Advance Journal of Food Science and Technology 5(10): 1355-1360, 2013
DOI:10.19026/ajfst.5.3110
ISSN: 2042-4868; e-ISSN: 2042-4876

Submitted: June 19, 2013 Accepted: July 05, 2013 Published: October 05, 2013

Research Article

Chemical Analysis and Antioxidant Activity *in vitro* of Polysaccharides Extracted from Lower Grade Green Tea

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Abstract: Tea is a well-known and important agricultural product in the world. The Crude Polysaccharides from tea leaves (CP) probably have good antioxidant activities. However, whether or not the antioxidant abilities of CP depend on tea polyphenols in the CP is not understanded. In this study, four CP fractions (TPF30, TPF50, TPF70 and TPF90) were isolated from CP and their antioxidant activities were compared. Meanwhile, Chemical and physical characteristics of CP and four CP fractions were investigated by the combination of chemical and instrumental analysis methods. Their antioxidant activities were investigated *in vitro* systems, including hydroxyl radical assay, 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH·) scavenging activity, reducing power and chelating activity. Among CP and these four polysaccharides, TPF90 showed more significant DPPH· scavenging activity and highest reducing power, chelating activity and inhibitory effects on hydroxyl radical. Thus, it can be concluded that polysaccharides extracted from the lower grade green tea might be employed as ingredients in healthy and functional food to alleviate the oxidative stress.

Keywords: Antioxidant activity, cheating activity, polysaccharides, reducing power, scavenging ability tea

INTRODUCTION

Tea, a product made from the leaf and bud of tea plant (*Camellia sinensis L.*), is one of the most widely consumed beverages worldwide. The chemical components in tea mainly consist of polyphenols and polysaccharides polysaccharides (Andrea and Michael, 1997; Raal *et al*., 2012; Schoenthal, 2011; Wang, 2012; Yu *et al*., 2007). Recently, great advances have been achieved in chemical and bioactive studies of polyphenols in tea leaves. However, Polysaccharides (TPS) from tea materials have been received much less consideration than Tea Polyphenols (TPPs) (Wang *et al*., 2013). In recent studies, polysaccharides used in the food industry and in medicine, have been attracted much attention for a long time, due to their biological activities. Tea Polysaccharide (TPS) has been found to be an important water-soluble polysaccharide exhibiting many bioactivities in the late 2000s (Mori *et al*., 1989).

TPS were ranged from 0.4 to 1.5% in the various grade tea leaves (Xiao, *et al*., 2011). Many studies has shown that the crude TPS have good antioxidant activities (Chen *et al*., 2008a, b, 2009; Han *et al*., 2011; Nie *et al*., 2008; Sun, 2011; Xiao *et al*., 2011). The bioactivity of polysaccharides depends on their chemical characteristics. TPS conjugates were found to exhibit antioxidant activities and there was a direct relationship between the uronic acid contents and the radical-scavenging effects of tea polysaccharide conjugates (Chen *et al*., 2004). Nie *et al*. (2008), found that the antioxidant abilities of TPS-protein conjugates depend on the protein content and with the increasing of the protein content, the antioxidant activities of TPS-protein conjugates enhanced. Shu-chi *et al*. (2010) studied the antioxidant activity of Anji white tea polysaccharides, red blood cells from oxidative hemolysis and oxidation of hydrogen peroxide induced erythrocyte hemolysis, when the concentration was up to 7 mg/mL, the antioxidant activity of tea polysaccharide was good as the same concentration of Vc solution (Shu-chi *et al*., 2010).

We all know that tea polyphenols have good antioxidant activities, whether or not the antioxidant abilities of crude TPS depend on tea polyphenols contained in the crude TPS is not understand. Chemical properties and biological activity of tea polysaccharide from different species show diversity and specificity. Ni *et al*. (2004) have compared Tea Polysaccharides (TPS) characteristics and their role in scavenging free radicals. TPS was extracted from green, Oolong and Black tea, which were made from the same fresh leaves from Hubei, Fujian and Yunnan. It turned out that there was a remarkable effect of region and process on physico-

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chemical characteristics, effect of scavenging radical (Ni *et al*., 2004). There has been many research on tea polysaccharide, but the difference of producing area, processing methods and the variety would result in the difference of molecular organization, structure and antioxidant activity of tea polysaccharide. This article studies the green tea of rude Longjing Tea, which was produced in Zhejiang Hangzhou.

There was no research about the green tea of Longjing, which is famous in Hangzhou and the annual output is very high. In this study, Crude tea Polysaccharide (CP) and TPS fractions separated from CP on different alcohol concentration were prepared and their antioxidant activities of CP and TPS fractions were assayed *in vitro*, such as hydroxyl radical, DPPH radical scavenging activity, reducing power and chelating ability. The relationship between chemical characteristics and antioxidant activity was reported as well.

EXPERIMENT

Materials and chemicals: Low-grade green tea was purchased from Longguan Tea Factory, Zhejiang Province, China. It was identified as grade six crude green tea, according to the Green Tea Quality Standard (GH016-84) of China.

1,1-Diphenyl-2-Picryl-Hydrazyl radical (DPPH·), ferrozine, deoxyribose, salicylic acid, Trifluoroacetic Acid (TFA), sodium borohydride ($NaBH_4$), l-rhamnose, d-glucose, d-xylose, d-fucose, d-galactose and d-mannose were purchased from Merck Co. (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO, USA), All the other reagents used were at analytical grade.

Preparation of crude polysaccharides: One hundred grams dried tea leaves were cut into small pieces and soaked with 1 L of 95% ethanol (v/v) for 16 h (2 times) to remove lipids. The residue was dried and extracted with boiling water for 2 h (3 times). The water-insoluble material was removed by filtration and the supernatant was then centrifuged at 4000 rpm for 15 min. Ethanol (95%) was added slowly to final alcohol concentration of 80% and precipitated at 4°C over night. The precipitate obtained by centrifugation (3000 rpm, 10 min, 4°C) was named CP; Deproteinization was performed with sevage' reagent (5 times). Ninety five percent Ethanol was continually added slowly to final concentration of 30%, the collected precipitate was termed TPF30; similarly, the precipitates TPF50, TPF70 and TPF90 were obtained by adding Ethanol to concentration of 50, 70 and 90% successively. Finally, four fractions of crude polysaccharides were obtained by lyophylization. The concentration of total sugar was determined by the method of phenol-sulfuric acid (Dubois *et al*., 1956) by using glucose as a standard.

Monosaccharide composition analysis: Twenty milligram sample (TPF30, TPF50, TPF70 or TPF90) was hydrolyzed with 2 moL trifluoroacetic acid (TFA, 5 mL) at 110°C for 3 h. The excess acid was removed by vacuum evaporation with methyl alcohol (MeOH) after the hydrolysis. The hydrolyzed products were reduced by $NaBH_4$ (60 mg) and acetylated with acetic anhydride (Albersheim *et al*., 1967; Johnes and Albersheim, 1972), the standard monosaccharide was derived with same method. The alditol acetates were analyzed by Gas Chromatography (GC) using an Agilent 7890N instrument equipped with an HP-5 capillary column (30 m×0.32 mm×0.25 μm) and a Flameionization Detector (FID). The applied temperature program was as follows: oven temperature was initially set at 120°C, increasing to 240°C at a rate of 10°C/min and then held at 240°C for 6.5 min. The heater temperatures of both injector and detector were kept at 250°C. Nitrogen was used as the carrier gas. Quantitation was calculated from the peak area using response factors.

ASSAY FOR ANTIOXIDANT ACTIVITY

Hydroxyl radical assay: The hydroxyl radical assay of samples was measured according to the method described by Ghiselli *et al*. (1998) with a minor modification. Samples were dissolved in distilled water at 0 (control), 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL. (0.2 mL) sample solution was mixed with 2.0 mL of deionized water; 0.2 mL of 9 mmol/L ferrous sulfate, 0.2 mL of 9.0 mmol/L salicylic acid and 0.2 mL of 8.8 mmol/L H_2O_2 were then added to the reaction solution. The reaction solution was incubated at 37°C for 30 min. The absorbance of the mixture was measured at 510 nm. The experiment was carried out in triplicate. Percent inhibition of hydroxyl radical was calculated as:

$$\text{Inhibition (\%)} = [A_0 - (A_1 - A_2)] / A_2 \times 100$$

where,

A_0 : The absorbance of the control containing salicylic acid, $FeSO_4$ and H_2O_2

A_1 : The absorbance of the test sample

A_2 : The absorbance of the sample only (sample without H_2O_2 solution)

DPPH radical-scavenging activity test: Radical-scavenging activity against the stable radical DPPH· was determined spectrophotometrically following the scientific literature (Morales and Jiménez-Pérez, 2001). In brief, the solution of DPPH· in ethanol (0.1 mM) was prepared daily, before UV measurements. An aliquot of sample (0.2 mL, 0.1-1.0 mg/mL) were thoroughly mixed with 2 mL of freshly prepared DPPH· and kept in the dark for 30 min at room temperature (25°C) and then the absorbance was measured (U-2000, Hitachi, Japan) at 517 nm. The experiment was carried out in triplicate. The ability to scavenge the DPPH· radical was calculated by the following formula:

Scavenging effect (%) = [1 - (A_s - A_0/A_1)] ×100

where,

A_1 : The absorbance of the control (DPPH· solution without sample)

A_s : The test sample (DPPH· solution plus test sample)

A_0 : The absorbance of the sample only (sample without DPPH· solution)

Reducing power assay: The reductive potential of samples was determined according to the method of (Oyaizu, 1986) with some modification. (0.4 mL) samples (0.1-1.0 mg/mL), 1.0 mL pH 6.6 phosphate buffer (0.2 mol/L) and 1.0 mL 1% (w/v) $K_3Fe(CN)_6$ were incubated at 50°C for 20 min. The reaction was terminated by adding 1.0 mL trichloroacetic acid (10%, w/v) and centrifuged at 2000 g for 10 min. The upper layer (2.0 mL) was mixed with 3.4 mL water and 1.0 mL 0.1% (w/v) $FeCl_3$. The mixture was shaken and its absorbance was measured at 700 nm against a blank. The reduction capability was calculated by the following formula:

Reduction capability (%) = [A_0 - (A_1 - A_2)] /A_2×100

where,

A_0 : The absorbance of the control

A_1 : The absorbance of the test sample

A_2 : The absorbance of the sample only (sample without $FeCl_3$ solution)

Ferrous metal ions chelating activity: The chelating of ferrous ions ability was determined according to the method described by Dinis *et al*. (1994). The samples at different concentration (0.1-1 mg/mL, 0.2 mL) were mixed with 2.25 mL of water and 0.05 mL of 2.0 mmol/L ferrous chloride. The reaction was initiated by adding 5.0 mmol/L ferrozine (0.2 mL). After reaction for 10 min at room temperature, absorbance of the solution was measured at 562 nm against a blank. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the following equation:

Metal chelateing effect (%) = [A_0 - (A_1 - A_2)] /A_2×100

where,

A_0 : The absorbance of the control

A_1 : The absorbance of the test sample

A_2 : The absorbance of the sample only (sample without $FeCl_2$ solution)

RESULTS AND DISCUSSION

Characterization and composition of the polysaccharides: The water-soluble crude polysaccharide named CP was obtained from the low-grade green tea. The total yield of CP was about 19.2 mg/g dried material and the yield of TPF30, TPF50, TPF70 and TPF90 were 0.06, 0.82, 0.33 and 0.17 mg/g respectively. The monosaccharide composition of polysaccharides are shown in Table 1.

Table 1: The monosaccharide composition of the tea polysaccharides

	Samples[1]			
Sugar components[2]	TPF30	TPF50	TPF70	TPF90
Rha	0.99	0.82	1.69	0.40
Fuc	1.05	0.75	1.32	0.73
Ara	3.67	4.67	4.33	2.29
Man	0.81	1.39	0.79	0.67
Glu	1.00	1.00	1.00	1.00
Gal	1.86	1.77	3.25	1.09
Glucuronic acid	13.63	27.43	6.04	1.20

[1]: TPF30, TPF50, TPF70, TPF90: crude polysaccarides isolated from CP; [2]: Rha, Rhama; Fuc, Fucose; Ara, Arabibinose; Man, Mannose; Glu, Glucose; Gal, Galactose

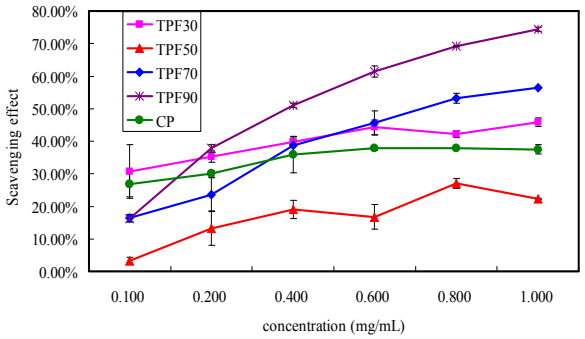

Fig. 1: Scavenging activities of CP, TPF30, TPF50, TPF70 and TPF90 on hydroxyl radicals

Hydroxyl radical assay: Hydroxyl radical is considered to be a high potent oxidant, which can react with all biomacromolecules functioning in living cells (İlhami *et al*., 2010). In this study, hydroxyl radical was generated by reaction of iron complex with H_2O_2 in the presence of salicylic acid, yield a pink tint. Added hydroxyl radical scavengers for the resulted hydroxyl radicals and diminish tint formation.

As shown in Fig. 1, all of the four CP fractions have the abilities to scavenge hydroxyl radicals at concentrations from 0.1 to 1.0 mg/mL. The scavenging abilities increased with the concentration increasing. The scavenging activity of TPF90 was the strongest among them, while polysaccharide content of TPF90 was not the highest. At concentration of 1 mg/mL, scavenging effect of CP, TPF30, TPF50, TPF70 and TPF90 was 37.44, 45.91, 22.24, 56.36 and 74.39%, respectively. It has been reported that scavenging effect on hydroxyl radical of Vitamin C was less than 20% at 0.63 mg, namely 6.3 mg/mL (Luo and Fang, 2008). Therefore, the polysaccharides from low-grade green tea had significant effect on scavenging hydroxyl radical.

DPPH· scavenging activity: DPPH· is a useful reagent for investigating the free radical-scavenging activities of materials. In the DPPH· test, the antioxidants were able to reduce the stable DPPH· to

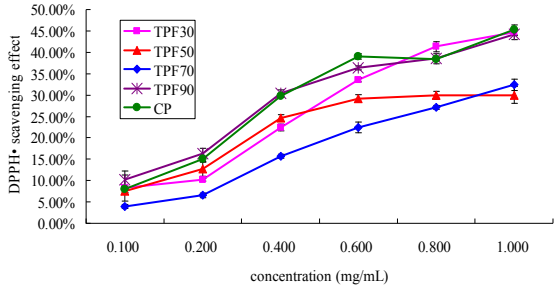

Fig. 2: Scavenging activities of CP, TPF30, TPF50, TPF70 and TPF90 on DPPH radicals

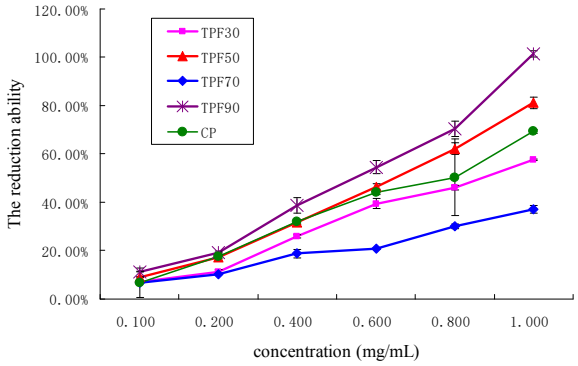

Fig. 3: Total reductive potential of CP, TPF30, TPF50, TPF70 and TPF90 from the low grade green tea

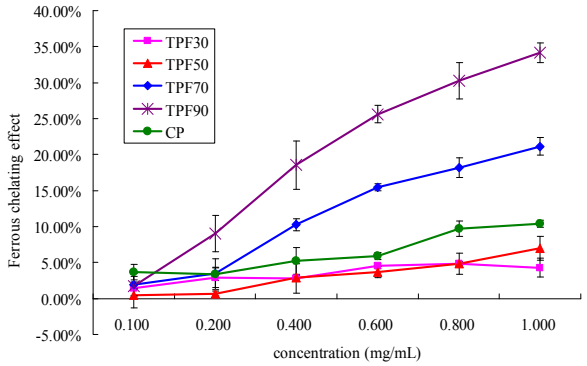

Fig. 4: Chelating activity of CP, TPF30, TPF50, TPF70 and TPF90 from the low grade green tea

the yellow-colored diphenylpricrylhydrazine. The DPPH· free radical-scavenging activities of tea polysaccharides with different concentration were shown in Fig. 2. As can be seen in Fig. 2, tea polysaccharides exhibited a rather strong concentration-dependent DPPH· radical-scavenging activity and the scavenging activity of CP, TPF30 and TPF90 were the stronger among them and the DPPH scavenging percentages of them were between 7.95 and 45.26% and increased with increasing concentration from 0.1 to 1.0 mg/mL. At concentration of 1 mg/mL, scavenging effects of CP, TPF30, TPF50, TPF70 and TPF90 were 45.26, 44.08, 30.00, 32.37 and 44.26%, respectively. It was noted that, when the concentration of tea polysaccharides were increased, the antioxidant activity increased.

Reducing power assay: It has been reported that the antioxidant activities is a positive correlation with the reducing power, prevention of chain initiation, radical scavenging and so on (Pellegrini *et al*., 1999; Diplock, 1997). The reducing properties are generally associated with the presence of reduction, which have been shown to exert antioxidant action by breaking the free radical chain with donating a hydrogen atom (Gordon, 1990). The Fe^{3+}-Fe^{2+} transformation was investigated in the presence of CP and CP fractions. Figure 3 showed the reducing power of CP, TPF30, TPF50, TPF70 and TPF90 was in concentration dependent manner. The reducing power of TPF90 was the strongest among them and at concentration of 1.0 mg/mL, the reducing power of CP, TPF30, TPF50, TPF70 and TPF90 was 69.30, 57.67, 81.17, 37.07 and 101.40%, respectively. The data on the reducing power of different contents suggested that they were likely to contribute the observed antioxidant effect.

Ferrous metal ions chelating activity ferrous chelating effect: Transition metal ions, such as Cu^{2+} and Fe^{2+}, can catalase the generation of reactive oxygen species and result in lipid peroxidation and DNA damage (Stohs and Bagchi, 1995). Therefore, the chelation of transition metal ions by antioxidant or antioxidative peptides would retard the oxidation reaction.

The effective Fe^{2+} chelators afford protection against oxidative damage by removing Fe^{2+} that may otherwise participate in HO• generating Fenton type reactions. This can protect against oxidative damage by inhibiting production of ROS and lipid peroxidation. The Fe^{2+} chelating capacity of tea polysaccharides were determined by measuring the iron-ferrozine complex. The results are shown in Fig. 4. At a concentration of 0.1-1.0 mg/mL, ferrous metal ions chelating activities of TPF90, TPF70 and CP increased with the concentration increasing. However, TPF50 and TPF30 showed very low ferrous metal ions chelating potential, which was hardly changed with increasing concentration. CP, TPF30, TPF50, TPF70 and TPF90 could be chelated with 10.38, 4.30, 7.02, 21.14 and 34.15% of Fe^{2+} at the concentration of 1.0 mg/mL. Figure 4 revealed that some CP fractions had an effective capacity for iron binding. It also suggests that its action as antioxidant might be related to its iron-binding capacity.

CONCLUSION

In this study, crude tea polysaccharide and four CP fractions were isolated from the low-grade green tea.

Several assays *in vitro* were applied to evaluate the antioxidant potential of these polysaccharides. Results indicated that CP and the four polysaccharide fractions from the low-grade green tea had different antioxidant activities in different evaluation system. TPF90 showed the more significant DPPH· scavenging activity; its inhibitory effects on hydroxyl radical and reducing power and chelating activity were also the highest, which showed much stronger antioxidant potential than crude tea polysaccharide. Tea polysaccharide as food antioxidants had important applications for food industries, while the antioxidant mechanisms and in vivo antioxidant activity need to be further studied.

ACKNOWLEDGMENT

The financial support provided by the Key innovation team Project of Science and Technology Department of Zhejiang Province of China (No. 2010R50028) is greatly appreciated.

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