Phenolic Antioxidants from the Leaves of *Corchorus olitorius* L.

Keiko Azuma,* Masayoshi Nakayama, Masaji Koshioka, Katsunari Ippoushi, Yuichi Yamaguchi, Katsunari Kohata, Yuji Yamauchi, Hidekazu Ito, and Hisao Higashio

National Research Institute of Vegetables, Ornamental Plants and Tea, Ano, Mie 514-2392, Japan

Six phenolic antioxidative compounds [5-caffeoylquinic acid (chlorogenic acid), 3,5-dicaffeoylquinic acid, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-(6-malonylglucoside), and quercetin 3-(6-malonylgalactoside) (tentative)] were identified from the leaves of *Corchorus olitorius* L. (moroheiya) by NMR and FAB-MS. The contents of these phenolic compounds, ascorbic acid, and α-tocopherol in *C. olitorius* leaves were determined, and their antioxidative activities were measured using the radical generator-initiated peroxidation of linoleic acid. The results obtained showed that 5-caffeoylquinic acid was a predominant phenolic antioxidant in *C. olitorius* leaves.

Keywords: *Corchorus olitorius; antioxidants; phenolics; caffeoylquinic acid; quercetin glycosides*

INTRODUCTION

In recent years, the physiological functionality of foods has received much attention due to the increasing interest in human health and has been studied in vitro and in vivo by many researchers. The antioxidative action, one of the important physiological functions of foods, is supposed to protect living organisms from oxidative damages, resulting in the prevention of various diseases such as cancer, cardiovascular diseases, and diabetes.

Food plants including fruits, vegetables, and spices are the primary sources of naturally occurring antioxidants for humans. There are many studies on the antioxidative activity and antioxidative mechanism of naturally occurring antioxidants such as α-tocopherol, ascorbic acid, and carotenoids. Furthermore, the antioxidative activity of many plants has been evaluated, and their antioxidants have been identified. The occurrence of various kinds of antioxidative phenolic compounds including flavonoids and cinnamic acid derivatives in food plants was reviewed by Herrmann (1976, 1988, 1989). Recently, novel antioxidative phenolics have been isolated from food plants such as perilla (Tada et al., 1996) and garland (Chuda et al., 1996). Plant flavonoids have been shown to be powerful antioxidants in vitro (Vinson et al., 1995), and the close relations of phenolics to the antioxidative activity of vegetables have been reported (Tsushida et al., 1994; Velioglu et al., 1998; Vinson et al., 1998).

The young leaves of *Corchorus olitorius* L. are edible and used as a vegetable for soup in Egypt. In recent years, this plant, important as a nutritive vegetable rich in K, Ca, P, Fe, ascorbic acid, and carotene, and so on, has been cultivated and consumed under the name "moroheiya" in Japan. We have already found that the leaves of *C. olitorius* exhibited significantly high antioxidative activities in both a linoleic acid emulsion system and a liposome system among various vegetables (Azuma et al., 1999). Furthermore, the responsibility of phenolic antioxidants for the activities of these vegetables was suggested from their activity patterns (Azuma et al., 1999).

The purpose of the present study was to isolate and identify phenolic antioxidants from *C. olitorius* leaves and to investigate their relationship to the antioxidative activity of this vegetable.

MATERIALS AND METHODS

Plant Material. *C. olitorius* L. was cultivated at the National Research Institute of Vegetables, Ornamental Plants and Tea. In early August 1997 (for isolation of antioxidants) and 1998 (for quantitative analysis), when the plants were ∼1.5 m in height, young leaves branching from the stem <20 cm from the top were harvested and immediately used.

Antioxidative Activity. The antioxidative activity was assayed by using a linoleic acid system. Each sample, dissolved in 0.2 mL of distilled water and 0.5 mL of 0.2 M phosphate buffer (pH 7.0), was mixed with 2.5% (w/v) linoleic acid in ethanol (0.5 mL). The peroxidation was initiated by the addition of 50 μL of 0.1 M 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) and carried out at 37 °C for 5 h in the dark. The degree of oxidation was measured according to the thiocyanate method (Mitsuda et al., 1966) for measuring peroxides by reading the absorbance at 500 nm after coloring with $FeCl_2$ and ammonium thiocyanate. The purification of antioxidative compounds was done with the guidance of the antioxidative activity.

Isolation of Antioxidants. *C. olitorius* leaves (300 g of fresh weight) were extracted with MeOH and concentrated to aqueous phase in vacuo. The aqueous residue was ajusted to pH 2 and partitioned with n-BuOH. The n-BuOH fraction was partitioned with 20 mM carbonate buffer (pH 9.5). The buffer fraction was adjusted to pH 2 and extracted with n-BuOH again. The acidic n-BuOH fraction was subjected to a silica gel column and eluted 10% stepwise with MeOH in EtOAc. The fractions eluted with 0 and 10% MeOH in EtOAc were passed through an ODS Sep-Pak cartridge (Waters) and then purified by ODS-HPLC using the following conditions: column, Develosil ODS-5 column (10 mm ∅ × 150 mm, Nomura Chemicals); solvent, 0−5 min, MeOH/H_2O/AcOH (25:70:5), 5−40 min, linear gradient of MeOH/H_2O/AcOH (25:70:5)−MeOH; flow rate, 3.0 mL/min; column temperature, 40 °C; UV detection, 254 and 300 nm.

FAB-MS and NMR. The FAB-MS data were obtained with a JEOL JMS-SX102A system. ^{1}H and ^{13}C NMR, $^{1}H-^{1}H$ COSY, $^{1}H-^{13}C$ COSY, and HMBC data were obtained with JEOL JNM-LA500 and JMN-EX270 systems in DMSO-d_6 at 35 °C.

* Author to whom correspondence should be addressed (e-mail azumak@nivot.affrc.go.jp).

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Quantitative Analysis of Phenolic Compounds. *C. olitorius* leaves (50 g of fresh weight) were freeze-dried and pulverized in a sample mill. Phenolic compounds were extracted by suspending 20 mg of the sample in 2 mL of 70% MeOH for 24 h at room temperature with occasional vigorous shaking. After centrifugation at 9000*g* for 1 min, the extraction was repeated one more time. Each supernatant was combined, filtered through a filter (45 μm), and made up to 10 mL with 70% MeOH. Phenolic compounds of the extract were analyzed by HPLC monitoring UV 300 nm using the following conditions: column, Capcell Pak C_{18} UG120 (4.6 mm ∅ × 250 mm, Shiseido); solvent, 0−5 min, MeOH/H_2O/AcOH (25:70:5), 5−40 min, linear gradient of MeOH/H_2O/AcOH (25:70:5)−MeOH; flow rate, 1.0 mL/min; column temperature, 40 °C.

Quantitative Analysis of Ascorbic Acid. *C. olitorius* leaves were homogenized in 9 times volume of 5% metaphosphoric acid and centrifuged at 9000*g* for 10 min. Ascorbic acid in the extracts was analyzed by HPLC under the following conditions: column, Shim-pack SCR-102H (8 mm ∅ × 300 mm, Shimadzu); solvent, 2 mM perchloric acid; flow rate, 1.0 mL/min; column temperature, 40 °C. For more selective detection of ascorbic acid (reduced form, AsA) and dehydroascorbic acid (DHAA), these were derivatized to compounds having a maximum absorption wavelength of 300 nm under alkaline conditions by the postcolumn derivatization method developed by Yasui and Hayashi (1991). Ascorbic acid content was described as the total amount of AsA and DHAA, detected by the absorbance at 300 nm.

Quantitative Analysis of α-Tocopherol. The α-tocopherol content of *C. olitorius* leaves was determined by HPLC following the methods described by Igarashi and Ueda (1996).

RESULTS

Identification of Phenolic Compounds. Five phenolic antioxidative compounds **1**−**5** were obtained from the acidic n-BuOH fraction of the MeOH extract of *C. olitorius* leaves.

FAB-MS: (**1**) *m/z* 354, (**2**) *m/z* 516, (**3**) *m/z* 464, (**4**) *m/z* 464, (**5**) *m/z* 550.

^{1}H NMR (270 MHz, DMSO-d_6): (**1**) δ 7.42(1H, d, J = 16 Hz, H-7 caffeoyl), 7.03 (1H, d, J = 2 Hz, H-2 caffeoyl), 6.98 (1H, dd, J = 2 and 8 Hz, H-6 caffeoyl), 6.77 (1H, d, J = 8 Hz, H-5 caffeoyl), 6.15 (1H, d, J = 16 Hz, H-8 caffeoyl), 5.08 (1H, br d, J = 5 Hz, H-3 quinic), 3.94 (1H, br s, H-5 quinic), 3.55 (1H, br d, J = 4 Hz, H-4 quinic), 1.7−2.1 (2H, m, H-6 quinic), 1.98 (2H, br d, J = 5 Hz, H-2 quinic); (**2**) 7.48 (1H, d, J = 16 Hz, H-7 caffeoyl linked in the 3-position of quinic acid), 7.45 (1H, d, J = 16 Hz, H-7 caffeoyl linked in the 5-position of quinic acid), 7.05 (1H, br s, H-2 caffeoyl linked in the 3-position of quinic acid), 7.03 (1H, br s, H-2 caffeoyl linked in the 5-position of quinic acid), 6.99 (2H, d, J = 8 Hz, H-6 caffeoyl linked in the 3- and 5-positions of quinic acid), 6.77 (2H, d, J = 8 Hz, H-5 caffeoyl linked in the 3- and 5-positions of quinic acid), 6.23 (1H, d, J = 16 Hz, H-8 caffeoyl linked in the 3-position of quinic acid), 6.18 (1H, d, J = 16 Hz, H-8 caffeoyl linked in the 5-position of quinic acid), 5.18 (2H, m, H-3 and H-5 quinic acid), 3.79 (1H, m, H-4 quinic acid), 1.8−2.3 (4H, m, H-2 and H-6 quinic acid).

^{13}C NMR (125 MHz, DMSO-d_6): (**1**) δ 174.9 (C-7 quinic), 165.7 (C-9 caffeoyl), 148.3 (C-4 caffeoyl), 145.5 (C-3 caffeoyl), 144.8 (C-7 caffeoyl), 125.6 (C-1 caffeoyl), 121.2 (C-6 caffeoyl), 115.7 (C-5 caffeoyl), 114.7 (C-2 caffeoyl), 114.3 (C-8 caffeoyl), 73.6 (C-1 quinic), 70.9 (C-3 quinic), 70.6 (C-4 quinic), 68.3 (C-5 quinic), 37.2 (C-6 quinic), 36.5 (C-2 quinic); (**3**) 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.2 (C-2, C-9), 148.4 (C-4′), 144.7 (C-3′), 133.5 (C-3), 121.9 (C-6′), 121.0 (C-1′), 115.9 (C-5′), 115.1 (C-2′), 103.9 (C-10), 101.8 (C-1 galactose), 98.6 (C-6), 93.4 (C-8), 75.8 (C-5 galactose), 73.2 (C-3 galactose), 71.2 (C-2 galactose), 67.9 (C-4 galactose), 60.1 (C-6, galactose); (**4**) 177.3 (C4), 164.2 (C-7), 161.2 (C-5), 156.3, 156.1 (C-2, C-9), 148.4 (C-4′), 144.7 (C-3′), 133.3 (C-3), 121.5 (C-6′), 121.1 (C-1′), 116.1 (C-5′), 115.1 (C-2′), 103.8 (C-10), 100.9 (C-1 glucose), 98.6 (C-6), 93.4 (C-8), 77.4 (C-5 glucose), 76.5 (C-3 glucose), 74.0 (C-2 glucose), 69.9 (C-4 glucose), 60.9 (C-6 glucose); (**5**) 177.3 (C-4), 167.7, 166.7 (CO malonyl), 164.1 (C-7), 161.1 (C-5), 156.6, 156.3 (C-2, C-9), 148.5 (C-4′), 144.8 (C-3′), 133.1 (C-3), 121.4 (C-1′), 121.0 (C-6′), 116.2 (C-5′), 115.1 (C-2′), 103.8 (C-10), 101.1 (C-1 glucose), 98.6 (C-6), 93.5 (C-8), 76.2 (C-3 glucose), 73.9 (C-2 glucose, C-5 glucose), 69.5 (C-4 glucose), 63.5 (C-6 glucose), 41.3 (CH_2 malonyl).

^{1}H NMR and ^{13}C NMR data of **1** were identical to those of authentic 5-caffeoylquinic acid (chlorogenic acid). ^{1}H NMR and ^{13}C NMR data of **2**−**5** coincided with those of previous reports of 3,5-dicaffeoylquinic acid (Chuda et al., 1996), quercetin 3-galactoside (Bennini et al., 1992), quercetin 3-glucoside (Bennini et al., 1992), and quercetin 3-(6-malonylglucoside) (Wald et al., 1989; Ferreres et al., 1997), respectively. Therefore, **1**−**5** were identified as these respective compounds (Figure 1).

The HPLC fraction of compound **5** was hydrolyzed with 1% HCl in MeOH and analyzed by HPLC, and quercetin 3-galactoside was detected as a minor compound (12%) as well as quercetin 3-glucoside (88%) (data not shown). This suggests the occurrence of acyl quercetin 3-galactoside(s) in the fraction. We could find no proton signals of acyl groups other than the malonyl group. Among sugar proton signals, only methylene proton signals were shifted to low field. Therefore, the quercetin galactoside derivative might be acylated by malonic acid at 6-H of the galactoside (**6**).

Table 1. Contents of Antioxidative Compounds in *C. olitorius* Leaves[a]

compound	content (mg/100 g of fresh wt)
5-caffeoylquinic acid	383.9 ± 20.4
3,5-dicaffeoylquinic acid	102.1 ± 8.3
quercetin 3-galactoside	53.3 ± 5.0
quercetin 3-glucoside	376.8 ± 2.8
quercetin 3-(6-malonylglucoside)	126.2 ± 10.4
quercetin 3-(6-malonylgalactoside)	16.7 ± 1.0
ascorbic acid	257.8 ± 14.7
α-tocopherol	14.0 ± 0.7

[a] Values are mean ± SD of three replications.

Contents of Antioxidants in *C. olitorius* Leaves and Their Antioxidative Activities. Table 1 shows the contents of the isolated antioxidative compounds, ascorbic acid, and α-tocopherol in *C. olitorius* leaves. The primary compound was 5-caffeoylquinic acid; its content was as high as 384 mg/100 g of fresh weight. The content of 3,5-dicaffeoylquinic acid was approximately a fourth of that of 5-caffeoylquinic acid. The total content of the four quercetin glycosides was 233 mg/100 g of fresh weight, and quercetin 3-(6-malonylglucoside) was predominant among them. *C. olitorius* leaves also contained significantly high amounts of ascorbic acid (258 mg/100 g of fresh weight), consisting of 95% AsA and 5% DHAA. The α-tocopherol content was 14 mg/100 g of fresh weight.

The effects of these antioxidative compounds on the AAPH-induced peroxidation of linoleic acid are shown in Figure 2. Although there were no large differences in antioxidative activities among the isolated phenolic

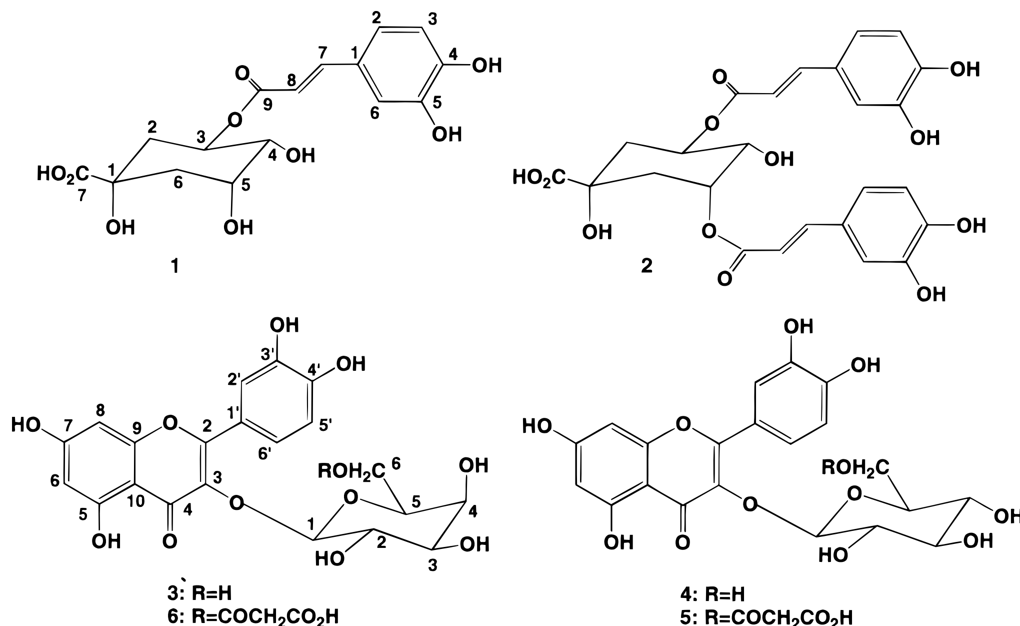

Figure 1. Chemical structures of phenolic antioxidants isolated from *C. olitorius* leaves.

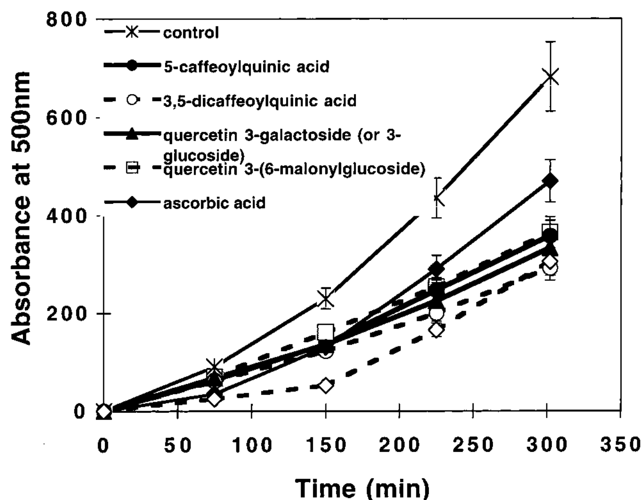

Figure 2. Effect of antioxidative compounds on the AAPH-initiated peroxidation of linoleic acid. The reaction system consisting of 1.0% (w/v) linoleic acid, 0 μM (control) or 10 μM antioxidants, and 4 mM AAPH in 40% ethanol, 80 mM phosphate buffer (1.25 mL, pH 7.0) was incubated at 37 °C in the dark. Tested quercetin 3-(6-malonylglucoside) contained 12% quercetin 3-(6-malonylgalactoside). Reported values are mean ± SD of three replications.

compounds, 3,5-dicaffeoylquinic acid was more active than 5-caffeoylquinic acid. Among the isolated quercetin glycosides, 3-galactoside and 3-glucoside were slightly more active compared with 3-(6-malonylglucoside) containing 12% 3-(6-malonylgalactoside). The antioxidative activities of these phenolic compounds were characterized by their extended stability. On the other hand, AsA showed a high antioxidative activity at an initial stage of the peroxidation, but then it rapidly lost activity. α-Tocopherol had the highest antioxidative activity, although the activity was reduced during the latter period of the peroxidation, similar to AsA.

DISCUSSION

Compounds **1**−**6** were isolated as phenolic antioxidants of *C. olitorius* leaves. These compounds were divided into cinnamic acid derivatives and flavonoids. Esters of hydroxycinnamic acid including 5-caffeoylquinic acid and 3,5-dicaffeoylquinic acid are widely distributed in the plant kingdom (Sondheimer, 1958; Herrmann, 1989). High amounts of caffeoylquinic acids and dicaffeoylquinic acids are found in coffee beans (6.57−9.04% of dried beans) (Clifford, 1985). Caffeoylquinic acids and their derivatives are also reported as antioxidants in sweet potatoes (Hayase and Kato, 1984), burdock roots (Maruta et al., 1995), garland leaves (Chuda et al., 1996), and prunes (Donovan, 1998). 5-Caffeoylquinic acid is found in fruits such as peaches (18.6 mg/100 g of fresh weight), apples (13.9 mg/100 g of fresh weight), and pears (13.4 mg/100 g of fresh weight) and is rich in vegetables such as corn salad (*Valerianella olitoria*) (101.6 mg/100 g of fresh weight), eggplant (57.5−63.2 mg/100 g of fresh weight), and artichoke (43.3 mg/100 g of fresh weight) (Herrmann, 1989). *C. olitorius* leaves contained significantly greater amounts of 5-caffeoylquinic acid compared with these fruits and vegetables (Table 1). 3,5-Dicaffeoylquinic acid is reported as one of the predominant phenolic antioxidants in the young leaves of garland (22.9 mg/g of dry weight) (Chuda et al., 1998). This compound was not so rich in *C. olitorius* leaves compared with garland leaves.

Quercetin glycosides are flavonoids with widespread occurrence in plants (Herrmann, 1988). Several malonyl esters of flavonoids have subsequently been reported, and one of these, quercetin 3-(6-malonylglucoside), has been isolated from some plants such as pears and red pigmented lettuce (Wald et al., 1989; Ferreres et al., 1997). Quercetin 3-(6-malonylgalactoside) had been reported to be isolated from *Adiantum capillus-veneris* L. (Imperato, 1981). The content of quercetin glycosides in *C. olitorius* leaves is also remarkable—approximately twice that of onion, one of the major sources of quercetin glycosides (Price and Rhodes, 1997).

All of the isolated phenolic compounds showed relatively high and lasting antioxidative activities (Figure 2). The difference in the antioxidative activities between 5-caffeoylquinic acid and 3,5-dicaffeoylquinic acid might be attributed to the number of caffeic acid residues in

their structures. From the result that 3-galactoside and 3-glucoside showed the same antioxidative activity, it is supposed that the activities of 3-(6-malonylglucoside) and 3-(6-malonylgalactoside) might be identical. The antioxidative activity of AsA was found only during an initial period of linoleic acid peroxidation, although its content in *C. olitorius* leaves is high. The loss of antioxidative activity of AsA is thought to occur when all of the molecules of AsA are oxidized. α-Tocopherol has high antioxidative activity, but it is found at much lower levels than the phenolic antioxidants and AsA (Table 1 and Figure 2). These results suggested that the phenolic antioxidants might greatly contribute to the antioxidative activity of *C. olitorius* leaves and that 5-caffeoylquinic acid could be the most predominant antioxidant.

The presence of antioxidative compounds other than isolated phenolic antioxidants is suggested from the fact that relatively high antioxidative activity was also found in the neutral aqueous fraction of the MeOH extract of *C. olitorius* leaves. The isolation of these compounds from *C. olitorius* leaves is in progress in our laboratory.

Studies on the absorption, metabolism, and antioxidative actions in vivo of flavonoids such as quercetin and its glycosides (Hollman et al., 1995; Manach et al., 1997) and epicatechin (Piskula and Terao, 1998; Silva et al., 1998) have been developed. On the other hand, there are few findings on the behaviors in vivo of caffeoylquinic acids and dicaffeoylquinic acids, although inhibition of human low-density lipoprotein oxidation in vitro by them has been reported (Meyer et al., 1998). Further studies on the physiological functions of these antioxidants are required.

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