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Characterization and antioxidant activities of polysaccharides from leaves, flowers and seeds of green tea

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ABSTRACT

The chemical characterization and antioxidant activities of tea leaves polysaccharides (TLPS), tea seed polysaccharide (TSPS), tea flower polysaccharide (TFPS) were investigated after isolated from the leaves, flowers and seeds of camellia sinensis. Results indicated that TLPS, TFPS and TSPS were all composed of Rha, Ara, Gal, Glu, Xyl, GalA and GluA, besides, TLPS was also contained Man and Rib, and TFPS was also contained Man. The molecular weight distribution of TLPS, TFPS and TSPS ranged from 3.67×10^3 to 7.58×10^5 Da, 2.56×10^3 to 1.46×10^6 Da and 3.66×10^3 to 9.61×10^5 Da, respectively. Antioxidant activity tests revealed that TLPS, TFPS and TSPS exhibited strong antioxidation in a concentration-dependent manner. Furthermore, TLPS and TFPS had higher antioxidant activity than TSPS. The nature of monosaccharide and the higher molecular weight in TLPS and TFPS molecules probably contribute to the activities.

1. Introduction

Polysaccharides and their conjugates, used in the food industry and in medicine for a long time, have attracted much attention, in recent years, due to their biological activities. Tea polysaccharide conjugate is one of the main bioactive components of green tea (*Camellia sinensis*) (Chen & Xie, 2002). Chemical analysis of tea polysaccharide conjugates revealed that they contained neutral sugars, uronic acid and protein. Tea polysaccharides have been found to be an important water soluble polysaccharide with certain bioactivities in the late 1980s (Kardosova & Machova, 2006; Warrand, 2006), including immunostimulation, antitumor, antioxidant activities, anti-inflammatory, hypoglycemic, etc.

The tree of *C. sinensis*, an evergreen shrub or small tree, produces glossy green leaves, white flowers and brown seeds. Tea is one of the world's most popular beverages and green tea appears to be one of the healthiest as well. In recent years, tea polysaccharide 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, and have caused great interests among researchers (Chen, Zhang, & Xie, 2005; Yu, Sheng, Xu, An, & Hu, 2007). For a long time, however, the research of tea leave polysaccharide (TLPS) and tea flower polysaccharide (TFPS) was more than tea seed polysaccharides (TSPS). There are very few studies about the tea seed polysaccharides (TSPS). In this study, chemical characterization and antioxidant activities of crude tea polysaccharides all obtained from the tree of *C. sinensis* have been evaluated. The studies and application of different sources of crude tea polysaccharides are also becoming valuable.

2. Materials and methods

2.1. Materials and reagents

Tea seeds, tea leaves and tea flowers were obtained commercially from Hubei province of China. Ascorbic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA) and D-mannitol were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Nicotinamide adenine dinucleotide (NADH) and Ferrozine were obtained from Sangon Biotech (Shanghai) Co. Ltd. (Shanghai, China). 2-Deoxy-D-ribose, nitrotetrazolium blue chloride (NBT), phenazine methosulfate (PMS), linoleic acid, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Aladdin Reagent Int. (Shanghai, China). All other reagents and solvents were of analytical reagent grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double distilled water.

Abbreviations: Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man, mannose; Rib, ribose; GalA, galacturonic acid; GlcA, glucuronic acid; MW, molecular weight; TLPS, tea leaves polysaccharide; TSPS, tea seed polysaccharide; TFPS, tea flower polysaccharide.

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2.2. Analytical methods of components in three polysaccharides

The total sugars were determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with D-glucose as standard. The soluble protein (SP) was determined by the Coomassie brilliant blue G-250 method (Bradford, 1976) with bovine serum albumin as a standard. Uronic acid content was determined according to a meta-hydroxydiphenyl colorimetric method (Blumenkrantz & AsboeHansen, 1973) with galacturonic acid as standard. Total phenolic content in the crude tea polysaccharide was determined with the Folin-Ciocalteu's reagent (FCR) according to Khokhar's method (Khokhar and Magnusdottir, 2002).

2.3. Preparation of crude polysaccharides

2.3.1. Preparation of TLPS

Lower grade green tea (250 g) was mixed with 2000 ml of 80% ethanol (v/v) and refluxly heated at 30 °C for 24 h. This treatment was repeated twice. After filtration, the tea leaf residues were extracted with 2000 ml distilled water in bath at 90 °C for 2 h. After filtered, the residues were extracted again with 2500 ml distilled water at the same temperature for another 2 h. Then the extracts were centrifuged to remove the contaminants. The supernatant was concentrated via rotary evaporation and precipitated with 95% ethanol. Then the precipitation was dissolved with water and dialyzed to remove the small molecules. The dialyzed solution was freeze-dried to yield polysaccharide powder.

2.3.2. Preparation of TFPS

The dry flowers, cut into small pieces (50 g), were mixed with 2000 ml of 80% ethanol (v/v) and refluxly heated at 30 °C for 24 h. This treatment was repeated twice. After filtration, the tea flower residues were extracted with 400 ml distilled water in water bath at 90 °C for 2 h. After filtered, the flowers were extracted again with 300 ml distilled water in water bath at the same temperature for another 1 h. Then the extracts were centrifuged to remove the contaminants. The supernatant was concentrated via rotary evaporation method and precipitated with 95% alcohol. Then the precipitation was dissolved with water and dialyzed to remove the small molecules. The dialyzed solution was freeze-dried to yield polysaccharide powder.

2.3.3. Preparation of TSPS

The outer covering of tea seeds was removed and the kernels of tea seeds were ground into powder. The dry ground tea seeds (80 g) were treated by aqueous vapor for 20 min, then extracted with Na-citric acid buffer (pH 4.0) in bath at 55 °C, and the solution were extracted with 0.5% (m/v) water at 55 °C for 6 h. After centrifugation, the oil phase was extracted by petroleum ether and the extraction phase was demulsificated. After vacuum distillation, the oil and the tea seeds meal were obtained, respectively. Tea seeds meal and distilled water were put into Beaker at a proportion of 1:6 (v/v). The extraction performed at 90 °C for 1.5 h respectively. Then the combined filtrate solution was centrifuged to remove the contaminants. The supernatant was concentrated and precipitated with 75% alcohol, and then the precipitation was dissolved with water and dialyzed to remove the small molecules. The dialyzed solution was freeze-dried to yield polysaccharide powder.

2.4. Determination of molecular weight – HPGPC

The molecular weight of TLPS, TFPS and TSPS were determined by gel permeation chromatography. Sample (10 mg) were dissolved in 1 ml of 0.02 M phosphate buffer solution and centrifuged at 15,000 rpm for 10 min, and then passed through a 0.45 μm filter. 20 μl of the supernatant was injected into a Shodex SB-804 HQ GPC column (300 mm × 8 mm) with a Shodex SB-G guard column (50 mm × 6 mm) from Showa Denko K.K. (Tokyo, Japan). GPC system was maintained at 45 °C and eluted with phosphate buffer solution at a rate of 0.3 ml/min. The molecular weight was calculated by the calibration curve obtained by using various standard dextrans with different molecular weight (T3, T6, T10, T40, T100, T500, and T1000).

2.5. Analysis of monosaccharide composition

Polysaccharides samples (2 mg) were dissolved in 4 ml of 2 M trifluoroacetic acid solution (TFA) and hydrolyzed at 110 °C for 6 h. The hydrolysate of sample was evaporated to dry under reduced pressure. Then, TFA was removed by washing with methanol (3 ml) four times in order to remove TFA absolutely. The dried hydrolysates were dissolved with ultra-pure water and diluted to 100 ml, and then measured by diluting 10-fold again (Yang, Zhang, Tang, & Pan, 2005). Ion chromatography (IC) was used for the identification and quantification of monosaccharide. IC experiment was performed on a Dionex ICS2500 chromatographic system (CA, USA) with a Dionex pulsed amperometric detector equipped with an Au electrode, a Dionex Carbopac PA20 column (150 mm × 3 mm). The temperature was kept at 30 °C and the injection volume was 25 μl. The eluents were NaOH (2 mM) at a flow rate of 0.45 ml/min. D-Fuc, D-GalA, D-GluA, D-Man, D-Xyl, D-Rib, D-Glu, D-Gal, D-Fru, L-Rha, L-Ara were used as references.

2.6. IR spectroscopy

The IR spectra of the polysaccharides were determined using a Fourier transform infrared spectrophotometer. The sample was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for FT-IR measurement in the frequency range of 4000–400 cm^{-1} (Mid infrared region).

2.7. Antioxidant activity assay

2.7.1. Total antioxidant activity

The total antioxidant activity of crude polysaccharide by use of linoleic system was determined according to the literature (Ardestani & Yazdanparast, 2007) with some modifications. The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid and 0.2804 g of Tween 20 emulsifier into 50 ml of phosphate buffer (0.2 M, pH 7.0). The mixture was then homogenized 0.5 ml of different concentrations of samples (25, 50, 100, 200, 400 μg/ml) were mixed with 2.5 ml of linoleic acid emulsion (0.2 M, pH 7.0) and 2 ml of phosphate buffer (0.2 M, pH 7.0), then the reaction mixture was incubated at 37 °C in the dark to accelerate the per-oxidation process. The levels of per-oxidation were determined according to the thiocyanate method by adding ethanol (5 ml, 75%) sequentially, ammonium thiocyanate (0.1 ml, 30%), 0.1 ml of above mixed solution, and ferrous chloride (0.1 ml, 20 mM in 3.5% HCl). The absorbance was measured at 500 nm after mixing for 3 min. Ascorbic acid was used as positive control.

2.7.2. Scavenging effect on superoxide radicals assay

The superoxide radical scavenging abilities of all the samples were assessed by the method of Nishimiki, Rao, and Yagi (1972), In this experiment, superoxide anion radicals were generated in 3 ml of sodium phosphate buffer (100 mM, pH 7.4) containing 1 ml of (NBT) nitroblue tetrazolium (150 μM) solution, 1 ml of (NADH) nicotinamide adenine dinucleotide (468 μM) solution and different concentrations of samples (0.5–400 μg/ml) in water. The reaction was started by adding 1 ml PMS (60 μM) solution to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by a spectrophotometer

against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide radical scavenging activity. The capability of scavenging the superoxide anion radicals was calculated using the following equation:

$$\text{Superoxide anion scavenging activity}(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100,$$

where A_{control} is the absorbance of the control only without sample or standard sample, and A_{sample} is the absorbance of sample or standard sample.

2.7.3. Metal chelating assay

The metal chelating effect of the crude tea polysaccharide was determined according to the ferrous ion chelating assay modified from the method of Carter (1971) with some modifications. A reaction solution composed of 1 ml of samples at different concentrations (0.5–5 mg/ml) and 0.1 ml of $FeCl_2$ (2 mM), shook well and stood for 30 s), then was activated by the addition of 0.2 ml of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, here after referred as ferrozine (5 mM) and 2.7 ml of ultrapure water. After vortex, the reaction mixture was incubated at room temperature for 10 min, and the absorbance of the mixture was measured at 562 nm against a blank. In the control, sample was substituted with EDTA. The ability of different concentrations of samples to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating ability}(\%) = \left[1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}}\right] \times 100$$

where A_{control} is the absorbance of the control only without sample or standard sample, A_{sample} is the absorbance of sample or standard sample, and A_{blank} is the absorbance of the sample only without $FeCl_2$.

2.7.4. Hydroxyl radical scavenging activity

The deoxyribose method for determining the rate of reaction of hydroxyl radical with antioxidant was performed, as described by Wang et al. (2003). Briefly, the reaction mixture, adding 1 ml of different concentrations of samples (0.5–400 μg/ml), was incubated with 0.7 ml of deoxyribose (2.8 mM), 1 ml of phosphate buffer (20 mM, PH 7.4) containing $FeCl_3$ (0.1 mM), ascorbic acid (0.1 mM), EDTA (0.1 mM) and H_2O_2 (1 mM) for 1 h at 37 °C. The extent of deoxyribose degradation was measured by TBA method. The reaction was terminated by adding 1 ml of TCA (1%, w/v) and 0.3 ml of TBA (2.8%, w/v), and then heating the tubes in a boiling water bath for 20 min. After cooling to room temperature, the absorbance of the mixture was measured at 532 nm against a blank. Mannitol was used as positive control. The capability of scavenging to hydroxyl radical was calculated using the following formula:

$$\text{Hydroxyl radical scavenging activity}(\%) = \left[1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}}}\right] \times 100$$

where A_{control} is the absorbance of the control only without samples or standard sample, A_{sample} is the absorbance of samples or the standard sample, and A_{blank} is the absorbance of samples only without addition of deoxyribose.

2.7.5. DPPH radicals assay

The antioxidant activity of crude polysaccharide was measure on the basis of the scavenging activity of DPPH free radical (Blois, 1958). Briefly, 1 ml of the crude polysaccharides extracts at variable concentrations (25–400 μg/ml) was added to 1 ml of DPPH solution (0.2 mM in ethanol) as the free radical source. The decrease in the solution absorbance, due to proton donating activity by TLPS, TFPS and TSPS, was measured at 517 nm after 30 min (Yamaguchi, Takamura, Matoba, & Terao, 1998). The DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging activity}(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{control} is the absorbance of the control only without samples or standard sample, and A_{sample} is the absorbance of samples or standard sample.

2.7.6. Reducing power assay

The reducing power was determined as described previously by Kong et al. (2010). Briefly, 1 ml different concentrations of samples (25–400 μg/ml) in phosphate buffer (0.2 M, pH 6.6) was mixed with 2 ml potassium ferricyanide (1%, w/v), and was incubated at 50 °C for 20 min. Afterwards, 2 ml trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction. After cooling rapidly, the reaction was terminated by adding 2 ml of TCA solution (10%, w/v) and centrifuged at 1750 rpm for 10 min. The supernatant (2.5 ml) mixed with 2.5 ml of distilled water and 1 ml of 0.1% ferric chloride. The absorbance of the reaction mixture was read at 700 nm. Ascorbic acid was used as positive control. Increased absorbance of the reaction mixture indicated increased reducing power.

2.7.7. Lipid peroxidation inhibition assay

The assay was performed by using the method described by Mee, Han, and Ha (2001), but with some modifications. The most prominent assay currently being used as an index for lipid peroxidation in biological systems is the measurement of the thiobarbituric acid reactive substances (TBARS) in the rat liver homogenate according to the thiobarbituric acid (TBA) test (Garcia, Rodrguez-Malaver, & Pealoza, 2005; Gutteridge & Halliwell, 1990). Male rats, weighing 18–25 g (purchased from Sino-British Sippr/BK Lab. Animal Ltd., Co.), were sacrificed, and the livers were excised, rapidly washed with 0.15 M NaCl and homogenate 10% (w/v) was prepared in phosphate buffer (50 mM, pH 7.4) using a pestle in a mortar. The liver homogenate was spun at 10,000 rpm for 10 min. All these steps were carried out at 4 °C. Supernatant of the liver homogenate was collected. Reaction mixtures contained 0.5 ml of the tissue homogenate, 0.9 ml of phosphate buffer (50 mM, pH 7.4), 0.25 ml of $FeSO_4$ (0.01 mM), 0.25 ml of ascorbic acid (0.1 mM), and 0.1 ml of different concentrations of samples. The reaction mixture was incubated at 37 °C for 30 min and then terminated by adding BHT (1 ml of 2% (w/v) in 95% (v/v) ethanol), followed by addition of 1 ml of TCA (20%, w/v) to the mixture, followed by centrifugation (3000 rpm, 10 min). After the addition of 1 ml of the TBA (0.67%) reagent to the supernatant, the tubes were placed in a boiling water bath for 15 min. Absorbance was then measured at 532 nm. BHT was used as positive control.

2.8. Statistics

All the data were expressed as means (standard deviation (SD) of three replications, and Student's test was used for the statistical analysis. The values were considered to be significantly different when the P value was less than 0.05.

3. Results and discussion

3.1. Chemical composition and molecular weight of three polysaccharides

The neutral sugar, uronic acid, protein, polyphenol contents and molecular weight of crude tea polysaccharides are

Table 1
Composition and molecular weight of three polysaccharides.

Sample	Neutral sugar (%)	Uronic acid (%)	Protein (%)	Total phenolic (%)	Molecular weight		
					Mw[a]	Mn[b]	Mw/Mn
TLPS	59.62	24.32	1.87	12.18	758,257	741,382	1.02276
					55,425.6	30,995.4	1.78818
					5520.27	5440.71	1.01462
					3667.34	3636.23	1.00855
TFPS	62.84	14.32	3.64	10.27	1,460,280	1,435,680	1.01714
					765,752	754,624	1.01475
					56694.2	25929.1	2.18651
					2561.32	2439.889	1.04977
TSPS	47.58	22.78	6.72	8.53	961,141	914,860	1.05059
					20445.3	13153.8	1.55432
					3664.11	3617.36	1.01292

[a] Mw, weight-average molecular weight.
[b] Mn, number-average molecular weight.

summarized in Table 1. The neutral sugar of TFPS was higher (62.84%) than TLPS (59.62%) and TSPS (47.58%). The content of polyphenol was relatively lower in TSPS (8.53%) than TFPS (10.27%) and TLPS (12.18%). The uronic acid contents evaluated in TLPS, TFPS and TSPS were 24.32%, 14.32% and 22.78%, respectively. The molecular weight distribution of TLPS, TFPS and TSPS ranged from 3.67×10^3 to 7.58×10^5 Da, 2.56×10^3 to 1.46×10^6 Da and 3.66×10^3 to 9.61×10^5 Da, respectively.

3.2. Monosaccharide composition of three tea polysaccharides

To further investigate effect of different sources on monosaccharide compositions in TLPS, TFPS and TSPS, IC analysis were used. As shown in Fig. 1, IC analysis showed difference of crude tea polysaccharides, with the presence of fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, ribose, GalA, GluA in the molar ratio of 0.29:0.87:1.27:1.00:1.77:0.07:0.11:0.3:2.54:0.24 (Fig. 1A) for TLPS; and rhamnose, arabinose, galactose, glucose, xylose, mannose, GalA, GluA in the molar ratio of 0.42:0.97:1.00:0.36:0.11:0.17:0.71:0.08 (Fig. 1B) for TFPS, also rhamnose, xylose, arabinose, glucose and galactose, GalA, GluA in the molar ratio of 0.35:0.15:0.95:1.95:1.00:0.23:0.07 (Fig. 1C) for TSPS. There was no mannose detected in TSPS.

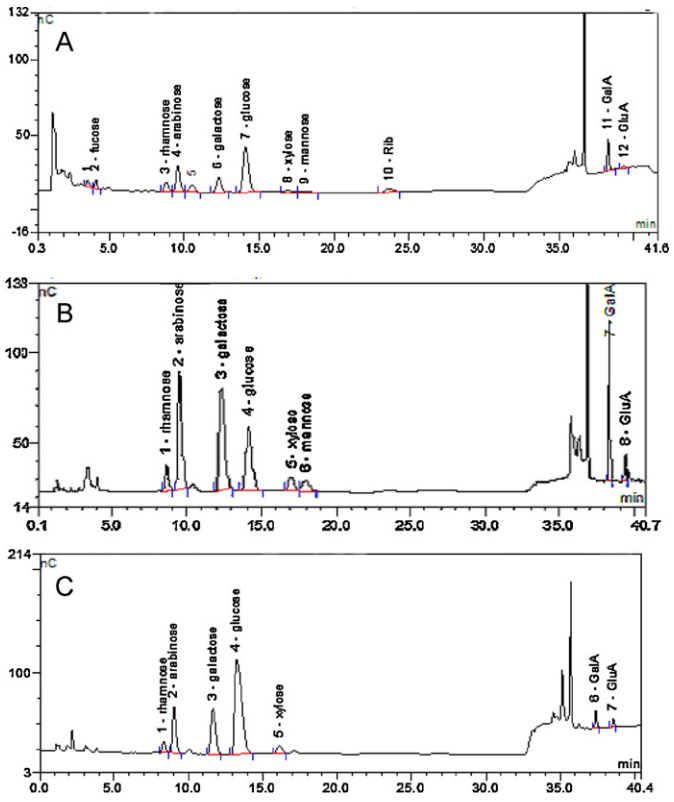

Fig. 1. Ion chromatograms of tea polysaccharides (A) ion chromatograms of TLPS, (B) ion chromatogram of TFPS, (C) ion chromatograms of TSPS.

3.3. IR analysis of three tea polysaccharides

The IR spectra of TLPS, TFPS and TSPS were basically indistinguishable only with some difference in the intensity of bands (Fig. 2). The absorption bands within the range of 3600–3200 cm^{-1}, 3000–2800 cm^{-1}, 1400–1200 cm^{-1} and 1200–1000 cm^{-1} were the characteristic absorption peaks of polysaccharides. The intensity of bands around 3419.1 cm^{-1} was assigned to —OH stretching frequency and as expected it was broad. The weak intensity of band attributed to C—H group stretching (2931.41 cm^{-1}) could also be observed. New bands in the region of 1743.8 cm^{-1} appeared, corresponding to C—O stretching vibration of carbonylic products. The peak at around 1643 cm^{-1} was attributed to N—H vibration or C═O asymmetric vibration of carboxyl group, which was also the characteristic IR absorption of polysaccharide. The absorption at 1433 and 1380 cm^{-1} was possibly due to non-symmetric and symmetric CH_3 bending, respectively. A band at 1250 cm^{-1} due to non-symmetric C—O—C stretching vibration could be observed. The band in the region of 1740 cm^{-1} was acetyl and that at

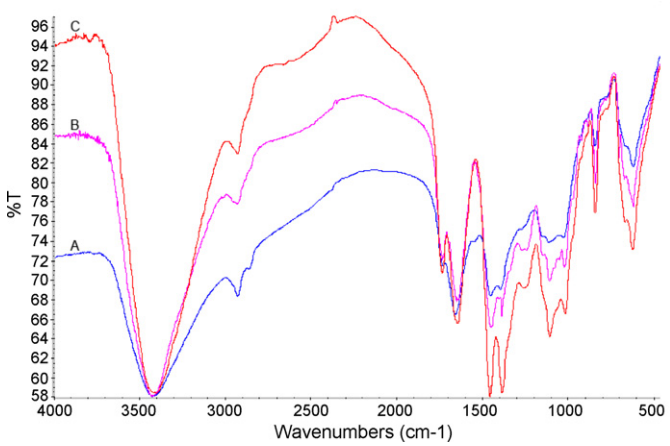

Fig. 2. The IR spectrum of tea polysaccharides (A) the IR spectrum of TLPS, (B) the IR spectrum of TFPS, (C) the IR spectrum of TSPS.

1643 cm^{-1} and 1416 cm^{-1} corresponds to acylamino, which showed that TLPS, TFPS and TSPS were glycoprotein containing polysaccharide and protein.

3.4. Total antioxidant activity

Total antioxidant capacity is an easily and increasingly broadly used parameter employed in clinical studies and in food science, useful in comparison of the antioxidant content of body fluids, cell and tissue homogenates, food, and beverages (Bartosz, 2003). The total antioxidant of TLPS, TFPS and TSPS compared with that of the control which contained no antioxidant component. Fig. 3 shows that the changes of TLPS, TFPS and TSPS (25–400 μg/ml) in the absorbance at 37 °C, compared to ascorbic acid as a positive control during 84 h. Total antioxidant activities of TLPS, TFPS and TSPS increased gradually as time extends. TLPS and TFPS exhibited definite antioxidant activity compared to the control. Moreover, the activities of them were higher than that of ascorbic acid. The antioxidant activities of the polysaccharides can be attributed to the mechanism, the linoleate free radical and other free radicals formed in this model system.

3.5. Superoxide anion scavenging activity

Superoxide anions are a precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage. Superoxide anion would play important roles in the formation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA (Halliwell & Gutteridge, 1984). As shown in Fig. 4B, superoxide scavenging activities of TLPS, TFPS and TSPS increased very significantly ($P < 0.01$) with increasing concentrations in the range of 0.5–100 μg/ml, and the superoxide radical scavenging rate of TLPS, TFPS and TSPS at 400 μg/ml was as high as 90.45%, 78.58% and 58.34%, respectively, with EC_{50} value of TLPS, TFPS and TSPS was 11.37 μg/ml, 18.29 μg/ml and 211.35 μg/ml, respectively. Scavenging activity of TFPS was significantly higher ($P < 0.05$) than that of TLPS, but they significantly higher ($P < 0.01$) than that of TSPS. The EC_{50} value of TLPS, TFPS and TSPS was 11.37 μg/ml, 18.29 μg/ml and 211.35 μg/ml, respectively, which indicated that the scavenging activity of TLPS and TFPS against superoxide radical was stronger than that of ascorbic acid, the latter's EC_{50} value being 57.22 μg/ml. In the system of pyrogallol's autoxidation, TLPS, TFPS and TSPS could inhibit the autoxidation of pyrogallol, which indicated that TLPS, TFPS and TSPS played important roles in antioxidation. The possible mechanism of TLPS, TFPS and TSPS scavenging superoxide radical is that they can combine with superoxide radical ions and themselves forms a stable radical to terminate the radical chain reaction.

3.6. Metal chelating assay

Chelation is an important parameter in the sense that iron is essential for life being required for oxygen transport, respiration and activity of many enzymes. It was reported that the compounds with structures containing two or more of the following functional groups: —OH, —SH, —COOH, $—PO_3H_2$, C═O, $—NR_2$, —S— and —O— in a favorable structure–function configuration can show metal chelation activity (Yuan, Bone, & Carrington, 2005). Moreover, the chelation efficiency of some compounds carrying phenyl group on Fe^{2+} was dependent on the number of hydroxyl groups on benzene ring, and hydroxyl substitution in the ortho position results in an increasing chelating effect. However, iron is an extremely reactive metal and will catalyze oxidative changes in lipids, proteins and other cellular components (Smith, Halliwell, & Aruoma, 1992). Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$). Fe^{3+} ion also produces radicals from peroxides although the rate is less than tenth that of Fe^{2+} ion. Fe^{2+} ion is the most powerful prooxidant among the various species of metal ions (Halliwell & Gutteridge, 1984). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red color of the complex. Measurement of color reduction therefore allows estimating the metal chelating activity of the co-existing chelator. Fig. 5A shows chelating activities of TLPS, TFPS and TSPS to Fe^{2+}. Among the range of 0.5–5 mg/ml, there was no significant difference ($P > 0.05$) in chelating activity between TLPS and TFPS, but ferrous metal ions chelating activity of TSPS was significantly less ($P < 0.05$) than that of TLPS and TFPS. At the concentration of 5 mg/ml, TLPS, TFPS and TSPS could chelated 75.41%, 71.46% and 54.37% of Fe^{2+}. The EC_{50} value of TLPS, TFPS and TSPS was 0.654 mg/ml, 1.428 mg/ml and 4.327 mg/ml, respectively, which indicated that the scavenging activity of TLPS and TFPS against superoxide radical was stronger than that of TSPS. Metal chelating capacity is significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. TLPS, TFPS exhibited a stronger chelating activity, which suggested that hydroxyl and acetyl substitution of polysaccharide is desirable for metal chelating activity. The number of acetyl substitution of TLPS and TFPS was more than that of TSPS, which led to that TLPS and TFPS showed stronger $Fe2^{+}$-chelating ability than TSPS.

3.7. Hydroxyl radical scavenging activity

Except for superoxide radical, hydroxyl radical is considered to be a highly potent oxidant, which can react with all biomacromolecules functioning in living cells (Gülçin, 2006). As shown in Fig. 4A, scavenging activities of TSPS against hydroxyl radical was significantly less than that of TLPS and TFPS in the range of 25–400 μg/ml ($P < 0.01$). The scavenging rate of TLPS, TFPS and TSPS against hydroxyl radical was 64.51%, 80.24% and 40.91% at 400 μg/ml, with EC_{50} value of TLPS, TFPS and TSPS was 88.32 μg/ml, 102.37 μg/ml and 487.25 μg/ml, respectively. These results showed that induced deoxyribose cleavage in a concentration-dependent manner. The scavenging effect of TLPS, TFPS and TSPS increased with increasing sample concentration. The scavenging activity of TFPS was close to mannitol at 400 μg/ml. As shown from Fig. 4A, TLPS and TFPS had a stronger hydroxyl radical scavenging activity, but TSPS had a moderate hydroxyl radical scavenging activity. Previous studies of the antioxidant activity of various natural plant derived biomolecules have suggested that the scavenging activity for hydroxyl radicals was not due to directly scavenging but due to inhibition of hydroxyl radical generation by chelating ions such as Fe^{2+} and Cu^{+} (Diplock, 1997). In the former experiment, we demonstrated that TFPS and TLPS had higher chelating activity for Fe^{2+} than that of TSPS, which was in accorded with our results that TFPS and TLPS exhibited a higher scavenging rate against hydroxyl radical than TSPS. It is likely that this chelating effect might be responsible for the inhibition of Fe^{2+} oxidation.

3.8. DPPH radical scavenging assay

DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al., 1998). Further it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability. DPPH is a free radical compound and has been used widely to test the free radical-scavenging ability of various samples.

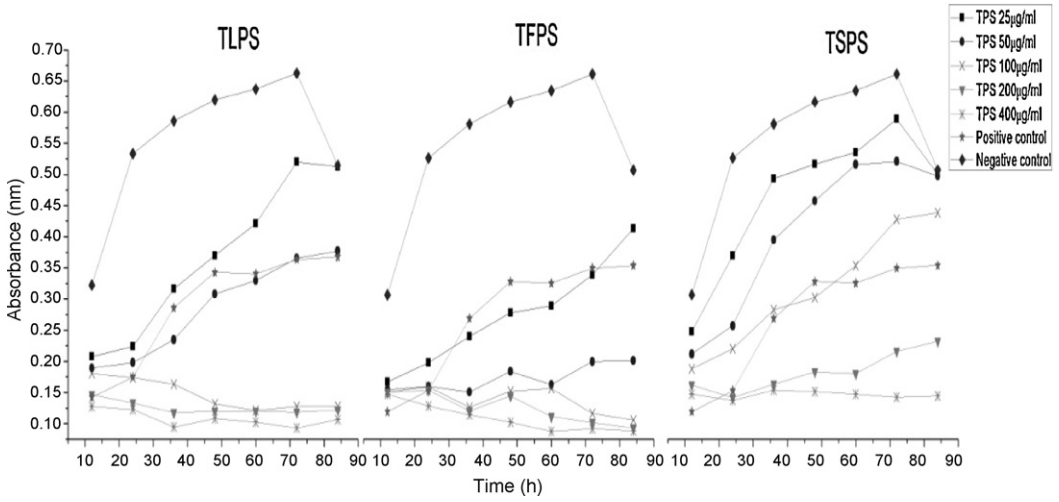

Fig. 3. Total antioxidant activity of TLPS, TFPS and TSPS. Each value represents the mean ± SD ($n = 3$).

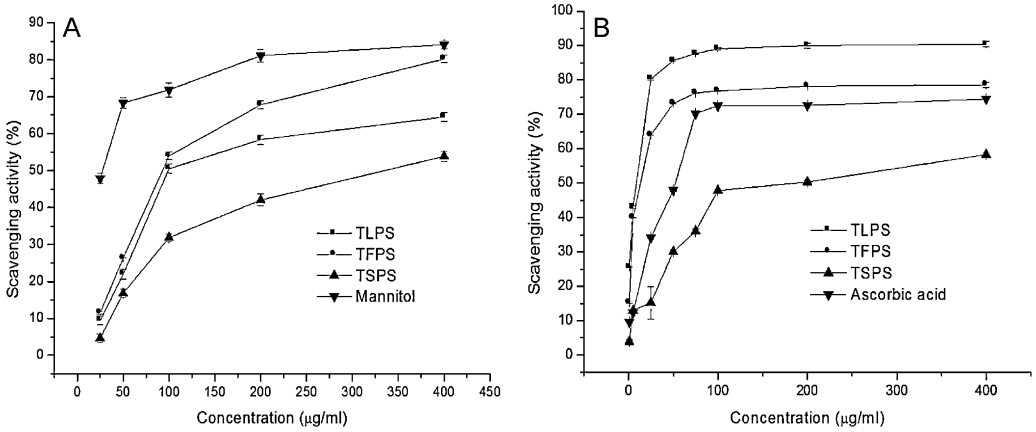

Fig. 4. (A) Hydroxyl radical scavenging activity of TLPS, TFPS and TSPS. Each value represents the mean ± SD ($n = 3$). (B) Superoxide anion scavenging activity of TLPSTFPS and TSPS. Each value represents the mean ± SD ($n = 3$).

Antioxidants react with DPPH, reducing a number of DPPH molecules equal to the number of available hydroxyl groups (Matthaus, 2002). Total DPPH scavenging effects of all samples at varying concentrations were measured and the results were depicted in Fig. 6A, The scavenging effects of TLPS, TFPS and TSPS extracts increased with increasing concentration between 25 and 400 μg/ml and the superoxide radical scavenging rate of TLPS, TFPS and TSPS at 400 μg/ml was as high as 93.11%, 88.72% and 78.47%, respectively. The EC_{50} value of TLPS, TFPS and TSPS was 64.17 μg/ml, 92.27 μg/ml and 176.36 μg/ml, respectively. In the range of 5–200 μg/ml, scavenging activity of TFPS was higher ($P < 0.05$) than that of TLPS in the range of 200–400 μg/ml. However, the concentration of total phenolic in them was decreased in the order of TLPS > TFPS, indicating that the DPPH radical scavenging activity of them may be due to the carboxy group in hexuronic acid but not to phenolic compounds.

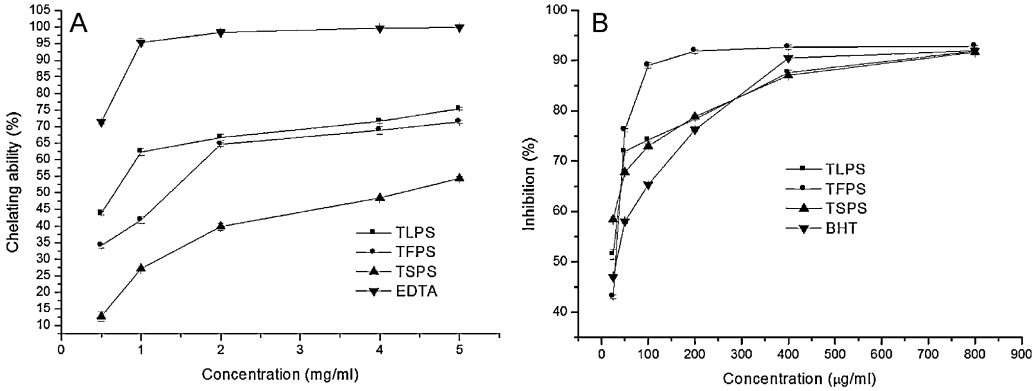

Fig. 5. (A) Metal chelating ability of TLPS, TFPS and TSPS. Each value represents the mean ± SD ($n = 3$). (B) Inhibition by TLPS, TFPS and TSPS on Fe^{2+}/ascorbate induced lipid peroxidation of mouse liver homogenates. Each value represents the mean ± SD ($n = 3$).

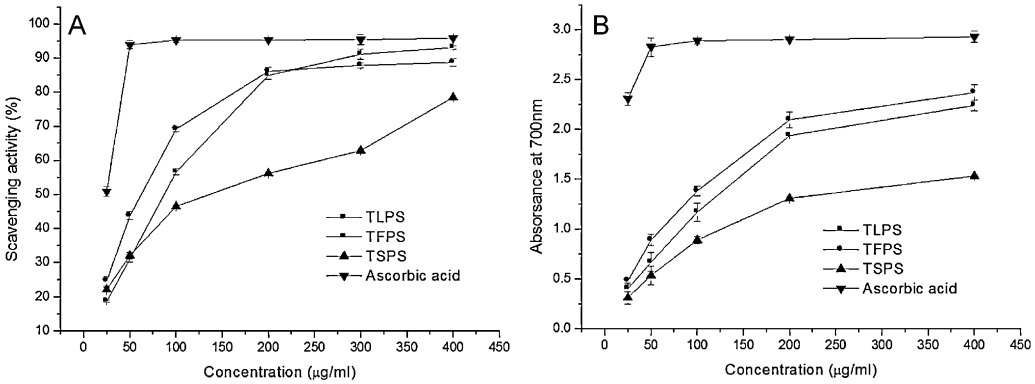

Fig. 6. (A) DPPH radical scavenging activity of TLPS, TFPS and TSPS. Each value represents the mean ± SD ($n = 3$). (B) Reducing power of TLPS, TFPS and TSPS. Each value represents the mean ± SD ($n = 3$).

3.9. Reducing power assay

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities (Meir, Kanner, Akiri, & Hadas, 1995). Fig. 6B shows the reductive activity of TLPS, TFPS and TSPS using the potassium ferricyanide reduction method. The reducing power of TLPS and TFPS increased significantly ($P < 0.01$) with increasing concentration of samples. Among the range of 25–400 μg/ml, there was no significant difference ($P > 0.05$) in reductive power between TLPS and TFPS, but reductive power of TLPS and TFPS was significantly higher ($P < 0.01$) than that of TSPS. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. TLPS, TFPS and TSPS have been found consist of fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, ribose, GalA and GluA; rhamnose, arabinose, galactose, glucose, xylose, mannose, GalA and GluA; rhamnose, xylose, arabinose, glucose, galactose, GalA and GluA, respectively. These monosaccharides are actually potent reductive agents as they have a hidden aldehyde moiety as illustrated in Fig. 2. Accordingly, the antioxidant mechanism of TLPS, TFPS and TSPS is apparently based on the reductive nature of these monosaccharides.

3.10. Non-enzymatic lipid peroxidation induced by Fe^{2+}/ascorbate

As shown in Fig. 5B, in the mouse microsomal lipid peroxidation system as well as in the iron-induced microsomal lipid peroxidation system, Fig. 5B shows that the inhibiting effects on the lipid peroxidation of TLPS, TFPS and TSPS was similar, the inhibiting effects of the tested samples were concentration-dependent, and they showed significant antioxidant activity. Inhibition rose from 43.09% to 92.82% for TLPS, 51.44% to 91.90% for TFPS, and 58.44% to 91.65% for TSPS with the concentration increasing in the lipid spontaneous peroxidation system. In the iron-induced mouse microsomal lipid peroxidation system, TLPS, TFPS and TSPS possessed higher lipid peroxidation inhibiting ability than that of BHT.

Lipid peroxidation (LPO) is associated with various pathological events, such as inflammation, postischemic reperfusion injury, atherosclerosis, ethanol toxicity, and cancer. These pathological phenomena of the body may be the result of ability of MDA to covalently bond and to crosslink a variety of biological macromolecules (Nair, Cooper, Vietti, & Turner, 1986). The free radical chemistry of lipid peroxidation is complex. The generation of the initiator molecule is followed by chain initiation, propagation, branching and termination reactions in this complex system. Lipid peroxidation can be initiated through free radical reactions. In the preliminary assay of iron-induced lipid peroxidations, the presence and involvement of metal ions and oxygen induced generation of free radicals. The results suggested that there was a combination between TLPS, TFPS and TSPS and metal ions, which could interfere with the free radical reaction chains.

On the basis of the results above, it can be obviously concluded that TLS and TFPS had stronger antioxidant activity, but TSPS had lower antioxidant activity, suggesting the activity of them may be ascribed to the higher molecular weight. TFPS had higher activity for DPPH, lipid peroxidation inhibition, scavenging activity of hydroxyl radical and reducing power than TLPS. However, the concentration of total phenolic in them was decreased in the order of TLPS > TFPS, indicating polysaccharide parts in them played key role in their activity. Thus, the antioxidant and free radical scavenging activities of the polysaccharide were not a function of a single factor but a combination of several factors. The exact mechanism underlying the radical scavenging activity and antioxidant activity exerted by polysaccharides needs to be further investigated.

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References

Ardestani, A., & Yazdanparast, R. (2007). Antioxidant and free radical scavenging potential of *Achillea santolina* extracts. *Food Chemistry*, *104*(1), 21–29.

Bartosz, G. (2003). Total antioxidant capacity. *Advances in Clinical Chemistry*, *37*, 219–292.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181*(4617), 1199–1200.

Blumenkrantz, N., & AsboeHansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*(2), 484–489.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

Carter, P. (1971). Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Analytical Biochemistry*, *40*(2), 450–458.
Chen, H. X., & Xie, B. J. (2002). The preventive and curative effect on diabetic mice of tea polysaccharides. *Acta Nutrimenta Sinca*, *24*, 85–86.
Chen, H. X., Zhang, M., & Xie, B. J. (2005). Components and antioxidant activity of polysaccharide conjugate from green tea. *Food Chemistry*, *90*(1–2), 17–21.
Diplock, A. T. (1997). Will the 'good fairies' please prove to us that vitamin E lessens human degenerative of disease? *Free Radical Research*, *27*, 511–532.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.
Garcia, Y. J., Rodrguez-Malaver, A. J., & Pealoza, N. (2005). Lipid peroxidation measurement by thiobarbituric acid assay in rat cerebellar slices. *Journal of Neuroscience Methods*, *144*(1), 127–135.
Gülçin, I. (2006). Antioxidant and antiradical activities of L-carnitine. *Life Science*, *78*, 803–811.
Gutteridge, J. M. C., & Halliwell, B. (1990). The measurement and mechanism of lipid peroxidation in biological systems. *Trends in Biochemical Science*, *15*(4), 129–135.
Halliwell, B., & Gutteridge, J. M. C. (1984). Oxygen toxicology, oxygen radicals, transition metals and disease. *Journal of Biochemistry*, *219*, 1–4.
Kardosova, A., & Machova, E. (2006). Antioxidant activity of medicinal plant polysaccharides. *Fitoterapia*, *77*, 367–373.
Khokhar, S., & Magnusdottir, S. G. M. (2002). Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *Journal of Agricultural and Food Chemistry*, *50*(3), 565–570.
Kong, F., Zhang, M., Kuang, R., Yu, S., Chi, J., & Wei, Z. (2010). Antioxidant activities of different fractions of polysaccharide purified from pulp tissue of litchi (Litchi chinensis Sonn). *Carbohydrate Polymer*, *81*(3), 612–616.
Matthaus, B. (2002). Antioxidant activity of extracts obtained from residues of different oilseeds. *Journal of Agricultural and Food Chemistry*, *50*, 3444–3452.
Mee, H. K., Han, J. H., & Ha, C. S. (2001). Identification and antioxidant activity of novel chlorogenic acid derivatives from bamboo (phyllostachys edulis). *Journal of Agricultural and Food Chemistry*, *49*, 4646–4655.
Meir, S., Kanner, J., Akiri, B., & Hadas, S. P. (1995). Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *Journal of Agricultural and Food Chemistry*, *43*, 1813–1815.
Nair, V., Cooper, C. S., Vietti, D. E., & Turner, G. A. (1986). The chemistry of lipid peroxidation metabolites: Crosslinking reactions of malondialdehyde. *Lipids*, *21*(1), 6–10.
Nishimiki, M., Rao, N. A., & Yagi, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine metho sulfate and molecular oxygen. *Biochemical and Biophysical Research Communications*, *46*, 849–853.
Smith, C., Halliwell, B., & Aruoma, O. I. (1992). Protection by albumin against the prooxidant actions of phenolic dietary components. *Food Chemistry Toxicology*, *30*, 483–489.
Wang, Q. J., Ding, F., Zhu, N. N., Li, H., He, P. G., & Fang, Y. Z. (2003). Determination of hydroxyl radical by capillary zone electrophoresis with amperometric detection. *Journal of Chromatography A*, *1016*, 123–128.
Warrand, J. (2006). Healthy polysaccharides. *Food Technology and Biotechnology*, *44*(3), 355–370.
Yamaguchi, T., Takamura, H., Matoba, T., & Terao, J. (1998). HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Bioscience, Biotechnology, and Biochemistry*, *62*, 1201–1204.
Yang, R. Z., Zhang, J. S., Tang, Q. J., & Pan, Y. J. (2005). High performance anion exchange chromatography method to determine the monosaccharide composition of polysaccharides. *Edible Fungi (China)*, *24*, 42–44.
Yu, F., Sheng, J., Xu, J., An, X., & Hu, Q. (2007). Antioxidant activities of crude tea polyphenols, polysaccharides and proteins of selenium-enriched tea and regular green tea. *European Food Research and Technology*, *225*(5–6), 843–848.
Yuan, Y. V., Bone, D. E., & Carrington, M. F. (2005). Antioxidant activity of dulse (*Palmaria palmata*) extract evaluated in vitro. *Food Chemistry*, *91*, 485–494.