a nutraceutical and functional food.

KEYWORDS: *adaptation, caffeoylquinic acid, chlorophylls, Ligularia fischeri, phenolic compound, radical scavenging activity*

INTRODUCTION

Ligularia fischeri (Ledeb.) Turcz is a perennial vegetable plant found mainly in damp shady regions in Europe and Asia.[1] In Korea, this plant has been consumed for a long time as a food and the area of cultivation of this plant has been steadily increasing in response to its consumption in the market. Recently, *L. fischeri* leaf tea prepared by blanching fresh leaves in boiling water has been recognized as a value-added functional food and the chemical constituents of this type of tea infusion have been analyzed.[2] This plant has been used pharmaceutically for the treatment of jaundice, scarlet fever, rheumatoid arthritis, and hepatic function failure.[3] A number of biological activities including cancer prevention[3] and antimutagenic and antigenotoxic activities[4] have been reported for *L. fischeri* extracts. Antioxidant activity of these extracts has been demonstrated by several independent methods, indicating that the plant contains high amounts of antioxidant constituents.[5−7] Therefore, this plant can be regarded as an important source of dietary antioxidants, especially considering its high radical scavenging activity.[2] Several biological constituents, including terpenoids,[8−11] flavonoids,[12,13] and phenolic acid,[14,15] have been documented in different parts of this plant. In particular, a number of caffeoylquinic acid derivatives (CQAs) have been isolated, and these are suggested to represent the major phenolic constituents in the leaves.[2,14,15]

The CQAs form a widely recognized group of antioxidant compounds characterized by caffeoyl groups bound to the hydroxyl group of quinic acid in the form of an ester bond. The classification of CQAs depends on the position and number of caffeoyl groups bound to quinic acid.[16] CQAs are a common constituent in a variety of plants and especially in green coffee beans. The concentration of CQAs in the dried green beans of *Coffea arabica* and *C. canephora*, two commonly cultivated coffee trees, is 4.1% and 11.3%, respectively.[17] Although a number of studies have investigated the biological activities of CQAs from various plants, their biosynthetic pathways and functions in the life of the plant have not yet been established. The biosynthetic routes leading to the production of di-*O*-caffeoylquinic acids (diCQAs) and tri-*O*-caffeoylquinic acids (triCQAs) from mono-*O*-caffeoylquinic acids (monoCQAs) have not yet been completely defined, but the prior accumulation of CQAs, which can be synthesized through three distinct routes in the phenylpropanoid pathway, does appear to be necessary.[18] CQAs are believed to play important roles in free radical scavenging, enzymatic browning of fruits and vegetables, defense against fungal pathogens, and resistance to pathogenic insects.[16]

The physiology and antioxidant content of plants vary considerably with the growth and management conditions used. Antioxidants such as CQAs accumulate as part of complex defense mechanism against a wide range of stresses.[19] This means that the health-promoting benefits of plant-based foods can conceivably be enhanced by using regulated environmental stresses during cultivation. However, some caution has to be considered when applying this type of cultural practice because of the potential adverse effects on quality and production of the plants.

In this study, the variation in CQAs was analyzed in *L. fischeri* collected from several different areas. Two triCQAs not previously identified in this plant were isolated and characterized from the leaves. In addition, the effect of sunlight

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was investigated on leaf growth, and on chlorophyll, flavonoid, and phenolic content of young leaves of *L. fischeri*. The changes in amounts of major CQAs (5-monoCQA, 3,4-, 3,5- and 4,5-diCQA) were monitored during the experimental period. The results are discussed with respect to potential uses of this plant.

MATERIALS AND METHODS

Plant Materials. *L. fischeri* used in this study for the screening of CQAs was collected in June 2011 from three different areas: National forest (Pyungchang, Korea), KIST (Gangneung, Korea), and Alps farm (Gangneung, Korea) and three different markets in Gangneung, Korea. All voucher specimens were deposited in the Herbarium at KIST Gangneung Institute, Korea. For the test of sunlight effects, at least three-year-old roots of *L. fischeri* were planted in the field (Alps farm, Gangneung, Korea) in April, 2011. After one month, the plants with two or three leaves were divided into two groups; one continued to grow under sunlight, and the other was grown under shading conditions (around 75% shading of full sun). The leaves from both groups were harvested every week for the next month. The fresh leaves were weighed just after harvest and air-dried under shade for one week. The weight of dried leaves was measured and expressed as % value of fresh weight. The dried leaves were kept at room temperature until use. The average temperatures (the highest and the lowest) of each week were obtained from the Gangwon Regional Meteorological Administration (http://web.kma.go.kr/aboutkma/intro/gangwon).

Chemicals and Reagents. All solvents for extraction and chromatography were analytical grade and purchased from Dae-Jung Chemical (Kyung-gi, Korea). All HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Methanol-d_4 was purchased from Cambridge Isotope Laboratory (Andover, MA, USA). 5-CQA and three DCQAs purified in our previous work were used for quantification.[15]

Isolation of Compounds. Two additional compounds not detected in previous work were isolated from *L. fischeri* collected from National forest. A total of 300 g of dried leaves was extracted in 9 L of methanol for 18 h at room temperature. The extract was filtered and concentrated in a vacuum evaporator to yield 18.9 g of crude extract. The extract was dissolved in 1 L of water and serially fractionated with *n*-hexane (1 L × 3), methylene chloride (1 L × 3), ethyl acetate (1 L × 3), and *n*-butanol (1 L × 3). Ethyl acetate fractions were analyzed by HPLC and concentrated to yield 3.5 g of ethyl acetate fractionate. Two hundred milligrams of this fractionate was separated on a Jasco preparative HPLC (Japan Spectroscopic, Tokyo, Japan) equipped with an ultraviolet detector (MD 2015), pump (PU2089), column oven (CO 2065), and YMC Pack Pro C_{18} RP column (250 × 20 mm i.d., 5 μm particle size) to isolate the two additional peaks detected in the crude extract. The mobile phase consisted of acetonitrile and water containing 0.1% trifluoroacetic acid with 10 mL min^{-1} flow rate. The gradient was run as follows: acetonitrile at 20% for the first 10 min, then increased to 35% for the next 50 min. The chromatogram peaks were detected at 330 nm. Two compounds collected at elution times of 37−38 min and 48−50 min were analyzed by HPLC and concentrated to produce 17 mg of compound **1** and 6 mg of compound **2**. These compounds were then analyzed further using mass spectrometry and NMR spectroscopy.

Mass Spectroscopy and Nuclear Magnetic Resonance. For the structural determination, 1-dimensional and 2-dimensional NMR and ESI/MS spectral data were collected and compared with published data. Mass data were collected using a model Varian 1200 L LC−MS system (Walnut Creek, CA, USA), with an ESI source (negative mode). A 1 μL volume of the isolated compounds (1 mg mL^{-1} in methanol) was directly injected into the mass spectrometer and mass spectra were acquired in a range of *m/z* 50−1000. The mass spectrometer conditions were as follows: needle voltage, −4500 V; nebulizing gas pressure (air), 60 psi; drying gas (N_2) flow rate, 4 L/min; and drying gas temperature, 300 °C. NMR data was collected with a 500 MHz Varian NMR system (Varian, Palo Alto, CA, USA), operating at 500 MHz for ^1H and 125 MHz for ^{13}C. Conventional pulse sequences were used to obtain $^1H-^1H$ correlation spectroscopy (COSY), $^1H-^{13}C$ heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond coherence (HMBC). The data were processed by the MestReNova program (Mestrelab Research, Santiago de Compostela, Spain). All data were obtained in methanol-d_4 and the chemical shifts are expressed as δ values from tetramethylsilane.

1,3,4-tri-*O*-Caffeoylquinic Acid (1,3,4-triCQA) (1). Brownish amorphous power: ESI-MS *m/z* 677 $[M - H]^-$; ^1H NMR (500 MHz, CD_3OD, ppm) δ 1.98 (dd, 1H, *J* = 13.6, 11.4 Hz, H-6a), 2.50 (dd, 1H, *J* = 16.1, 3.3 Hz, H-2a), 2.62 (ddd, 1H, *J* = 13.6, 4.5, 3.4 Hz, H-6e), 2.91 (dt, 1H, *J* = 16.1, 3.4, 3.3 Hz, H-2e), 4.49 (ddd, 1H, *J* = 11.4, 9.9, 4.5 Hz, H-5), 5.00 (dd, 1H, *J* = 3.5, 9.9 Hz, H-4), 5.67 (q, 1H, *J* = 3.3, 3.3, 3.5 Hz, H-3) (quinic acid moiety); δ 6.10, 6.24, 6.34 (d, 1H each, *J* = 15.9 Hz, H-8′, 8″, 8‴), 6.48, 6.71, 6.74 (d, 1H each, *J* = 8.2 Hz, H-5′, 5″, 5‴), 6.53, 6.85, 6.91 (dd, 1H each, *J* = 2.0, 8.2 Hz, H-6′, 6″, 6‴), 6.80, 7.01, 7.05 (d, 1H each, *J* = 2.0 Hz, H-2′, 2″, 2‴), 7.45, 7.54, 7.60 (d, 1H each, *J* = 15.9 Hz, H-7′, 7″, 7‴) (caffeoyl groups); ^{13}C NMR (125 MHz, CD_3OD, ppm) δ 31.37 (C-2), 40.30 (C-6), 64.02 (C-5), 68.51 (C-3), 75.44 (C-4), 79.33 (C-1), 172.84 (C-7) (quinic acid moiety); δ 113.27, 113.40, 113.69 (C-8′, 8″, 8‴), 113.49, 113.99, 115.04 (C-2′, 2″, 2‴), 115.01, 115.24, 115.28 (C-5′, 5″, 5‴), 120.39, 121.79, 121.84 (C-6′, 6″, 6‴), 125.80, 126.02, 126.26 (C-1′, 1″, 1‴), 145.06, 145.34, 145.46 (C-3′, 3″, 3‴), 145.93, 146.16, 146.42 (C-7′, 7″, 7‴), 147.93, 148.19, 148.43 (C-4′, 4″, 4‴), 166.36, 166.83, 167.17 (C-9′, 9″, 9‴) (caffeoyl groups).

3,4,5-tri-*O*-Caffeoylquinic Acid (3,4,5-triCQA) (2). Yellowish amorphous power: ESI-MS *m/z* 677 $[M - H]^-$; ^1H NMR (500 MHz, CD_3OD, ppm) δ 2.09 (dd, 1H, *J* = 14.3, 5.2 Hz, H-2a), 2.18 (dd, 1H, *J* = 13.6, 3.6 Hz, H-6e), 2.27 (dd, 1H, *J* = 13.6, 9.0 Hz, H-6a), 2.36 (dd, 1H, *J* = 14.3, 3.7 Hz, H-2e), 5.23 (dd, 1H, *J* = 8.1, 3.5 Hz, H-4), 5.56 (td, 1H, *J* = 3.6, 8.1, 9.0 Hz, H-5), 5.58 (dt, 1H, *J* = 5.2, 3.7, 3.5 Hz, H-3) (quinic acid moiety); δ 6.12, 6.12, 6.22 (d, 1H each, *J* = 15.9 Hz, H-8′, 8″, 8‴), 6.61, 6.66, 6.67 (d, 1H each, *J* = 8.2 Hz, H-5′, 5″, 5‴), 6.75, 6.83, 6.85 (dd, 1H each, *J* = 2.0, 8.2 Hz, H-6′, 6″, 6‴), 6.90, 6.93, 6.96 (d, 1H each, *J* = 2.0 Hz, H-2′, 2″, 2‴), 7.42, 7.45, 7.50 (d, 1H each, *J* = 15.9 Hz, H-7′, 7″, 7‴) (caffeoyl groups); ^{13}C NMR (125 MHz, CD_3OD, ppm) δ 37.33 (C-6), 39.90 (C-2), 70.09 (C-5), 70.49 (C-3), 70.80 (C-4), 75.57 (C-1), 176.41 (C-7) (quinic acid moiety); δ 115.11, 115.16, 115.25 (C-8′, 8″, 8‴), 115.90, 115.95, 116.00 (C-2′, 2″, 2‴), 117.32, 117.35, 117.39 (C-5′, 5″, 5‴), 124.09, 124.14, 124.31 (C-6′, 6″, 6‴), 128.37, 128.39, 128.59 (C-1′, 1″, 1‴), 147.60, 147.62, 147.65 (C-3′, 3″, 3‴), 149.49, 149.74, 149.87 (C-7′, 7″, 7‴), 150.52, 150.64, 150.67 (C-4′, 4″, 4‴), 168.62, 168.93, 169.33 (C-9′, 9″, 9‴) (caffeoyl groups).

Online HPLC-ABTS Antioxidant Assay. The radical scavenging activity of CQAs isolated from *L. fischeri*, was measured by online HPLC-ABTS antioxidant assay system as described in a previous report with slight modification.[20] A solution containing 200 μg mL^{-1} of each compound was prepared in methanol and injected into the system. All experiments were repeated in triplicate, and the data were expressed as mean value.

Characterization of *L. fischeri* Leaves. Leaves of *L. fischeri* were characterized by measuring chlorophyll, total phenolic, and total flavonoid contents with a Lambda 3B UV/vis spectrophotometer (Perkin-Elmer, Norwalk, CT). Two hundred milligrams of dried leaves was extracted with 10 mL of methanol at room temperature for 12 h. After filtration, the extract solution was used for characterization analysis and quantification.

Total phenolic contents were determined according to the Folin−Ciocalteu method using gallic acid as the standard.[21] The total phenolic content was expressed as gallic acid equivalents (GAE) in mg g^{-1} fresh weight.

Total flavonoid contents were determined by the aluminum chloride colorimetric method as described in the literature.[22] Quercetin was used as the standard, and the total flavonoid content was expressed as quercetin equivalents (QE) in mg g^{-1} fresh weight.

The chlorophyll content was measured as described in the literature with slight modification.[23] In brief, the absorbance of a methanol

extract was measured at 470, 652.4, and 665.2 nm and the amounts of chlorophylls *a* and *b* were calculated according to the following equation:

$$\text{chlorophyll } a\ (\mu g\ mL^{-1}) = 16.72A_{665.2} - 9.16A_{652.4}$$

$$\text{chlorophyll } b\ (\mu g\ mL^{-1}) = 34.09A_{652.4} - 15.28A_{665.2}$$

The amount of chlorophyll was then expressed on a fresh weight basis (mg g^{-1} FW).

Quantification of CQAs by HPLC. A change in major CQAs (5-monoCQA, 3,4-, 3,5-, and 4,5-diCQAs) in the *L. fischeri* plants was quantified by an HPLC method. HPLC analysis was performed with an Agilent 1200 analysis HPLC system (Agilent Technologies, Santa Clara, CA, USA), consisting of binary pump (G1312A), auto sampler (G1367B), diode array detector (G1315D), and degasser (D1379B). The separation of each compound was carried out on an Alltech Prevail C_{18} analytical column [250 × 4.6 mm i.d., 5 μm particle size (Alltech Associates, Lokeren, Belgium)] with 1 mL min^{-1} flow rate in the solvent system of acetonitrile and 0.1% aqueous trifluoroacetic acid (TFA) (flow rate: 1 mL min^{-1}). The gradient program started with 15% acetonitrile initially and increased to 40% acetonitrile in 28 min, then 90% acetonitrile in 33 min. The chromatogram was detected at 330 nm. Calibration curves were constructed with each compound in a range of 10−500 μg mL^{-1}. Each experiment was repeated in triplicate.

RESULTS AND DISCUSSION

Variations in CQA Levels in the Extracts of *L. fischeri*. A previous report documented that the major phenolic compounds found in the leaves of *L. fischeri* were CQAs.[15] In addition, most of the radical scavenging activity in the leaf extract could be attributed to these compounds. Therefore, the antioxidant activities reported in previous research on this plant might also arise from the presence of CQAs. In the current study, *L. fischeri* plants were collected from several different areas and markets in order to investigate the amounts of CQAs in the leaves. Chromatograms from each extract are shown in Figure 1. The most abundant CQA among the four major CQAs was 3,5-diCQA based on a peak area. However, the relative ratios of each peak in the chromatograms varied considerably in each sample and minor flavonoid peak could also be detected in the plants from the Alps farm.

In addition to the four major CQAs, two additional compounds with similar UV spectra could be detected in the chromatograms of the plants collected in the National forest and from market 1. The UV spectra of these compounds scanned during HPLC analysis showed a distinctive pattern where λ_{max} was 210, 245, and 330 nm. These patterns were coincident with those of CQAs, indicating the presence of certain *trans* cinnamic acids in the structure.[17] These results taken together, the content of CQAs in *L. fischeri* showed a significant variation according to the location of cultivation.

Isolation and Identification of Tri-*O*-caffeoylquinic Acids. Two additional compounds were isolated by extraction and preparative HPLC of the plants from the National forest. The structures of the purified compounds were resolved with MS and various NMR techniques.

The MS data for two compounds showed a parent ion of *m/z* 677 $[M - H]^-$ in the negative mode, which is identical with that of triCQAs. The carbon numbers of these compounds in ^{13}C NMR spectra were also identical with those of triCQAs. Therefore, it could be highly suggested that these compounds are triCQAs.[24,25] Four triCQA isomers occur in nature with structures that vary based on the esterification position of caffeoyl groups in the quinic acid moiety (1,3,4-, 1,3,5-, 1,4,5-, and 3,4,5-triCQA). The triCQA isomers were distinguished by

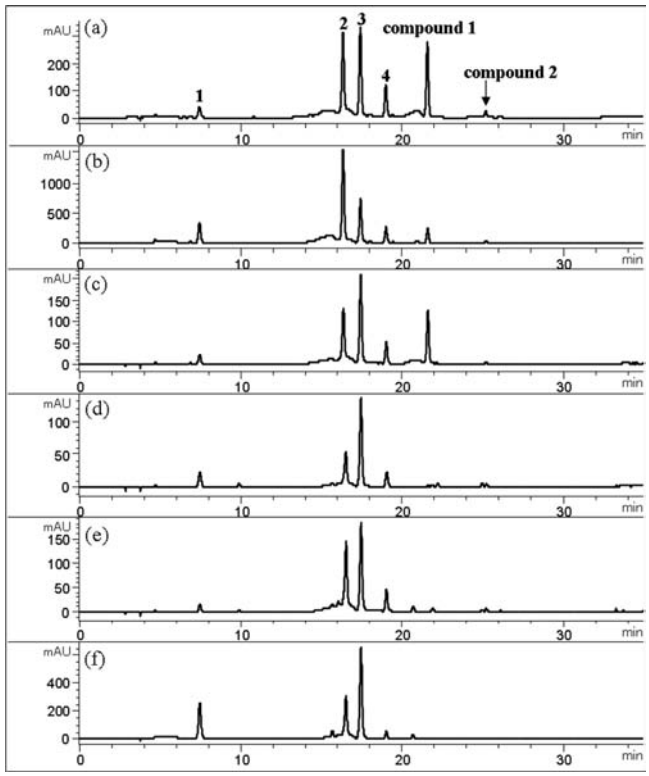
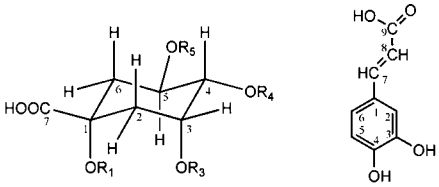


Figure 1. HPLC chromatograms of *Ligularia fischeri* leaf extracts. All samples were collected in different areas (a, National forest; b, KIST, f, Alps farm) or purchased from different markets (c−e) in Gangneung City, Korea. Numbers indicate caffeoylquinic acid derivatives (1, 5-monoCQA; 2, 3,4-diCQA; 3, 3,5-diCQA; 4, 4,5-diCQA). Compounds **1** and **2** represent caffeoylquinic acid derivatives newly isolated in this study.

^1H and various 2-dimensional NMR data archived for the two compounds. As expected, the proton signals from caffeoyl groups could be detected between δ 6.1 and 7.6 ppm and the signals of 4 protons in the C-2 and C-6 positions of quinic acid could be detected between δ 2.0 and 3.0 ppm. One important clue in the structural elucidation of CQAs is the position of the caffeoyl group in quinic acid. As described in our previous work,[15] the deshielding effect caused a significant downfield movement of the chemical shift of the proton geminal to the hydroxyl group connected to one caffeoyl group by an ester bond in quinic acid.[26] The signals of the ring protons at C-3, C-4, and C-5 in quinic acid have chemical shifts in the range of δ 3.49−4.11 ppm (δ 4.11 for H-3, δ 3.49 for H-4, δ 3.99 for H-5 in D_2O) in the literature.[27] Based on this information, two caffeoyl groups of compound **1** are bound to the hydroxyl groups at C-3 and C-4. The remaining caffeoyl group is bound to the hydroxyl group at C-1, as shown by the ^{13}C signal of C-1 (δ 79.33 ppm), which shifts downfield from that of 3,4-diCQA (δ 73.2 ppm in DMSO-d_6) in the literature.[25] Therefore, compound **1** could be suggested as 1,3,4-triCQA, and 2-dimensional NMR data also supported this suggestion. In a like manner, compound **2** could be concluded to be 3,4,5-triCQA, whose proton signals at C-3, C-4, and C-5 in quinic acid shifted downfield from those of quinic acid. Compound **1** is a quite rare derivative of triCQA. Of these four triCQA isomers, three have been isolated from some composite species and tomato species.[24,28] To the best of our knowledge, this is the first report of the NMR data for 1,3,4-triCQA.

Antioxidant Activity of CQAs. The purification of CQAs from *L. fischeri* identified 6 isomers in the leaves.[15] The chemical structures of the CQAs isolated from *L. fischeri* are listed in Figure 2 according to their number of caffeoyl groups



Name and abbreviation	R_1	R_3	R_4	R_5
5-mono-*O*-caffeoylquinic acid (5-monoCQA)	H	H	H	C
3,4-Di-*O*-caffeoylquinic acid (3,4-diDCQA)	H	C	C	H
3,5-Di-*O*-caffeoylquinic acid (3,5-diCQA)	H	C	H	C
4,5-Di-*O*-caffeoylquinic acid (4,5-diCQA)	H	H	C	C
1,3,4-Tri-*O*-caffeoylquinic acid (1,3,4-triCQA)	C	C	C	H
3,4,5-Tri-*O*-caffeoylquinic acid (3,4,5-triCQA)	H	C	C	C

Figure 2. Chemical structures of caffeoylquinic acid derivatives isolated from *Ligularia fischeri* leaves.

and the position. The 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of each compound was investigated using the online HPLC-ABTS assay system, and the result is shown in Table 1.[15] As described in our previous report, the low reaction rate of analytes in the online HPLC system generated lower Trolox equivalent antioxidant capacity (TEAC) values than has been reported for an off-line assay system in the literature.[15,29] However, TEAC values clearly increased according to the number of caffeoyl groups, indicating that increased radical scavenging activity is proportional to the number of caffeoyl groups in quinic acid (Table 1). Because the ABTS method is a radical scavenging method that reduces radicals primarily by single electron transfer,[30] the increased number of caffeoyl groups might have a positive effect on the single electron transfer reaction in the reaction mixture.

In the structure of CQAs, caffeic acid is a well-known radical scavenger while quinic acid is not. The radical scavenging activity of CQAs therefore can be proportional to the number of caffeoyl groups at the same molar concentration.[31] Similar to the antioxidant activity, the responses in activity in other biological tests among CQAs also show an ordered pattern. The order of antimutagenicity of CQAs was 3,4,5-triCQA > 3,4-diCQA = 3,5-diCQA = 4,5-diCQA > 3-monoCQA.[28] Another group studied ATP production in human neuroblastoma SH-SY5Y cells and suggested that caffeoyl groups bound to quinic acid are important for activity and that activity increases as more caffeoyl groups are bound to quinic acid.[32]

No conclusion could be drawn regarding a positional effect of the caffeoyl group in quinic acid on radical scavenging activity, since some variations have appeared in the literature with respect to the order of antioxidant activity among monoCQAs and diCQA isomers.[31,33,34]

Influence of Sunlight on Growth and Chlorophyll Content of *L. fischeri*. *L. fischeri* prefers a shady and wet soil environment for growth, and strong sunlight is known to cause growth inhibition. The physiological changes in *L. fischeri* in response to sunlight were examined in one-month plantlets grown in the field in natural sunlight. The plants were divided into two groups: a sunlight group and a shaded group (75% of the sunlight was shielded by a shade net) (Figure 3a). During the 4 weeks, effects of solar radiation and temperature were seen on the growth of *L. fischeri* in every week. The effect of solar radiation was investigated by comparing leaves growing in sunlight and shade during the same week, whereas the effect of temperature was deduced by comparing the same group since the average temperature increased during the experiment. As shown in Figure 3b, after 1 week of shading, a 38.7% decrease was seen in the dry weight of shade leaves compared to leaves from 0 week plants. In contrast, sun leaves showed an increase of 14.1% in dry weight after 1 week of shading. The dry weight of the shade leaves after shading was almost half that of sunlight leaves every week (Figure 3b). The observation could arise from the fact that the sunlight leaves were thicker and harder than shade leaves. Therefore, solar radiation affected leaf growth. A similar phenomenon has been reported for *Chenopodium album*.[35] Because sunlight leaves have a high photosynthetic capacity and need to have a large number of chloroplasts in the mesophyll cells, sunlight leaves form thick mesophyll layers that have chloroplasts arranged along the mesophyll cell surface. Temperature also affected leaf growth in *L. fischeri*. The dry weight of sunlight leaves increased by 45.4% during the 4 weeks. In the shaded plants, the first week of shading presumably represented an adaptation period. In the following 3 weeks, the dry weight of shade leaves also increased by 19.7%. Therefore, temperature also had positive effects on the growth of *L. fischeri*.

Leaf chlorophyll content showed opposite results for the two groups. Chlorophyll in sunlight leaves was slightly reduced over the 4 weeks whereas it slightly increased in shade leaves (Figure 3c), in agreement with previous literature.[36] However, sunlight leaves commonly have a higher photosynthetic capacity and,

Table 1. Regression Equations and Antioxidant Activities of Caffeoylquinic Acid Derivatives from *Ligularia fischeri*

			online HPLC-ABTS antioxidant anal.		
compd	regression eq	correlation coeff (r^2)	Trolox equiv[a] (μM)	concn[a] (μM)	TEAC value[b]
5-monoCQA	$y = 16.744x - 7.788$	1.000	156.496	564.971	0.277
3,4-diCQA	$y = 22.447x + 37.195$	0.998	183.797	387.597	0.484
3,5-diCQA	$y = 28.453x - 237.520$	0.993	205.243	387.597	0.525
4,5-diCQA	$y = 13.496x - 158.190$	0.991	126.232	387.597	0.342
1,3,4-triCQA	$y = 28.201x - 74.974$	0.998	204.114	295.421	0.708
3,4,5-triCQA	$y = 21.109x + 4.288$	0.999	200.978	295.421	0.699

[a]These values were determined from the regression equation of Trolox by online HPLC-ABTS antioxidant analysis with 200 μg mL^{-1} solution of each compound. [b]Trolox equivalent antioxidant capacity, defined as the concentration of Trolox (mM) having the same activity as 1 mM of the test compound.

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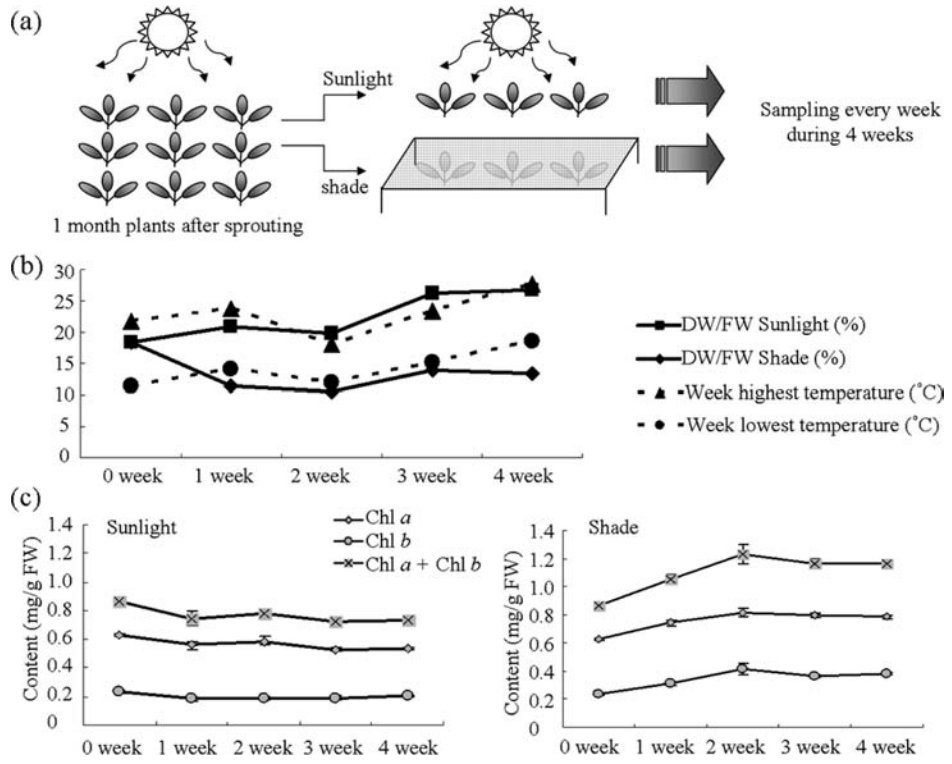

Figure 3. The experimental scheme for *Ligularia fischeri* (a) and characteristics of fresh leaves (b and c). One month old plants after sprouting were treated under sunlight or shaded conditions, and the leaves were harvested every week for 4 weeks (a). The dry weights of the leaves from sunlight and shade were expressed as % of the weight of fresh leaves based on the highest and the lowest weekly average temperature (b). The chlorophyll content was also monitored on a fresh weight basis during the 4 week experiment (c).

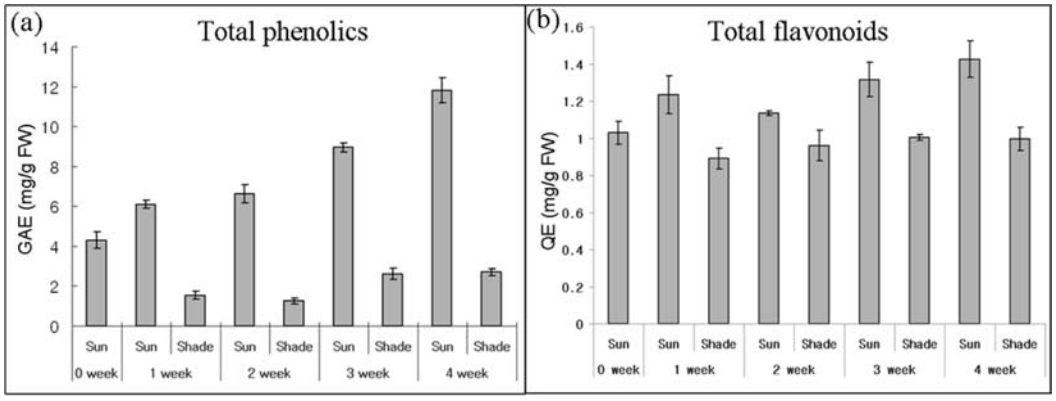

Figure 4. The changes in total phenolics (a) and total flavonoids (b) in the leaves from two different groups (sunlight and shade) of *Ligularia fischeri*.

thus, a higher chlorophyll content than shade leaves.[35] The opposite condition seen in *L. fischeri* might be due to the greatly increased dry weight. The chlorophyll content might be increased in sunlight leaves and decreased in shade leaves, but this effect would be missed when the values are calculated on a weight basis rather than a surface area basis.

Influence of Sunlight on the Phenolic Compounds in *L. fischeri*. The main soluble phenolic classes in *L. fischeri* leaves were flavonoids and phenolic acids. Two flavonoids, hyperoside and 2″-acetylhyperoside, were reported previously in this plant,[12] and CQAs were also isolated from the leaves of *L. fischeri*.[15] The impact of sunlight on phenolic compounds was investigated by determining the total phenolic and flavonoid contents in the collected samples. As shown in Figure 4, the total phenolic content gradually increased in sunlight leaves, from 4.29 mg g^{-1} FW to 11.80 mg g^{-1} FW, almost a 3-fold increase over the 4 weeks. However, shade leaves showed a dramatic decrease to 1.53 mg g^{-1} FW during the first week, and then the phenolic content gradually increased to 2.69 mg g^{-1} FW over the next three weeks. The total flavonoid content showed little change in sunlight. During 4 weeks, the total flavonoids increased only 38.5% in sunlight leaves and showed no significant difference from the shade leaves. These observations suggest that phenolic compounds other than flavonoids were significantly affected by sunlight.

A number of studies have reported a correlation between sunlight and phenolic content of plants.[37,38] In most cases, phenolics are induced by increased sunlight, especially UV-B irradiance, as a defense mechanism against light stresses. The phenylpropanoid pathway is a well-known target for environ-

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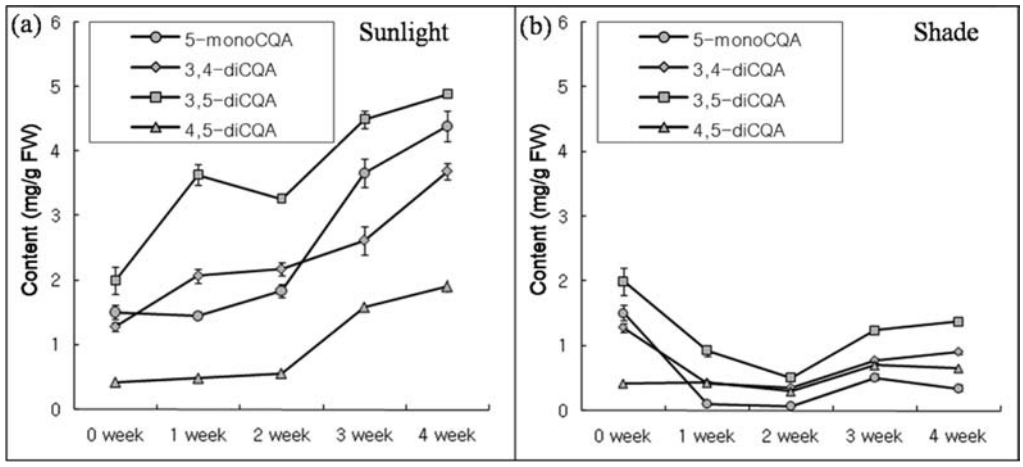

Figure 5. The changes in caffeoylquinic acid derivatives in the leaves of *Ligularia fischeri* from sunlight (a) and shade (b).

mental stress responses in plants.[19] Various environmental stresses such as high light/UV, wounding, pathogen attack, low temperature, and low nitrogen can affect this pathway to change the content of flavonoids, as well as anthocyanins, coumarin, phenolic acids, and lignin.

In the present study, CQAs therefore may represent the target phenolic compounds that are induced by sunlight. The change in content of the four main CQAs found in the leaves during the experimental period is shown in Figure 5. All four CQAs were induced together by sunlight and reduced together by shading. These patterns of change were highly similar to the pattern seen for total phenolics (Figure 4). Therefore, the main phenolic components responding to sunlight exposure appear to be the CQAs.

The influence of light on the concentration of CQAs has been investigated previously by several researchers. For example, CQAs accounted for up to 90% of the total phenolic compounds in potato tubers and were highly induced by light exposure.[39] Globe artichoke (*Cynara cardunculus* L.), also known for a high CQA content in the leaves, shows a steady increase in levels of diCQAs following UV-C exposure.[40] Because CQAs act as antioxidants in plants, the induction of CQAs in *L. fischeri* in response to direct exposure to sunlight might be a main defense response to oxidative stress.

In summary, the concentration of each CQA in *L. fischeri* was found to vary according to the cultivation conditions. Two additional triCQA compounds were isolated, and their structures were determined. In addition, changes in leaf growth, chlorophyll content, and phenolics were investigated by shading sunlight leaves. The level of CQAs was highly induced by sunlight. This plant is cultivated for commercial purposes in Korea and mostly used as a food as a raw or brined vegetable. Particularly, fresh *L. fischeri* leaves are consumed mostly as "wrap-up vegetable" which is used for wrapping other foods such as boiled rice or meat. Therefore, eating quality is the first priority in cultivating this plant and thick and hard textures of leaves grown under sunlight are not suitable for wrap-up vegetable. For this reason, plants are typically grown in the shade and harvested at a young plant stage to maximize eating quality. However, if new formulations such as teas, nutraceuticals, and functional foods are developed from this plant, different cultivation conditions, such as varying light intensity or temperature, etc., can be applied to this plant to maximize functional constituents such as CQAs. Therefore, the data obtained in this study can be valuable information in cultivating this plant for extending the application of *L. fischeri*.

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Notes

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ABBREVIATIONS USED

CQA, caffeoylquinic acid; monoCQA, mono-*O*-caffeoylquinic acid; diCQA, di-*O*-caffeoylquinic acid; triCQA, tri-*O*-caffeoylquinic acid; QE, quercetin equivalents; GAE, gallic acid equivalents; ROS, reactive oxygen species; NMR, nuclear magnetic resonance; MS, mass spectrometry; ESI, electrospray ionization

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