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Characterization of the constituents and antioxidative activity of cocoa tea (*Camellia ptilophylla*)

Li Peng, Xiujuan Wang, Xianggang Shi, Chengren Li, Chuangxing Ye, Xiaohong Song *

School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, PR China

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ABSTRACT

Three main types of cocoa tea derived from *Camellia ptilophylla*, white cocoa tea (WCT), green cocoa tea (GCT) and black cocoa tea (BCT) were prepared. A comparison between cocoa tea and commonly consumed tea Biluochun cha (BLC), Longjing cha (Lj), Qimenhong cha (Qmh) and Yinghong 9# (Yh9) was studied. Levels of catechins and purine alkaloids were determined by HPLC. Total polyphenol contents were determined by ferrous tartrate assay. Antioxidative activity was evaluated using ABTS decolorization assay, DPPH radical assay and ferric thiocyanate (FTC) method. The results showed that WCT contained the highest polyphenol content and showed the highest antioxidative activity. The radical scavenging ability of the tea extracts followed this order: WCT > GCT > Blc > Lj > BCT > Yh9 > Qmh. Tea extracts measured by the linoleic acid system also showed the same patterns of activity as the ABTS and DPPH method. A high correlation between polyphenol content and antioxidative activity of tea extracts was observed.

1. Introduction

Cocoa tea (*Camellia ptilophylla*) is a natural decaffeinated tea plant which grows in southern China. The plant was discovered by Professor Chang Hung-ta in 1981 (Chang, Ye, & Zhang, 1988). The local people have habitually drunk cocoa tea for a long time, not only as a healthy beverage but also as a traditional remedy for ailments. Earlier study showed that cocoa tea had significant cytotoxic effect on Hela, CNE2 and MGC-803 cell lines and had inhibitory effect against Ehrlich solid carcinoma in mice (Xie, Liu, & Pan, 1992). It also had inhibitory effect on the DNA polymerase of Ehrlich ascite tumor cells (Xian, Liu, Pan, & Li, 1997). A recent study showed cocoa tea had hypolipemic activity and changed the ambulatory behaviors combined administration with green tea (He, Xie, Yao, & Kurihara, 2009; Kurihara et al., 2006). Our previous study showed that cocoa tea had an inhibitory ability against prostate cancer both *in vitro* and *in vivo* (Peng, Khan, Afaq, Ye, & Mukhtar, 2010).

Cocoa tea belongs to *Camellia* Sect. *Thea* (L.) Dyer, and has a similar chemical profile as tea (*Camellia sinensis*). However, cocoa tea contains theobromine instead of caffeine, and the major catechin is (−)-gallocatechin gallate (GCG) instead of (−)-epigallocatechin gallate (EGCG) (Peng, Song, Shi, Li, & Ye, 2008; Yang, Ye, Xu, & Jiang, 2007). Caffeine is the main purine alkaloid in traditional teas. It can induce excitation in the central nervous system, and it also causes irritation of the gastrointestinal tract and sleeplessness for certain people (Nehlig, Daval, & Debry, 1992). It was shown that excessive caffeine ingestion from tea increased the seizure frequency of heart disease, and the avoidance of excessive caffeine in patients with epilepsy was recommended (Kaufman & Sachdeo, 2003). A daily caffeine intake of 4 mg kg^{-1} body weight for adult and 1 mg kg^{-1} body weight for children was suggested (Baronea & Roberts, 1996). So, cocoa tea as a natural decaffeinated tea may become a choice for many tea lovers.

Many studies focused on green tea polyphenols and its antioxidative activity, and among the polyphenols, EGCG was the most abundant catechin and was reported to possess the most important action (Cai et al., 2002; Chen & Chan, 1996; Zhang, Chan, Luk, Ho, & Chen, 1997). GCG, the epimer of EGCG, plays less important roles in traditional teas because of its little amount. However, it was shown that GCG had a higher antioxidative activity than EGCG, based on their chemical structure (Guo, Zhao, & Hou, 1999; Zhao, Guo, & Xin, 2001). Since the major catechin in cocoa tea is GCG, and there is no report on antioxidative activity of cocoa tea, it is of great interest to find out the antioxidative activity of cocoa tea as well as the comparison between cocoa tea and traditional teas.

In the present study, we prepared three main type of cocoa tea, white cocoa tea (WCT), green cocoa tea (GCT) and black cocoa tea (BCT). The constituents of fermented and unfermented cocoa teas were determined by HPLC. In addition, the antioxidative activity and the total polyphenols content were determined and compared with the values of green tea and black tea (*Camellia sinensis*).

* Corresponding author. Tel.: +86 20 84112874; fax: +86 20 84036215.
E-mail address: songxh@mail.sysu.edu.cn (X. Song).

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2. Materials and methods

2.1. Chemicals and reagents

Pure standards of (+)-Catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-catechin gallate (CG), (−)-epicatechin gallate (ECG), (−)-gallocatechin gallate (GCG), (−)-epigallocatechin gallate (EGCG), Theophylline (Tp), theobromine (Tb), theanine (Ta), gallic acid (GA), ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased from Sigma (St. Louis, MO, USA). Caffeine (Caf) and Linoleic acid (99%) were purchased from Wako Pure Chemical Industries Ltd. Company (Osaka, Japan). HPLC-grade acetonitrile (ACN) was purchased from Merck Company (Darmstadt, Germany). HPLC-grade water (18 mΩ) prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA, USA) was used for the mobile phases and to prepare all solutions. Ammonium thiocyanate and other reagents were of analytic reagent grade.

2.2. Preparation of tea water extracts

WCT, GCT and BCT were obtained from Tea Institute of Guangdong Province, Yingde, China. Commonly consumed teas, including green tea [Biluochun cha (Blc) and Longjing cha (Lj)] and black tea [Qimenhong cha (Qmh) and Yinghong 9# (Yh9)] were purchased from supermarket.

The ground dry tea leaves (0.5 g) were added to 250 ml boiling water and steeped for 45 min. The infusion was cooled to room temperature, filtered and made up to 500 ml with distilled water. Then it was diluted 5-, 10-, and 15-fold with distilled water for further research.

2.3. Compositional analysis of teas by HPLC

The content of catechins and purine alkaloids in tea water extracts were analyzed according to previously published methods (Peng et al., 2008), using a Waters HPLC system. A Discovery RP-Amide C_{16} guard column (4.0 × 20 mm, 5 μm), fitted with a Discovery RP-Amide C16 guard column, two Waters 515 LC pumps, a HT-230A column oven and a Waters 2487 UV detector were employed.

In brief, the separation was carried out with 5% ortho-phosphoric acid in water (elute A) and acetonitrile (elute B). The gradient was as follows: 0–4 min, 2% B; 4–21 min, linear gradient from 2% B to 9% B; 21–32 min, linear gradient from 9% B to 23% B; 32–45 min, 23% B. Post-run time was 10 min. Elution was performed at a solvent flow rate of 0.8 ml/min (20 μl injection volume) and the column oven was set at 35 °C. Stock solutions of pure standards of catechins and purine alkaloids were stored at 4 °C and used within 1 week. Detection was carried out at 210 nm; all peaks were plotted and integrated using the dedicated software. The area and the retention time of the analyzed peak were compared with those of respective standards. The quantification of alkaloids and catechins was performed using standard calibration curves. Data are average values for three experiments.

2.4. Determination of polyphenol content

The content of total tea polyphenols (TP) in tea water extracts was determined by a spectrophotometer. 1 ml of tea extract was transferred into a 25 ml volumetric flask to react with 5 ml dye solution (1 g ferrous sulfate and 5 g potassium sodium tartrate tetrahydrate dissolved in 1000 ml distilled water), 4 ml distilled water and 15 ml buffer (0.067 M potassium phosphate, pH 7.5). Several minutes were required for color development. Absorbance readings were made at 540 nm by a PGENERAL TU-1901 spectrophotometer, using a blank solution prepared with distilled water replacing the tea extract. The content of TP was calculated by the following equation:

$$\mathrm{TP}(\%) = 2A \times 1.957 \times (L1/L2 \times M/1000) \times 100\%$$

where $L1$ is the total volume of extract solutions in ml; $L2$ is the volume of the extract solutions used for analysis in ml; M is the weight of tea leaves for extraction in g; A is the absorbance of diluted and dyed extract solutions at 540 nm; 1.957 means that when the concentration of TP in tea extraction, which was treated with the method mentioned above was 1.957 mg/ml, its absorbance at 540 nm will be 0.5.

2.5. Determination of antioxidative activity

2.5.1. ABTS decolorization assay

The total antioxidative activity of the tea infusions were measured by the $ABTS^{\cdot+}$ radical cation decolorisation assay (Lorenzo, Francesca, & Pilergiorgio, 2003). $ABTS^{\cdot+}$ radical cation was prepared by reacting 10 ml of 2 mM ABTS water solution with 100 μl of 70 mM potassium persulfate, and the mixture was allowed to stand in the dark at room temperature for 12 h before use. Prior to the assay, the solution was diluted in ethanol to give an absorbance at 734 nm of 0.70 ± 0.02 in a 1-cm cuvette, and was equilibrated at 30 °C. 1 ml of ethanolic $ABTS^{\cdot+}$ radical cation solution was added to

Table 1
Contents of TP, catechins and purine alkaloids in different types of tea.

Compound	Content (%, w/w)						
	WCT	GCT	BCT	Lj	Blc	Qmh	Yh9
Ta	0.59 ± 0.01[a]	0.55 ± 0.03	0.29 ± 0.02	0.52 ± 0.04	2.11 ± 0.04	0.74 ± 0.04	0.76 ± 0.03
GA	0.24 ± 0.03	0.19 ± 0.01	0.69 ± 0.01	0.09 ± 0.01	0.13 ± 0.01	0.32 ± 0.03	0.35 ± 0.02
Tb	6.70 ± 0.05	6.50 ± 0.07	6.47 ± 0.05	0.01 ± 0.01	0.28 ± 0.01	0.04 ± 0.01	0.2 ± 0.02
GC	1.87 ± 0.03	1.54 ± 0.02	0.06 ± 0.01	0.64 ± 0.03	0.89 ± 0.03	nd	nd
Caf	nd[b]	nd	nd	3.41 ± 0.06	4.69 ± 0.04	3.78 ± 0.03	5.39 ± 0.04
EGC	0.19 ± 0.02	0.17 ± 0.02	nd	4.02 ± 0.05	4.82 ± 0.05	nd	nd
C	3.60 ± 0.02	3.40 ± 0.01	0.42 ± 0.01	0.12 ± 0.02	0.39 ± 0.03	nd	0.01 ± 0.01
EC	0.13 ± 0.02	0.11 ± 0.03	nd	0.89 ± 0.02	1.19 ± 0.03	0.04 ± 0.01	0.01 ± 0.01
EGCG	1.07 ± 0.07	0.82 ± 0.04	nd	6.07 ± 0.05	8.27 ± 0.06	nd	nd
GCG	7.70 ± 0.02	7.35 ± 0.04	0.48 ± 0.11	0.19 ± 0.02	0.61 ± 0.03	nd	nd
ECG	0.24 ± 0.03	0.19 ± 0.02	0.02 ± 0.03	1.28 ± 0.04	2.30 ± 0.03	0.01 ± 0.01	0.36 ± 0.02
CG	0.07 ± 0.03	0.06 ± 0.01	0.02 ± 0.01	0.06 ± 0.01	0.12 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
TP	33.02 ± 0.32	32.97 ± 0.47	19.47 ± 0.13	19.62 ± 0.23	24.85 ± 0.21	7.15 ± 0.42	10.67 ± 0.43

[a] Data were expressed as means ± SD (n = 3).
[b] nd = not detected.

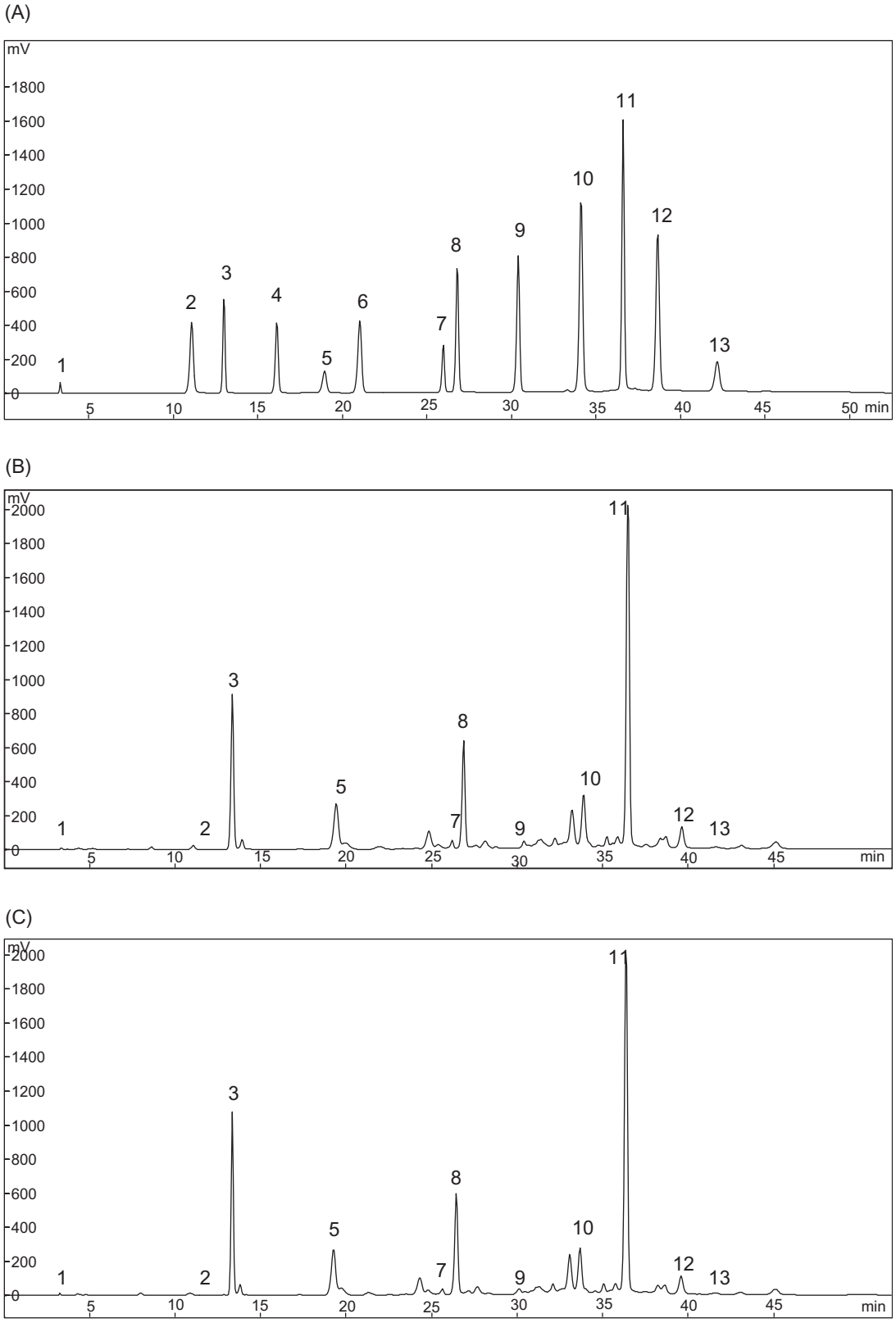

Fig. 1. The chromatograms of different types of tea. Detection was carried out with UV at 210 nm. (A) Standards, (B) WCT, (C) GCT, (D) BCT, (E) Blc, (F) Lj, (G) Qmh, (H) Yh9. Peak identification: 1.Ta, 2.Ga, 3.Tb, 4.Tp, 5.GC, 6.Caf, 7.EGC, 8.C, 9.EC, 10.EGCG, 11.GCG, 12.ECG, 13.CG.

aliquots of 10 μl of different tea infusions, stirred for 30 s, and the absorbance of tea samples (*A*i) were read at 734 nm. The blank Absorbance (*A*0) was measured using methanol. All the tests were performed in triplicate and the results were averaged. Antioxidant activity was expressed as percentage inhibition of the $ABTS^{\cdot+}$ radical cation and was determined by the following equation:

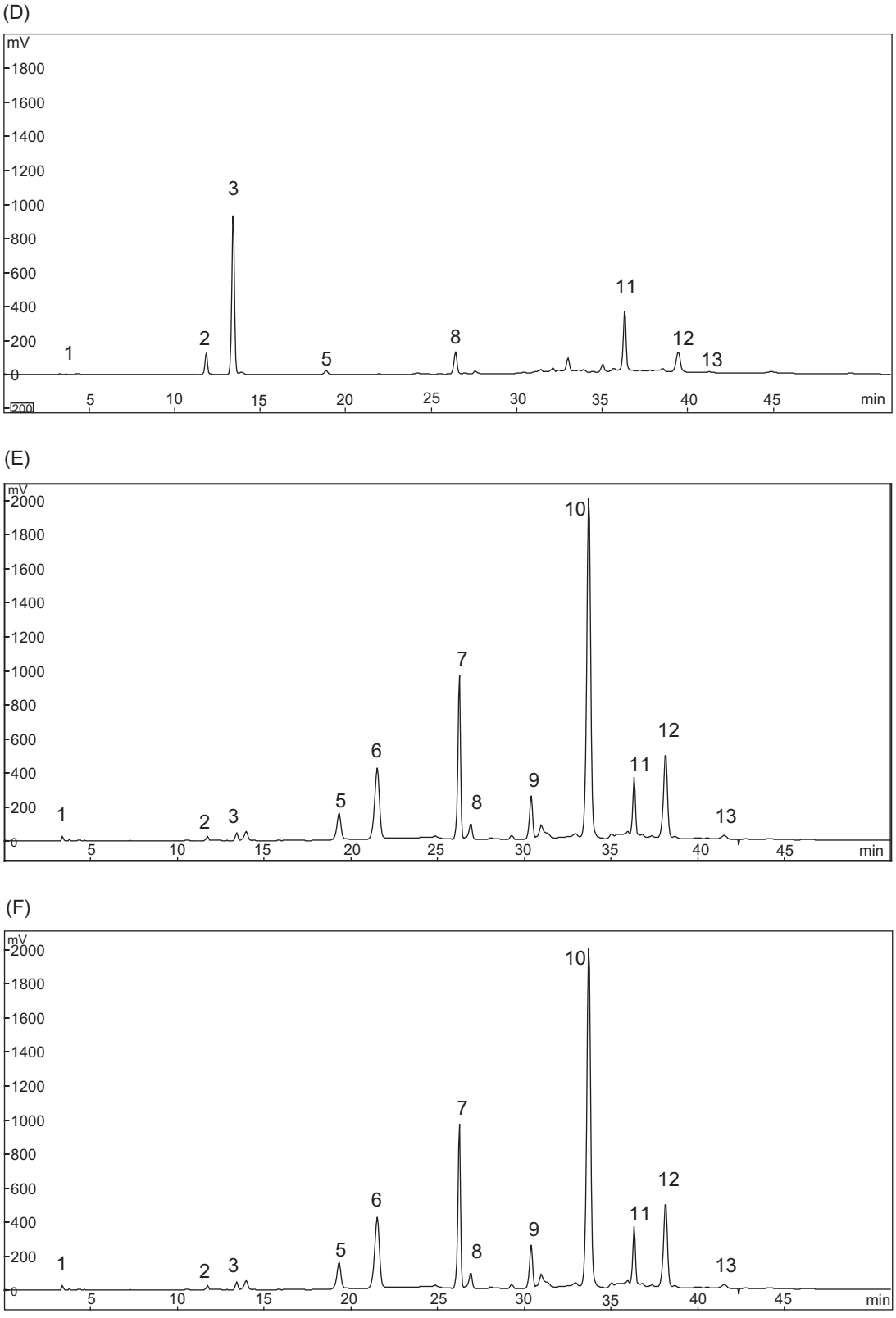

Fig. 1 (*continued*)

$$AA(\%) = [1 - (Ai/A0)] \times 100\%. \quad (1)$$

Meanwhile, using the trolox as the control antioxidant, the TEAC (Trolox-equivalent antioxidant capacity) value which represent the scavenging capacity of the $ABTS^{\cdot+}$ radical relative to Trolox was also calculated. The TEAC value was determined by the following equation:

$$TEAC = AA(\text{tea samples})/AA(\text{Trolox}). \quad (2)$$

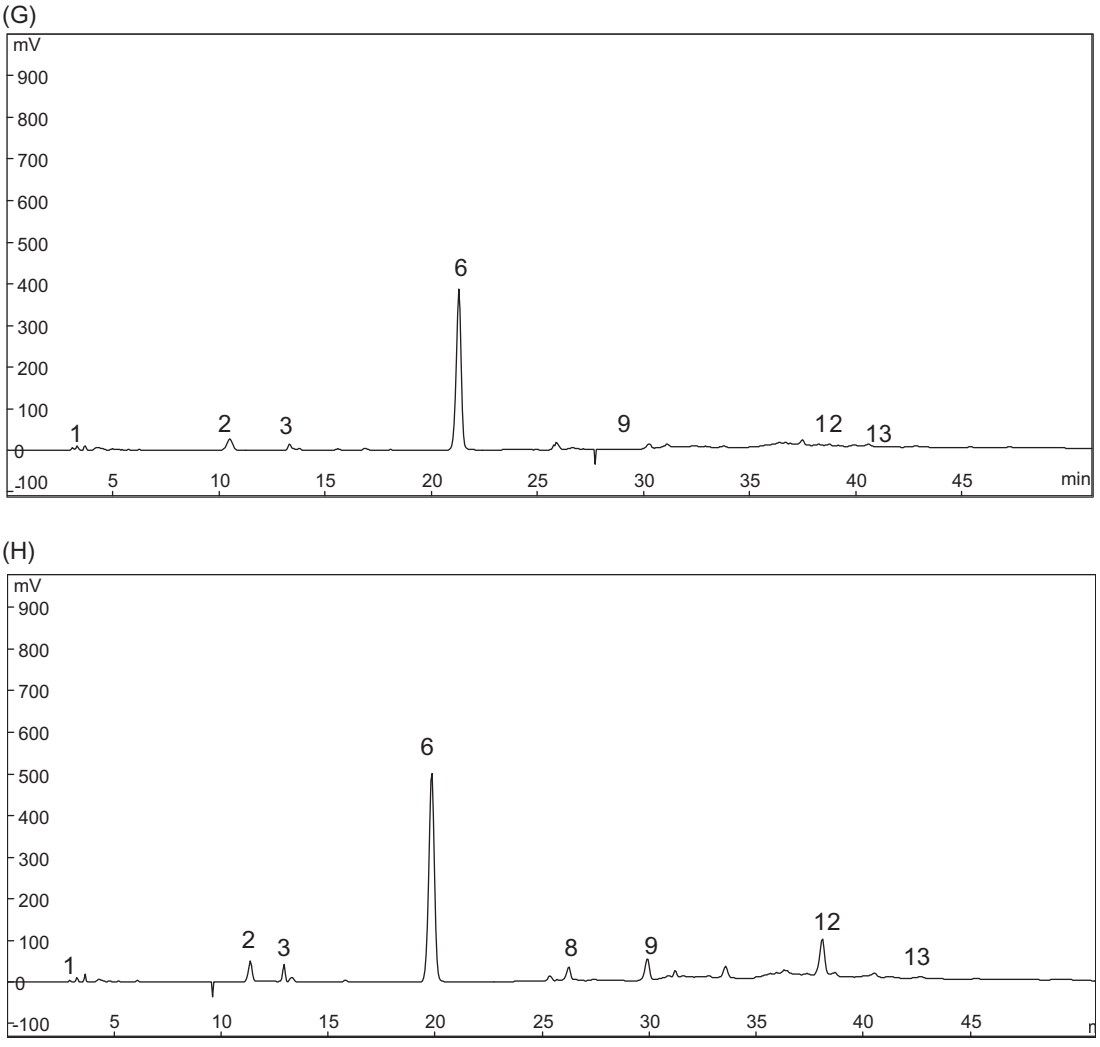

Fig. 1 (*continued*)

2.5.2. DPPH radical assay

According to Borse, Kumar, and Rao (2007), 20 μl of sample was placed in a cuvette, and 2 ml of 0.06 mM ethanol solution of DPPH was added. Distilled water was used as a control instead of extract. The reaction mixture was vortex-mixed and let to stand at 25 °C in the dark for 20 min. Absorbance (*A*i) of tea samples at 517 nm was measured using a spectrophotometer and the blank absorbance (*A*0) was measured using methanol. The DPPH solution was freshly prepared daily, stored in a flask covered with aluminum foil, and kept in the dark at 4 °C between measurements. Antioxidant activity was expressed as percentage inhibition of the DPPH radical and was determined by the following equation:

$$\mathrm{AA}(\%) = [1 - (Ai/A0)] \times 100\%. \quad (3)$$

2.5.3. Ferric Thiocyanate (FTC) method

Tea water (2 ml) extracts (1 mg/ml), 2 ml of 2.51% (w/v) linoleic acid in ethanol, 4 ml of 0.05 M of phosphate buffer (pH 7.0), and 2 ml of distilled water were mixed in a vial of 10 ml with a screw cap and then kept in an 40 °C water bath in the dark; 0.1 ml of this mixture was mixed with 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. After 5 min, 0.1 ml of 0.02 M ferrous chloride in 3.5% (v/v) hydrochloric acid was added to the mixture and then kept in a 40 °C water bath in the dark. The absorbance of the mixture was measured at 500 nm every 24 h. The FTC method was described in detail by Kikuzaki and Nakatani (1993).

2.6. Statistical analysis

Results were presented as means ± standard errors of triplicate measurements and analyzed by SPSS for Windows. Student's *t*-test was used to determine the level of significance ($p < 0.05$).

3. Results

3.1. Total polyphenol, catechins and purine alkaloids

The total polyphenols (TP), catechins and purine alkaloids contents of cocoa tea and commercial teas were examined and presented in Table 1 and Fig.1. The level of TP in selected green tea samples varied between 19.62 ± 0.23% and 33.02 ± 0.32% of dry weight and was significantly lower (7.15 ± 0.42% ~19.47 ± 0.13%) in selected black tea samples. The content of TP in WCT sample was the highest (33.02 ± 0.32%), whereas the content of TP in Qmh sample was the lowest (7.15 ± 0.42%). The amount of TP in tea extracts followed this order: WCT > GCT > Blc > Lj > BCT > Yh9 > Qmh.

As shown in Fig. 1, EGCG was the major catechin in commercial green teas, which was 6.07 ± 0.05% and 8.27 ± 0.06% in Lj and Blc, respectively. But it was not detected in black teas. While in WCT

and GCT, the major catechin was GCG, which was 7.70 ± 0.02% and 7.35 ± 0.04%, respectively. The trends of individual catechin contents (EGCG > EGC > ECG > EC > GC > GCG > C > CG) were similar in commercial green teas, while the trends in WCT and GCT were GCG > C > GC > EGCG > ECG > EGC > EC > CG. There was very less catechins in black teas.

The amount of caffeine was similar to all commonly consumed commercial teas but it was not detected in cocoa tea. While in cocoa tea the major purine alkaloids was theobromine, which was up to 6.70 ± 0.05%, 6.50 ± 0.07% and 6.47 ± 0.05% in WCT, GCT and BCT, respectively.

3.2. Antioxidant activity

3.2.1. Scavenging of ABTS$^{\cdot+}$

The scavenging activities of teas against hydrophilic ABTS$^{\cdot+}$ radicals were determined by measuring the decolorization of the ABTS$^{\cdot+}$ radicals at 734 nm. The extent of scavenging of the ABTS$^{\cdot+}$ was plotted as a function of antioxidant concentration, as shown in Fig. 2, cocoa tea and other teas scavenged ABTS$^{\cdot+}$-free radicals dose-dependently. It was found that WCT had very significant scavenging activity on the ABTS$^{\cdot+}$ radicals, having more than 50% inhibitory activity at a low concentration of 0.5 mg/ml. The EC_{50} values for WCT, GCT, BCT, Lj, Blc, Qmh, and Yh9 in scavenging ABTS$^{\bullet+}$ radical were 0.47, 0.54, 0.97, 0.79, 0.62, 1.95 and 1.26 mg/ml, respectively. The TEAC values for WCT, GCT, BCT, Lj, Blc, Qmh and Yh9 in scavenging ABTS$^{\cdot+}$ radicals were 0.63, 0.55, 0.30, 0.48, 0.37, 0.23 and 0.15, respectively. The ability of scavenging of ABTS$^{\cdot+}$ radicals was as follows: WCT > GCT > Blc > Lj > BCT > Yh9 > Qmh.

3.2.2. Scavenging of DPPH

This method is based on the bleaching of the stable radical of DPPH. The extent of scavenging of DPPH radicals was plotted as a function of antioxidant concentration. As shown in Fig. 3, cocoa tea

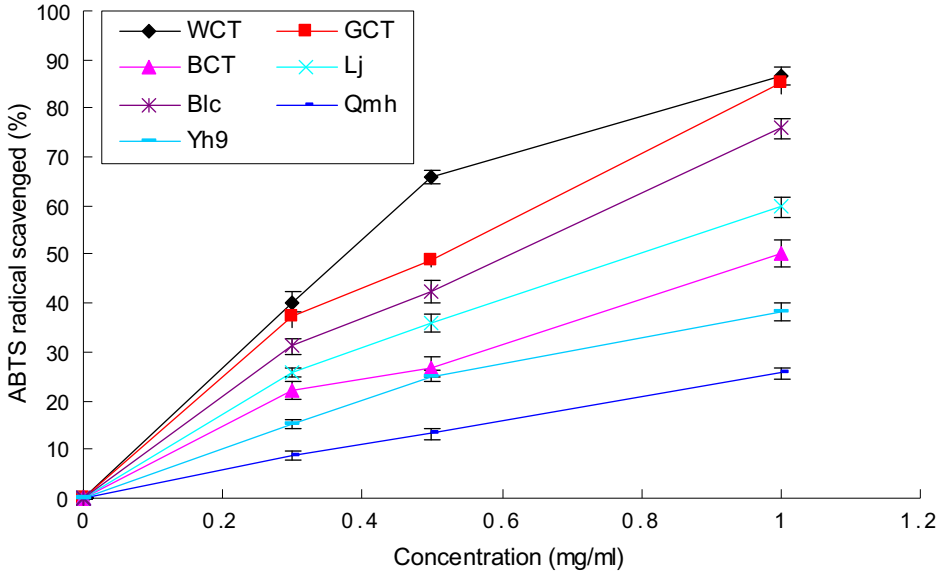

Fig. 2. Scavenging of ABTS$^{\cdot+}$ radicals by different types of tea. ABTS$^{\cdot+}$ solution was mixed with indicated concentrations of teas for 30 s and the absorbance was determined at 734 nm. The scavenging effects were calculated from the absorbance at 734 nm and plotted against the concentration of teas. Data are means of three samples.

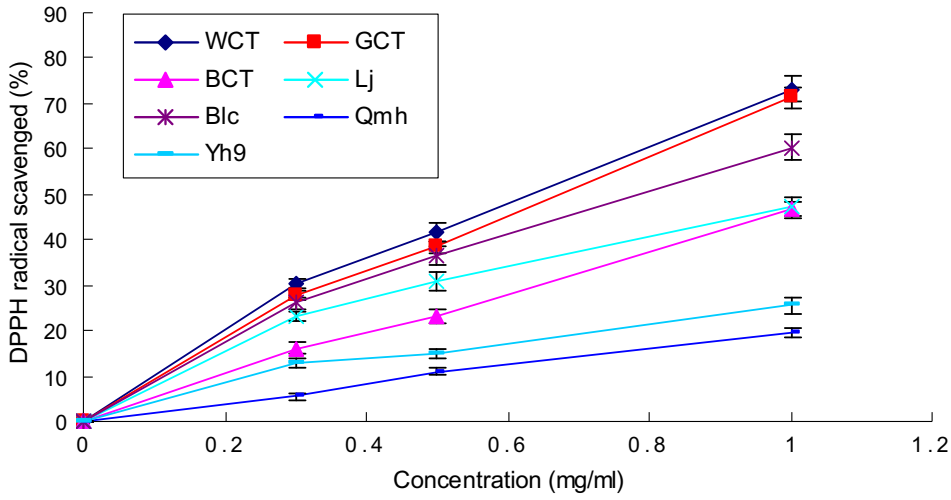

Fig. 3. Scavenging of DPPH radicals by different types of tea. The DPPH solution was mixed with indicated concentrations of teas for 20 min and the absorbance was determined at 517 nm. The scavenging effects were calculated from the absorbance at 517 nm and plotted against the concentration of teas. Data are means of three samples.

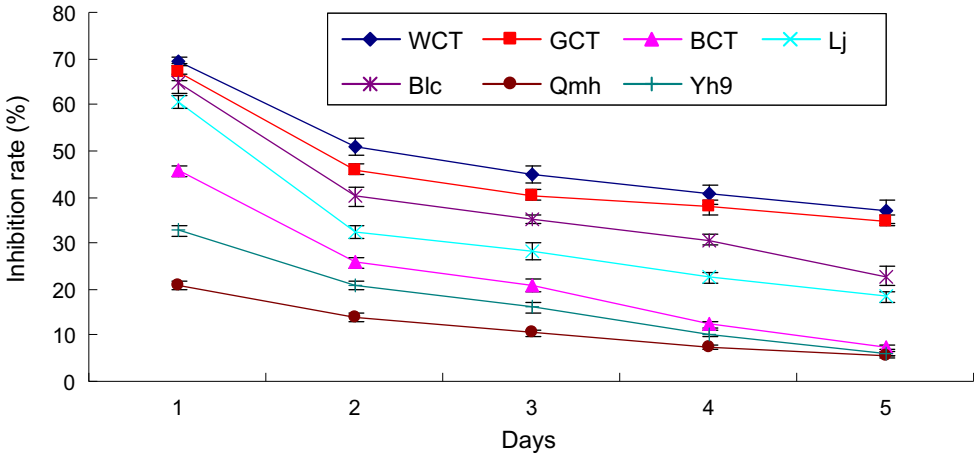

Fig. 4. The antioxidant activity of different tea extracts as assessed by linoleic acid. Data are means of three samples.

and other teas scavenged DPPH radicals dose-dependently. The EC_{50} values for WCT, GCT, BCT, Lj, Blc, Qmh, and Yh9 in scavenging DPPH radicals were 0.65, 0.67, 1.07, 0.78, 0.99, 1.96 and 2.51 mg/ml, respectively. The TEAC values for WCT, GCT, BCT, Lj, Blc, Qmh and Yh9 in scavenging DPPH radicals were 0.58, 0.56, 0.35, 0.48, 0.38, 0.19 and 0.15, respectively. The ability of scavenging of DPPH radicals was as follows: WCT > GCT > Blc > Lj > BCT > Yh9 > Qmh.

3.2.3. FTC method

The FTC method was used to measure the amount of peroxide in the initial stages of lipid oxidation. The individual activity of tea extracts showed that a low absorbance value indicated a high level of antioxidative activity. All of the tea extracts manifested almost the same patterns of activity as in the $ABTS^{\cdot+}$ and DPPH method (Fig. 4): WCT > GCT > Blc > Lj > BCT > Yh9 > Qmh. WCT still had the highest inhibition activity against lipid oxidation, whereas Qmh showed the lowest inhibition activity.

4. Discussion

According to the manufacturing process, tea can be classified as six types: white tea, yellow tea, green tea, oolong tea, black tea and pu-erh tea. White tea, yellow tea and green tea belong to unfermented tea, oolong tea belong to semi-fermented tea, black tea and pu-erh tea belong to fully fermented tea. In this study, GCT, Blc and Lj are classified as green tea. BCT, Qmh and Yh9 are classified as black tea, while WCT classified as white tea.

In contrast to green and black tea, white tea is manufactured only from the buds or first leaves of *Camellia* that are plucked and dried with minimal processing. Therefore, the concentrations of catechins and also methylxanthines (like caffeine) are enriched in white tea compared to green and black tea (Hilal & Engelhardt, 2007). In this study, WCT showed the highest TP content compared to GCT or BCT as well as other green tea or black tea (Table 1). Besides, white tea and green tea prior to black tea manufacturing generally contain a high catechin content, which may also account for some of the variation in the catechin contents. Manzocco, Anese, and Nicoli (1998) found that green tea contained higher total phenols (95.4 mg/g) than black tea (80.1 mg/g). The present results confirmed that the level of total phenols in green tea is richer than in black tea. On the other hand, Khokhar and Magnusdottir (2002) found that the level of total phenols in selected black teas varied between 80.5 and 134.9 mg/g and was significantly lower (65.8–106.2 mg/g) in green tea. Hoff and Singleton (1977) have also reported similar levels of total phenols in both black (67.7 mg/g) and green teas (62.3 mg/g). This could be due to varying amounts of oxidation during fermentation and handling.

In previous studies from other authors, TP was considered to be the main antioxidant contributing greatly to the potent antioxidative activity of tea. The antioxidative effect of TP was concentration-dependent (Shahidi & Wanasundara, 1992; Yokozawa et al., 1998). The present study showed a high correlation between the polyphenol content and the antioxidative activity of tea extracts.

Guo et al. (1999) found that the scavenging effects of galloylated catechins (EGCG and GCG) on the four free radicals were stronger than those of non-galloylated catechins (EGC, GC, EC, (+)-C), and that the scavenging abilities of GCG, GC and (+)-C were stronger than those of their corresponding epimers (EGCG, EGC and EC). The differences between their stereo structures played a more important role in their abilities to scavenge large free radicals. Takako (Yokozawa, Cho, Hara, & Kitani, 2000) also found EGCG and GCG had higher antioxidative activity than EGC and GC, respectively, suggesting that the *O*-dihydroxy structure in the B ring and the galloyl groups are important determinants for radical scavenging and antioxidative potential. In this study, we found that cocoa tea showed the strongest antioxidative activity compared to other teas, whose predominant catechin was GCG. Whether GCG contributed the most to antioxidative activity in cocoa tea needs to be studied further.

In conclusion, unfermented cocoa tea, especially WCT is characterized by a higher content of polyphenols, and displays a higher antioxidative activity in comparison to the regular tea.

Acknowledgments

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