Carbohydrate Polymers 101 (2014) 990–997

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Characterization, antioxidant and hepatoprotective activities of polysaccharides from *Ilex latifolia* Thunb

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ARTICLE INFO

Article history:
Received 11 September 2013
Received in revised form 6 October 2013
Accepted 12 October 2013
Available online 21 October 2013

Keywords:
Kudingcha
Ilex latifolia Thunb
Polysaccharide
Antioxidant activity
Hepatoprotective activity

ABSTRACT

Crude polysaccharides from the leaves of *Ilex latifolia* Thunb (ILPS) was fractionated by DEAE cellulose-52 chromatography, affording four fractions of ILPS-1, ILPS-2, ILPS-3 and ILPS-4 in the recovery rates of 32.3, 20.6, 18.4 and 10.8%, respectively, based on the amount of crude ILPS used. The four fractions were mainly composed of arabinose and galactose in monosaccharide composition. Compared with ILPS-1 and ILPS-2, ILPS-3 and ILPS-4 had relative higher contents of sulfuric radical and uronic acid. The antioxidant activities *in vitro* of ILPS decreased in the order of crude ILPS > ILPS-4 > ILPS-3 > EPS-2 > ILPS-1. Furthermore, the administration of crude ILPS significantly prevented the increase of serum alanine aminotransferase and aspartate aminotransferase levels, reduced the formation of malondialdehyde and enhanced the activities of superoxide dismutase and glutathione peroxidase in carbon tetrachloride-induced liver injury mice. The results suggested that ILPS should be a potent natural polymer with antioxidant and hepatoprotective activities.

1. Introduction

Reactive oxygen species (ROS), including free radicals such as superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), play important roles in degenerative and pathological processes such as cancer, aging, inflammation and fibrosis (Grivennikov, Greten, & Karin, 2010; Sánchez-Valle, Chávez-Tapia, Uribe, & Méndez-Sánchez, 2012). It has been reported that the mammalian cells constantly exposed to excessively high level of free radicals or ROS may lead to detrimental effects such as lipid peroxidation of cellular membranes, enzyme inactivation, DNA breakage, alteration of lipid–protein interaction and eventually the promotion of mutations that initiate tumor progression (Kim et al., 2013; Marczak & Bukowska, 2013). Although almost all organisms possess antioxidant and repair systems to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely. Therefore, more attentions have been paid to natural antioxidants that can effectively prevent senescence. In the past two decades, some natural compounds such as polyphenols, flavonoids and polysaccharides from Chinese traditional tea beverages including green tea, Oolong tea, Pu-erh tea, sweet tea and kudingcha have been demonstrated to have antioxidant activity and could be explored as natural potential antioxidants to prevent the damage of ROS (Chen et al., 2011; Sugiyama, He, Wada, & Saeki, 1999; Xie et al., 2010; Xu, Ye, Sun, Tu, & Zeng, 2012; Zhu et al., 2009).

Kudingcha, a kind of herbal tea, is a popular functional tea beverage in China and other Southeast Asia countries including Singapore, Malaysia and Vietnam. As an ancient traditional medicinal tea, it has been consumed for nearly 2000 years in China. In traditional Chinese medicine, kudingcha has been used in the formulae for treating obesity, hypertension, cardiovascular disease, hyperlipidemia and various other diseases. There are more than 30 known species from 13 genera original plants for making kudingcha in China (Zhu et al., 2009), and the main species are *Ilex kudingcha*, *I. latifolia* and *I. cornuta*, which belong to the same genus as mate (*I. paraguariensis*) (Filip, Lotito, Ferraro, & Fraga, 2000; Hao et al., 2013). Recently, more attentions have been paid to the extraction and identification of main chemicals in kudingcha such as triterpenoids, phenolic acids, flavonoids and essential oils and their potential healthy benefits in antioxidant, anti-obesity, neuroprotective, antidiabetic, hepatoprotective and anti-inflammatory effects (Chen, Li, & Xie, 1995; Fan et al., 2012; Hao et al., 2013; Huang, Ogihara, Shimizu, Takeda, & Akiyama, 2000; Kim et al., 2012; Li et al., 2011, 2013; Liu et al., 2009; Nishimura, Fukuda, Miyase, Noguchi, & Chen, 1999; Song, Xie, Zhou, Yu, & Fang, 2012;

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http://dx.doi.org/10.1016/j.carbpol.2013.10.037

Zheng et al., 2009; Zhou et al., 2012). To our knowledge, little information about the compositions and bioactivity of polysaccharides from *I. latifolia* Thunb (ILPS) (Sun et al., 2010) is available compared with that of polysaccharides from *I. kudingcha* or *Camellia sinensis* (Lin, Chen, Su, & Chen, 2008; Nie & Xie, 2011; Sun, Hu, Zhang, Ye, & Zeng, 2009; Wang, He, & Liu, 2008). In fact, polysaccharides that distribute widely in animals, plants and microorganisms have been demonstrated to play an important role as dietary free radical scavenger in the prevention of oxidative damage in living organism and can be exploited as novel potential antioxidants (Kardošová & Machová, 2006; Tsiapali et al., 2001; Wang et al., 2013). In addition, it has been reported that polysaccharides possess a wide range of biological properties such as hypoglycemic, immunomodulatory and anti-cancer activities (Nie & Xie, 2011; Zong, Cao, & Wang, 2012). The objectives of present study, therefore, were to characterize the preliminary structure and investigate the *in vitro* antioxidant activity and *in vivo* protective effects against carbon tetrachloride (CCl_4)-induced hepatic damage in mice of ILPS.

2. Materials and methods

2.1. Materials

The sample of kudingcha made from the leaves of *I. latifolia* Thunb was kindly provided by the Department of Tea Science, Zhejiang University (Hangzhou, China). The sample was ground into powder using a milling machine and the material that passed through an 80-mesh sieve was kept in sealed polyethylene bags at room temperture until the use for extraction. The female Kunming mice were purchased from the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). Arabinose, rhamnose, fucose, xylose, galactose, glucose, glucuronic acid, mannose, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), ferrozine, nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), reduced nicotinamide adenine dinucleotide (NADH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Assay kits for protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were of analytical grade.

2.2. Preparation of ILPS

The preparation of crude ILPS was carried out according to the reported method with some modifications (Xu et al., 2012). Briefly, the powder of *I. latifolia* Thunb was pre-extracted two times with 85% aqueous ethanol solution (v/v) in a ratio of 1:15 (material to ethanol solution, g/mL) at 80 °C for 1 h each, and the supernatant (containing colored materials, oligosaccharides and small molecule compounds) was removed. The resulting residues were extracted three times with distilled water in a ratio of material to water 1:20 (g/mL) at 90 °C for 3 h each, and the extracts were centrifuged at 5000 rpm for 15 min. The supernatants were combined and concentrated by a rotary evaporator to a proper volume. The resulting concentrate was mixed with three times volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitates were then collected by centrifugation at 5000 rpm for 15 min, dissolved in distilled water, dialyzed against distilled water and lyophilized, affording the crude ILPS.

The crude ILPS was purified by chromatography of DEAE cellulose-52 according to the reported method (Qiao et al., 2009a; Ye, Wang, Zhou, Liu, & Zeng, 2008). Briefly, 150 mg of crude ILPS was dissolved in 7.5 mL deionized water, and the solution was filtered through a 0.45 μm membrane filter. The resulting ILPS solution was then loaded onto a column (2.6 cm × 30 cm) of DEAE cellulose-52, and the column was stepwise eluted with deionized water, 0.1, 0.3 and 0.5 M sodium chloride (NaCl) solution at a flow rate of 1.0 mL/min. The eluate was collected automatically (10 mL/tube) and the carbohydrate in each tube was determined by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The resulting fractions were pooled, concentrated, dialyzed against deionized water and lyophilized, respectively, affording four fractions, named ILPS-1, ILPS-2, ILPS-3 and ILPS-4.

2.3. Preliminary characterization of ILPS

2.3.1. Determination of contents of carbohydrate, sulfuric radical, protein and uronic acid

The contents of carbohydrate in crude ILPS and its purified fractions were determined by phenol–sulfuric acid method using glucose as the standard. The content of protein was determined according to the reported method (Bradford, 1976) using bovine serum albumin as the standard. The content of sulfate radical was determined according to the reported method (Doigson & Price, 1962), and the content of uronic acid was determined according to the method reported by Blumenkrantz and Asboe-Hansen (1973) using D-glucuronic acid as the standard.

2.3.2. Analysis of monosaccharide composition

The monosaccharide compositions of crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4 were analyzed by gas chromatography (GC) according to the reported method with slight modifications (Qiao et al., 2009a). Briefly, the polysaccharide sample was hydrolyzed with 2 mL trifluoroacetic acid (2 M) for 2 h in an oven (120 °C). The hydrolyzate was repeatedly co-concentrated with methanol to dryness and acetylated by the addition of a mixture of methanol, pyridine and acetic anhydride. The monosaccharide standards of rhamnose, arabinose, fucose, xylose, mannose, glucose and galactose were acetylated in a similar manner. All the derivatives were then analyzed by a 6890N GC (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector and an HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The operation conditions of GC were as following: flow rates of N_2, H_2 and air were 25, 30 and 400 mL/min, respectively; the temperatures of oven, detector and inlet were set at 210, 280 and 250 °C, respectively. The injection volume was 1 μL aliquot for each run.

2.3.3. Fourier-transform infrared spectrometric analysis

The fourier-transform infrared (FT-IR) spectra of all ILPS samples were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The dried polysaccharide sample was ground with potassium bromide powder and pressed into pellet for spectrometric measurement in the frequency range of 4000–400 cm^{-1}.

2.4. Assay of antioxidant activity in vitro of ILPS

There are several methods for determination of antioxidant activity *in vitro*. In the present study, four of the methods, assays of scavenging activities on DPPH free radical, $O_2^{\bullet -}$ and •OH, based on reaction with electron-donating or hydrogen radicals (H·) producing compounds/antioxidants according to the reaction R· + Aox-H → RH + Aox·, and assay of metal ion chelating ability, were used.

2.4.1. Assay of scavenging activity on DPPH free radical

The scavenging activity on DPPH free radicals was measured by using the reported method with some modifications (Luo et al., 2010). Briefly, 0.3 mL DPPH• solution (400 μmol/L in dehydrate

alcohol) was added to 3.0 mL of ILPS solution, and the mixture was incubated at 30 °C for 30 min in the dark. Then, the absorbance (Abs) at 517 nm was measured. Deionized water and ascorbic acid (V_C) were used as the blank and positive control, respectively, and the scavenging activity was calculated by the following equation:

$$\text{Scavenging activity}(\%) = [\text{Abs}_0 - (\text{Abs}_1 - \text{Abs}_2)]/\text{Abs}_0 \times 100$$

where Abs_0 is the Abs of the control (water instead of sample solution), Abs_1 is the Abs of the sample and Abs_2 is the Abs of the sample under identical conditions as Abs_1 with dehydrate alcohol instead of DPPH• solution.

2.4.2. Assay of superoxide anion radical scavenging activity

Assay of scavenging activity on $O_2^{\bullet -}$ was performed based on the reported method (Qiao et al., 2009b) with some modifications. Each 1.0 mL of sample solution, NBT solution (156 μM of NBT in 0.2 M phosphate buffer, pH 7.4) and NADH solution (468 μM of NADH in 0.2 M phosphate buffer, pH 7.4) were mixed. The reaction was started by adding 1.0 mL of PMS solution (60 μM PMS in 0.2 M phosphate buffer, pH 7.4) to the mixture. Then, the reaction mixture was incubated at room temperature for 5 min, and the Abs at 560 nm was read against a blank (water and 0.2 M phosphate buffer instead of sample solution and NBT solution, respectively). The scavenging activity on $O_2^{\bullet -}$ was calculated using the following equation:

$$\text{Scavenging activity}(\%) = [\text{Abs}_0 - (\text{Abs}_1 - \text{Abs}_2)]/\text{Abs}_0 \times 100$$

where Abs_0 is the Abs of the control (water instead of sample solution), Abs_1 is the Abs of the sample solution and Abs_2 is the Abs of the sample solution under identical conditions as Abs_1 with 0.2 M phosphate buffer instead of NBT solution.

2.4.3. Assay of hydroxyl radical scavenging activity

The hydroxyl radical (•OH) scavenging activity was measured according to the reported method (Jin, Cai, Li, & Zhao, 1996) with some modifications. The •OH was generated in a mixture of 1.0 mL of 0.75 mM 1,10-phenanthroline, 2.0 mL of 0.2 M sodium phosphate buffer (pH 7.4), 1.0 mL of 0.75 mM $FeSO_4$ and 1.0 mL of H_2O_2 (0.01%, v/v). After addition of 1.0 mL sample solution, the mixture was incubated for 30 min at 37 °C. Then, the Abs of the mixture at 536 nm was measured. Deionized water and V_C were used as the blank and positive control, respectively. The scavenging activity on •OH was calculated by the following equation:

$$\text{Scavenging activity}(\%) = (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})/(\text{Abs'} - \text{Abs}_{\text{blank}}) \times 100$$

where Abs' is the Abs of the deionized water instead of H_2O_2 and sample in the assay system.

2.4.4. Assay of metal ion chelating activity

The metal ion chelating activity of ILPS in terms of chelating ferrous ion (Fe^{2+}) in the iron–ferrozine complex was measured according to the reported method (Decker & Welch, 1990) with minor modifications. Briefly, 1.0 mL ILPS solution, 0.05 mL ferrous chloride solution ($FeCl_2$, 2.0 mmol/L), 0.2 mL ferrozine solution (5.0 mmol/L) and 2.25 mL deionized water were mixed and the mixture was kept at room temperature for 10 min. Then, the Abs at 562 nm was measured against a blank (deionized water instead of ILPS and $FeCl_2$ solution). The metal ion chelating activity was calculated as follows:

$$\text{Chelating activity}(\%) = [\text{Abs}_0 - (\text{Abs}_1 - \text{Abs}_2)]/\text{Abs}_0 \times 100$$

where Abs_0 is the Abs of the control (deionized water instead of ILPS solution), Abs_1 is the Abs of the test sample and Abs_2 is the Abs of the sample under identical conditions as Abs_1 with deionized water instead of $FeCl_2$ solution.

2.5. Assay of hepatoprotective effects in vivo of crude ILPS

2.5.1. Animal grouping and experimental design

In the present study, the female Kunming mice (8-week-old, 20 ± 2 g of body weight (BW) and free of specific pathogens) were used to evaluate the hepatoprotective activity of crude ILPS against CCl_4-induced acute liver injury. All procedures involving animals were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals during the entire experimental period. Briefly, the mice were handled under standard laboratory conditions of a 12 h light/dark cycle in a room maintained at a temperature of 22 ± 0.5 °C and the animals had access to food and water ad labium. After adapting to their environment for 1 week, the mice were randomly divided into six groups (eight mice for each group). Mice in Group I (normal control) and Group II (CCl_4 model control) were treated with 0.9% sodium chloride (25 mL/kg BW per day). Mice in Group III (positive control) were treated with a hepatoprotective drug (silymarin, 100 mg/kg BW per day). Mice in Groups IV (low dose of crude ILPS), V (medium dose of crude ILPS) and VI (high dose of crude ILPS) were treated with 100, 200 and 400 mg/kg BW of crude ILPS per day, respectively. All the treatments were conducted by gastric gavage for 14 consecutive days on daily basis. Then, CCl_4 was administrated *via* intraperitoneal injection at 150 μL/100 g BW of a 1:1 (v/v) mixture of CCl_4 and corn oil to induce acute liver injury (Simile et al., 2001). The mice in Groups II–VI received a single dose of CCl_4 on the 15th day, while Group I received an equal amount of corn oil instead of CCl_4. The blood samples were collected by retro-orbital bleeding into 1.5 mL sharp-bottomed epoxy centrifuge tubes, and all the mice were sacrificed by cervical dislocation at 24 h post-injection of CCl_4 or corn oil. The collected blood samples were immediately centrifuged at 4000 *g* for 10 min at 4 °C to afford the serums. Then, the resulting serums were stored at −80 °C until the use of ALT and AST assay. The liver was excised, homogenized immediately in 0.1 g/mL wet weight of ice-cold physiological saline, centrifuged at 3000 rpm for 10 min, and the supernatant was collected for biochemical analysis.

2.5.2. Biochemical assay

The activities of ALT and AST in serum, protein content, level of MDA and activities of SOD and GSH-Px in liver were determined by using commercial reagent kits according to the instruction manuals.

2.6. Statistical analysis

Data were expressed as mean ± standard deviation (SD) (at least three replicates except for the animal study) and evaluated by one way analysis of variance (ANOVA) followed by the Duncan's multiple-range tests. All statistical analysis was carried out by using SAS for Windows. Difference was considered to be significant at a level of $P < 0.05$.

3. Results and discussion

3.1. Preparation and preliminary characterization of ILPS

In the present study, ILPS was extracted by using hot water since the traditional water extraction was an ideal way to extract plant polysaccharides from a variety of biological materials (Wei et al., 2010). As a result, crude ILPS was obtained in an overall yield of 6.3%. As shown in Fig. 1, the crude ILPS was fractionated into four polysaccharide fractions by anion-exchange chromatography of DEAE cellulose-52. Moreover, the recovery rates of ILPS-1, ILPS-2, ILPS-3 and ILPS-4 were 32.3, 20.6, 18.4 and 10.8%, respectively, based on the amount of crude ILPS used. The contents of carbohydrate, sulfate, protein and uronic acid in crude ILPS and its

Table 1
The contents of carbohydrate, protein, uronic acid and sulfuric radical for crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4.

Sample	Crude ILPS	ILPS-1	ILPS-2	ILPS-3	ILPS-4
Carbohydrate (%)	61.3 ± 1.3	81.8 ± 2.1	64.5 ± 1.5	72.2 ± 1.7	65.6 ± 1.6
Protein (%)	5.8 ± 0.3	1.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	6.0 ± 0.5
Uronic acid (%)	21.8 ± 1.1	1.6 ± 0.1	11.8 ± 0.1	20.4 ± 1.2	23.2 ± 1.3
Sulfuric radical (%)	2.5 ± 0.2	2.2 ± 0.1	2.6 ± 0.2	3.3 ± 0.3	3.7 ± 0.4

four purified fractions are shown in Table 1. Notably, ILPS-4 contained the highest contents of sulfuric radical (3.7%) and uronic acid (23.2%), while ILPS-1 contained the highest carbohydrate content (81.8%). Accordingly, ILPS-1 should be a neutral polysaccharide as it was eluted by water from a DEAE cellulose-52 anion-exchange column, while ILPS-2, ILPS-3 and ILPS-4 should be acidic polysaccharides (Mukhiddinov, Khalikov, Abdusamiev, & Avloev, 2000) since they were eluted by 0.1, 0.3 and 0.5 M NaCl solutions, respectively. These results could be verified by the fact that ILPS-2, ILPS-3 and ILPS-4 had much higher contents of uronic acid than ILPS-1.

The monosaccharide compositions of crude ILPS and its purified fractions (ILPS-1, ILPS-2, ILPS-3 and ILPS-4) were determined and are presented in Fig. 2 and Table 2. It was observed that crude ILPS was composed of rhamnose, arabinose, fucose, xylose, mannose, glucose and galactose in a molar percentage of 19.9, 44.6, 1.7, 1.5, 4.4, 4.7 and 23.2%, respectively. There is difference between the monosaccharide compositions of ILPS and polysaccharide from the leaves of *I. kudincha* C.J. Tseng which was reported to be composed of arabinose, xylose, mannose, galactose and glucose in a molar ratio of 10.2:1.0:1.2:8.0:5.4, respectively (He, Chen, & Lan, 2007). Compared with crude ILPS, mannose was not detected in ILPS-1, fucose was not detected in ILPS-2 and ILPS-3, mannose, xylose, glucose and fucose were not found in ILPS-4. Accordingly, ILPS-1, ILPS-2, ILPS-3 and ILPS-4 were mainly composed of arabinose and galactose.

As shown in Fig. 3, strong and broad absorption peaks at 3500–3000 cm^{-1} for O—H stretching vibrations, peaks at 3000–2800 cm^{-1} for C—H stretching vibrations, and a strong extensive absorption in the region of 1000–1200 cm^{-1} for stretching vibrations of C—OH side groups and the C—O—C glycosidic band vibrations (Kacurakova, Capek, Sasinkova, Wellner, & Ebringerova, 2000) were observed in the FT-IR spectra of crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4. All these characteristic absorptions indicated that crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4 were polysaccharides (Peng, Li, Ou, Jiang, & Zeng, 2010). Furthermore, absorption peaks at 1650 and 1420 cm^{-1} for the asymmetric and symmetric stretching of carboxylate anions groups indicated that there were carboxyl groups in ILPS. Besides, absorption peak at 1240 cm^{-1} was assigned to the stretching vibrations of S=O (Percival & Wold, 1963), indicating ILPS was sulfated polysaccharides. All the results of FT-IR characterization are in coincidence with the results of Table 1.

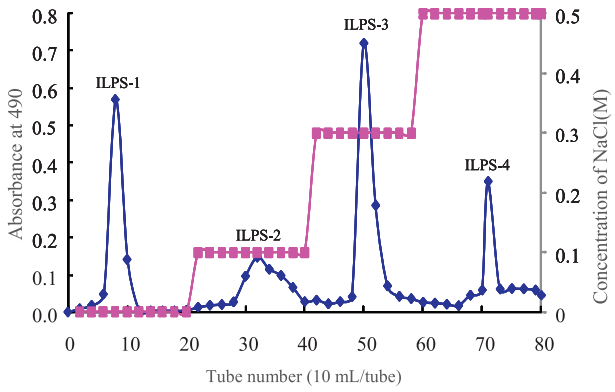

Fig. 1. Stepwise elution curve of crude ILPS on anion-exchange chromatography of DEAE-52 cellulose.

3.2. Antioxidant activity in vitro of ILPS

Different *in vitro* chemical-based assays have been developed to determine the antioxidant activity of natural products, including the popular oxygen radical absorbance capacity (ORAC), DPPH• scavenging method, ferric reducing capacity, *etc.* and more recently the use of nanoprobes to evaluate the metal-reducing capacity of antioxidants (López-Alarcón & Denicola, 2013). To study the potential antioxidant health-protecting effects of natural products, however, a single *in vitro* chemical method is not enough to evaluate and compare their antioxidant properties by considering the complexity involved in their *in vivo* mechanisms of action. Among the free radical species, DPPH• is more stable and has the ability to become a stable diamagnetic molecule by accepting an electron or hydrogen. Therefore, it has been widely used to evaluate the antioxidative activity of natural antioxidants (Ahn et al., 2007). In addition, •OH, $O_2^{\bullet-}$ and its subsequent radicals are also considerated as the most harmful ROS and responsible for the oxidative injury of biomolecules (Ozyurek, Bektasoglu, Guclu, & Apak, 2008). Besides, as the most powerful prooxidant with high reactivity, Fe^{2+} can stimulate lipid peroxidation by generating hydroxyl radicals through Fenton reaction (Liu et al., 2010; Sun & Kennedy, 2010). Therefore, in the present study, the scavenging activities against DPPH•, •OH, $O_2^{\bullet-}$ and Fe^{2+} chelating activity were selected to evaluate the antioxidant activity of ILPS *in vitro*.

Fig. 4A shows the scavenging activity of ILPS on DPPH•. Notably, crude ILPS exhibited the strongest scavenging activity, while ILPS-1 had the weakest scavenging activity. In addition, except ILPS-1 the scavenging activity of ILPS increased significantly with the increase of sample concentration ranging from 62.5 to 1000 μg/mL. The IC_{50} of the scavenging activities for crude ILPS, ILPS-3 and ILPS-4 were 118.1 ± 3.3, 632.3 ± 5.2, 205.7 ± 4.7 μg/mL, respectively. At the concentration of 1000 μg/mL, the scavenging activities of ILPS-1 and ILPS-2 were 24.7 ± 2.1 and 41.3 ± 2.7%. Similar results were reported by Qiao et al. (2009b).

Fig. 4B shows the scavenging activities of crude ILPS, ILPS-1, ILPS-2, ILPS-3, ILPS-4 and V_C on $O_2^{\bullet-}$. Notably, all the ILPS samples possessed $O_2^{\bullet-}$ scavenging activities, especially crude ILPS showed relative stronger activity. The IC_{50} of the scavenging activities for crude ILPS, ILPS-2, ILPS-3 and ILPS-4 were 503.1 ± 4.3, 2704.2 ± 15.9, 1824.4 ± 9.6, and 916.7 ± 6.7 μg/mL, respectively. At the concentration of 4000 μg/mL, the scavenging activity of ILPS-1 was 35.9 ± 1.9%. The mechanism for free radical scavenging of polysaccharides is still not fully understood. It has been reported that the mechanism of scavenging superoxide anion might be associated with dissociation energy of O-H bond and the addition of electron-donating substituents. For example, sulphated groups could increase radical scavenging activity as a result of increased electron density on the heterocyclic ring of carbons (Tsiapali et al., 2001). In addition, the acidic polysaccharide exhibited higher superoxide anion scavenging activity than the neutral polysaccharide due to the presence of some types of electrophilic groups like keto or aldehyde in acidic polysaccharide to facilitate liberation of hydrogen from O—H bond (Xu, Shang, & Li, 2011).

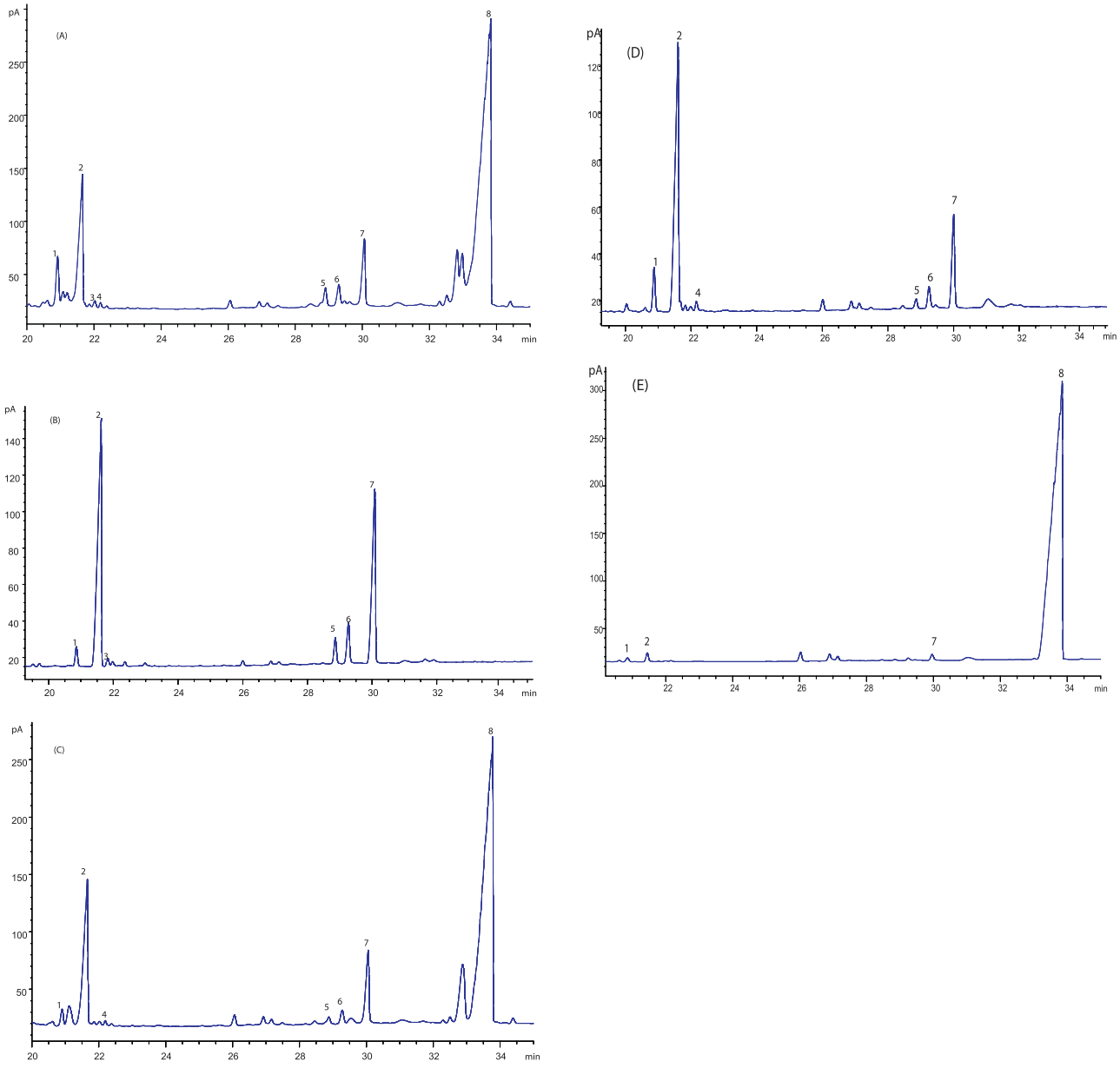

Fig. 2. GC chromatograms showing the monosaccharide compositions of crude ILPS (A), ILPS-1 (B), ILPS-2 (C), ILPS-3 (D) and ILPS-4 (E). 1, Rhamnose; 2, Arabinose; 3, Fucose; 4, Xylose; 5, Mannose; 6, Glucose; 7, Galactose; 8, Inositol.

Therefore, it is not clear whether the mechanism of superoxide radical scavenging by ILPS is attributed to that.

As shown in Fig. 4C, we found that the scavenging activity of ILPS against •OH increased steadily with the increase of concentration up to 4000 μg/mL. The IC_{50} of the scavenging activities for crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4 were 216.5 ± 4.1, 3376.5 ± 20.7, 1695.6 ± 15.6, 879.1 ± 9.2 and 510.8 ± 5.5 μg/mL, respectively. Although the mechanism on how polysaccharides possess the hydroxyl radical scavenging activity is unclear, it was proposed that polysaccharides could inhibit the formation of hydroxyl radicals, probably due to the hydrogen or electron abstraction mechanism (Xu et al., 2011). Furthermore, it has been

Table 2
The monosaccharide composition of crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4.

Sugar component (%)	Sample				
	Crude ILPS	ILPS-1	ILPS-2	ILPS-3	ILPS-4
Rhamnose	19.9 ± 0.2	4.0 ± 0.2	6.0 ± 0.5	11.8 ± 1.1	31.0 ± 1.9
Arabinose	44.6 ± 0.9	48.4 ± 2.1	47.3 ± 2.7	59.4 ± 1.3	34.0 ± 2.1
Fucose	1.7 ± 0.2	0.5 ± 0.1	–	–	–
Xylose	1.5 ± 0.1	4.3 ± 0.4	1.0 ± 0.1	1.8 ± 0.2	–
Mannose	4.4 ± 0.4	–	1.6 ± 0.1	1.7 ± 0.2	–
Glucose	4.7 ± 0.5	5.1 ± 0.4	2.5 ± 0.2	3.3 ± 0.7	–
Galactose	23.2 ± 1.9	37.7 ± 2.3	41.6 ± 2.9	22.1 ± 1.2	35.0 ± 1.9

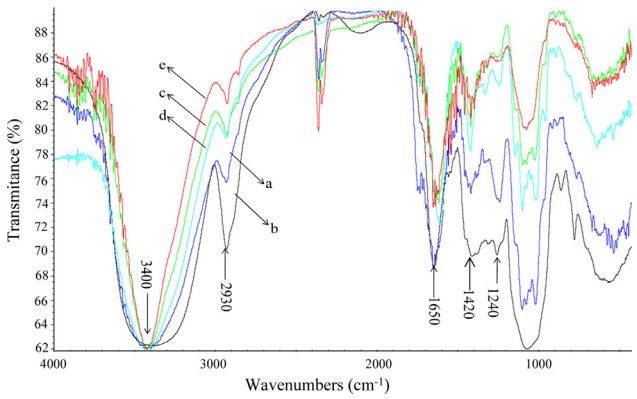

Fig. 3. FT-IR spectra of crude ILPS (a), ILPS-1 (b), ILPS-2 (c), ILPS-3 (d) and ILPS-4 (e).

confirmed that the uronic acid content and average molecular weight have significant effects on scavenging hydroxyl radical (Chen, Ju, Li, & Yu, 2012a). Accordingly, crude ILPS and ILPS-4 showed a higher hydroxyl radical scavenging activity might be related to their relative higher contents of uronic acid (21.8% and 23.2%, respectively, Table 1).

As shown in Fig. 4D, the Fe^{2+} chelating activities of crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4 were evident at all of the tested concentrations. In addition, their Fe^{2+} chelating activities were correlated well with the increase of concentration up to 4000 μg/mL. The IC_{50} of the Fe^{2+} chelating activities for crude ILPS and ILPS-4 were 1137.5 ± 7.3 and 1965.3 ± 8.1 μg/mL. At a concentration of 4000 μg/mL, the Fe^{2+} chelating activities for ILPS-1, ILPS-2 and ILPS-3 were 4.7, 11.3 and 46.7%, respectively. The results indicated that crude ILPS and ILPS-4 exhibited moderate chelating activities. It has been reported that compounds with metal ion chelating activities usually contain two or more of the following functional groups: —OH, —SH, —COOH, $—PO_3H_2$, CO, $—NR_2$, —S— and —O— (Yuan, Bone, & Carrington, 2005). Therefore, the chelating effect of ILPS-4 might be partly due to the presence of Fe^{2+} chelating groups such as —COOH and sulfuric radical in the structure.

3.3. Hepatoprotective activity in vivo of crude ILPS

In the liver, CCl_4 is mainly metabolized to highly reactive trichloromethyl radical and proxy trichloro-methyl radical (ROS). So it is widely used to induce toxic liver injury as medicinal model to evaluate the protective effect of various extracts (Chen et al., 2012b). As mentioned above, ILPS exhibited high radical scavenging activity *in vitro*, the hepatoprotective activity of crude ILPS, due to a great amount of crude ILPS available, was therefore investigated by using the model of CCl_4-induced acute liver damage in mice.

The effects of crude ILPS on the activities of ALT and AST in serums, activities of SOD, GSH-Px and level of MDA in livers of CCl_4-induced hepatotoxicity mice were determined and shown in Table 3. Apparently, significant increases in ALT and AST activities ($P < 0.05$) were observed in serums between Group I (normal control group) and Group II (CCl_4 model control group), indicating that the CCl_4-induced hepatotoxicity model in mice was well-established. In contrast, both crude ILPS and silymarin treatments significantly decreased ($P < 0.05$) the activities of ALT and AST in serums in a dose-dependent manner (Groups IV–VI), but there was no significant difference between Groups V and VI. Furthermore, a significant increase in MDA and marked decreases ($P < 0.05$) of antioxidant enzyme activities (SOD and GSH-Px) were observed in livers between Group I (normal control group) and Group II (CCl_4 model control group). In contrast, both crude ILPS and silymarin treatments significantly decreased ($P < 0.05$) the level of MDA and

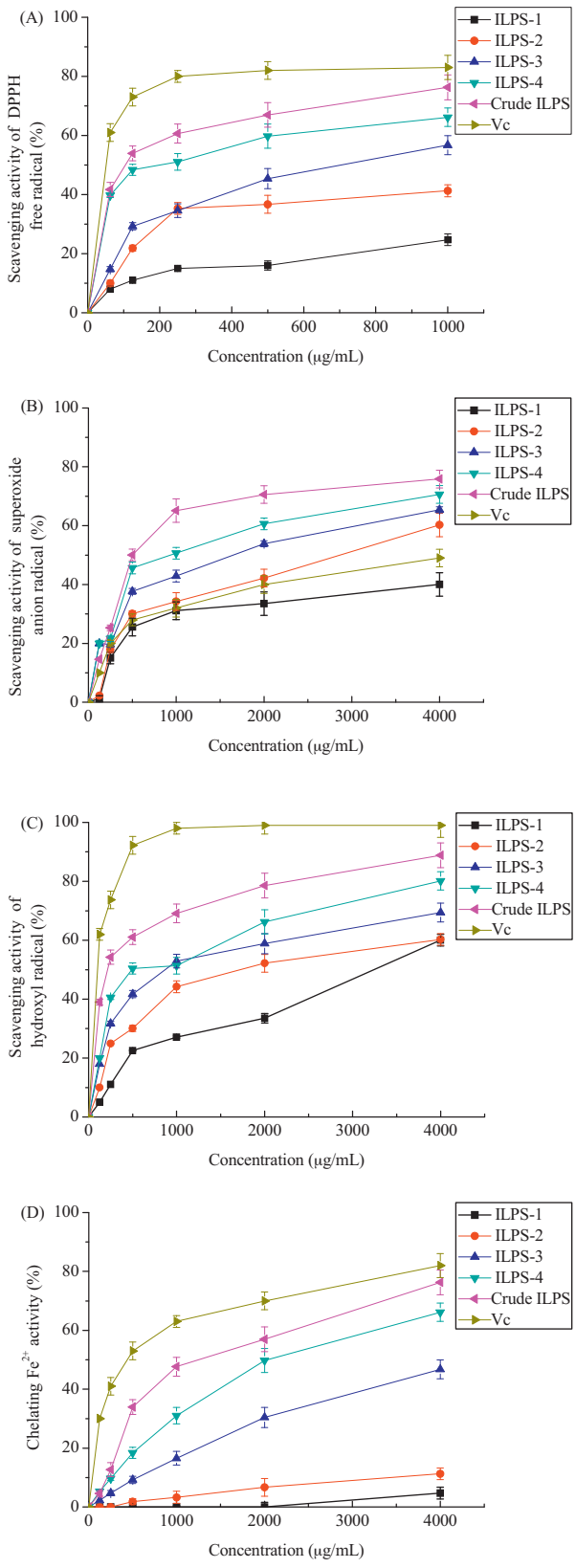

Fig. 4. Scavenging effects of crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4 on DPPH radical (A), superoxide radical (B), hydroxyl radical (C) and metal ion chelating activity (D).

Table 3
Effect of crude ILPS on the activities of ALT and AST in serum and activities of SOD, GSH-Px, and MDA level in liver of CCl_4-induced liver injury mice.

Group	ALT (IU/L)	AST (IU/L)	SOD (U/mg protein)	GSH-Px (U/mg protein)	MDA (nmol/mg protein)
I (Normal control group)	17.88 ± 2.2^{e}	22.38 ± 3.22^{c}	371.03 ± 6.87^{b}	598.31 ± 80.13^{b}	0.64 ± 0.04^{c}
II (CCl_4 model control group)	139.72 ± 9.02^{a}	73.91 ± 4.07^{a}	297.18 ± 9.36^{a}	285.81 ± 13.16^{a}	1.21 ± 0.09^{a}
III (Positive control group)	36.67 ± 2.93^{d}	28.72 ± 2.98^{c}	696.95 ± 35.90^{d}	1130.64 ± 75.70^{d}	0.63 ± 0.08^{c}
IV (ILPS treatment group, 100 mg/Kg)	79.03 ± 4.74^{c}	59.95 ± 1.13^{b}	378.32 ± 1.48^{b}	474.81 ± 12.64^{c}	0.83 ± 0.05^{b}
V (ILPS treatment group, 200 mg/Kg)	24.96 ± 0.57^{e}	25.87 ± 3.66^{c}	439.29 ± 35.40^{c}	629.79 ± 2.41^{b}	0.49 ± 0.08^{d}
VI (ILPS treatment group, 400 mg/Kg)	112.42 ± 18.30^{b}	68.46 ± 17.25^{a}	349.53 ± 41.97^{b}	426.34 ± 28.04^{c}	1.16 ± 0.05^{a}

[a–e] Data are presented as mean ± SD ($n = 8$) by one-way ANOVA followed by the Duncan's multiple-range tests, and different lowercase alphabet letters within each column are significantly different from CCl_4 model control group (II) at the level of $P < 0.05$.

raised the activities of antioxidant enzymes in mice livers. All the results suggested that crude ILPS might have protective effects against CCl_4-induced liver injury in mice through its antioxidant activity to protect biological systems against the oxidative stress in multiple mechanisms, including free radicals scavenging activity, increasing the activities of a number of antioxidant enzymes (SOD and GSH-Px) and decreasing the lipid peroxidation. Although the hepatoprotective activity of polysaaccharides has been demonstrated (Chen, Zhang, & Xie, 2004; Dhanasekaran, Ignacimuthu, & Agastian, 2009; Nada, Omara, Abdel-Salam, & Zahran, 2010), the antioxidant mechanism *in vivo* is not clear. Only Pang, Chen, and Zhou (2008) reported that the effects of polysaccharides on SOD, GSH-Px and catalase might be associated with the induction on gene expressions of SOD, GSH-Px and catalase. The hepatoprotective effect of crude ILPS might be related to its relative higher antioxidant activity. Thus, further and deep works is needed to elucidate the hepatoprotective mechanisms of ILPS and the relationship between structure and functionality.

4. Conclusions

In this study, preliminary characterization, antioxidant *in vitro* and hepatoprotective activity *in vivo* of ILPS were carried out. Moreover, the assay of the antioxidant activity *in vitro* demonstrated that crude ILPS, ILPS-1, ILPS-2, ILPS-3 and ILPS-4 showed moderate DPPH free radical scavenging activity, •OH scavenging activity, $O_2^{\bullet -}$ scavenging activity and strong Fe^{2+} chelating activity. The assay of hepatoprotective activity *in vivo* demonstrated that the administration of ILPS significantly decreased serum ALT and AST levels, inhibited MDA formation and enhanced the activities of liver SOD and GSH-Px in CCl_4-induced liver injury mice. These results suggest that ILPS has potent antioxidant activity and could be utilized as new natural antioxidant in food and therapeutics. Further works on the structure, function and mechanisms of action are in progress.

Acknowledgements

This work was supported by a grant-in-aid for scientific research from the National Natural Science Foundation of China (31201454), the Fundamental Research Funds for the Central Universities (KYZ201218) and a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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