JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Antioxidant and DNA-Protective Activities of Chlorogenic Acid Isomers

Jian-Guo Xu,*[,†] Qing-Ping Hu,[‡] and Yu Liu[†]

[†]College of Engineering and [‡]College of Life Sciences, Shanxi Normal University, Linfen City, 041004, China

ABSTRACT: Chlorogenic acid is a well-known antioxidant and has more isomers according to the difference in binding location and number of caffeic on quinic acid. In this study, we investigated and compared the profiles of antioxidant and DNA-protective activities of chlorogenic acid isomers including three caffeoylquinic acid isomers (3-*O*-caffeoylquinic acid, 3-CQA; 4-*O*-caffeoylquinic acid, 4-CQA; and 5-*O*- caffeoylquinic acid, 5-CQA) and three dicaffeoylquinic acid isomers (3,5-dicaffeoyl-quinic acid, ICAA; 3,4-dicaffeoylquinic acid, ICAB; and 4,5-dicaffeoyl-quinic acid, ICAC). The results showed that each of chlorogenic acid isomers studied exhibited antioxidant activities and DNA damage protective effects to various extents. On the whole, dicaffeoylquinic acids possessed better antioxidant activities, mostly because they have more hydroxyl groups than caffeoylquinic acids. Three caffeoylquinic acid isomers showed quite similar antioxidant activities, indicating that the position of esterification on the quinic moiety of caffeoylquinic acid had no effect on its antioxidant activities. Quite the contrary, a difference among dicaffeoylquinic acid isomers was observed, namely, ICAA and ICAB exhibited the same antioxidant activities, whereas ICAC had higher antioxidant activities than ICAA and ICAB in some assays, which implied that their antioxidant activities were probably influenced by the position of esterification on the quinic moiety. We speculated that this difference might be due to the fact that there may exist a steric hindrance effect in the ICAC. However, this assumption needs to be further confirmed.

KEYWORDS: *chlorogenic acid, isomer, antioxidant, DNA-protective activity*

INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by many redox processes that normally occur in the metabolism of aerobic cells, and at low-to-moderate concentrations, they are known to possess various physiological roles ranging from cellular signal transduction to defense against pathogens.[1] However, if not eliminated in time, the excessive amounts of ROS and RNS can attack important biological molecules such as carbohydrates, proteins, lipids, DNA, and RNA, which lead to cell death and tissue damage and the development of chronic diseases, such as cancer, nephritis, diabetes mellitus, and ischemic and cardiovascular diseases.[2,3] Antioxidants can delay or inhibit the oxidation of lipid or other molecules by inhibiting or eliminating the initiation or propagation of excess reactive species.[3] It is well-known that chlorogenic acid acts as antioxidant and is widely distributed in the leaves and fruits of dicotyledonous plants as an important plant polyphenol. Chemically, chlorogenic acids are a family of esters formed between quinic acid and 1−4 residues of certain *trans*-cinnamic acids, most commonly caffeic, ferulic, and *p*-coumaric acids.[4] In theory, caffeoylquinic acid has four isomers, and dicaffeoylquinic acid has six isomers according to the difference in binding location and binding number of caffeic on quinic acid. So far, the isomers in plants mainly include chlorogenic acid (3-*O*-caffeoylquinic acid, 3-CQA), neochlorogenic acid (5-*O*-caffeoylquinic acid, 5-CQA), cryptochlorogenic acid (4-*O*-caffeoylquinic acid, 4-CQA), isochlorogenic acid A (3,5-dicaffeoylquinic acid, ICAA), isochlorogenic acid B (3,4-dicaffeoylquinic acid, ICAB), isochlorogenic acid C (4,5-dicaffeoylquinic acid, ICAC), and cynarin (1,5-dicaffeoylquinic acid).[5] Structures of some isomers are shown in Figure 1.

Chlorogenic acid is widely recognized to have antioxidant activities[6−8] and a wide range of differing biological effects, such as antimutagenic, antiviral,[9] anticarcinogenic,[10] and enhancing cellular defense,[11] which is attributed to scavenging species of oxygen and nitrogen.[12] However, these studies on chlorogenic acid are mainly focused on 3-CQA[11,13,14] and 5-CQA.[15,16] As members of the family of chlorogenic acid, it is expected that other chlorogenic acid isomers, such as 4-CQA, ICAA, ICAB, and ICAC, may also show biological effects and might thereby exert different antioxidant activities in a food context. However, only meager information is available on the antioxidant activity of chlorogenic acid isomers.[6,15] To our knowledge, very little research has been conducted to investigate if there are differences in antioxidant activities among different chlorogenic acid isomers. Such comparisons are very important for the preparation of chlorogenic acid and its application in food and medicine because the isomerization can occur through hydrolysis and removal of the ester functionalities in intramolecular chlorogenic acid in some plants during extraction with different extraction methods, which may result in the change of its bioactivity.[6,17] Therefore, the objective of this research was to utilize six different assays to investigate and compare comprehensively and systematically the profiles of antioxidant activities and DNA damage protective effects of chlorogenic acid isomers over a wide concentration range (5−1000 μg/mL) and to further evaluate the structure−antioxidant activity relationships of chlorogenic acids on the basis of the biological results.

Received: September 1, 2012
Revised: November 7, 2012
Accepted: November 7, 2012
Published: November 7, 2012

dx.doi.org/10.1021/jf303771s | *J. Agric. Food Chem.* 2012, 60, 11625−11630

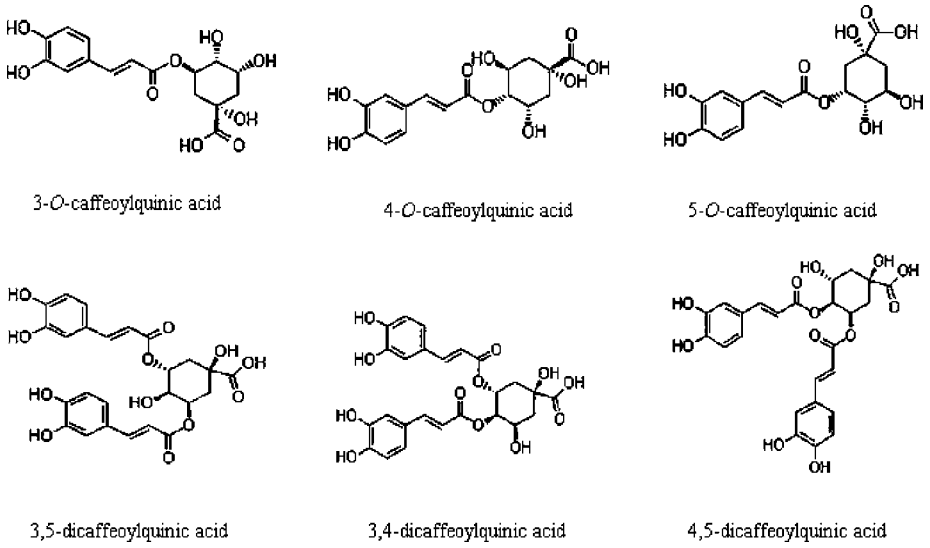

Figure 1. Structures of chlorogenic acid isomers.

MATERIALS AND METHODS

Chemicals. 2,4,6-Tri-(2-pyridyl)-s-triazine (TPTZ) and β-carotene were purchased from Fluka (Switzerland). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenothiazoline-6-sulfonic acid) diammonium salts (ABTS), 2,6-di-*tert*-butyl-4-hydroxytoluene (BHT), ferrozine, ethylenediamine tetra-acetic acid (EDTA), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Sigma (United States). 3-CQA, 4-CQA, 5-CQA, ICAA, ICAB, and ICAC were obtained from Chengdu Must Biotechnology Co. Ltd. (Chengdu, China), and the purity of these products was up to 98%.

DPPH Radicals Scavenging Activity Assay. The DPPH radical scavenging activity was determined according to the method of Hu and Xu[18] with some modifications. Briefly, each of the sample solutions (1 mg/mL in methanol) was serially diluted to various concentrations in methanol, respectively, and then, a 0.5 mL of samples was mixed with 2.5 mL of 60 μM DPPH dissolved in methanol. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was measured at 517 nm against a solvent blank. The scavenging rate on DPPH radicals was calculated according to the formula scavenging rate (%) = $[(A_o - A_1)/A_o] \times 100$, where A_o is the absorbance of the control solution and A_1 is the absorbance in the presence of samples in DPPH solution. The scavenging activity of the sample on DPPH radicals was expressed by EC_{50} value, and BHT was used as a positive control. The EC_{50} value is the effective concentration at which DPPH radicals are scavenged by 50% and is obtained by interpolation from regression analysis. The effectiveness of antioxidant activity inversely correlates with their EC_{50} values.

ABTS Free Radicals Scavenging Activity Assay. ABTS free radical scavenging activity was determined according to the method described by Re et al.[19] with slight modifications. Briefly, the ABTS cation radical was generated by a reaction of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate. The reaction mixture was allowed to stand in the dark at room temperature for 16−24 h before use and was used within 2 days. The $ABTS^+$ solution was diluted with methanol to an absorbance of 0.700 ± 0.050 at 734 nm. One hundred microliters of the diluted samples was mixed with 2.0 mL of diluted $ABTS^+$ solution. The mixture was allowed to stand for 6 min at room temperature, and the absorbance was immediately recorded at 734 nm. The scavenging rate and EC_{50} value were calculated using the equation described for DPPH assay. Trolox was used as the positive control.

Ferric Reducing Antioxidant Power (FRAP) Assay. The reducing ability was determined by using the FRAP assay described by Hu and Xu[18] with slight modifications. Briefly, the FRAP reagent was freshly prepared from 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in a water bath prior to use. Then, 0.1 mL of samples was mixed with 1.8 mL of FRAP reagent and 3.1 mL of ultra pure water. The absorption of the reaction mixture was measured at 593 nm after incubation for 30 min at 37 °C. The increased absorbance of the reaction mixture indicated increased reducing power. BHT was used as a positive control, and its reducing power was expressed as the concentration of BHT at which the absorbance of the reaction mixture is 0.5.

Metal Ion Chelating Assay. The ability of the sample to chelate iron(II) was estimated according to the method described by Shyu et al.[20] with minor modifications. An aliquot of each sample (100 μL) was mixed with 500 μL of $FeCl_2$ (0.5 mmol/L). After 5 min of incubation, the reaction was initiated by the addition of 200 μL of ferrozine (5.0 mmol/L), and the mixture was adjusted to a total volume of 3 mL with methanol. After 10 min of incubation at room temperature, the absorbance at 562 nm was recorded against a blank. The chelating ability of the sample to chelate ferrous ion was calculated using the formula given below: metal chelating ability (%) = $[A_o - A_1)/A_o] \times 100$, where A_o is the absorbance of the ferrozine−Fe^{2+} complex and A_1 is the absorbance of the test compound. EDTA was used as a positive control.

β-Carotene Bleaching Assay. The assay was performed as given by Shyu et al.[20] and modified slightly. First, 20 mg of β-carotene dissolved in 10 mL of chloroform was mixed with 200 mg of linoleic acid and 2 g of Tween 40 followed by chloroform removal under nitrogen, and 500 mL of distilled water was added with vigorous shacking to prepare a β-carotene linoleate emulsion. An aliquot of each sample (0.2 mL) was mixed with 3.8 mL of the emulsion, and then, the absorbance was determined at 470 nm at 45 °C for 2 h. β-Carotene bleaching inhibition was estimated as the following equation: bleaching inhibition (%) = $[1 - (A_o - A_1)/(C_o - C_1)] \times 100$, where A_o and C_o are the initial absorbance of sample and control, respectively, and A_1 and C_1 are the absorbance of sample after 2 h, respectively. BHT was used as a positive control, and the EC_{50} value is the concentration of sample that could give 50% bleaching inhibition.

DNA Damage Protective Effect Assay. The ability of samples to protect supercoiled pBR322 plasmid DNA against H_2O_2 was estimated with the DNA nicking assay as described by Jeong et al.[21] with minor modifications. The reaction mixtures (15 μL) contained 5 μL of phosphate buffer saline (PBS, 10 mM, pH 7.4), 1 μL of plasmid DNA (0.5 μg), 5 μL of the samples, 2 μL of 1 mM $FeSO_4$, and 2 μL of 1 mM H_2O_2 and were incubated at 37 °C for 30 min. After incubation, 2 μL of a loading buffer [50% glycerol (v/v), 40 mM EDTA, and 0.05% bromophenol blue] was added to stop the reaction, and the reaction mixtures were electrophoresed (DYCP-31A Agarose Electrophoresis Instrument, Beijing Liuyi Instrument Factory, Beijing, China) on 1% agarose gel containing 0.5 μg/mL ethidium bromide in Tris/acetate/

Table 1. DPPH Radicals Scavenging Activity of Different Chlorogenic Acid Isomers[a]

	scavenging rate (%) at different concns (μg/mL)						
sample	5	10	20	40	60	regression eq (5−40 μg/mL)	EC_{50} (μg/mL)
3-CQA	13.8 ± 0.9 c	38.6 ± 1.7 c	62.8 ± 2.0 b	93.4 ± 1.6 a	95.1 ± 1.4 a	$y = 37.943\ \mathrm{Ln}(x) - 48.367$ $R^2 = 0.9969$	13.4 ± 1.1 a
4-CQA	15.1 ± 1.0 c	40.2 ± 2.6 c	63.2 ± 1.8 b	92.2 ± 2.4 a	94.9 ± 1.8 a	$y = 36.688\ \mathrm{Ln}(x) - 44.517$ $R^2 = 0.9978$	13.2 ± 1.0 a
5-CQA	13.4 ± 0.8 c	36.6 ± 3.1 c	61.2 ± 2.6 b	92.0 ± 2.0 a	94.6 ± 2.2 a	$y = 37.568\ \mathrm{Ln}(x) - 48.723$ $R^2 = 0.9954$	13.8 ± 1.3 a
ICAA	29.6 ± 1.5 b	52.8 ± 3.0 b	75.3 ± 2.2 a	94.3 ± 3.5 a	95.6 ± 2.6 a	$y = 31.249\ \mathrm{Ln}(x) - 19.783$ $R^2 = 0.9980$	9.3 ± 0.5 b
ICAB	28.4 ± 2.2 b	52.2 ± 2.4 b	76.8 ± 2.1 a	94.9 ± 1.8 a	96.1 ± 1.6 a	$y = 32.331\ \mathrm{Ln}(x) - 2.574$ $R^2 = 0.9957$	9.4 ± 0.2 b
ICAC	36.5 ± 1.8 a	59.7 ± 1.9 a	81.2 ± 3.2 a	96.4 ± 3.6 a	97.5 ± 1.2 a	$y = 29.027\ \mathrm{Ln}(x) - 8.447$ $R^2 = 0.9916$	7.5 ± 0.6 b

[a]Values represent means of three independent replicates ± SDs. R^2 refers to the regression coefficients. Different letters within a column indicate statistically significant differences between the means ($p < 0.05$) for different chlorogenic acid isomers.

Table 2. ABTS Cation Radicals Scavenging Activity of Different Chlorogenic Acid Isomers[a]

	scavenging rate (%) at different concns (μg/mL)						
sample	50	75	100	125	150	equation (50−150 μg/mL)	EC_{50} (μg/mL)
3-CQA	20.3 ± 1.1 c	41.4 ± 1.5 c	54.8 ± 2.2 c	70.2 ± 1.8 c	93.4 ± 2.0 a	$y = 0.7x - 13.98$ $R^2 = 0.9913$	91.4 ± 3.5 a
4-CQA	23.7 ± 0.8 c	44.1 ± 1.9 c	57.3 ± 1.8 c	72.8 ± 1.4 bc	94.6 ± 1.8 a	$y = 0.682x - 9.7$ $R^2 = 0.993$	87.5 ± 1.2 a
5-CQA	21.6 ± 1.4 c	40.6 ± 1.0 c	54.2 ± 2.6 c	69.6 ± 1.6 c	93.5 ± 2.6 a	$y = 0.6904x - 13.16$ $R^2 = 0.9906$	91.5 ± 4.3 a
ICAA	32.9 ± 1.5 b	49.3 ± 2.1 b	64.6 ± 2.3 b	76.0 ± 1.9 b	94.4 ± 2.2 a	$y = 0.5988x + 3.56$ $R^2 = 0.9959$	77.6 ± 2.3 b
ICAB	33.4 ± 2.2 b	49.8 ± 1.8 b	63.5 ± 2.0 b	75.6 ± 1.2 b	94.3 ± 2.4 a	$y = 0.5904x + 4.28$ $R^2 = 0.9954$	77.4 ± 2.6 b
ICAC	38.4 ± 1.8 a	55.5 ± 2.4 a	71.5 ± 1.9 a	81.5 ± 2.3 a	97.4 ± 1.5 a	$y = 0.576x + 11.26$ $R^2 = 0.9933$	67.3 ± 4.9 c

[a]Values represent means of three independent replicates ± SDs. R^2 refers to the regression coefficients. Different letters within a column indicate statistically significant differences between the means ($p < 0.05$) for different chlorogenic acid isomers.

EDTA gel buffer for 60 min (60 V), and the DNA in the gel was visualized and photographed under ultraviolet light. The protective effect was expressed as a percentage content of the supercoiled form of plasmid DNA treated with samples in untreated plasmid DNA. Trolox (50 μM) was used as a positive control.

Statistical Analysis. All experiments were conducted three times independently, and the experimental data were expressed as means ± standard deviations (SDs). The correlation coefficient, regression analyzes, one-way analysis of variance (ANOVA), and a Duncan's test that is used for determining significant differences ($p < 0.05$) between the means were carried out by Data Processing System (DPS, version 7.05) and the Excel program.

RESULTS

DPPH Radicals Scavenging Activity. To evaluate variations in antioxidant activity of chlorogenic acid isomers, a method based on the reduction of DPPH was performed. The scavenging activity assayed herein on DPPH radicals is shown in Table 1. The EC_{50} values of chlorogenic acid isomers ranged from 7.5 (for ICAC) to 13.8 μg/mL (for 5-CQA), lower than that of BHT (EC_{50} was 39.62 μg/mL), indicating that the DPPH radicals scavenging activity of ICAC was the highest, the lowest for 5-CQA, and that all of the chlorogenic acid isomers exhibited a higher antioxidant activity than BHT. A significant difference ($p < 0.05$) in scavenging activity was detected between caffeoylquinic and dicaffeoylquinic acids, and the scavenging activity of dicaffeoylquinic acids was significantly higher than that of caffeoylquinic acids, which was in keeping with previous reports that the scavenging activity of ICAA was stronger than that of 5-CQA.[22,23] However, no significant difference in scavenging activity was observed among three caffeoylquinic acids as well as three dicaffeoylquinic acids. For each of the chlorogenic acid isomers, it showed a concentration-dependent scavenging of the DPPH radicals at lower concentrations, which was also consistent with the previous reports.[22,23] Also, there was a significant difference in scavenging rate among chlorogenic acid isomers when at the same concentration, which had a more similar trend to the EC_{50} values. Nevertheless, the difference gradually became nondistinctive with the increase of their concentrations; even the scavenging rate of these isomers was the same at concentrations above 40 μg/mL, which was mainly because the DPPH radicals in the test system were almost completely scavenged.

ABTS Cation Radicals Scavenging Activity. The scavenging activity of different chlorogenic acid isomers on ABTS cation radicals is shown in Table 2. The profile of scavenging activity of different chlorogenic acid isomers on ABTS was similar to the result of the scavenging activity on DPPH radicals. Somewhat differently, the EC_{50} values on scavenging ABTS cation radicals ranged from 67.3 (for ICAC) to 91.5 μg/mL (for 5-CQA), and the scavenging activity of ICAC was significantly higher than that of ICAA and ICAB, which was higher than of Trolox (EC_{50} was 131.1 μg/mL). Similarly, ABTS cation radicals scavenging activity of each chlorogenic acid isomer increased dose dependently at concentrations ranging from 50 to 150 μg/mL, which may be attributable to its hydrogen-donating ability. The difference in scavenging rates among chlorogenic acid isomers at the same concentration was similar to that in their EC_{50} values. However, the scavenging rate of these isomers on ABTS cation radicals was the same at concentrations above 150 μg/mL in this experimental test for the same reason from the DPPH assay. These differences in data between DPPH and ABTS assays were likely due to different experimental conditions.

FRAP. The FRAP may serve as a significant indicator of the potential of antioxidant activity.[24] Higher absorbance of the reaction mixture indicated greater reducing power. Figure 2 showed that the reducing power of each isomer was in a concentration-dependent manner and increased with concentration. However, the reducing power was different for different isomers at the same concentration, and the highest was ICAC, followed by ICAA and ICAB, the lowest for the three caffeoylquinic acids. These results suggested that chlorogenic acid isomers had a remarkable potency to donate electrons to reactive free radicals, converting them into more stable

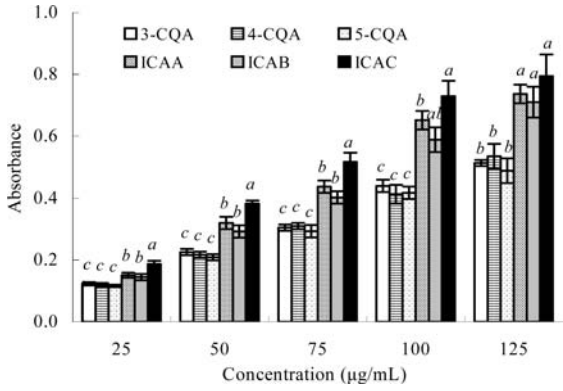

Figure 2. Reducing power of different chlorogenic acid isomers. Data are expressed as the mean values of three independent replicates ± SDs. Different letters indicate statistically significant differences between the means ($p < 0.05$) for different chlorogenic acid isomers at the same concentration.

nonreactive species and terminating the free radical chain reaction. However, the reducing power of each isomer was lower than that of BHT (55.6 μg/mL) at which the absorbance of the reaction mixture was 0.5.

β-Carotene Bleaching Inhibition and Metal Ion Chelating Ability. The antioxidant activities of chlorogenic acid isomers were also assessed by the estimation of β-carotene bleaching and metal ion chelating ability (Table 3). The different

Table 3. β-Carotene Bleaching and Chelating Ability of Different Chlorogenic Acid Isomers

sample	β-carotene bleaching (EC_{50}, μg/mL)	chelating ability (%)[a]
3-CQA	607 ± 26 a	19.4 ± 1.2 b
4-CQA	585 ± 31 a	20.2 ± 1.9 b
5-CQA	611 ± 27 a	18.6 ± 2.0 b
ICAA	559 ± 44 a	40.8 ± 2.5 a
ICAB	569 ± 38 a	39.3 ± 3.4 a
ICAC	547 ± 33 a	42.9 ± 2.9 a

[a]Chelating ability was determined at 1 mg/mL of each sample. Values represent means of three independent replicates ± SDs. Different letters within a column indicate statistically significant differences between the means ($p < 0.05$) for different chlorogenic acid isomers.

isomers showed different antioxidant activities. The EC_{50} values of β-carotene bleaching for chlorogenic acid isomers ranged from 547 (for ICAC) to 611 μg/mL (for 5-CQA), and the sequence for β-carotene bleaching inhibition was ICAC > ICAA > ICAB > 4-CQA > 3-CQA > 5-CQA, which indicated that chlorogenic acid can probably reduce the extent of β-carotene destruction by neutralizing the linoleate-free radical and other free radicals formed in the system. However, β-carotene bleaching inhibition of each chlorogenic acid isomer was much less than of BHT (EC_{50} was 92 μg/mL). Ohnishi et al.[22] reported that 5-CQA, ICAA, and caffeic acid inhibited the formation of conjugated diene from linoleic acid, and the inhibitory activity of ICAA was stronger than those of 5-CQA or caffeic acid; Nakatani et al.[15] suggested that 3-CQA, 4-CQA, and 5-CQA exhibited almost the same activity against oxidation of methyl linoleate and that their oxidative stability index ranged from 13.83 to 15.15, which supported our results. Furthermore, we also found no significant difference in β-carotene bleaching inhibition among chlorogenic acid isomers including caffeoylquinic and dicaffeoylquinic acids.

In the metal ion chelating assay, the EC_{50} value of EDTA was 0.28 mg/mL (not shown), but we did not obtain the EC_{50} values of chlorogenic acid isomers even if their concentrations reached 1 mg/mL, which is a relatively large dose in food as an additive, so we only investigated the chelating ability of chlorogenic acid isomers at this concentration on Fe^{2+} (Table 3). As observed in the DPPH radical scavenging activity measurements, no significant difference in the chelating ability was found among three dicaffeoylquinic acids as well as three caffeoylquinic acids. However, the chelating ability of dicaffeoylquinic acids (39.3−42.9%) were significantly higher than that of caffeoylquinic acids (18.6−20.2%). The results obtained were partly consistent with a previous study that also reported that 3-CQA could chelate ferrous ions, and at 1.0 mg/mL, there was about an 18% chelating effect on ferrous ions.[13] Chelating agents remove prooxidant metal ions from the environment, thereby inhibiting the Fenton reaction. Our results showed that chlorogenic acid inhibited the formation of ferrous and ferrozine complexes to a certain extent, suggesting that the higher concentration of chlorogenic acid isomers would be somewhat beneficial to protect against oxidative damage.

DNA Damage Protective Effect. The efficiency of different chlorogenic acid isomers in preventing oxidative damage of DNA induced by H_2O_2 was also evaluated, and the result (concentration of each sample at 50 μg/mL) is shown in Figure 3. The damage of plasmid DNA produces a relaxed open circular

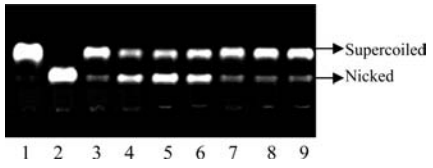

Figure 3. DNA damage protective effect of different chlorogenic acid isomers. Lane 1, the native DNA; lane 2, the DNA treated with 1 mM $FeSO_4$ and 1 mM H_2O_2; lane 3, the DNA treated with Trolox (50 μM), 1 mM $FeSO_4$, and 1 mM H_2O_2; and lanes 4−9, the DNA treated with 1 mM $FeSO_4$ and 1 mM H_2O_2 and treated with 3-CQA, 4-CQA, 5-CQA, ICAA, ICAB, and ICAC, respectively.

form and further a linear double-stranded DNA molecule. Therefore, the formation of circular form of DNA is indicative of single strand breaks, and the formation of linear form of DNA is indicative of double strand breaks.[25] The plasmid DNA was mainly of the supercoiled form in the absence of Fe^{2+} and H_2O_2 (Figure 3, lane 1). During the addition of Fe^{2+} and H_2O_2, the supercoiled form of DNA converted into the open circular and linear forms (Figure 3, lane 2), indicating that hydroxy radicals generated from iron-mediated decomposition of H_2O_2 produced both single strand and double strand DNA breaks. From the gel analysis, the protection offered against DNA damage by chlorogenic acid isomers (10−50 μg/mL) was concentration dependent (data not shown). At a concentration of 50 μg/mL, the DNA damage protective effect measured in different samples ranged from 43.1 to 62.4%, and the order was ICAC > ICAB > ICAA > 5-CQA > 4-CQA > 3-CQA. Only the protective effect of ICAC (62.4%) was more effective and slightly close to that of 50 μM Trolox (65.5%). Combining the results obtained from metal ion chelating tests, chlorogenic acids might prevent the reaction of Fe ions with H_2O_2, on the other hand, and it probably quenched hydroxy radicals by donating hydrogen atoms or electrons, therefore protecting the supercoiled plasmid DNA from hydroxy radical-dependent strand breaks.[25] Kasai et al.[26]

demonstrated that chlorogenic acid induced oxidative DNA damage in vitro and in a rat carcinogenesis model. Shibata et al.[14] reported that 3-CGA prevented a stepwise conversion of plasmid pUC18 DNA, and its EC_{50} value was 64.7 μM. Tang and Liu[11] also reported that 3-CGA protects DNA perfectly when the concentration of 3-CGA was as high as 500 μM. These were consistent with our results except in numerical values for 3-CGA in this work. However, these results seem contrary to a report that the oxidative damage of DNA was accelerated by chlorogenic acid in the presence of Cu(II) ions,[27] which might be because chlorogenic acid reduces Cu(II) to form Cu(I), and the Cu(II)/Cu(I) redox cycle leads to the formation of hydroxyl radical, but some researchers have pointed out that different determination methods may lead to controversy results.[28]

DISCUSSION

Polyphenols play multiple biological functions, and many of these functions have been attributed to its antioxidant activity.[29] In the present study, to investigate and compare the profiles of antioxidant activities and DNA damage protective effects of the chlorogenic acid isomers, six assays were performed because the use of any single method for measurement of antioxidant activity can yield rather misleading results.[30,31] From the above results, all of the chlorogenic acid isomers studied exhibited better antioxidant activities resulting from the oxidizable *o*-diphenolic functionality, which can act as a hydrogen atom donor, thereby inhibiting the propagation of radical chain reactions and other biological oxidants, as a transition metal chelator preventing Fenton type processes[32,33] and as an efficient trap for electrophilic nitrosating agents.[34] Their potential as antioxidants is dependent on the number and arrangement of hydroxyl groups, as well as the nature of the substituents in the ring structures. Dicaffeoylquinic acids possessed better antioxidative activities than caffeoylquinic acids, except for the antioxidative activity determined by the β-carotene bleaching assay, which indicated the number of esterification on the quinic acid molecule with caffeic acid had significant influence on the antioxidative activities. Generally, the antioxidant activity was shown to increase with an increase in hydroxyl groups and to enhance the presence of *ortho*-hydroxyl groups attached to the aromatic ring.[35] The dicaffeoylquinic acid has one more hydroxyl group or *ortho*-hydroxyl groups attached to the aromatic ring than the caffeoylquinic acid, which might be chiefly responsible for exhibiting better antioxidant activities. However, surprisingly, the antioxidant activities of dicaffeoylquinic and caffeoylquinic acids on the β-carotene bleaching inhibition were close, indicating that the amount of hydroxyl groups is not the sole factor important for the protection against β-carotene oxidation. The difficulty of predicting the antioxidant effectiveness in β-carotene linoleate emulsion could be due to measurement temperature and their ability to interact with association colloids such as the reverse micelles formed by different solvent systems in this study. This enables us to state again that it clearly does not make sense to just consider the magnitudes of the antioxidant capacities as being values universally applicable for all conditions.

In this study, three caffeoylquinic acid isomers showed quite similar antioxidant activities determined by each assay, which was in agreement with some earlier findings.[15,36] Thus, it seems that the position of esterification on the quinic moiety has no influence on the antioxidant activities of caffeoylquinic acids. Interestingly, when compared with the antioxidant activity of three dicaffeoylquinic acid isomers, some obvious differences among dicaffeoylquinic acid isomers were observed in some antioxidant activities, especially when the concentration of dicaffeoylquinic acids was low. Namely, there was no difference in antioxidant activities between ICAA and ICAB, whereas the antioxidant activities of ICAC were higher than those of ICAA and ICAB in some assays, such as the scavenging activity on DPPH and ABTS cation radicals, FRAP, and chelating ability on ferrous ions. This result implied that the antioxidant activities of dicaffeoylquinic acids were probably influenced by the position of esterification on the quinic moiety, which was just the reverse of what we observed in caffeoylquinic acids. Chemically, ICAA and ICAB are formed by two residues of caffeic binding on the sides of the quinic acid ring, while two residues of caffeic of ICAC locate in one side of the quinic acid ring. Apparently, the structural difference of dicaffeoylquinic acids affected the antioxidant activities. It might be expected that there may exist a steric hindrance effect in the ICAC because of the closer distance between two residues of caffeic locating, which can increase the chemical bonding energy and unstabilize its molecules relative to ICAA and ICAB, as well as enhancing activity. However, this assumption need to be further confirmed.

In conclusion, this work showed that the chlorogenic acid isomers studied exhibited antioxidant activities and DNA damage protective effects to various extents. On the whole, dicaffeoylquinic acids possessed better antioxidative activities, mostly because they have more hydroxyl groups than caffeoylquinic acids. Three caffeoylquinic acid isomers showed quite similar antioxidant activities, which implied that the position of esterification on the quinic moiety of caffeoylquinic acid had no influence on the antioxidant activities. Quite the contrary, some differences among dicaffeoylquinic acid isomers were observed in some antioxidant assays, indicating that their antioxidant activities might be influenced by the position of esterification on the quinic moiety. It might be expected that there may exist steric hindrance effects in the ICAC, which may result in this difference. However, this assumption needs to be further confirmed, as well as the mechanism and extent of antioxidant activity by structurally related dicaffeoylquinic acids.

AUTHOR INFORMATION

Corresponding Author

*Tel: +86-357-2051714. Fax: +86-357-2051000. E-mail: xjg71@163.com.

Funding

This work was financially supported by a project of the Natural Science Foundation of Shanxi Province, China (project no. 2012011031-3).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Chunya Gao and Jingbo Guo for their help and discussions.

ABBREVIATIONS USED

3-CQA, 3-*O*-caffeoylquinic acid; 4-CQA, 4-*O*-caffeoylquinic acid; 5-CQA, 5-*O*-caffeoylquinic acid; ICAA, 3,5-dicaffeoylquinic acid; ICAB, 3,4-dicaffeoylquinic acid; ICAC, 4,5-dicaffeoylquinic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis (3-ethylbenothiazoline-6-sulfonic acid) diammonium salts; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; EDTA, ethylenediamine tetra-acetic acid; BHT, 2,6-di-

tert-butyl-4-hydroxytoluene; TPTZ, 2,4,6-tri-(2-pyridyl)-s-triazine; FRAP, ferric reducing antioxidant power

REFERENCES

(1) Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44−84.

(2) Alexandrova, M. L.; Bochev, P. G. Oxidative stress during the chronic phase after stroke. *Free Radical Biol. Med.* **2005**, *39*, 297−316.

(3) Alamed, J.; Chaiyasit, W.; McClements, D. J.; Decker, E. A. Relationships between free radical scavenging and antioxidant activity in foods. *J. Agric. Food Chem.* **2009**, *57*, 2969−2976.

(4) Clifford, M. N. Chlorogenic acids and other cinnamates—Nature, occurrence, dietary burden, absorption and metabolism. *J. Sci. Food Agric.* **2000**, *80*, 1033−1042.

(5) Clifford, M. N.; Knight, S.; Kuhnert, N. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSn. *J. Agric. Food Chem.* **2005**, *53*, 3821−3832.

(6) Mullen, W.; Nemzer, B.; Ou, B.; Stalmach, A.; Hunter, J.; Clifford, M. N.; Combet, E. The antioxidant and chlorogenic acid profiles of whole coffee fruits are influenced by the extraction procedures. *J. Agric. Food Chem.* **2011**, *59*, 3754−3762.

(7) Sato, Y.; Itagaki, S.; Kurokawa, T.; Ogura, J.; Kobayashi, M.; Hirano, T.; Sugawara, M.; Iseki, K. *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. *Int. J. Pharm.* **2011**, *403*, 136−138.

(8) López-Giraldo, L. J.; Laguerre, M.; Lecomte, J.; Figueroa-Espinoza, M.-C.; Baréa, B.; Weiss, J.; Decker, E. A.; Villeneuve, P. Kinetic and stoichiometry of the reaction of chlorogenic acid and its alkyl esters against the DPPH radical. *J. Agric. Food Chem.* **2009**, *57*, 863−870.

(9) Wang, G.-F.; Shi, L.-P.; Ren, Y.-D.; Liu, Q.-F.; Liu, H.-F.; Zhang, R.-J.; Li, Zh.; Zhu, F.-H.; He, P.-L.; Tang, W.; Tao, P.-Z.; Li, C.; Zhao, W.-M.; Zuo, J.-P. Anti-hepatitis B virus activity of chlorogenic acid, quinic acid and caffeic acid *in vivo* and *in vitro*. *Antiviral Res.* **2009**, *83*, 186−190.

(10) Gordon, M. H.; Wishart, K. Effects of chlorogenic acid and bovine serum albumin on the oxidative stability of low density lipoproteins *in vitro*. *J. Agric. Food Chem.* **2010**, *58*, 5828−5833.

(11) Tang, Y.-Z.; Liu, Z.-Q. Chemical kinetic behavior of chlorogenic acid in protecting erythrocyte and DNA against radical-induced oxidation. *J. Agric. Food Chem.* **2008**, *56*, 11025−11029.

(12) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(13) Xiang, Z.; Ning, Z. Scavenging and antioxidant properties of compound derived from chlorogenic acid in South-China honeysuckle. *LWT—Food Sci. Technol.* **2008**, *41*, 1189−1203.

(14) Shibata, H.; Sakamoto, Y.; Oka, M.; Kono, Y. Natural antioxidant, chlorogenic acid, protects against DNA breakage caused by monochloramine. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 1295−1297.

(15) Nakatani, N.; Kayano, S.; Kikuzaki, H.; Sumino, K.; Katagiri, K.; Mitani, T. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). *J. Agric. Food Chem.* **2000**, *48*, 5512−5516.

(16) Clifford, M. N.; Wu, W.; Kirkpatrick, J.; Kuhnert, N. Profiling the chlorogenic acids and other caffeic acid derivatives of herbal chrysanthemum by LC-MS^n. *J. Agric. Food Chem.* **2007**, *55*, 929−936.

(17) Nakatani, N.; Jitoe, A.; Masuda, T.; Yonemori, S. Constituents of Zingiberaceae. 2. Flavonoid constituents of *Zingiber zerumbet* Smith. *Agric. Biol. Chem.* **1991**, *2*, 455−460.

(18) Hu, Q.-P.; Xu, J.-G. Profiles of carotenoids, anthocyanins, phenolics, and antioxidant activity of selected color waxy corn grains during maturation. *J. Agric. Food Chem.* **2011**, *59*, 2026−2033.

(19) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorisation assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(20) Shyu, Y.-S.; Lin, J.-T.; Chang, Y.-T.; Chiang, C.-J.; Yang, D.-J. Evaluation of antioxidant ability of ethanolic extract from dill (*Anethum graveolens* L.) flower. *Food Chem.* **2009**, *115*, 515−521.

(21) Jeong, J. B.; Park, J. H.; Lee, H. K.; Ju, S. Y.; Hong, S. C.; Lee, J. R.; Chung, G. Y.; Lim, J. H.; Jeong, H. J. Protective effect of the extracts from *Cnidium officinale* against oxidative damage induced by hydrogen peroxide via antioxidant effect. *Food Chem. Toxicol.* **2009**, *47*, 525−529.

(22) Ohnishi, M.; Morishita, H.; Iwahashi, H.; Toda, S.; Shirataki, Y.; Kimura, M.; Kido, R. Inhibitory effects of chlorogenic acids on linoleic acid peroxidation and haemolysis. *Phytochemistry* **1994**, *36*, 579−583.

(23) Morishita, H.; Kido, R. Antioxidant activities of chlorogenic acids. *Assoc. Sci. Int. Cafe, Colloq.* **1995**, 119−124.

(24) Yildirim, A.; Mavi, A.; Kara, A. A. Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *J. Agric. Food Chem.* **2001**, *49*, 4083−4089.

(25) Singh, B. N.; Singh, B. R.; Singh, R. L.; Prakash, D.; Dhakarey, R.; Upadhyay, G.; Singh, H. B. Oxidative DNA damage protective activity, antioxidant and antiquorum sensing potentials of moringa oleifera. *Food Chem. Toxicol.* **2009**, *47*, 1109−1116.

(26) Kasai, H.; Fukada, S.; Yamaizumi, Z.; Sugie, S.; Mori, H. Action of chlorogenic acid in vegetables and fruits as an inhibitor of 8-hydroxydeoxyguanosine formation in vitro and in a rat carcinogenesis model. *Food Chem. Toxicol.* **2000**, *38*, 467−471.

(27) Zheng, L.-F.; Dai, F.; Zhou, B.; Yang, L.; Liu, Z.-L. Prooxidant activity of hydroxycinnamic acids on DNA damage in the presence of Cu (II) ions: mechanism and structure-activity relationship. *Food Chem. Toxicol.* **2008**, *46*, 149−156.

(28) Dizdaroglu, M.; Jaruga, P.; Birincioglu, M.; Rodriguez, H. Free radical-induced damage to DNA: mechanism and measurement. *Free Radical Biol. Med.* **2002**, *32*, 1102−1115.

(29) Hollman, P. C. H. Evidence for health benefits of plant phenols: Local or systemic effects? *J. Sci. Food Agric.* **2001**, *81*, 842−852.

(30) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.

(31) Moon, J.-K.; Takayuki, S. Antioxidant assays for plant and food components. *J. Agric. Food Chem.* **2009**, *57*, 1655−1666.

(32) Jin, U. H.; Lee, J. Y.; Kang, S. K.; Kim, J. K.; Park, W. H.; Kim, J. G.; Moon, S. K.; Kim, C. H. A phenolic compound, 5-caffeoylquinic acid (chlorogenic acid), is a new type and strong matrix metalloproteinase-9 inhibitor: Isolation and identification from methanol extract of *Euonymus alatus*. *Life Sci.* **2005**, *77*, 2760−2769.

(33) Terao, J.; Karasawa, H.; Arai, H.; Nagao, A.; Suzuki, T.; Takama, K. Peroxyl radical scavenging activity of caffeic acid and its related phenolic compounds in solution. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 1204−1205.

(34) Napolitano, A.; d'Ischia, M. New insights into the acidpromoted reaction of caffeic acid and its esters with nitrite: Decarboxylation drives chain nitrosation pathways toward novel oxime derivatives and oxidation/fragmentation products thereof. *J. Org. Chem.* **2002**, *67*, 803−810.

(35) Fagerlund, A.; Sunnerheim, K.; Dimberg, L. H. Radical-scavenging and antioxidant activity of avenanthramides. *Food Chem.* **2009**, *113*, 550−556.

(36) Chun, O. K.; Kim, D.; Moon, H. Y.; Kang, H. G.; Lee, C. Y. Contribution of individual polyphenolics to total antioxidant capacity of plums. *J. Agric. Food Chem.* **2003**, *51*, 7240−7245.