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Antioxidative Capacity of Extracts and Constituents in *Cornus capitata* Adventitious Roots

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Radical scavenging activities of extracts and constituents in *Cornus capitata* adventitious root cultures were evaluated by using 1,1-diphenyl-2-pycrylhydrazyl (DPPH) and superoxide anion radicals. Inhibitory activity against peroxidation of linoleic acid was assayed by using the thiobarbituric acid (TBA) method. Ethyl acetate and aqueous fractions were prepared from adventitious roots cultured in Murashige−Skoog liquid medium with 0.1 μM Cu^{2+} (0.1CuMS) or 10 μM Cu^{2+} (10CuMS). The highest scavenging activities on DPPH and superoxide anion radicals were observed in the ethyl acetate fraction from 0.1CuMS. In the inhibitory activity against linoleic acid oxidation, the ethyl acetate fraction from 10CuMS was highest among the fractions tested. The ethyl acetate fraction of adventitious roots cultured in 0.1CuMS contained mainly galloylglucoses (1,2,3,6-tetragalloylglucose and 1,2,3,4,6-pentagalloylglucose). The ethyl acetate fraction of adventitious roots cultured in 10CuMS contained mainly ellagic acid derivatives [3,3′-di-*O*-methylellagic acid 4-(5″-acetyl)-α-L-arabinofuranoside and stenophyllin H1]. Aqueous fractions prepared from both media contained iridoid glycosides (dihydrocornin and cornin). Tetra- and pentagalloylglucoses showed strong inhibitory activities (61.9 and 85.2%, respectively) against linoleic acid oxidation relative to those of butylated hydroxytoluene (BHT) (91.1%) or α-tocopherol (49.5%) at 50 μM concentration. Although both ellagic acid derivatives had weak activities (<50%) on DPPH and superoxide anion radical scavenging, 3,3′-di-*O*-methylellagic acid 4-(5″-acetyl)-α-L-arabinofuranoside was stronger (74.7%) than α-tocopherol (49.5%) in inhibiting linoleic acid oxidation at 50 μM concentration. Iridoid glycosides exhibited little activity against DPPH and superoxide anion radicals or against oxidation of linoleic acid.

KEYWORDS: Antioxidant; galloylglucose; ellagic acid derivative; iridoid glycoside; adventitious root; *Cornus capitata*

INTRODUCTION

Plant natural products have been known to have various bioactivities such as antioxidative, antivirus, antibacterial, and radical scavenging actions (*1*). In vitro plant tissue cultures such as plant cell, callus, and adventitious root could be new sources of antioxidants (*2*, *3*). Rakotoarison et al. (*2*) reported on the antioxidative activity of proanthocyanidin B2 in cell suspension cultures of *Crataegus monogyna* and anthocyanins, catechins, and stilbenes isolated from *Vitis vinifera* cell cultures inhibited low-density lipoprotein (LDL) oxidation (*3*). *Cornus capitata* (Cornaceae) is native from China to The Himalayas and decorates the mountainsides with its bright yellow bracts. In China, the fruit of this plant is used to make an alcoholic beverage (*4*). The genus *Cornus* is well-known to contain a large amount of tannins, especially hydrolyzable tannins (*5*−*9*), and iridoid compounds (*10*−*14*). Tannins and related compounds have been reported to have antioxidative (*1*), antimicrobial (*15*), anticancer (*16*), and anti-HIV activities (*17*). We have investigated the polyphenol production and the antioxidative activity of adventitious roots of *C. capitata* (*18*). The production of polyphenols in the adventitious roots was regulated by the concentration of Cu^{2+} in the culture medium. The adventitious root cultures of *C. capitata* cultured in a medium with low concentrations of Cu^{2+} produced galloylglucoses (1,2,3,6-tetra-*O*-galloyl-β-D-glucose and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose). In contrast, a high concentration of Cu^{2+} (>1 μM) in a medium markedly inhibited the production of galloylglucoses. The 1,1-diphenyl-2-pycrylhydrazyl (DPPH) radical scavenging activities of the adventitious root extract increased with galloylglucose concentration of the extract (*18*). However, on the autoxidation of linoleic acid, the extract prepared from adventitious roots cultured a medium with a high concentration of Cu^{2+} showed a strong inhibitory effect (*18*). Therefore, the adventitious roots cultured in a medium with a high concentration of

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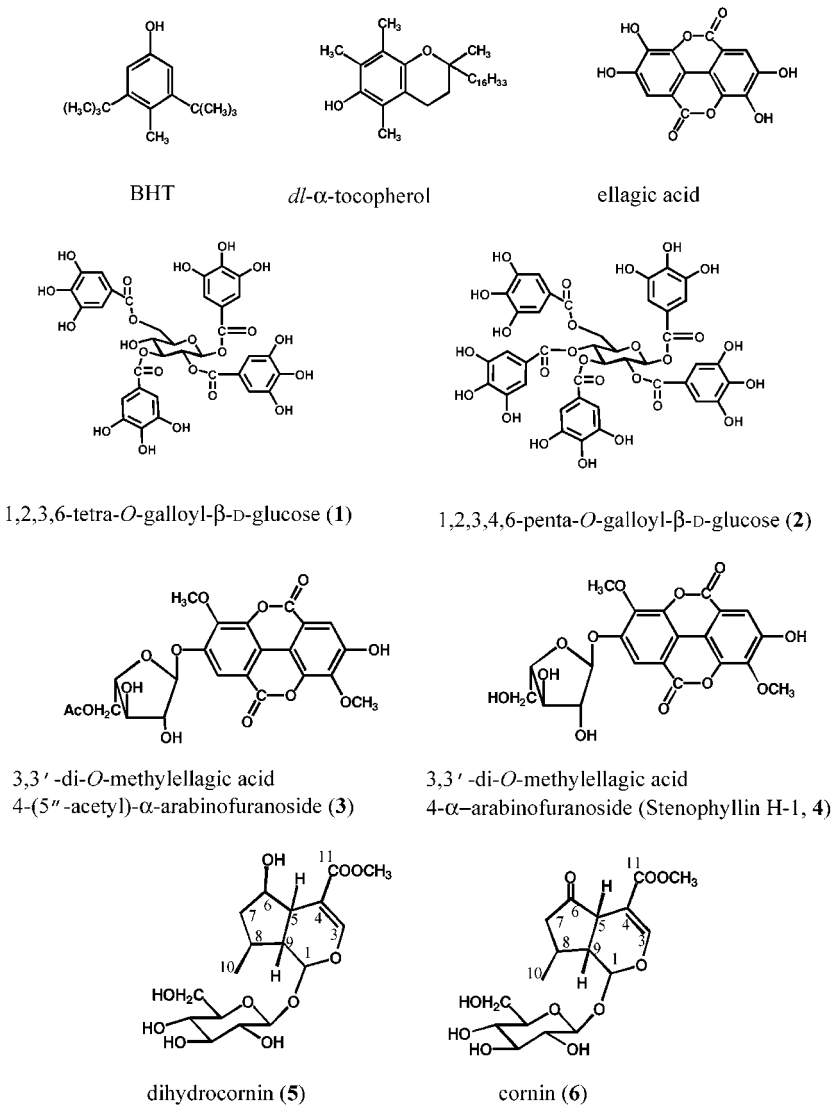

Figure 1. Antioxidant compounds and secondary metabolites (**1**–**6**) isolated from *C. capitata* adventitious roots.

Cu^{2+} seemed to suggest the possibility of the existence of other secondary metabolites having strong antioxidative activity on the autoxidation of linoleic acid. Recently, ellagic acid derivatives and iridoid glycosides were isolated from the root cultures cultured in a medium containing high concentrations of Cu^{2+} (*19*); the antioxidative capacity of these compounds has not been reported. The aim of this study is to evaluate the antioxidative activity of aqueous and ethyl acetate extracts from *C. capitata* adventitious roots cultured in a medium containing low or high concentrations of Cu^{2+}. Moreover, the antioxidative activities of secondary metabolites isolated from the adventitious roots cultured in a medium containing low or high concentrations of Cu^{2+} were compared with those of butylated hydroxytoluene (BHT), *dl*-α-tocopherol, and ellagic acid.

The antioxidative activity of each extract and isolated compound of *C. capitata* adventitious roots was examined by three assay systems. First, DPPH method was used for hydrogen-donating capacity. However, this method alone cannot estimate the antioxidative activity on the production of oxidative chain reactions. A second method assessed superoxide anion radical scavenging activity. Superoxide anion radicals are important as initiators of the chain reactions, producing hydrogen peroxide. A third method measured the inhibitory activity against lipid peroxidation in linoleic acid.

MATERIALS AND METHODS

Chemicals. 1,2,3,6-Tetra-*O*-galloyl-*β*-D-glucose (**1**), 1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose (**2**), 3,3′-di-*O*-methylellagic acid 4-(5″-acetyl)-α-arabinofuranoside (**3**), stenophyllin H-1 (**4**), dihydrocornin (**5**), and cornin (**6**) (**Figure 1**) were isolated from *C. capitata* adventitious roots (*19*). BHT and *dl*-α-tocopherol were purchased from Sigma-Aldrich Japan Co., and ellagic acid was purchased from Sigma Chemical Co. (St. Louis, MO).

Adventitious Root Culture. The adventitious root culture of *C. capitata* was established in Murashige−Skoog (MS) liquid medium as previously described (*19*). The roots (~0.8 g of fresh weight) were transferred to MS liquid media (0.1CuMS and 10CuMS) supplemented with different concentrations of cupric sulfate (0.1 and 10 μM), containing 30 g/L maltose and 3.0 mg/L 3-indoleacetic acid (IAA), and were cultured for 7 weeks at 25 °C in the dark on a rotary shaker (100 rpm). The adventitious roots were subcultured in the dark for >1 year at 8-week intervals.

Extraction and Fractionation. Lyophilized adventitious roots (2.0 g) were extracted with MeOH (20 mL × 5) at room temperature. The extracts were filtered and evaporated under reduced pressure, suspended in H_2O (20 mL), and extracted successively with EtOAc (20 mL × 3) at room temperature. The aqueous and EtOAc fractions were evaporated to dryness in vacuo to give brown and yellow residues, respectively. These residues (0.1CuMS-Aq, 10CuMS-Aq, 0.1CuMS-EtOAc, and 10CuMS-EtOAc) were used for radical-scavenging and antioxidative assays.

DPPH Radical-Scavenging Method. A solution of standards and samples prepared from adventitious roots in MeOH, DMSO, or H_2O (500 μL) was added to 500 μL of 500 μM DPPH solution. After the mixture had been shaken, the reaction solution was allowed to stand for 20 min at room temperature in the dark. The absorbance of the mixture was measured at 517 nm. Solvent (500 μL) containing 500 μM DPPH without mixing with sample solution was used as control. The radical-scavenging activity of the samples was calculated according to the following formula: DPPH radical scavenging activity (%) = 100 − (absorbance of sample/absorbance of control) × 100.

Superoxide Dismutase (SOD)-like Activity by the NBT Method. SOD-like activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT), according to the method of Beyer and Fridovich (*20*), with some modification. The reaction solution (1 mL) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 9.9×10^{-3} M l-methionine, 5.7×10^{-5} M NBT, 2.5×10^{-2} % (w/v) Triton X-100, 2.3×10^{-6} M riboflavin, and sample solution. The mixtures were kept for 7 min at 25 °C under light (5500 lx) and then measured at 560 nm. Reaction solutions without tested samples were used as control. Reaction solutions that were not illuminated were used as blank. The SOD-like activity of the samples was calculated according to the following formula: SOD-like activity (%) = 100 − [(absorbance of sample − absorbance of sample blank)/(absorbance of control − absorbance of control blank)] × 100. The level of the SOD-like activity was converted into that of SOD (EC.1.15.1.1) on the market. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT measured at 560 nm.

Antioxidative Assay in the Linoleic Acid/H_2O_2/$FeCl_2$ System. Autoxidation of linoleic acid in the H_2O_2−$FeCl_2$ system was carried out by using the modified method of Tamura and Takenaka (*21*). Each sample (50 μL) was added to a solution mixture (410 μL) of linoleic acid (200 mg) and sodium dodecyl sulfate (200 mg) in 30 mM Tris-HCl buffer (pH 7.4, 100 mL). The solution was sequentially added to 2.5 mM H_2O_2 (20 μL) and to 2.5 mM $FeCl_2$ solution (20 μL), which was incubated at 37 °C for 24 h.

Thiobarbituric Acid (TBA) Method. The TBA assay was performed according to the modified method of Ohkawa et al. (*22*) and Ando et al. (*23*). The incubated samples (200 μL) were mixed with 650 μL of a mixture of 5.2% (w/v) sodium dodecyl sulfate, 0.8% (w/v) BHT in acetic acid, 0.8% (w/v) TBA, and 150 μL of 20% (v/v) acetate buffer (pH 3.5) and heated at 100 °C for 1 h. After cooling, the mixture was centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 532 nm. The antioxidative activity of the samples was calculated according to the following formula: antioxidative activity (%) = 100 − (absorbance of sample/absorbance of control) × 100. The level of TBA-reactive substances (TBARS) by autoxidation of linoleic acid was calculated as the amount of malondialdehyde.

Quantification of Galloylglucoses, Ellagic Acid Derivatives, and Iridoid Glycosides by HPLC Analysis. A Shimadzu LC-10A system with a Shimadzu SPD-M10 was employed. Each sample prepared for analysis was filtered through a Millipore filter (0.45 μm) and subjected to HPLC analysis on a 250 mm × 4.6 mm i.d. ODS-120A column (TOSOH): mobile phase, 1 mM tetrabutylammonium chloride adjusted to pH 2.9 with acetic acid/acetonitrile (9:1 → 1:4, in 30 min); flow rate, 0.6 mL/min; column temperature, 40 °C; UV spectrophotometric detection, 254 nm.

RESULTS AND DISCUSSION

DPPH and Superoxide Radical-Scavenging Activities and Antioxidative Activity of Aqueous and Ethyl Acetate Fractions Prepared from *C. capitata* Adventitious Roots. To determine to which solvent fraction the secondary metabolites contained in the adventitious roots are distributed, the extracts of aqueous (0.1CuMS-Aq or 10CuMS-Aq) and ethyl acetate fractions (0.1CuMS-EtOAc or 10CuMS-EtOAc) were prepared from *C. capitata* adventitious roots cultured in MS liquid medium with 0.1 μM $CuSO_4$ (0.1CuMS) or 10 μM $CuSO_4$ (10CuMS) by partitioning between ethyl acetate and water. **Table 1** shows the concentrations of phenolic and iridoid compounds contained in each extract. Iridoid glycosides were mainly distributed in the aqueous fractions. The EtOAc extract from 0.1CuMS contained mainly tetra- and pentagalloylglucoses, and that from 10CuMS contained ellagic acid derivatives such as dimethylellagic acid acetyl-α-arabinofuranoside (**3**) and stenophyllin H-1 (**4**) at a high level.

Table 1. Secondary Metabolite Amounts in Extracts of *C. capitata* Adventitious Roots Used for DPPH, NBT Methods, and Linoleic Acid Oxidation System

	μg/100 μg of extract			
compound[a]	0.1CuMS-Aq[b]	0.1CuMS-EtOAc[b]	10CuMS-Aq[c]	10CuMS-EtOAc[c]
galloylglucoses				
1	0.02 ± 0.00	1.63 ± 0.17	nd[d]	nd
2	0.08 ± 0.00	1.60 ± 0.18	nd	nd
ellagic acid derivatives				
3	tr[e]	0.45 ± 0.06	0.05 ± 0.00	2.48 ± 0.31
4	0.02 ± 0.00	0.25 ± 0.07	0.06 ± 0.01	0.95 ± 0.06
iridoid glycosides				
5	5.69 ± 0.50	0.35 ± 0.15	4.39 ± 0.01	0.40 ± 0.04
6	6.45 ± 0.53	0.68 ± 0.23	13.80 ± 0.00	0.84 ± 0.04

[a] 1,2,3,6-Tetra-*O*-galloyl-β-D-glucose (**1**), 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (**2**), 3,3′-di-*O*-methylellagic acid 4-(5″-acetyl)-α-arabinofuranoside (**3**), stenophyllin H-1 (**4**), dihydrocornin (**5**), and cornin (**6**). [b] Extracts of aqueous and EtOAc extracts prepared from the cultures cultured in MS liquid medium containing 0.1 μM $CuSO_4$ for 6 weeks in the dark. Values indicate the mean of three replications ± SD. [c] Extracts of aqueous and EtOAc extracts prepared from the cultures cultured in MS liquid medium containing 10 μM $CuSO_4$ for 6 weeks in the dark. Values indicate the mean of three replications ± SD. [d] nd = not detected. [e] tr = trace.

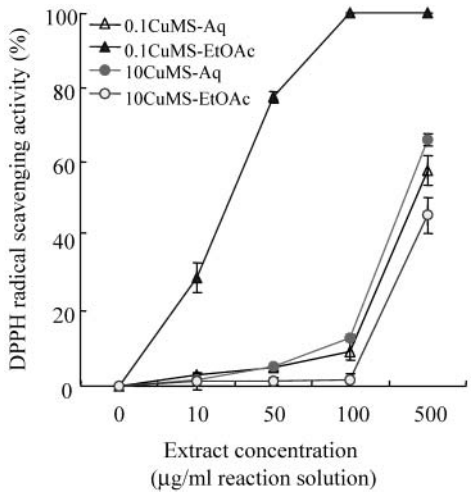

Figure 2. DPPH radical-scavenging activity of aqueous and ethyl acetate extracts from *C. capitata* adventitious roots: 0.1CuMS-Aq and 0.1CuMS-EtOAc, aqueous and EtOAc extracts prepared from the cultures cultured in MS liquid medium containing 0.1 μM $CuSO_4$ for 6 weeks in the dark; 10CuMS-Aq and 10CuMS-EtOAc, extracts from the cultures cultured in MS liquid medium containing 10 μM $CuSO_4$ for 6 weeks in the dark. Values indicate the mean of three replications.

The DPPH radical-scavenging activity of each extract is shown in **Figure 2**. In the extract (0.1CuMS-EtOAc) prepared from the roots cultured in 0.1CuMS, the DPPH radical-scavenging action increased as the concentrations of the extracts increased, and the DPPH radicals were scavenged 100% at the concentrations of 100 and 500 μg. However, the aqueous extract (0.1CuMS-Aq) prepared from the roots cultured in 0.1CuMS and the extracts (10CuMS-Aq and 10CuMS-EtOAc) from

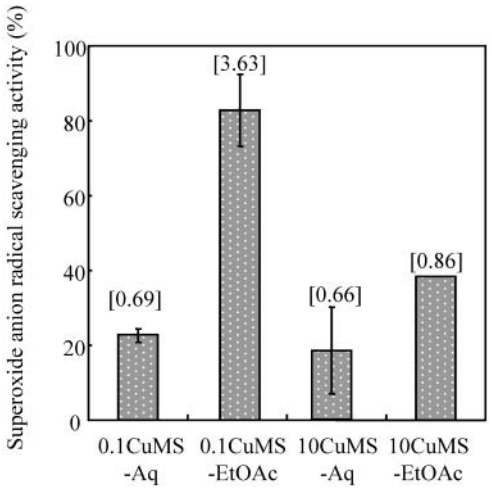

Figure 3. Superoxide anion radical-scavenging activity of aqueous and ethyl acetate extracts (100 μg) from *C. capitata* adventitious roots by the NBT method. SOD equivalent units/100 μg of extract are given in brackets.

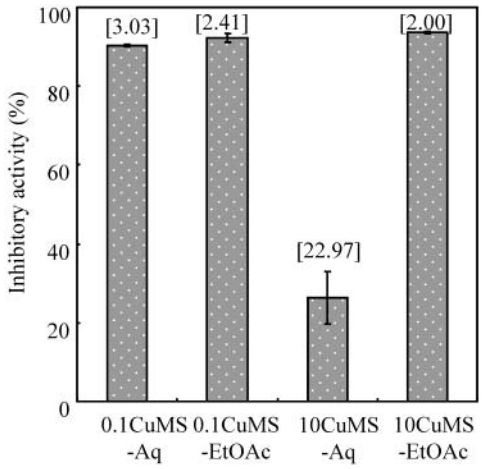

Figure 4. Inhibitory activity of extracts (100 μg) from *C. capitata* adventitious roots against linoleic acid oxidation by FeC_2/H_2O_2 system. TBARS values (nmol/mL reaction solution) are given in brackets.

10CuMS hardly scavenged the DPPH radical at concentrations of 10−100 μg. At a concentration of 500 μg, the scavenging activities of the extracts were 57.8, 65.9, and 45.8%, respectively. In a previous study, we reported that the DPPH radical-scavenging activity of the *C. capitata* root extracts increased with the galloylglucose content in the extract (*18*). Therefore, in the 0.1CuMS-EtOAc extract, it was thought that the DPPH radicals were scavenged by a high concentration of galloylglucoses.

The superoxide anion-scavenging activities (SOD-like activity) of various extracts (100 μg/mL reaction solution) were investigated (**Figure 3**). Among the extracts assayed, the 0.1CuMS-EtOAc extract showed the strongest SOD-like activity (3.63 SOD equivalent units/100 μg of extract). The SOD-like activity of the 10CuMS-EtOAc extract was slightly higher than those of 0.1CuMS-Aq and 10CuMS-Aq. The 10CuMS-EtOAc extract contained higher concentrations of ellagic acid derivatives. The level of **3** especially was 5.5 times higher than that of the 0.1CuMS-EtOAc extract (**Table 1**). The ellagic acid derivative concentrations in the extract may influence the SOD-like activity.

The antioxidative activity was evaluated by the TBA method as inhibitory effects of the aqueous and EtOAc extracts from *Cornus* root cultures on the oxidation of linoleic acid (**Figure 4**). The inhibitory effect on the linoleic acid oxidation in the EtOAc extract from the adventitious roots cultured in 10CuMS was highest among the extracts tested (93.6% inhibition, TBARS value = 2.00 nmol/mL linoleic acid reaction solution). The 0.1CuMS-Aq and 0.1CuMS-EtOAc extracts, which were prepared from the root cultures cultured in MS medium containing a low concentration of $CuSO_4$, were also observed to have a strong inhibitory effect on the oxidation of linoleic acid (90.3 and 92.3% inhibition, respectively), whereas the 10CuMS-Aq prepared from the roots cultured in MS medium containing a high concentration of $CuSO_4$ showed a low antioxidative activity (TBARS value = 22.97 nmol/mL linoleic acid solution). It is not apparent why the 0.1CuMS-Aq extract having a low content of secondary metabolites has a strong inhibitory effect against lipid peroxidation in linoleic acid. However, this suggests that there might exist other antioxidants in the 0.1CuMS-Aq extract.

DPPH and Superoxide Anion Radical-Scavenging and Antioxidative Activities of Secondary Metabolite Compounds from Adventitious Roots of *C. capitata*. The DPPH and superoxide anion radical-scavenging and antioxidative activities were investigated for six secondary metabolites, which were isolated from adventitious roots of *C. capitata* (**Table 2**). Their activities were compared to those of BHT, *dl*-α-tocopherol, and ellagic acid. As shown in **Table 2**, the DPPH radical-scavenging activity at the concentration of 50 μM was high for the galloylglucoses (**1** and **2**), ellagic acid, and α-tocopherol. The synthetic antioxidant BHT, the ellagic acid derivatives, **3** and **4**, and iridoid glycosides, **5** and **6**, hardly scavenged the DPPH radicals. As described in a previous study, the galloylglucoses such as **1** and **2**, with many phenolic OH groups in the molecules, strongly scavenged DPPH radicals (*18*, *24*). The ellagic acid derivatives, **3** and **4**, methylated at the 3- and 3′-positions, showed a low level of DPPH radical-scavenging action. Moreover, the radical-scavenging activity of the iridoid glycosides, lacking a phenolic OH group, was also weak.

Table 2. Radical-Scavenging Activities and Inhibitory Action on Linoleic Acid Oxidation of Secondary Metabolites from *C. capitata* Adventitious Roots

compound[a] (50 μM)	DPPH scavenging[b] (%)	superoxide anion scavenging[b] (%)	inhibitory effect on linoleic acid oxidation[b] (%)
BHT	1.38 ± 0.12	25.32 ± 3.45	91.17 ± 3.23
α-*dl*-tocopherol	34.20 ± 5.22	34.06 ± 0.18	49.53 ± 5.48
ellagic acid	82.57 ± 4.14	49.49 ± 3.45	82.08 ± 2.24
1	100	77.25 ± 2.36	61.94 ± 4.20
2	100	89.59 ± 2.36	85.25 ± 12.12
3	1.07 ± 0.38	41.26 ± 2.36	74.75 ± 21.18
4	0.91 ± 0.12	37.28 ± 1.45	44.26 ± 1.90
5	1.46 ± 0.76	11.57 ± 9.09	14.24 ± 0.46
6	1.41 ± 0.87	2.06 ± 1.82	1.25 ± 0.10

[a] BHT, butylated hydoxytoluene; **1**, 1,2,3,6-tetra-*O*-galloyl-β-D-glucose; **2**, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose; **3**, 3,3′-di-*O*-methylellagic acid 4-(5″-acetyl)-α-arabinofuranoside arabinofuranoside; **4**, stenophyllin H-1; **5**, dihydrocornin; **6**, cornin. [b] Values indicate the mean of three replications ± SD.

The SOD-like activity was in the order **2** > **1** > ellagic acid > **3** > **4**, α-tocopherol > BHT > **5** > **6** at a concentration of 50 μM. The superoxide anion radical-scavenging activity of the phenolic compounds also increased in accordance with the number of phenolic hydroxyl groups in the molecule. Compound **2** with 15 phenolic OH groups showed the maximum level of the activity, whereas iridoid glycosides without a phenolic hydroxyl group showed a low radical-scavenging activity. The inhibitory action on the superoxide anion radicals by methyl-

ellagic acid acetylarabinoside (**3**) was a little more efficient than that by methylellagic acid arabinoside (**4**).

The antioxidative activity of secondary metabolites from *C. capitata* was in the order BHT > **2**, ellagic acid > **3** > **1** > α-tocopherol > **4** > **5** > **6** at a concentration of 50 μM. Moreover, the antioxidative activity of **3** was ∼1.7 times stronger that that of **4** at the concentration of 50 μM. Compound **3** differs from **4** by the presence of an acetyl group (**Figure 1**). Zafrilla et al. reported on the DPPH radical-scavenging activity of ellagic acid derivatives isolated from raspberries (*25*). Their results showed that the DPPH radical-scavenging action of ellagic acid 4-(4″-acetyl)-arabinoside was slightly higher than that of ellagic acid 4-arabinoside. Therefore, the acetyl residue in ellagic acid glycoside or methylellagic acid glycoside might also be important for the inhibitory effect on linoleic acid oxidation. In linoleic acid−H_2O_2/$FeCl_2$ systems, iridoid glycosides that did not contain a phenolic OH group in the molecule exhibited weak antioxidative activity. However, when the activities of two iridoids was compared, the effect of **5** (14.2% inhibition) was much greater than that of **6** (1.3% inhibition) at a concentration of 50 μM. It is thought that the hydroxyl group at C-6 of **5** also took part in the antioxidative action on linoleic acid.

In conclusion, galloylglucoses were important metabolites that acted as radical scavengers against DPPH and superoxide anion radicals and as antioxidants of peroxidation in linoleic acid in extracts prepared from *C. capitata* adventitious roots cultured in a medium with a low concentration of $CuSO_4$. On the other hand, in extracts prepared from adventitious roots cultured in a medium with a high concentration of $CuSO_4$, ellagic acid derivatives might be important metabolites, which act as radical scavengers and/or antioxidants against the superoxide anion radicals and the peroxidation of linoleic acid.

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