Food Chemistry 112 (2009) 35–41

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Determination of polyphenolic content and antioxidant activity of kudingcha made from *Ilex kudingcha* C.J. Tseng

Lixiang Liu [a], Yi Sun [a,1], Tanguy Laura [a,b], Xingfei Liang [a], Hong Ye [a], Xiaoxiong Zeng [a,*]

[a] College of Food Science and Technology, Nanjing Agricultural University, 1 Weigang, Nanjing 210095, Jiangsu, PR China
[b] Department of Food Engineering, Polytech'Lille, Avenue Paul langevin, 59655 Villenevve d'Asqc, France

ARTICLE INFO

Article history:
Received 23 January 2008
Received in revised form 10 March 2008
Accepted 8 May 2008

Keywords:
Antioxidant activity
DPPH
FRAP
HPLC
Ilex kudingcha C.J. Tseng
Kudingcha
Polyphenol content
TEAC

ABSTRACT

The total polyphenol content and the antioxidant activity of kudingcha made from *Ilex kudingcha* C.J. Tseng were determined by Folin–Ciocalteu reagent, and DPPH, FRAP, and TEAC methods, respectively. The crude extract (CE) of kudingcha and its four fractions of chloroform (CfF), ethyl acetate (EaF), n-butanol (nBF), and water (WtF) were prepared and subjected to antioxidant evaluation and analysis by high performance liquid chromatography. The extracts of kudingcha contained large amounts of caffeoylquinic acid (CQA) derivatives, including 3-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA, and showed potent antioxidant activity. The antioxidant activities of CE and its fractions decreased in the order of EaF > nBF > CE > WtF > CfF, according to the DPPH assay and FRAP assay, which were the same, with the exception of the rank order of CfF and WtF, as the TEAC assay. Furthermore, a satisfactory correlation between total polyphenol content and antioxidant activity was observed.

1. Introduction

Kudingcha is a bitter tea of Chinese origin. The taste of kudingcha is much more bitter than that of tea made from the leaves of *Camellia sinensis*. Kudingcha has been consumed traditionally as a kind of herbal tea in China and Southeastern Asia. It has been reported that triterpenoids from kudingcha have inhibitory effects on acyl-CoA cholesteryl acyl transferase, thus potentially serving as new types of medicines to treat arteriosclerosis and obesity (Nishimura, Fukuda, Miyase, Noguchi, & Chen, 1999; Nishimura, Miyase, & Noguchi, 1999). The original plants for producing kudingcha have about 10 known species: *Ilex kudingcha*, *Ilex latifolia*, *Ilex cornuta*, *Ligustrum pedunclare*, *Ligustrum purpurascens*, *Ligustrum japonicum* var. *pubescens*, *Ligustrum robustrum*, *Cratoxylum prunifolium*, *Ehretia thyrsiflora*, and *Photinia serruiata* (He et al., 1994; Lau, He, Dong, Fung, & But, 2002). The main species are *I. kudingcha*, *I. latifolia*, and *I. cornuta*, which belong to the same genus as mate (*Ilex paraguariensis*) (Filip, Lotito, Ferraro, & Fraga, 2000). *Ilex kudingcha* is one of the main plants for kudingcha production in China, and there are some reports about its chemical composition and pharmaceutical functions (Chen, Li, & Xie, 1995; Nishimura et al., 1999; Nishimura et al., 1999). However, little information about the content of phenolic compounds and antioxidant activities of kudingcha made from *I. kudingcha* is available, compared with that of mate (*I. paraguariensis*, St. Hil) (Bravo, Goya, & Lecumberri, 2007; Filip, Lopez, Giberti, Coussio, & Ferraro, 2001; Filip et al., 2000; Turkmen, Sari, & Velioglu, 2006).

Phenolic compounds are ubiquitous bioactive compounds and a diverse group of secondary metabolites universally present in higher plants. Accordingly, bioactive polyphenols have attracted special attention because they can protect the human body from the oxidative stress which may cause many diseases, including cancer, cardiovascular problems, and aging (Robards, Prernzler, Tucker, Swatsitang, & Glover, 1999). Therefore, the main purpose of the present study was to determine the total polyphenol content (TPC) by Folin–Ciocalteu method and to determine the antioxidant activity of kudingcha made from *I. kudingcha* C.J. Tseng, by use of the three commonly used spectrophotometric methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging assay, TEAC (Trolox equivalent antioxidant capacity) assay, and FRAP (ferric ion reducing antioxidant power) assay. Furthermore, the main phenolics in the extract of kudingcha were analysed by high performance liquid chromatography (HPLC) and HPLC-mass spectroscopy with electrospray ionisation source.

* Corresponding author. Fax: +86 25 84396791.
E-mail address: zengxx@njau.edu.cn (X. Zeng).
[1] Co-first author.

0308-8146/$ - see front matter Crown Copyright © 2008 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.05.038

2. Materials and methods

2.1. Materials

Kudingcha made from *I. kudingcha* C.J. Tseng was obtained from Hainan Yexian Bio-Science Technology Co., Ltd. (Hainan, China). The samples were ground using a domestic blender, stored in sealed polyethylene bags and kept in a refrigerator at −20 °C until use. (−)-Epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Caffeine, chlorogenic acid, DPPH, 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonate) diammonium salt (ABTS), theobromine, and theophylline were obtained from Sigma Chemical Co. (St. Louis, MO). Folin–Ciocalteu reagent, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were obtained from Fluka (Buchs, Swiltzerland). All other chemicals were of analytical grade.

2.2. Preparation of extracts of kudingcha

The kudingcha was extracted with 10 times its volume of boiling water for 30 min at 95 °C. After the solution was centrifuged at 5000*g* for 10 min; the residue was re-extracted twice with boiling water, as described above. The combined supernatants were concentrated in a rotary evaporator and lyophilised to afford the crude extract (CE). The CE was further fractionated through solvent-solvent partitioning to afford different fractions. The four solvents used to achieve high to low polarity for solvent-solvent partitioning were water, *n*-butanol, ethyl acetate, and chloroform. The CE and its four fractions of chloroform (CfF), ethyl acetate (EaF), *n*-butanol (nBF), and water (WtF) were stored in dark bottles at 4 °C after solvent evaporation and freeze-drying. The CE and its four fractions were then subjected to the measurement of TPC and antioxidant activity, and HPLC analysis.

2.3. Determination of total polyphenol content

TPC was measured by the Folin–Ciocalteu method described by Nurmi with slight modification (Nurmi, Ossipov, Haukioja, & Pihlaja, 1996). Briefly, an aliquot of 0.5 ml of sample solution (with appropriate dilution to obtain absorbance in the range of the prepared calibration curve) was mixed with 1.0 ml of Folin–Ciocalteu reagent (10 times dilution) and allowed to react at 30 °C for 5 min in the dark. Then 2.0 ml of saturated Na_2CO_3 solution was added and the mixture was allowed to stand for 1 h before the absorbance of the reaction mixture was read at 747 nm. A calibration curve of chlorogenic acid (ranging from 0.02 to 0.10 mg/ml) was prepared, and TPC was standardised against chlorogenic acid and expressed as mg chlorogenic acid equivalent per gram of sample on a dry weight basis (DW).

2.4. Antioxidant activity determination

Three methods, DPPH, TEAC, and FRAP, based on reaction with electron-donating or hydrogen radicals (H) producing compounds/antioxidants according to the reaction R+Aox-H→RH+Aox, were used.

2.4.1. DPPH assay

The DPPH free radical-scavenging activity of each sample was determined (Hu, Lu, Huang, & Ming, 2004; Leong & Shui, 2002). Briefly, a 0.1 mM solution of ethanolic DPPH solution was prepared. The initial absorbance of the DPPH in ethanol was measured at 517 nm and did not change throughout the period of assay. An aliquot (0.1 ml) of each sample (with appropriate dilution if necessary) was added to 3.0 ml of ethanolic DPPH solution. Discolorations were measured at 517 nm after incubation for 30 min at 30 °C in the dark. Measurements were performed at least in triplicate. The percentage of DPPH· which was scavenged (%DPPH·$_{sc}$) was calculated using:

$$\%\mathrm{DPPH}^{\cdot}_{sc} = (A_{cont} - A_{samp}) \times 100/A_{cont},$$

where A_{cont} is the absorbance of the control, and A_{samp} is the absorbance of the sample. IC_{50} values calculated denote the concentration of a sample required to decrease the absorbance at 517 nm by 50%.

2.4.2. TEAC assay

The ABTS free radical-scavenging activity of each sample was determined according to the method described by Pavel and Vlastimil (2006). The radical cation ABTS·$^+$ was generated by persulfate oxidation of ABTS. A mixture (1:1, v/v) of ABTS (7.0 mM) and potassium persulfate (4.95 mM) was allowed to stand overnight at room temperature in the dark to form radical cation ABTS·$^+$. A working solution was diluted with phosphate buffer solution to absorbance values between l.0 and 1.5 at 734 nm (constant initial absorbance values must be used for standard and samples). An aliquot (0.1 ml) of each sample (with appropriate dilution if necessary) was mixed with the working solution (3.9 ml), and the decrease of absorbance was measured at 734 nm after 10 min at 37 °C in the dark. Aqueous phosphate buffer solution (3.9 ml, without ABTS·$^+$ solution) was used as a control. The ABTS·$^+$ scavenging rate was calculated, to express the antioxidant ability of the sample. IC_{50} values calculated denote the concentration of sample required to decrease the absorbance at 734 nm by 50%.

2.4.3. FRAP assay

The ability to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain (1996). An aliquot (0.2 ml) of each sample (with appropriate dilution if necessary) was added to 3.8 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution, and 1 part of 20.0 mM $FeCl_3{\cdot}6H_2O$ solution), and the reaction mixture was incubated at 37 °C for 30 min. The increase in absorbance at 593 nm was measured. Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of sample (DW).

2.5. Analysis of the main phenolic constituents of kudingcha extracts

2.5.1. HPLC analysis

The kudingcha extracts were analysed using an Agilent HPLC series 1100 equipped with Chemstation software, a model G1379A degasser, a model G1311A quatpump, a model G1316A column oven, and a model G1315B diode-array detector (Agilent, Santa Clara, CA). The separation was completed on a TSK gel ODS-80TsQA column (2.0 × 250 mm, 5 μm, Tosoh Corp., Tokyo, Japan). The column oven temperature was set at 40 °C. The mobile phase consisted of ultra-pure water (A), methanol (B), and 1.0% (v/v) formic acid (C). Elution was performed with the linear gradient as follows: 0–60 min, A from 85 to 65%, B from 5 to 25%, C 10%; 60–100 min, A from 65 to 45%, B from 25 to 45%, C 10%; 100–110 min, A 45%, B 45%, and C 10%. The flow rate was 0.2 ml/min.

2.5.2. LC-MS analysis

HPLC-MS analysis was performed with a Shimadzu LC-MS 2010A system (Shimadzu Co., Ltd., Kyoto, Japan) consisting of a LC-10ADvp solvent delivery pump, SIL-10Advp autosampler,

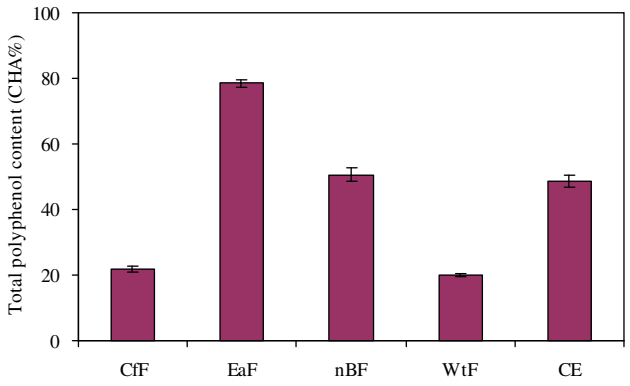

Fig. 1. The total polyphenol contents of CE and its four fractions of kudingcha made from *Ilex kudingcha* C.J. Tseng.

CTO-10Avp Oven, SPD-10AV detector, SCL-10Avp controller, LC-MS 2010A single quadrupole mass spectrometer equipped with an ESI interface and a Q-array-Octapole-Quadrupole mass analyzer. The HPLC conditions were the same as for the HPLC analysis described above. The curve dissolution line (CDL) temperature and the block temperature were maintained at 250 °C and 200 °C, respectively. The probe voltage, CDL voltage and detector voltage were fixed at 4.5 kV, −25 V, and 1.2 kV, respectively. Nitrogen was used as the source of nebuliser gas at a flow rate of 2.0 ml/min. Mass spectra were recorded in the range of *m/z* 0–1000.

2.5.3. Isolation of 4,5-dicaffeoylquinic acid (4,5-diCQA)

4,5-DiCQA was isolated from kudingcha extract by HPLC with a semi-preparative HPLC column of Zorbax SB-C18 (9.4 × 250 mm, 5 μm; Agilent). The fraction containing 4,5-diCQA was combined, concentrated, and dried. The structure was confirmed by NMR, MS, and HPLC. The data were identical to those of 4,5-diCQA reported (Islam et al., 2002). ^{1}H NMR (400 MHz, CD_3OD): δ 2.09 (2H, m) and 2.20 (2H, m), 4.35 (1H, br s), 5.10 (1H, dd, *J* 2.8 and 8.8 Hz), 5.59 (1H, m), 6.16 and 6.26 (each 1H, d, *J* 16 Hz), 6.73 and 6.75 (each 1H, d, *J* 8 Hz), 6.88 and 6.91 (each 1H, dd, *J* 1.6 and 6.8 Hz), 6.99 and 7.01 (each 1H, d, *J* 2.0 Hz), 7.49 and 7.57 (each 1H, d, *J* 16.0 Hz); LC-MS: *m/z* 515 for $[M-H]^-$.

2.6. Statistical analysis

Values shown in tables and graphs were means ± standard deviations of three parallel measurements. The IC_{50} values were calculated from linear regression analysis. The correlation coefficients between total phenolics and the three methods of radical-scavenging activity were demonstrated by employing SAS (Version 8.02; SAS).

3. Results and discussion

3.1. Preparation of crude extract and its fractions of kudingcha made from I. kudingcha C.J. Tseng and determination of the total polyphenol content

Due to the complicated constituents and pharmacological diversities of plant materials, *in vitro* bioassay-guided fractionation has been effectively applied to screen the biological activities that provide important indications for investigating the characteristics of active components (Yesilada, Tsuchiya, Takaishi, & Kawazoe, 2000). The CE of kudingcha made from *I. kudingcha* C.J. Tseng with hot water was fractionated through solvent-solvent partitioning to obtain four fractions of WtF, nBF, EaF, and CfF. The total recovery of

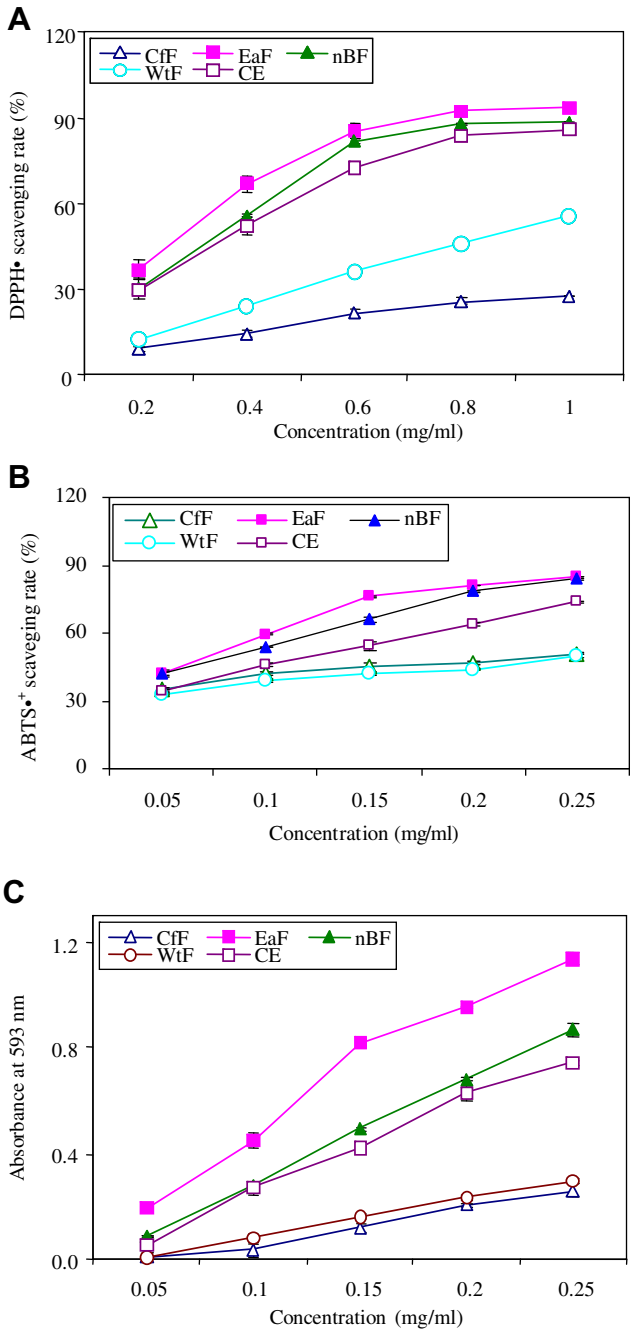

Fig. 2. Antioxidant activities of CE and its four fractions of kudingcha made from *Ilex kudingcha* C.J. Tseng determined by DPPH free radical-scavenging assay (A), TEAC assay (B), and FRAP assay (C).

Table 1
Antioxidant activities of CE and its four fractions of kudingcha made from *Ilex kudingcha* C.J. Tseng using the DPPH assay, TEAC assay, and FRAP assay

Samples	$IC_{50/DPPH}$ (μg/ml)[a]	$IC_{50/TEAC}$ (μg/ml)[b]	FRAP value (mmol $FeSO_4$/g DW)
CfF	not found	245 ± 1.4	1.4 ± 0.0
EaF	265 ± 3.7	67.0 ± 1.1	6.7 ± 0.0
nBF	3156 ± 1.9	74.5 ± 0.7	4.7 ± 0.0
WtF	892 ± 2.7	299 ± 1.5	1.5 ± 0.1
CE	359 ± 2.2	108 ± 0.9	4.2 ± 0.2

Data are presented as the mean ± standard deviation ($n \geqslant 3$).
[a] The antioxidant activity was evaluated as the concentration of the test sample required to decrease the absorbance at 517 nm by 50%.
[b] The antioxidant activity was evaluated as the concentration of the test sample needed to decrease the absorbance at 734 nm by 50%.

the four fractions was 98.01%, and the residual 1.99% was lost during the solvent-solvent partition, concentration, and lyophilisation. The recoveries of WtF, nBF, EaF, and CfF were 23.03%, 66.90%, 4.56%, and 3.52%, respectively.

The Folin–Ciocalteu reagent is used to obtain a crude estimate of the amount of phenolic compounds present in an extract. In the present study, TPC of CE and its four fractions were determined using the Folin–Ciocalteu method. As results, TPC of the extract and fractions decreased in the following order, as shown in Fig. 1: EaF > nBF > CE > CfF > WtF. The TPC of EaF was much higher than those of CE and the other fractions. Ethyl acetate seems to be the best solvent to concentrate phenolic substances of intermediate polarity. This is in accordance with the findings of other authors (Chung et al., 1999; Parejo et al., 2002). Lower polyphenol values were found in CfF and WtF, which showed similar total polyphenol contents.

3.2. Determination of antioxidant activity of extracts

3.2.1. Determination by DPPH assay

DPPH free radical-scavenging activities of CE and its four fractions are shown in Fig. 2. For each sample, five concentrations (mg/ml) were tested. EaF and nBF exhibited considerably higher DPPH radical-scavenging activities than other fractions, and this trend was similar to that observed for the total polyphenol content. Lowest concentration-dependent manner of DPPH-scavenging rate was found in CfF. By comparing CE and its fractions, the free radical-scavenging activities decreased in the order of EaF > nBF > CE > WtF > CfF. The free radical-scavenging activity of CE was less than those of EaF and nBF, which may result from the active components through condensation effects during the solvent-solvent partitioning processes. In order to quantify the antioxidant activity further, the IC_{50} was calculated and shown as in Table 1. The lower the IC_{50} value is, the greater the free radical-scavenging activity is. IC_{50} values of the DPPH radical-scavenging activity ranged from 265 to 892 μg/ml for CE and its fractions. IC_{50} value of CfF was not found for the DPPH-scavenging rate.

3.2.2. Determination by TEAC assay

The $ABTS^{\cdot+}$ radical formed from the reaction ABTS-e^{-}→$ABTS^{\cdot+}$ reacts quickly with electron/hydrogen donors to form colourless ABTS. The reaction is pH-independent. A decrease of the $ABTS^{\cdot+}$ concentration is linearly dependent on the antioxidant concentration, including Trolox as a calibrating standard (van den Berg, Haenen, van den Berg, van der Vijgh, & Bast, 2000). The results correlate very well with biological redox properties of phenolic substrates. The antioxidant abilities of the crude extract and its fractions of kudingcha determined by TEAC method were shown in Fig. 2. For each sample, five concentrations (mg/ml) were tested. The highest $ABTS^{\cdot+}$-scavenging rate was found for EaF while, the lowest was found for WtF. The antioxidant activity values decreased in the order of EaF > nBF > CE > CfF > WtF. The rank order of CfF and WtF was different compared with that of DPPH assay. The IC_{50} was further calculated and the values ranged from 67.0 to 299 μg/ml for CE and its four fractions (Table 1).

3.2.3. Determination by FRAP assay

FRAP assay is based on the ability of antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content (Benzie & Strain, 1996). In the present study, the trend for ferric ion reducing activities of CE and different fractions of kudingcha is shown in Fig. 2. For EaF, nBF, and CE, the absorbance clearly increased, due to the formation of the Fe^{2+}-TPTZ complex with increasing concentration.

Being expressed in $FeSO_4$ equivalents, the FRAP value was applied, to determine the antioxidant ability of kudingcha. The highest reducing activity was for EaF, compared to those of the other fractions (Table 1). Similar to the results obtained from the DPPH assay and TEAC assay, EaF and nBF showed relatively strong ferric ion-reducing activities. CfF and WtF showed lower ferric ion-reducing activities.

3.3. Phenolic constituents of kudingcha extracts

The kudingcha extracts showed strong antioxidant activities as mentioned above. Therefore, the extracts were analysed by HPLC and HPLC-MS with a TSK gel ODS-80TsQA column, as shown in Fig. 3. We did not find caffeine and catechins (EGCG, EGC, ECG, and EC) that are characteristic for tea made from the leaves of the *C. sinensis* plant in kudingcha extracts. In contrast,

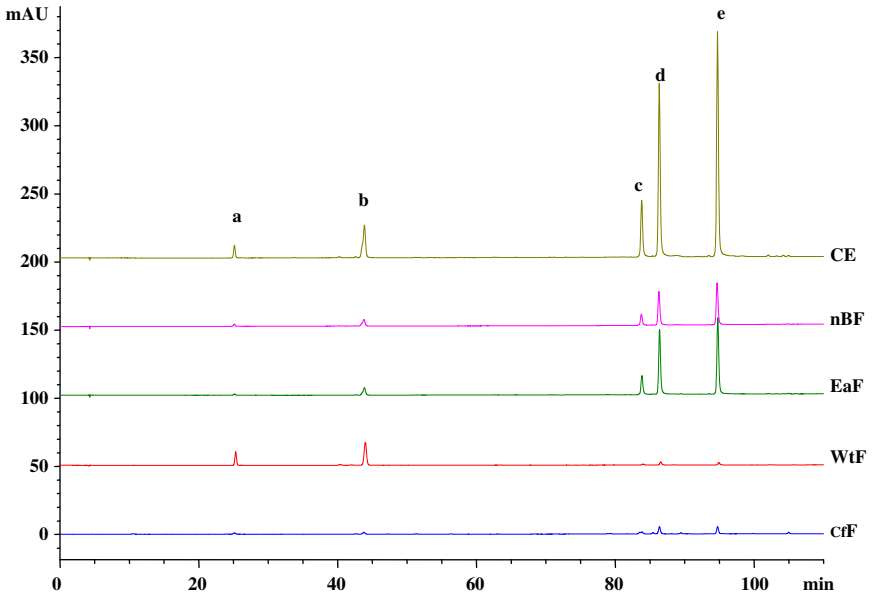

Fig. 3. HPLC chromatograms of CE, CfF, EaF, nBF, and WtF of kudingcha made from *Ilex kudingcha* C.J. Tseng detected at 326 nm.

we found a lot of caffeoylquinic acid (CQA) derivatives. Peak **a** and peak **b** showed similar UV spectra (Fig. 4A), with a maximum near 326 nm and a shoulder at 296 nm, characteristic of the caffeic acid moiety. Also, the mass spectra of the two peaks were similar, with an $[M–H]^-$ ion at *m/z* 353 in the ESI–MS spectrum obtained in negative ion mode (Fig. 4B). The HPLC retention time, UV and MS spectra of peak **b** were identical to those of standard chlorogenic acid (5-CQA). Peak **a** would correspond to the isomer of 5-CQA. Therefore, they were identified as 3-CQA and 5-CQA, respectively, considering the elution profile of CQA

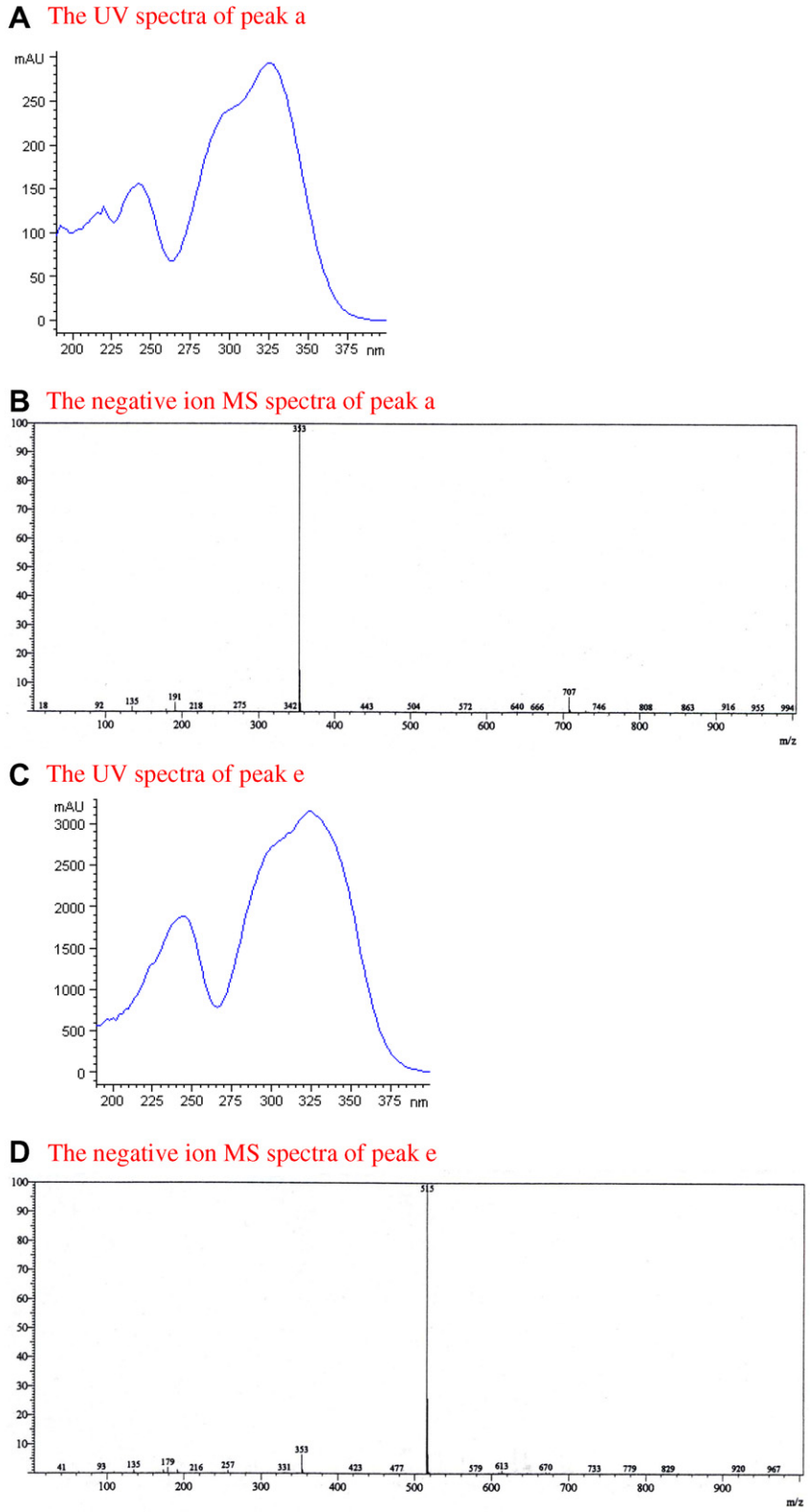

Fig. 4. The UV spectra (A,C) and negative ion MS spectra (B,D) for 3-O-caffeoylquinic acid (peak **a**) and 4,5-di-O-dicaffeoylquinic acid (peak **e**) respectively.

isomers reported in the literature on C_{18} HPLC columns (Negishi, Negishi, Yamaguchi, & Sugahara, 2004; Wang & Clifford, 2008). All data for CQA derivatives presented in this article use the recommended IUPAC numbering system (IUPAC, 1976). Peaks **c**, **d**, and **e** all had the same UV spectra (Fig. 4C), similar to that of chlorogenic acid (5-CQA). They also showed an $[M–H]^-$ ion at *m/z* 515 in the ESI–MS spectrum (Fig. 4D). The molecular ion at *m/z* 515 is indicative of diCQA isomers, which have been reported as the major constituents of mate and leaves from *I. latifolia* and *I. cornuta* (Negishi et al., 2004). Furthermore, the isolated and characterised 4,5-diCQA from kudingcha extract in the present study had the same HPLC pattern, UV, and MS spectra as peak **e**. Thus, peaks **c**, **d**, and **e**, were identified as 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA, respectively (Clifford, Knight, & Kuhnert, 2005; Negishi et al., 2004).

As shown in Fig. 3, most of the CQA derivatives were found in EaF and nBF, while little was found in CfF and WtF. The results are similar to those of the Folin–Ciocalteu method. Furthermore, the contents of chlorogenic acid and 4,5-diCQA in extracts were quantified by HPLC. 56.76% and 36.86% of chlorogenic acid in CE were found in EaF and nBF, and 89.45% and 5.77% of 4, 5-diCQA in CE were in EaF and nBF, respectively. The results indicate that more than 90% of the CQA derivatives in CE of kudingcha has gone to Eaf and nBF through solvent-solvent partitioning. May be it is the reason why EaF and nBF have higher antioxidant activity, when compared with the other two fractions.

3.4. Correlations of DPPH, TEAC, and FRAP with the total polyphenol content

Polyphenols have been reported to be responsible for the antioxidant activities of botanical extracts. The DPPH assay, TEAC assay, and FRAP assay have been used to measure antioxidant activity and the results of methods should correlate with those of the total phenol contents. A direct correlation between radical-scavenging activity and phenolic content of the extracts was demonstrated by linear regression analysis. With reference to Table 2, the correlations of the total polyphenol content against the antioxidant activity based on the DPPH assay, TEAC assay, and FRAP assay were satisfactory ($r > 0.812$). The results indicate that polyphenols in kudingcha extracts are largely responsible for the antioxidant activities. A strong correlation between the mean values of the total polyphenol content and FRAP deserves detailed attention, as it implies that polyphenols in kudingcha are capable of reducing ferric ions. Some authors have reported similar correlations between polyphenols and antioxidant activity measured by various methods (Awika, Rooney, Wu, Prior, & Cisneros-Zevallos, 2003; Zheng & Wang, 2001). The Folin–Ciocalteu method for the determination of phenolic compounds is, such as the methods of antioxidant activity determination, based on redox properties of the compounds. Thus, the values could partially express the antioxidant activity. This confirms a highly significant correlation between the values of Folin–Ciocalteu method and the values of individual methods for antioxidant activity. The results prove that the content of phenolic compounds and antioxidant activity correlate very well for kudingcha extracts.

Table 2
Correlation coefficients, *r*, for relationships between assays

	$IC_{50/DPPH}$	$IC_{50/TEAC}$	FRAP value
$IC_{50/TEAC}$	0.996		
FRAP value	0.857	0.872	
Total polyphenol content	0.829	0.812	0.984

3.5. Correlations of DPPH, TEAC, and FRAP

In a comparison of methods used in this study, all of the methods showed the capability to determine the antioxidant activity of kudingcha. The TEAC assay and FRAP assay were found to be more sensitive because the concentration measured was one order of magnitude less than the DPPH free radical-scavenging assay, which is in agreement with results reported by Parejo, Codina, Petrakis, & Kefalas (2000)). The lower sensitivity of the DPPH assay may be due to the relatively higher stability of DPPH·. Nevertheless, the three methods are capable of prescreening antioxidant activities.

As shown in Table 2, a direct correlation between the three methods was demonstrated by linear regression analysis. The strong correlation ($r > 0.857$) between the mean values of two kinds of IC_{50} and FRAP values deserves detailed attention. This could be explained from the basic concept that antioxidants are reducing agents. Antioxidants are compounds capable of donating a single electron or hydrogen atom for reduction. The compounds present in different fractions of kudingcha capable of scavenging DPPH· radicals and $ABTS^{\cdot+}$ radicals are also able to reduce ferric ions. Arnous, Makris, and Kefalas (2002) have reported a strong correlation between DPPH free radical-scavenging ability and ferric ion-reducing ability in wines. The antioxidant activities of polyphenols determined using different free radical methods showed similar results to those obtained using the FRAP assay. The results suggest that the reducing ability of polyphenols seems to be an important factor dictating free radical-scavenging capacity of these compounds.

4. Conclusions

In conclusion, the total polyphenol contents of CE and its four fractions of kudingcha made from *I. kudingcha* C.J. Tseng were determined by Folin–Ciocalteu method, and their antioxidant activities were determined by the DPPH, TEAC, and FRAP methods. Here, we demonstrate that kudingcha extract has potent antioxidant activity by the DPPH assay, TEAC assay, and FRAP assay. From the data obtained in these three methods, the antioxidant activities decreased in the order of EaF > nBF > CE > WtF > CfF, according to the DPPH assay and FRAP assay, which were the same with the exception of the rank order of WtF and CfF obtained from the TEAC assay. The antioxidant activity of kudingcha may be related to its phenolic substrates, especially CQA derivatives. The extract of kudingcha contained a large amounts of CQA derivatives, including 3-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA. The results provide useful information on pharmacological activities associated with free radicals of this traditional herbal tea. From a health point of view, kudingcha made from *I. kudingcha* C.J. Tseng is a meaningful component of herbal drinks, due to its antioxidant activity.

Acknowledgements

This work was supported by a grant-in-aid from 863 Program, Ministry of Science and Technology, P.R. China (2007AA10Z351 and 2007AA100403) and a grantin-aid from Nanjing Agricultural University for the Introduction of Outstanding Scholars (804066).

References

Arnous, A., Makris, D. P., & Kefalas, P. (2002). Correlation of pigment and flavanol content with antioxidant properties in selected aged regional wines from Greece. *Journal of Food Composition and Analysis, 15*, 655–665.

Awika, J. M., Rooney, L. W., Wu, X., Prior, R. L., & Cisneros-Zevallos, L. (2003). Screening methods to measure antioxidant activity of Sorghum (Sorghum bicolor) and Sorghum products. *Journal of Agricultural and Food Chemistry, 51*(23), 6657–6662.

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Journal of Analytical Biochemistry, 239*(1), 70–76.

Bravo, L., Goya, L., & Lecumberri, E. (2007). LC/MS characterization of phenolic constituents of mate (*Ilex paraguariensis*, St. Hil) and its antioxidant activity compared to commonly consumed beverages. *Food Research International, 40*, 393–405.

Chen, Y., Li, K. S., & Xie, T. G. (1995). Hypotensive action of the extract of kudingchadongqingye (*Ilex kudingcha*). *Chinese Traditional Herb Drugs, 26*, 250–252.

Chung, H. S., Chang, L. C., Lee, S. K., Shamon, L. A., van Breemen, R. B., Mehta, R. G., et al. (1999). Flavonoid constituents of *chorizante diffusa* with potential cancer chemopreventive activity. *Journal of Agricultural and Food Chemistry, 47*(1), 36–41.

Clifford, M. N., Knight, S., & Kuhnert, N. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. *Journal of Agricultural and Food Chemistry, 53*(10), 3821–3832.

Filip, R., Lopez, P., Giberti, G., Coussio, J., & Ferraro, G. (2001). Phenolic compounds in seven South American *Ilex* species. *Fitoterapia, 72*, 774–778.

Filip, R., Lotito, S. B., Ferraro, G., & Fraga, C. G. (2000). Antioxidant activity of *Ilex paraguariensis* and related species. *Nutrition Research, 20*(10), 1437–1446.

He, Z. D., Ueda, S., Akaji, M., Fujita, T., Inoue, K., & Yang, C. R. (1994). Monoterpenoid and phenylethanoid glycosides from *Ligustrum pedunculare*. *Phytochemistry, 36*, 709–716.

Hu, F. L., Lu, R. L., Huang, B., & Ming, L. (2004). Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medicinal plants. *Fitoterapia, 75*, 14–23.

Islam, M. S., Yoshimoto, M., Yahara, S., Okuno, S., Ishiguro, K., & Yamakawa, O. (2002). Identification and characterization of foliar polyphenolic composition in sweet potato (*Ipomoea batatas* L.) genotypes. *Journal of Agricultural and Food Chemistry, 50*(13), 3718–3722.

IUPAC. (1976). IUPAC Commission on the Nomenclature of Organic Chemistry (CNOC) and IUPAC-IUB Commission on Biochemical Nomenclature (CBN). Nomenclature of cyclitols. Recommendations, 1973. Biochemical Journal, 153(1), 23–31.

Lau, K. M., He, Z. D., Dong, D., Fung, K. P., & But, P. P. H. (2002). Anti-oxidative, anti-inflammatory and hepato-protective effects of *Ligustrum robustum*. *Journal of Ethnopharmacology, 83*, 63–71.

Leong, L. P., & Shui, G. (2002). An investigation of antioxidant capacity of fruits in Singapore markets. *Food Chemistry, 76*, 69–75.

Negishi, O., Negishi, Y., Yamaguchi, F., & Sugahara, T. (2004). Deodorization with Kuding-cha containing a large amount of caffeoyl quinic acid derivatives. *Journal of Agricultural and Food Chemistry, 52*(17), 5513–5518.

Nishimura, K., Fukuda, T., Miyase, T., Noguchi, H., & Chen, X. M. (1999). Activity-guided isolation of triterpenoid acyl CoA cholesteryl acyl transferase (ACAT) inhibitors from *Ilex Kudingcha*. *Journal of Natural Products, 62*(7), 1061–1064.

Nishimura, K., Miyase, T., & Noguchi, H. (1999). Triterpenoid saponins from *Ilex Kudincha*. *Journal of Natural Products, 62*(8), 1128–1133.

Nurmi, K., Ossipov, V., Haukioja, E., & Pihlaja, K. (1996). Variation of total phenolic content and individual low-molecular-weight phenolics in foliage of mountain birch trees (*Betula pubescens* ssp. tortuosa). *Journal of Chemistry and Ecology, 22*, 2023–2040.

Parejo, I., Codina, C., Petrakis, C., & Kefalas, P. (2000). Evaluation of scavenging activity assessed by Co (II)/EDTA-induced luminol chemiluminescence and DPPH free radical assay. *Journal of Pharmacological and Toxicological Methods, 44*, 507–512.

Parejo, I., Viladomat, F., Bastida, J., Rosas-Romero, A., Flerlage, N., & Burillo, J. (2002). Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled mediterranean herbs and aromatic plants. *Journal of Agricultural and Food Chemistry, 50*(23), 6882–6890.

Pavel, S., & Vlastimil, K. (2006). Determination of total content of phenolic compounds and their antioxidant activity in vegetables — Evaluation of spectrophometic methods. *Journal of Agricultural and Food Chemistry, 54*, 607–616.

Robards, K., Prernzler, P. D., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry, 66*, 401–436.

Turkmen, N., Sari, F., & Velioglu, S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food Chemistry, 99*, 835–847.

van den Berg, R., Haenen, G. R. M. M., van den Berg, H., van der Vijgh, W., & Bast, A. (2000). The predictive value of the antioxidant capacity of structurally related flavonoids using the Trolox equivalent antioxidant capacity (TEAC) assay. *Food Chemistry, 70*, 391–395.

Wang, Z., & Clifford, M. N. (2008). Profiling the chlorogenic acids of sweet potato (*Ipomoea batatas*) from China. *Food Chemistry, 106*, 147–152.

Yesilada, E., Tsuchiya, K., Takaishi, Y., & Kawazoe, K. (2000). Isolation and characterization of free radical scavenging flavonoid glycosides from the flowers of *Spartium junceum* by activity-guided fractionation. *Journal of Ethnopharmacology, 73*, 471–478.

Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry, 49*(11), 5165–5170.