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A comparative study on the antioxidant activities of an acidic polysaccharide and various solvent extracts derived from herbal *Houttuynia cordata*

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ABSTRACT

This study is designed to compare the antioxidant potential of a water-soluble polysaccharide (HCP) with solvent extracts (water, ethanol, ethyl acetate and chloroform) from *Houttuynia cordata* Thunb. The results showed that polar water extract exhibited the highest reducing power and scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide radical and hydroxyl radical, which were correlated with its high level of biopolymer HCP. Furthermore, the active HCP was identified as an acid hetero-polysaccharide by a rapid HPLC technology within 20 min, and galacturonic acid (29.4%) and galactose (24.0%) were approved as the prominent components of HCP, followed by rhamnose (17.2%), arabinose (13.5%), glucuronic acid (6.8%), glucose (5.3%), xylose (2.1%) and mannose (1.8%) in the molar percentages. This finding suggests that HCP is one of the main active ingredients responsible for antioxidant effect of *H. cordata*, which might be valuable as a natural antioxidant source applied in both healthy medicine and food industry.

1. Introduction

Reactive oxygen species (ROS), such as superoxide anion radical ($O_2\bullet^-$), hydroxyl radical (HO•) and H_2O_2, can cause oxidative damage of DNA, proteins, lipid, and small cellular molecules. Increasing evidence suggests that many human diseases, such as cancer, cardiovascular disease and neurodegenerative disorders, are the results of the oxidative damage by ROS (Halliwell, Gutteridge, & Cross, 1992; Lai, Wen, Li, Wu, & Li, 2010). Recently, great interest in finding natural antioxidants from plant materials has been considered. Numerous crude extracts and pure natural compounds from plants were reported to have antioxidant and radical-scavenging activities. Within the antioxidant compounds, polysaccharides and polyphenols with a large distribution in nature have received the greatest attention, and have been investigated extensively since they are assumed to be less toxic than synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are suspected of being carcinogenic and causing liver damage (Lin & Tang, 2007; Song, Zhang, Zhang, & Wang, 2010).

Houttuynia cordata Thunb. as a herbal plant is widely distributed throughout the southeast area of Asia. In southern China, green leaves and young roots of *H. cordata* are also popular vegetable or agricultural products, and the dry leaves of *H. cordata* are used to prepare drink by boiling decoction. Recently, *H. cordata* is increasing popular for health promotion and adjuvant therapy because it has been shown to possess a variety of pharmacological functions of clearing heat, eliminating toxins, reducing swelling, discharging pus and relieving stagnation (State Pharmacopoeia Commission of People's Republic of China, 2005). Therefore, a lot of medicines and food products derived from *H. cordata* have been marked (e.g. injection, drinking, etc.), and the demand and applied area of *H. cordata* are being extended in Asia.

In recent years, *H. cordata* was found to be an important source of natural polysaccharides and flavonoids (Choi et al., 2002; Meng et al., 2005; Nuengchamnong, Krittasilp, & Ingkaninan, 2009; State Pharmacopoeia Commission of People's Republic of China, 2005). Although the water or ethanol extracts of *H. cordata* have been proven to exhibit significant antioxidant activity, there is no available information to clarify the dependence of the antioxidant effect on different solvent properties during extraction, and whether the polysaccharides or flavonoids rich in hydroxyl groups are responsible for the antioxidant activity of *H. cordata* is still unclear (Choi et al., 2002; Nuengchamnong et al., 2009). Therefore, the objective of this work was to compare the antioxidant and radical-scavenging activities of the water-soluble polysaccharide (HCP) with other four extracts (different polar water, ethanol, ethyl acetate and chloroform) of air-dried *H. cordata* in different antioxidant test systems. Furthermore, the monosaccharide composition of the

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isolated acidic HCP was also identified by a rapid HPLC–UV analysis. Because of the important roles of the total phenolics and total flavonoids as antioxidants, the amounts of total phenolics and total flavonoids in the extracts were determined using spectrophotometric techniques. Such information would facilitate the sustainable use of *H. cordata* agricultural source.

2. Materials and methods

2.1. Plant materials

The aerial parts (stems and leaves) of the wild *H. cordata* plants were collected from Hunan province of China in June, 2009. All the samples were authenticated by Prof. Xianhua Tian in College of Life Sciences, Shaanxi Normal University, China. The materials were thoroughly washed with tap water, air-dried at room temperature, and finely powdered.

2.2. Chemicals and reference compounds

D-Mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-glucose, D-xylose, D-galactose, L-arabinose, D-fucose, BHT and ascorbic acid (Vc) were obtained from Sigma (St. Louis, USA). Gallic acid and rutin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), potassium ferricyanide [$K_3Fe(CN)_6$] and trichloroacetic acid (TCA) were purchased from Sigma (Sigma–Aldrich GmbH, Sternheim, Germany). Nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH) and phenazine methosulfate (PMS) were purchased from Applichem (Darmstadt, Germany). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Ferrozine was purchased from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Beijing Reagent Plant (Beijing, China). HPLC grade acetonitrile and methanol were purchased from Honeywell (USA). Other chemicals used in the study were of analytic grade.

2.3. Preparation of the polysaccharide and solvent extracts from *H. cordata*

A known quantity of air-dried *H. cordata* powder (100 g) was extracted with different solvents (each 500 mL) independently, namely water, ethanol, ethyl acetate and chloroform at room temperature for 24 h using a magnetic agitator. The extraction was repeated for 3 times. The crude extracts were evaporated to dryness under a reduced pressure at 30–35 °C and re-dissolved in a known volume of the respective solvent system.

HCP was isolated from the water extract of *H. cordata* by repeated ethanol precipitation as previously described (Lv et al., 2009). Briefly, the dried *H. cordata* powder (100 g) was extracted with distilled water (1:10, w/v) at 80 °C for 4 h, followed by a centrifugation for 15 min at 3000 rpm. The water extracts were collected, and the residues were extracted again for three cycles. The combined extract was pooled, and then condensed to about 200 mL under a reduced pressure. Subsequently, 4 volume of 95% alcohol was added slowly by stirring to precipitate the polysaccharides. This polysaccharide sediment was further refined by repeatedly dissolution and precipitation for 3 times, followed by washing with ethanol, acetone and ether alternately. After dialysis (cut-off MW 10,000 Da), the retentate portion was deproteinized by the freeze–thaw process for repeating 10 times followed by freeze dehydration.

2.4. Determination of total phenolics and total flavonoids

Total phenolic compounds in the extracts were estimated by the Folin–Ciocalteu method as gallic acid equivalents (GAE), expressed as mg of gallic acid/g extract (Lin & Tang, 2007). In addition, the total flavonoids of the samples were measured as rutin equivalents (RE) using a modified colorimetric method described previously by Jia, Tang, and Wu (1999).

2.5. Measurement of carbohydrate and protein contents in HCP

Total carbohydrate content in HCP was measured by phenol–sulfuric acid colorimetric method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The quantification of uronic acid was performed by vitriol–carbazole method using glucuronic acid as the standard (Bitter & Muir, 1962). Fourier transform infrared (FT-IR) spectroscopy was also conducted to further obtain the compositional information by the program that HCP sample was incorporated into KBr powder, and then pressed into a 1.0 mm pellet. FT-IR measurement was performed in the frequency range of 4000–500 cm^{-1} on a Bruker Equinox55 FT-IR spectrometer. In addition, proteins in HCP were quantified according to the Bradford method using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.6. HPLC analysis for the monosaccharide composition of HCP

HCP sample (20 mg) together with 0.82 mg fucose as internal standard was hydrolyzed with 2 mL of 3 M TFA at 100 °C for 8 h in an ampoule (5 mL) sealed under a nitrogen atmosphere. The released monosaccharides were derivatized with PMP to gain strong UV absorption as described previously (Lv et al., 2009). Briefly, the hydrolyzed samples of HCP (100 μL) were dissolved in 0.3 M aqueous NaOH (300 μL), and a 0.5 M methanol solution of PMP (200 μL) was added. Each mixture was allowed to react for 60 min at 70 °C, and subsequently neutralized with 300 μL of 0.3 M HCl. The resulting solution was extracted with 1 mL chloroform, and the aqueous layer was filtered through a 0.45 μm membrane for HPLC analysis. The analysis of PMP-labeled monosaccharides was performed on a Shimadzu LC-2010A HPLC system. The analytical column used was a RP-C_{18} column (4.6 mm i.d. × 250 mm, 5 μm, Venusil, USA). The mobile phase A consisted of acetonitrile, and mobile phase B was 3.3 mM KH_2PO_4–3.9 mM triethylamine buffer containing 10% acetonitrile using a gradient elution of 93–93–91–91% B by a linear decrease from 0–7–9–30 min. Elution was carried out at a flow rate of 1.0 mL/min, and the wavelength for UV detection was 250 nm. The injection volume was 20 μL. All separations were performed at 35 °C.

2.7. Determination of scavenging activity on DPPH radical

The ability of HCP and the extracts to scavenge DPPH• was determined by the method of Mohsen and Ammar (2009), with slight modification. 1.0 mL of the tested samples at various concentrations (0–1.2 mg/mL) was mixed with 3.0 mL of 0.1 mM DPPH in aqueous methanol. Absorbance at 517 nm was determined after 20 min, and the percentage of inhibition activity was calculated according to the following formula: scavenging activity against DPPH• (%) = $[1 - (A_s - A_b)/A_o] \times 100$, where A_o was the absorbance without sample, A_s was the absorbance with sample, and A_b was the absorbance of ground color. The percentage of scavenging activity was plotted against the sample concentration to obtain the IC_{50}, defined as the concentration of sample necessary to cause 50% inhibition.

Table 1
The contents of total phenolics, flavonoids and carbohydrates in the acidic polysaccharide (HCP) and various solvent extracts from the aerial part of *H. cordata*.[a]

Extract/fraction	Total phenolic amount (μg GAE/mg extract)	Total flavonoid amount (μg RE/mg extract)	Total carbohydrate amount (%, w/w)
HCP	nd[b]	nd[b]	78.6 ± 1.42a
Water extract (WE)	36.79 ± 0.59a	26.23 ± 0.89a	21.5 ± 1.17b
Ethanol extract (EE)	24.41 ± 0.44b	18.21 ± 0.91b	nd[b]
Ethyl acetate extract (EAE)	5.95 ± 0.15d	5.19 ± 0.49d	nd[b]
Chloroform extract (CE)	12.02 ± 0.35c	7.70 ± 0.85c	nd[b]

[a] Values (mean ± SD, $n = 3$) in the same column followed by a different letter are significantly different ($p < 0.05$).
[b] nd: not detected.

2.8. Determination of scavenging activity on superoxide anion

The modified PMS-NADH system was used to measure the capacity of HCP and different extracts to inhibit the photochemical reduction of NBT (Chen, Tsai, Huang, & Chen, 2009). Briefly, the reaction was carried out in a mixture containing 1.0 mL of 81 μM NBT in 20 mM potassium phosphate buffer (PBS, pH 7.4), 1.0 mL of 468 μM NADH in 20 mM PBS (pH 7.4), and 1.0 mL of an appropriately diluted samples (0–3.0 mg/mL). The reaction was initiated by the addition of 0.4 mL of 88 μM PMS in 20 mM PBS (pH 7.4) into the mixture. Then the reaction mixture was incubated at ambient temperature for 5 min, and the absorbance was recorded at 560 nm against blank (mixture without PMS was used as blank). The scavenging activity was calculated as follows: Scavenging activity (%) = [1 − (Abs. of sample − Abs. of blank)/Abs. of control] × 100. The IC_{50} was calculated according to the relationship of concentration and scavenging activity.

2.9. Measurement of scavenging activity on hydroxyl radical

The scavenging activity of extracts on HO• was measured by an improved Fenton-type reaction (Sminoff & Cumbes, 1989). Reaction mixture contained 1.0 mL of 6 mM $FeSO_4$, 1.0 mL of sample solutions in water at different concentrations (0–8.0 mg/mL), and 2.0 mL of 6 mM H_2O_2. After 10 min, 1.0 mL of 6 mM salicylic acid was added, and then the reaction mixture was incubated at ambient temperature for 30 min. The absorbance was recorded at 510 nm, and the scavenging activity of HCP and the extracts was calculated according to the equation: scavenging activity on HO• (%) = [1 − (Abs. of sample − Abs. of blank)/Abs. of control] × 100.

2.10. Assessment of reducing power

The reducing power of HCP and the extracts was determined according to the method of Oyaizu (Oyaizu, 1986). Briefly, 1.0 mL of different concentration samples (0–2.5 mg/mL) were mixed with 2.5 mL of 0.2 M PBS buffer (pH 6.6), and then 2.5 mL of 1% (w/v) $K_3Fe(CN)_6$ solution was added. The mixture as incubated in a water bath at 50 °C for 20 min. Afterward, 2.5 mL of a 10% (w/v) TCA solution was added, and the mixture was then centrifuged at 3000 rpm for 10 min. 2.0 mL of the upper layer was combined with 2.0 mL of distilled water and 0.5 mL of 0.1% (w/v) $FeCl_3$ solution, following by the absorbance analysis at 700 nm (Vc was used as a positive control). Increased absorbance of the reaction mixture indicates greater reducing power.

2.11. Iron(II) chelation activity

The ability of the samples to chelate Fe^{2+} was carried out as described by Jeong, Seo, and Jeong (2009)). 400 μL of each sample was added into a mixture of 200 μL of 0.5 mM aqueous $FeCl_2$ and 1800 μL of methanol. The controls contained all the reaction reagents except the samples. After 5 min incubation, the reaction was initiated by 800 μL of 5.0 mM ferrozine, followed by 10 min equilibrium and a further centrifugation. Finally, the absorbance at 562 nm was recorded (EDTA was used as a positive control). The ferrous ion-chelating activity was given by the following equation: Chelating ability (%) = [1 − (Abs. of sample − Abs. of blank)/Abs. of control] × 100.

2.12. Statistical analysis

All determination was performed in triplicate, and data were expressed as means ± SD. The data were subjected to an analysis of variance (ANOVA, $p < 0.05$), and Duncan's multiple range tests. A significant difference was judged to exist at a level of $p < 0.05$.

3. Results and discussion

3.1. Total phenolic and total flavonoid contents in the extracts and HCP

The aerial part of *H. cordata* was extracted with different solvents in an order of decreasing polarity such as water, ethanol, ethyl acetate, and chloroform. Furthermore, the water-soluble polysaccharide HCP was also prepared from the water extract. HCP and all solvent extracts were investigated for the total phenolics and total flavonoids. According to the data presented in Table 1, the significant difference in the amount of the total phenolics or total flavonoids was observed among these extracts, respectively ($p < 0.05$). As expected, no phenols and flavonoids were detected in HCP, suggesting that the small molecular phenolic compounds in the isolated polysaccharides had successfully been removed via dialysis processing (cut-off MW > 10,000 Da). The richest amount of total phenolics was found in the water extract (WE, 36.79 μg GAE/mg extract), followed by ethanol extract (EE, 24.41 μg GAE/mg extract), chloroform extract (CE, 12.02 μg GAE/mg extract) and ethyl acetate extract (EAE, 5.95 μg GAE/mg extract). Similarly, the highest flavonoid content (26.23 μg RE/mg extract) was observed in WE, while EAE had the lowest content (5.19 μg RE/mg extract), and the amounts of total flavonoids were affected by the extraction solvents with the following order: WE > EE > CE > EAE ($p < 0.05$ among the extracts).

As can be seen in Table 1, it is of interest that more than 60% of the extracted phenolic substances in the four extracts were of flavonoids. The polar water is the best solvent in extracting the flavonoids from *H. cordata*, indicating that most of the flavonoids exist in a conjugated form through their hydroxyl groups with glycosides, led to the increasing polarity and solubility in water (Mohsen & Ammar, 2009). However, according to another

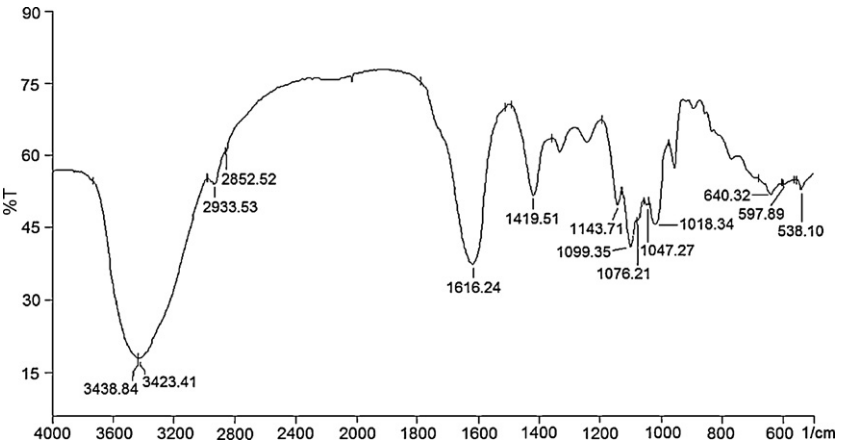

Fig. 1. FT-IR spectra of HCP in the frequency range 4000–500 cm^{-1}.

investigation conducted by Sahreen, Khan, and Khan (2010), a less polar solvent such as chloroform could extract more phenols from plant materials than more polar solvents (e.g. ethyl acetate). In agreement with this report, we also found that CE contained more phenolics than EAE, and the distinctions may be due to the deference in the types of flavonoid compounds in different plant materials. As well-known from the literatures, polysaccharides and flavonoids were found as the principal component in plant species including *H. cordata*, and it has been suggested that these compounds are possibly responsible for their antioxidant activities (Choi et al., 2002; Nuengchamnong et al., 2009).

3.2. Physicochemical properties of the acidic polysaccharides

HCP was further isolated from water extract of *H. cordata* by means of ethanol precipitation. Physicochemical analysis showed that HCP was an acid hetero-polysaccharide, and its total carbohydrate content and total uronic acid content were 78.6% (w/w) and 26.2% (w/w), respectively. In addition, FT-IR spectroscopy from

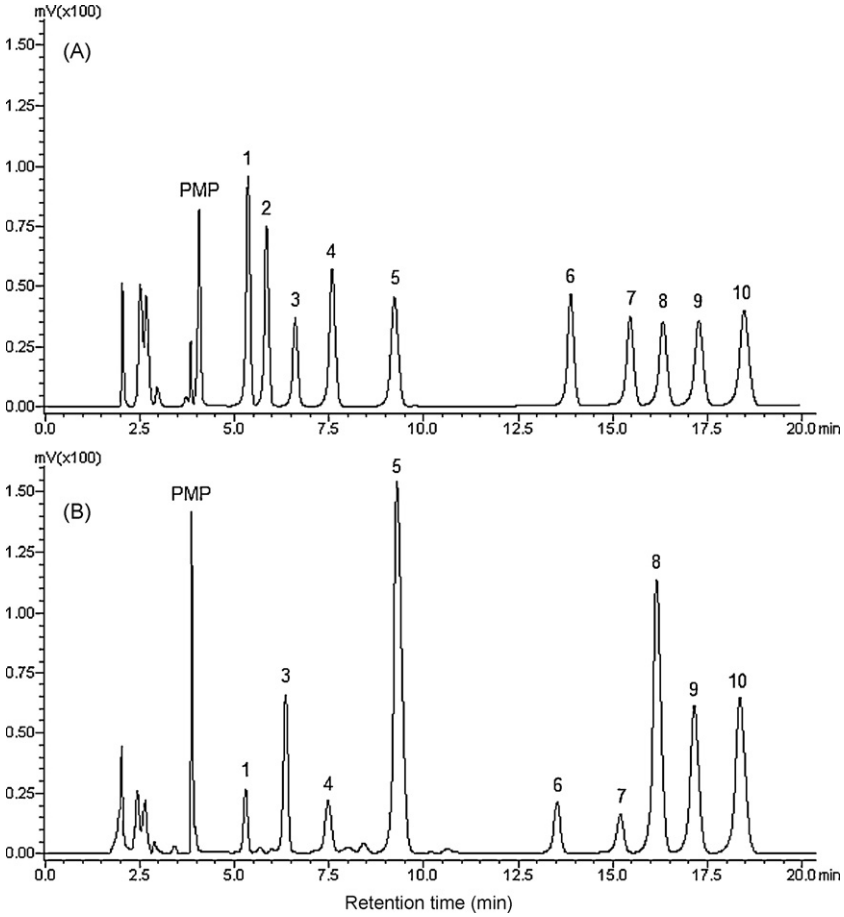

Fig. 2. The HPLC chromatograms of PMP derivatives of 10 standard monosaccharides (A) and component monosaccharides released from HCP (B). The polysaccharide was hydrolyzed with TFA at 100 °C for 8 h and then was labeled with PMP. The HPLC analysis was carried out as described in the experimental section. Peaks: (1) mannose; (2) ribose; (3) rhamnose; (4) glucuronic acid; (5) galacturonic acid; (6) glucose; (7) xylose; (8) galactose; (9) arabinose; and (10) fucose (internal standard).

Fig. 1 showed that the IR spectra of HCP displayed a broad stretching intense characteristic peak for the hydroxyl groups at around 3438 cm^{-1} and 3423 cm^{-1}, and two weak C–H stretching bands at 2933 cm^{-1} and 2852 cm^{-1}, respectively. Another two strong peaks towards 1616 cm^{-1} and 1419 cm^{-1} were attributed to the absorbance of the –COOH, suggesting that HCP was of uronic acid-rich polysaccharides. Furthermore, the only trace protein (0.41%, w/w) in HCP was detected, which was consistent with the FT-IR analytical results that it did not be detected the characteristic absorbance peaks of acid amides (Fig. 1). From these results, it could be concluded that HCP was approved as the high-purity acid polysaccharides.

3.3. Monosaccharide composition analysis of HCP by HPLC

In our previous work, we have developed a validated HPLC–UV method for assessing the quality of the polysaccharides, where 10 PMP-labeled monosaccharides were successfully separated within 40 min (Lv et al., 2009). Herein, a rapid analysis method within 20 min, for the first time, was established through the use of triethylamine as a modifier in sodium phosphate–acetonitrile mobile phase (Fig. 2A). In this test, triethylamine was found to acutely improve the separation of the PMP-labeled monosaccharides in the traditional RP-C_{18} column. The typical chromatogram of the HCP sample was shown in Fig. 2B (fucose as internal standard). As can be seen, HCP was composed of mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose in the molar contents of 8.7 μM, 84.6 μM, 33.5 μM, 144.6 μM, 26.1 μM, 10.2 μM, 118.0 μM and 66.3 μM, respectively, and their corresponding mole percentages were 1.8%, 17.2%, 6.8%, 29.4%, 5.3%, 2.1%, 24.0% and 13.5% in the mole percentages, respectively. It was very clear that HCP was the typically acidic polysaccharides rich in galacturonic acid. The result was strongly agreed with the conclusion obtained by the colorimetric analysis of uronic acid and FT-IR spectroscopy (Fig. 1). Moreover, the results from the developed HPLC analysis showed that the recoveries of all the tested nine monosaccharides ranged 95.2–111.2%, and the RSD values fell within 2.1–5.6%. Taken together, this rapid HPLC–UV method was showed to be a powerful analytical technique that has excellent sensitivity, and requires relatively simple and inexpensive instrumentation, and can be carried out in common laboratory.

3.4. Scavenging activity of HCP and various extracts on DPPH radical

The capability of scavenging the stable DPPH radicals was estimated by measuring the decrease in its absorbance induced by antioxidants (Choi et al., 2002). As can be seen in Fig. 3, the tested samples dose-dependently exhibited a DPPH•-scavenging effect at all the investigated concentrations ranged from 0 mg/mL to 1.2 mg/mL, and WE was found to be the most effective DPPH• scavenger of all the extracts and HCP. At 1.0 mg/mL, the DPPH•-scavenging abilities were 87.2%, 76.8%, 70.2%, 59.1%, 36.7% for WE, EE, HCP, EAE and CE, respectively, where the activity can be ranked as WE > EE > HCP > EAE > CE. However, no significant difference in the IC_{50} values among HCP, EE and EAE was observed ($p > 0.05$). It is also noteworthy that none of the extracts was as active as the positive controls, Vc and BHT ($p < 0.05$). As a result, the polar extracts were found to be more active for free radical activity than nonpolar extracts.

3.5. Scavenging activity of HCP and various extracts on superoxide radical

Among different ROS, $O_2\bullet^-$ is one of the precursors of HO• or singlet oxygen, and also can produce other kinds of oxidizing

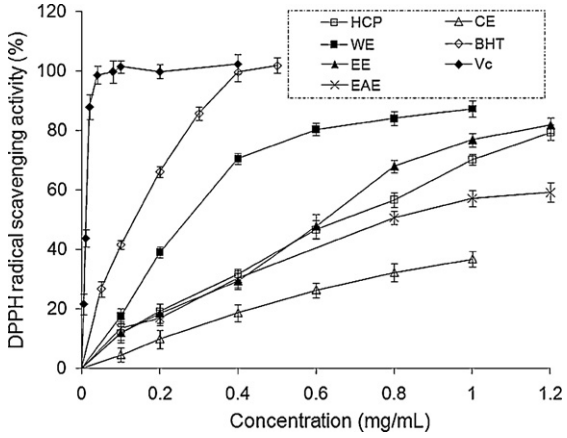

Fig. 3. DPPH radical-scavenging activity of HCP and various extracts from *H. cordata*. Vc and BHT were used as reference antioxidants. Data are presented as mean ± SD of three replicates ($n = 3$).

agents, where it indirectly initiates lipid peroxidation and magnifies cellular damage. Therefore, it was of great importance to characterize the $O_2\bullet^-$-scavenging potential of different antioxidants (Cotelle et al., 1996; Meyer & Isaksen, 1995). As expected, the tested samples significantly exerted the scavenging effects on $O_2\bullet^-$ in a concentration-dependent manner (0–3.0 mg/mL). As can been seen from Fig. 4, $O_2\bullet^-$-scavenging activity of WE and HCP at an amount of 2.0 mg/mL was 96.1% and 94.7%, respectively. The activities were almost equal to Vc at the same concentration ($p > 0.05$). The IC_{50} was found to be 0.135 mg/mL, 1.791 mg/mL, 1.350 mg/mL, 1.702 mg/mL, 0.264 mg/mL and 0.134 mg/mL for WE, EE, EAE, CE, HCP and Vc, respectively. Evidently, the order of $O_2\bullet^-$-scavenging activity was WE > HCP > EAE > CE > EE, where the IC_{50} values among WE, HCP and Vc did not reach statistical significance ($p > 0.05$). The present results suggested that HCP and WE, similar to the reference antioxidant Vc, were strong superoxide radical inhibitors that could help prevent or ameliorate oxidative damage.

3.6. Scavenging activity of HCP and various extracts on hydroxyl radical

Among the oxygen radicals, HO• is the most active and can induce oxidative damage to almost any biomolecule it touches, resulting in aging, cancer, and several diseases (Jeong et al., 2009; Kitada, Igarashi, Hirose, & Kitagawa, 1979). Fig. 5 shows the dose–response curves of HO•-scavenging activities of the tested

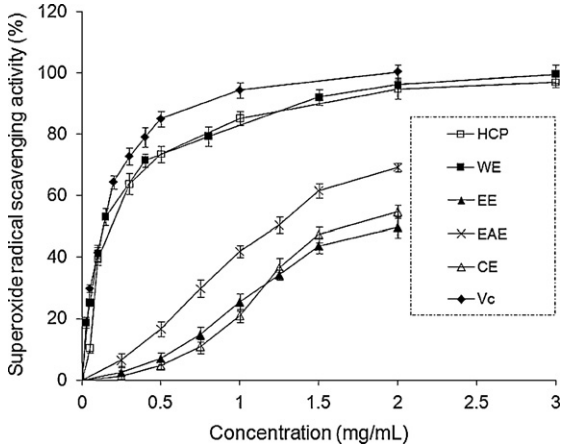

Fig. 4. Superoxide radical-scavenging activity of HCP and various extracts from *H. cordata*. Vc was used as a positive control. Data are presented as mean ± SD ($n = 3$).

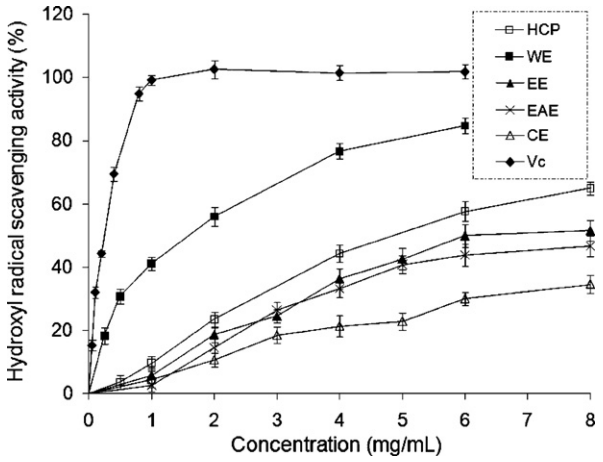

Fig. 5. Hydroxyl radical-scavenging activity of HCP and various extracts from *H. cordata*. Vc as a reference antioxidant. Data are presented as mean ± SD ($n = 3$).

samples. At the concentration of 6.0 mg/mL, WE presented the highest scavenging activity (84.8%) among the extracts, while the activity of HCP, EE, EAE and CE was 57.7%, 52.0%, 43.9% and 30.0%, respectively. However, Vc as a positive compound displayed the strongest effect with an IC_{50} of 0.271 mg/mL, and then followed by 1.239 mg/mL, 5.064 mg/mL, 6.161 mg/mL, 6.210 mg/mL, and 8.0 mg/mL for WE, HCP, EE, EAE, and CE, respectively. In agreement with the scavenging effects on DPPH• and $O_2•^-$, polar water extract and its water-soluble polysaccharide HCP also showed stronger HO•-scavenging activity than other less polar extracts (EE, EAE and CE). The data presented here indicated that the markedly antiradical ability of *H. cordata* extracts seems to be due to the presence of polysaccharides or polyphenols, which may act by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction (Gordon, 1990; Halliwell, Murcia, Chirco, & Aruoma, 1995).

3.7. Iron(III) to iron(II) reduction activity

The reducing capacity is a significant reflection of the antioxidant activity in assessing potential antioxidants (Lai et al., 2010; Mateos-Aparicio, Mateos-Peinado, Jiménez-Escrig, & Rupérez, 2010; Song et al., 2010). In this assay system, the presence of antioxidants causes the reduction of the $Fe^{3+}/K_3Fe(CN)_6$ complex to the ferrous form (Fe^{2+}), and consequently, the Fe^{2+} can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm. Fig. 6A shows the reducing powers of different crude extracts and HCP. As anticipated, all samples showed some degree of reducing power, and their effects were inferior to positive BHT and Vc. WE and HCP at 2.0 mg/mL exhibited the good reducing powers of 0.728 ± 0.037 and 0.465 ± 0.034, respectively, which are significantly higher ($p < 0.05$) than EE (0.362 ± 0.038), EAE (0.135 ± 0.030) and CE (0.147 ± 0.029) in absorbance values. The sequence for reducing power was Vc > BHT > WE > HCP > EE > CE > EAE, which is corresponding with the content of polysaccharides or phenolics and flavonoids in the solvent extracts (Table 1). Like the scavenging activity on DPPH•, $O_2•^-$ and HO•, polar WE and HCP also were proven to have strong reducing ability.

3.8. Fe^{2+} chelating ability

Ferrozine can quantitatively form the complexes with Fe^{2+} (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). In the presence of chelating agents, the complex formation is disrupted and a decrease in the red-color of the complex can be measured. As shown in Fig. 6B, all the samples could display different magnitudes

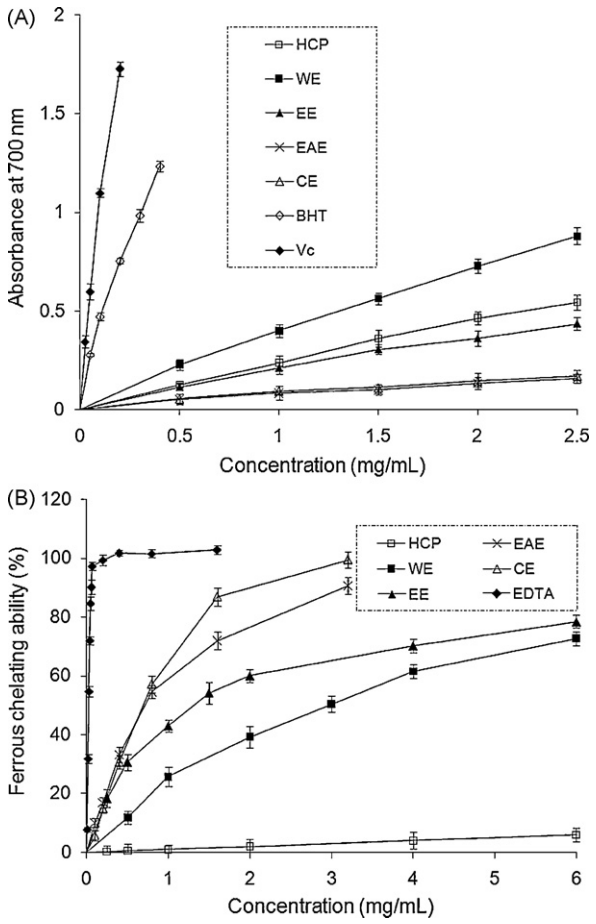

Fig. 6. Iron(III) to iron(II) reducing power of HCP and various extracts from *H. cordata*. Vc and BHA were used as positive control (A) and Fe^{2+} chelation activity of HCP and various extracts from *H. cordata*. EDTA was used as the positive control (B). Data are presented as mean ± SD ($n = 3$).

of Fe^{2+} chelating potency in a dose-dependent manner. Unexpectedly, polar WE had relatively weak Fe^{2+} chelating activity, and HCP was found to be almost inactive *in vitro* in comparison with less polar EE, EAE, and CE. At 3.2 mg/mL, CE and EAE chelated ferrous ions by 99.4% and 90.7%, respectively. At 6.0 mg/mL, chelating activity of EE and WE with ferrous ion was 78.4% and 72.8%, respectively. However, EDTA, used as a positive control, showed the strongest chelating ability of 97.3% at a concentration as low as 0.07 mg/mL. From the estimated IC_{50} values, it can be seen that the most effective Fe^{2+} chelating extract was CE (0.718 ± 0.033 mg/mL), followed by EAE (0.762 ± 0.047 mg/mL), EE (1.758 ± 0.173 mg/mL) and WE (27.8 ± 0.57 mg/mL) in decreasing order. It is well known that Fe^{2+} can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decompositing lipid hydroperoxides into peroxyl and alkoxyl radicals (Halliwell, 1991; Rice-Evans et al., 1995). The results of the present work revealed that nonpolar CE and EAE were found to be more active for Fe^{2+} chelating activity than polar WE and HCP. In practical applications, our findings suggest using water as the best means to extract the active free radical-scavenging ingredients from *H. cordata*. On the other hand, chloroform and ethyl acetate would be best employed to extract the active ingredients associated with Fe^{2+}-chelating ability.

It can be seen that *H. cordata* extracts prepared by different solvents exhibited various degrees of antioxidant activities. Among all the extracts, water extract showed the strongest free radical-scavenging activity as well as reducing power, and the water-soluble polysaccharides as primary metabolites were iden-

tified as the most abundant component (21.5%, Table 1) in WE, followed by phenolics and flavonoids (Table 1). Furthermore, the radicals-scavenging activities of the purified HCP from water extract were found to be higher than the organic solvent extracts, but slightly weaker than WE. Especially for scavenging $O_2\bullet^-$, it was no significant difference with Vc ($p > 0.05$, Fig. 4). These results suggested that the polysaccharides rich in hydroxyl groups were responsible for the antioxidant activity of *H. cordata*. Moreover, it is interest that a consistent decrease in Fe^{2+} chelating effects was observed in WE and HCP, possibly due to the presence of different amount of polysaccharides as main ingredient in the extracts. It was also suggested that the polysaccharides from *H. cordata* exhibited antioxidant effects mainly via the mechanism of scavenging free radicals and enhancing reducing power. Recently, several studies have reported on the relationship between phenolic content and antioxidant activity (Chen & Ho, 1997; Halliwell, 1991; Rice-Evans et al., 1995). However, it can be seen that the amount of total phenolics or total flavonoids of *H. cordata* is not very correlated with their antioxidant activity. This can be explained by the fact that there is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidants (Chen & Ho, 1997).

It is well known that biological activities of plant polysaccharides should depend on their detailed structures (Chen et al., 2009; Tsiapali et al., 2001). Unfortunately, the overall complexity of macromolecular polysaccharide has hindered the elucidation of actual structure–function relationships because so far there is not an ideal method available for assessing the full structures of polysaccharides. However, some polysaccharides rich in uronic acids were shown to exhibit high biological effects because uronic acid residues could alter polysaccharides' properties and modify the solubility, suggesting that varieties or contents of monosaccharides possibly contribute to the biological effects (Chen, Zhang, & Xie, 2004). For these reasons, it is of great importance to achieve a reliable analytical method to assay the monosaccharide composition of functional polysaccharides (Chen et al., 2004; Tsiapali et al., 2001). In this work, HCP was successfully approved as an acid polysaccharide, rich in galacturonic acid and glucuronic acid of up to 36.2% in the mole percentages by the proposed HPLC analysis (Fig. 2B). This can explain the strong antioxidant activity encountered in water extract and HCP, where the carboxyl groups of the uronic acid of the polysaccharides might play the role of hydrogen-donating and electron-transfer agent. However, the mechanism of free radical scavenging of the polysaccharide HCP is still not fully understood. Therefore, the further investigation on antioxidant activity *in vivo* and its mechanisms should be investigated in future works.

4. Conclusions

On the basis of the above results, it could be concluded that water-soluble ingredients of *H. cordata* were approved to possess the strongest antioxidant potential, which should be mainly attributed to the effect of acidic polysaccharides. Therefore, water extract, consumed as a drink in China, can be used as an accessible source of natural antioxidants with consequent health benefits. This is the first report on the evaluation of the composition and antioxidant properties of *H. cordata* polysaccharides. This perspective deserves to be explored by food and pharmaceutical industries to obtain material that can be used either as nutriments, food additives, or antioxidant supplements.

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