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Characterization and antioxidant activity of *Ginkgo biloba* exocarp polysaccharides

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ABSTRACT

Ginkgo biloba exocarp polysaccharide (GBEP) was obtained by hot water extraction, the crude polysaccharide was deproteinized by Sevag method and fractionized by a DEAE Sepharose fast flow anion-exchange column. Five fragments were obtained, including neutral polysaccharide (GBEP-N) and four acidic polysaccharides (GBEP-A1, GBEP-A2, GBEP-A3 and GBEP-A4). GBEP-N and GBEP-A3 were further purified by Superdex 200 gel column chromatography. The resulted two fractions GBEP-NN, and GBEP-AA were characterized by FT-IR, and HPGFC (high pressure gel filtration chromatography). Monosaccharide composition was determined by RP-HPLC method of precolumn derivatization with 1-phenyl-3-5-pyrazolone. GBEP-NN was mainly composed of rhamnose, arabinose, mannose, glucose and galactose, while GBEP-AA was mainly made up of mannose, rhamnose, glucuronic acid, galacturonic acid, galactosamine, glucose, galactose, xylose, arabinose, and fucose. The crude GBEP exhibited certain antioxidant activity. At the concentration of 5 mg/mL, the hydroxyl radical scavenging effect of GBEP was 90.52%, greater than 77.37% for the positive control ascorbic acid.

1. Introduction

Ginkgo biloba has been existed on earth since 200 million years and is considered as a "living fossil" (Singh, Kaur, Gopichand, Singh, & Ahuja, 2008). It is among the most sold medicinal plants with the annual consumption of dried leaves between 4.5 million pounds and 5.1 million pounds in 2001 (van Beek, 2002). Fifty million *Ginkgo biloba* trees are planted, especially in China, France, and USA, producing 8000 tons of dried leaves each year to meet the commercial demand for *Ginkgo biloba* products (Nakanishi, 2005). In China, the natural habitat of *Ginkgo biloba*, most of the exocarps are discarded as plant waste after seeds were collected, causing tremendous pollution and jeopardizing the ecology of soil as well as fish in the river. Therefore, proper usage of *Ginkgo biloba* exocarp will bring about not only economical but also environmental benefits (Wang, Wang, & Wei, 2008).

Ginkgo biloba exocarp contains a significant amount of polysaccharides. There are several reports about the therapeutic effect of GBEP. Xu and Chen have reported that GBEP had good clinical therapeutic effectiveness on gastric cancer (Xu et al., 2003). Sun and Dong have studied the larvicidal activity of exocarp extracts (Sun et al., 2006). Influence of *Ginkgo biloba* L. exocarp polysaccharides on serum superoxide dismutase activity and malondialdehyde level in mice under different conditions was also investigated (Xu, Chen, Wang, & Wang, 1998). Clinical study was focused on treatment of patients with upper digestive tract malignant tumors of middle and late stage with *Ginkgo biloba* exocarp polysaccharides capsule preparation (Chen et al., 2003). Xu et al. reported GBEP (40–160 mg/L, 48 h) could inhibit HL-60 cells proliferation and the expression of proliferation-promotion gene c-myc, induce HL-60 cells apoptosis and down-regulate the expression of apoptosis-inhibitory gene bcl-2 (Xu, Chen, & Sun, 2004). Yet, the antioxidant activity of GBEP has not been reported. Synthetic antioxidants such as BHA, BHT and gallates were introduced in the 1940s. In recent years, there has been an enormous demand for natural antioxidants, mainly because of adverse toxicological reports on many synthetic compounds. Thus, most of the recent investigations have been targeted towards identification of novel antioxidants from natural sources (Pokorny, Yanishlieva, & Gordon, 2001). Many polysaccharides derived from plants and other natural resources were reported to have antioxidant activities (Garde, Catala, Gavara, & Hernandez, 2001; Kong et al., 2010; Li, Zhou, & Han, 2006; Liu, Wang, Pang, Yao, & Gao, 2010; Qiao et al., 2009; Xu et al., 2009). The objective of this study is to evaluate the antioxidant properties of hot water extracted *Ginkgo biloba* exocarp and to characterize the basic structure information of purified GBEP.

2. Experimental

2.1. Materials

The exocarp of *Ginkgo biloba* was collected from Taixing, Jiangsu Province, China in September, 2009. DEAE-Sepharose Fast Flow and

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Superdex 200 were purchased from GE Healthcare (Fairfield, CT, USA). 1,1-Diphenyl-1-picrylhydrazyl (DPPH), ferrozine, mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, arabinose, fucose were obtained from Sigma–Aldrich Chemical Co. (St. Louis, USA). 1-Phenyl-3-methyl-5-pyrazolone (PMP), glucosamine and galactosamine were obtained from Acros Organics Co. (Liège Area, Belgium). Other chemicals were analytical grade reagents, bought from Sinopharm Chemical Reagent Company (Shanghai, China).

2.2. Isolation and purification of GBEP

The raw material was dried in shades then pulverized and refluxed with petroleum ether for 12 h to remove fat, stored at −4 °C for future use. 100 g of dried powder of *Ginkgo biloba* exocarp was extracted with 1 L of boiling water for three times. The supernatants were combined and concentrated to 300 mL with a IKA RV 10 Rotary Evaporator (IKA® Works, Guangzhou, China) at 70 °C, then precipitated with ethanol (1:4, v/v), stored under 4 °C for 24 h. The precipitate was separated by centrifugation on Beckman Coulter Avanti J-E at 8000 rpm for 30 min (Beckman Coulter Inc., Brea, USA), then washed with ethanol and ether, vacuum dried. The crude polysaccharide was re-dissolved in ultrapure water produced by Millipore Lab water purification system (Millipore Corporation, Bedford, USA) and treated with Sevag reagent (n-butanol:chloroform = 1:4, v/v) to remove proteins (Sevag, Lackman, & Smolens, 1938), repeated this treatment until there was no absorption under 260 nm on Cary 50 UV–Vis spectrophotometer (Varian Inc., Palo Alto, USA).

300 mg GBEP was dissolved in 30 mL ultrapure water, centrifuged at 10000 rpm for 10 min. The supernatant was loaded on a DEAE Sepharose Fast Flow column (2.6 cm × 30 cm). The column was eluted with 0 M, 0.05 M, 0.1 M, 0.15 M, and 0.2 M NaCl, respectively at a flow rate of 1 mL/min. Eluate was collected every 6 min and monitored with phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) for polysaccharide content. The appropriate fractions were combined, dialyzed against ultrapure water and lyophilized. Five polysaccharides were obtained, i.e. GBEP-N, GBEP-A1, GBEP-A2, GBEP-A3 and GBEP-A4.

GBEP-N and GBEP-A3 were further purified by gel-filtration chromatography on a Superdex 200 column (1.5 cm × 50 cm), eluted with 0.15 M NaCl solution at a flow rate of 0.5 mL/min. The polysaccharide content was determined by phenol–sulphuric acid method. The fractions with high polysaccharide content were combined, dialyzed and lyophilized.

2.3. Molecular weight determination

The molecular weight of GBEP-AA and GBEP-BB were determined by high pressure gel filtration chromatography (HPGFC), which was performed on Waters 600 HPLC system (Waters Corporation, MA, USA) with Ultrahydrogel™ Linear 300 mm × 7.8 mm i.d. × 2 mm gel filtration column. Standard dextrans of T-2000 (2×10^6), T-133.8 (1.338×10^5), T-21.4 (2.14×10^4), T-2.5 (2.5×10^3) were also analyzed with this system. Samples were eluted with sodium nitrate solution at the flow rate of 0.9 mL/min. Molecular weight distribution of GBEP, GBEP-AA and GBEP-NN were determined by comparison with the retention time of standard dextrans under the same condition.

2.4. Infrared analysis

The basic compositions of GBEP were determined by the FTIR spectrum. 1 mg sample (GBEP-NN and GBEP-AA) was mixed with potassium bromide, thoroughly ground and then was pressed into 1 mm pellet. Spectrums were recorded at the absorbance mode from 4000 cm^{-1} to 400 cm^{-1} on a Thermo Nicolet NEXUS 470 FTIR (Thermo Fisher Scientific, Waltham, USA).

2.5. Monosaccharide composition determination

The monosaccharide of GBEP was analyzed by the RP-HPLC method of precolumn derivation with 1-phenyl-3-5-pyrazolone (PMP) (Dai et al., 2010). 100 μL sample (5.7 mg/mL) and 100 μL trifluoroacetic acid (4 M) were added into a small ampoule, then sealed under nitrogen atmosphere, and kept in an oven at 110 °C for 2 h. After cooling to room temperature, 200 μL methanol was added into the ampoule. The mixture was dried by nitrogen. Same amount of methanol was added into the ampoule, with the same drying method as described before. Repeat this operation for three times to remove trifluoroacetic acid. 100 μL ultrapure water was added into the ampoule to dissolve the hydrolyzed polysaccharide.

Standard monosaccharide (mannose, glucosamine, ribose, rhamnose, glucuronic acid, galacturonic acid, galactosamine, glucose, galactose, xylose, arabinose, and fucose) mixture (100 μL) or hydrolyzed polysaccharide was mixed with 100 μL NaOH (0.6 M). 50 μL of this mixture was added into a small tube with lid, mixed with 50 μL methanolic solution of PMP (0.5 M). The tube was kept under 70 °C for 100 min. After cooling to room temperature, 50 μL HCl (0.3 M) was added into the solution and then dried. 1 mL of water and chloroform were added into the tube, shacked vigorously, the chloroform layer was discarded. This procedure was repeated for three times. The aqueous layer was filtered through a 0.45 μm pore membrane, then analyzed on Agilent HPLC ChemStation (Agilent, USA).

ZORBAX Eclips XDB-C_{18} HPLC column (250 mm × 4.6 mm i.d., 5 μm) was used at ambient temperature of 30 °C. The PMP derivatives elution was performed with a mixture of phosphate buffer (0.1 M, pH = 6.7) and acetoniltrile in the ratio of 83:17 (v/v) at a flow rate of 1 mL/min, and UV absorbance of the effluent was monitored at 245 nm.

2.6. Assay of in vitro antioxidant activity of GBEP

2.6.1. DPPH radical-scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity of GBEP was investigated with the method mentioned by Braca et al. (2001). 1 mL sample (0.1–5 mg/mL) was mixed with 3 mL MeOH solution of DPPH (0.004%, w/v). Absorbance was measured at 517 nm on Spectrum 722E spectrometer (Spectrum Instruments, China) 30 min later. The antioxidant activity was calculated with the following equation:

$$\text{Scavenging effect (\%)} = \left(\frac{1 - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{sample} is the absorbance of test sample (DPPH solution with sample or positive control) and A_{control} is the absorbance of control (DPPH solution with no sample).

2.6.2. Hydroxyl radical-scavenging activity

Hydroxyl radical-scavenging activity was measured according to Smirnoff's work (Smirnoff & Cumbes, 1989). 0.5 mL $FeSO_4$ (1.5 mM) was mixed with 0.35 mL H_2O_2 (6 mM), 0.15 mL sodium salicylate (20 mM) and 1 mL sample (0.1–5 mg/mL), then incubated for 1 h at 37 °C. The absorbance of the hydroxylated salicylate complex was measured at 562 nm. Ascorbic acid was used as the positive control. The antioxidant activity was calculated with the following equation:

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

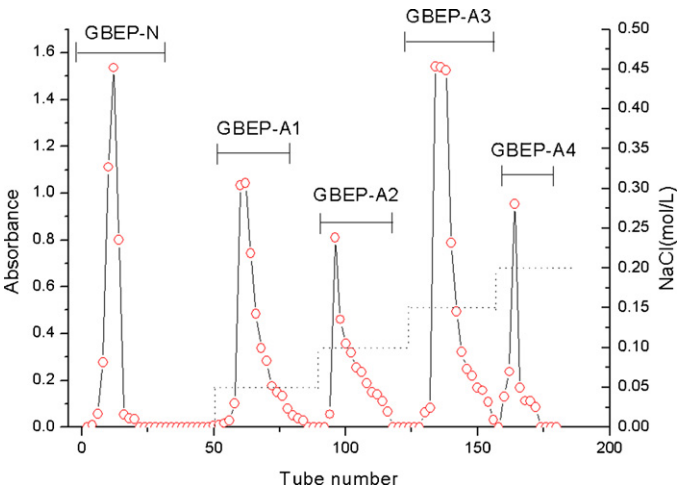

Fig. 1. Elution profile of GBEP on DEAE Sepharose fast flow column (eluted by a stepwise gradient of NaCl at the concentration of 0, 0.05 M, 0.1 M, 0.15 M and 0.2 M).

where A_{sample} was the absorbance of the test (sample or ascorbic acid), $A_{control}$ was the absorbance of the solvent control, and A_{blank} was the absorbance of the reagent blank without sodium salicylate.

2.6.3. Superoxide anion-scavenging activity

Superoxidate anion-scavenging activity GBEP was conducted according to (Marklund & Marklund, 1974). 4.2 mL sample (0, 0.05 mg/mL and 0.1 mg/mL) solution was mixed with 4.5 mL Tris–HCl buffer (pH 8.2, 50 mM), incubated at 25 °C for 20 min, then 0.3 mL 25 °C pre-heated pyrogallol (10 mM) was added to the reaction system, mix thoroughly. Absorbance of the mixture was determined at 562 nm every 30 s for 5 min immediately on Cary 50 UV–Vis spectrophotometer (Varian Inc., USA). Ascorbic acid was used as positive control at the same concentration. The ability of different scavenging ability for self-oxidation of pyrogallol was calculated using the equation below:

$$\text{Scavenging effect (\%)} = \left(\frac{1 - A_{sample}}{A}\right) \times 100,$$

where A_{sample} is the slope of sample, A is the slope of control.

2.6.4. Reducing power

Reducing power assay was carried out in reference to Yan, Li, Chung, Tam, and Lin (2005). 1 mL sample (0.1–5 mg/mL) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1%), then incubated at 50 °C for 20 min. 2.5 mL trichloroacetic acid (10%, w/v) was added to the mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 mL) was mixed with 2.5 mL water and 0.5 mL $FeCl_3$ (0.1%, w/v). The absorbance was measured at 700 nm. Ascorbic acid served as positive control at the same concentration.

3. Results and discussion

3.1. Fractionation by DEAE-Sepharose fast flow

The profile showed unbound portion of crude polysaccharide was eluted with water, the bounded portion were eluted with stepwise gradient of NaCl. Fig. 1 showed the peaks of different fragments were clearly separated, thus indicated this method was ideal for GBEP separation. Five different polysaccharides were obtained, namely GBEP-N, GBEP-A1, GBEP-A2, GBEP-A3 and GBEP-A4, account for 17.75%, 21.50%, 14.26%, 33% and 0.8% of the total polysaccharide. respectively.

3.2. Molecular weight of purified polysaccharide

After Superdex 200 column treatment, two polysaccharides were obtained, namely GBEP-NN and GBEP-AA with the purity of 92.47% and 98.12%, respectively. High-pressure gel filtration chromatography result indicated GBEP-NN and GBEP-AA had the average molecular weight of 4567 Da and 5679 Da, respectively.

3.3. IR spectroscopy

The spectrum in Fig. 2a showed the characteristic absorption of carbohydrate. The stretching vibration of O–H falls into the wave number between 3600 cm^{-1} and 3200 cm^{-1} (Silverstein, Webster, & KiemLe, 2005). The broad and pure peak at 3413.97 cm^{-1} indicated intermolecular hydrogen bonding. The bands in the region of 2920.64 cm^{-1} was due to C–H stretching vibration. The strong peak at 1038.99 cm^{-1} was the stretching vibration of C–O. The FTIR spectrum of GBEP-NN featured a strong absorption at 912 cm^{-1} due to asymmetric vibration of the pyranose ring. In the anomeric region (950–700 cm^{-1}), the spectrum exhibited the characteristic absorption at 811.43 cm^{-1} due to the presence of mannose.

The acidic polysaccharide have similar absorption band in the spectrum, but it has more featured area that natural polysaccharide does not possess. In Fig. 2b, the absorption peak at 1736.92 cm^{-1}, 1412.86 cm^{-1} and 1249.72 cm^{-1} were the characteristic absorption of C=O stretching vibration, C–O stretching vibration and O–H bending, indicated the presence of –COOH. The strong absorption at 1612.77 cm^{-1} was the stretching vibration of carbonyl bond of the N–H, while 1018.10 cm^{-1} was the stretching vibration of the C–N, which indicated the presence of amines. These observations further confirmed that GBEP-AA was a polysaccharide containing protein and uronic acid. This may caused by the incomplete removal of protein by the Savag method.

3.4. Monosaccharide composition

Neutral polysaccharide GBEP-NN was composed of rhamnose, arabinose, mannose, glucose and galactose, with the molar ratio of 1.88:2.41:1.53:1:2. Acidic polysaccharide GBEP-AA was mainly composed of ten monosaccharides, i.e. mannose, rhamnose, glucuronic acid, galacturonic acid, galactosamine, glucose, galactose, xylose, arabinose, and fucose, with the molar ratio of 11.85:15.98:1:5.05:1:5.98:193.52:3.34:14.56:2.67, indicating GBEP contains more monosaccharide than just glucose, fructose, galactose and rhamnose reported by Song et al. (Song, Xu, Chen, & Wang, 1997).

3.5. Antioxidant activities of GBEP

3.5.1. DPPH radical-scavenging activity

DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavenger (Song, Zhang, Zhang, & Wang, 2010). On interacting with DPPH, antioxidants transfer either an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character (Naik et al., 2003). The DPPH radical-scavenging ability of GBEP was shown in Fig. 3. At the concentration from 0.1 mg/mL to 1 mg/mL, the DPPH radical scavenging activity increase with the polysaccharide concentration, this increase became less obvious when the concentration exceeded 2 mg/mL, whereas the overall DPPH radical scavenging activity was not as strong as ascorbic acid.

3.5.2. Hydroxyl radical-scavenging activity

The hydroxyl radical has a very short in vivo half-life of approximately 10^{-9} s and a high reactivity. This makes it a very dangerous

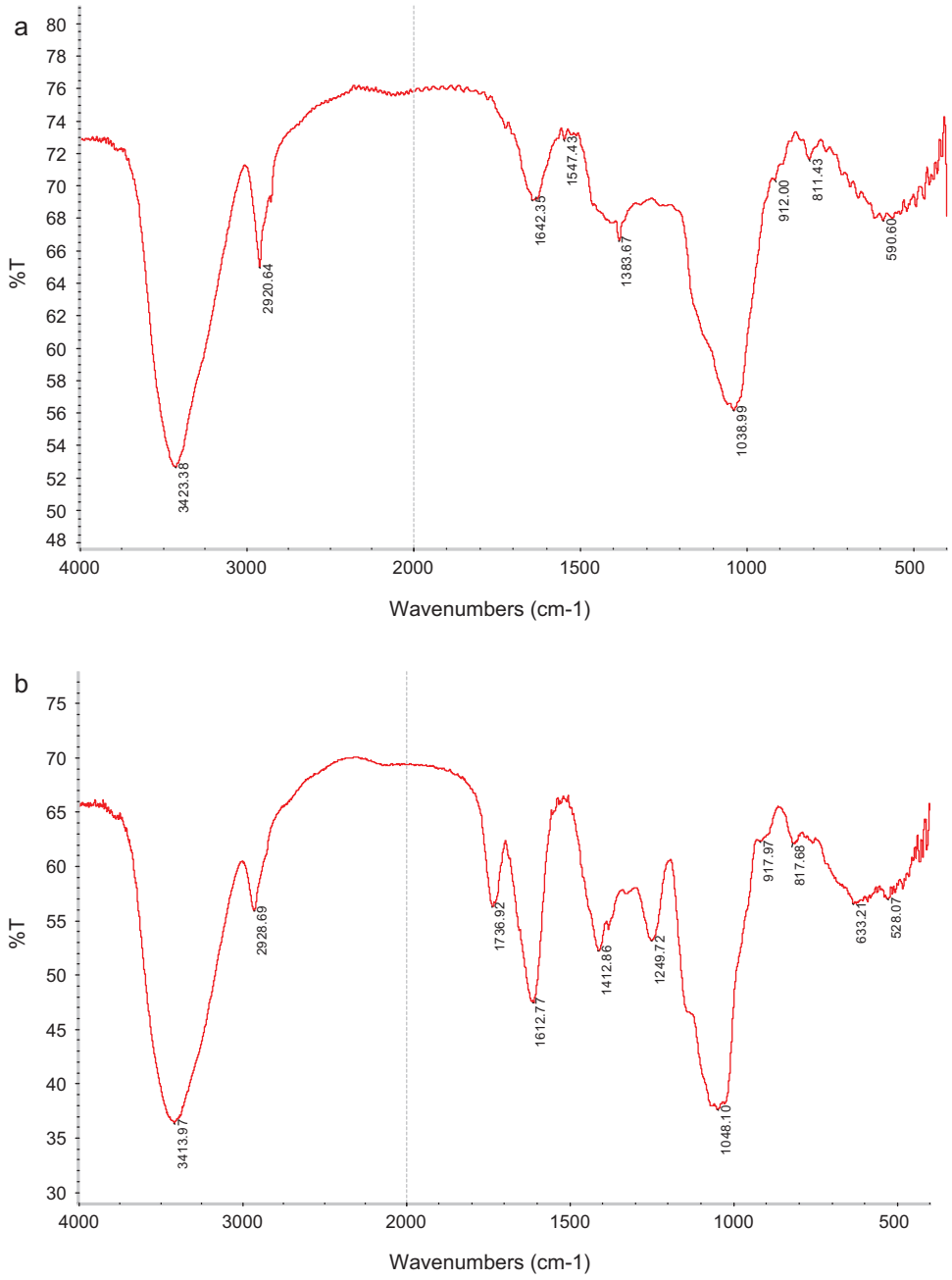

Fig. 2. (a) Infrared spectrum of GBEP-NN; (b) infrared spectrum of GBEP-AA.

compound to the organism (Sies, 1993). Most hydroxyl radicals are produced from the decomposition of hydro peroxides (ROOH). In this experiment, hydroxyl radicals were generated from 2,3-dihydroxybenzoic acid, generated by sodium salicylate in this reaction system. As shown in Fig. 4, the scavenging capacity of GBEP is week at low concentration, with only 3.67% at the concentration of 0.1 mg/mL, but increase quickly with higher concentration. Results show that GBEP has a high level of hydroxyl radical-scavenging effect, it is even stronger than the positive control ascorbic acid at a dose more than 4 mg/mL.

3.5.3. *Superoxide anion-scavenging activity*

The superoxide radical is a highly toxic species can be generated by numerous biological and photochemical reactions. In addition to directly attacking important biological molecules, superoxide radical may also decompose to form singlet oxygen and hydroxyl radicals, which may increase local oxidative stress and initiate cellular damage or lipid peroxidation and pathological incidents such as arthritis and Alzheimer's disease (Liu et al., 2010). The superoxide radical is one of the precursors of singlet oxygen and the hydroxyl radical. Superoxide radicals can be generated by pyrogallol auto oxidation. Pyrogallol (1,2,3-benzenetriol) has long been known to auto oxidize rapidly, especially in alkaline solution and the reaction has been employed for the removal of oxygen from gases (Marklund & Marklund, 1974). From Fig. 5a, the slope of pyrogallol became small after GBEP was added, which also suggests auto oxidation rate of pyrogallol was decreased by GBEP, while ascorbic acid has a higer inhibition capacity towards the atuo oxidation of pyrogallol. As can be seen in Fig. 5b, the superoxide anion-scavenging activity of GBEP was 90.46% for asorbic acid, while 18.82% for GBEP.

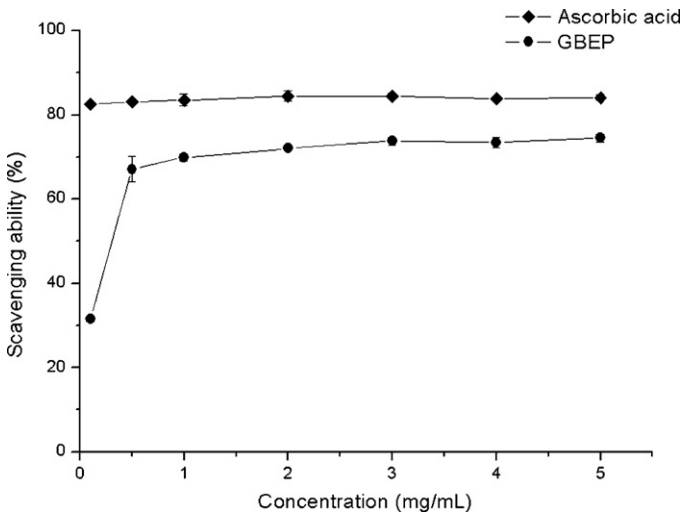

Fig. 3. DPPH radical scavenging activity of GBEP.

3.5.4. Reducing power

Reducing power of a compound is also a supporting feature for its antioxidant activity. The reducing properties are generally associated with the presence of reductones, which have been shown to exhibit antioxidant action by breaking the chain reactions by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation (Hikmet, Burhan, Gokhan, Selim, & Ismet, 2005). As shown in Fig. 6, GBEP exhibited higher reducing power as its concentration increased, this suggested GBEP may contain reductone-associated and hydroxide groups of polysaccharides can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reaction. GBEP exhibited a weaker reducing power than ascorbic acid.

4. Conclusion

High pressure gel filtration chromatography analysis indicated GBEP were low molecular weight polysaccharides. The average molecular weights of GBEP-NN and GBEP-AA were 4567 Da and 5679 Da, respectively. IR spectrum indicated GBEP-NN and GBEP-AA contains α-D-galactose and mannose, which was confirmed by monosaccharide analysis. The crude GBEP has certain antioxidant capability. It has good scavenging ability towards DPPH radical, hydroxyl radical. It can be exploited as antioxidant products and become a valuable bioresource, meanwhile it may solve the environmental problem caused by large amount of wasted material.

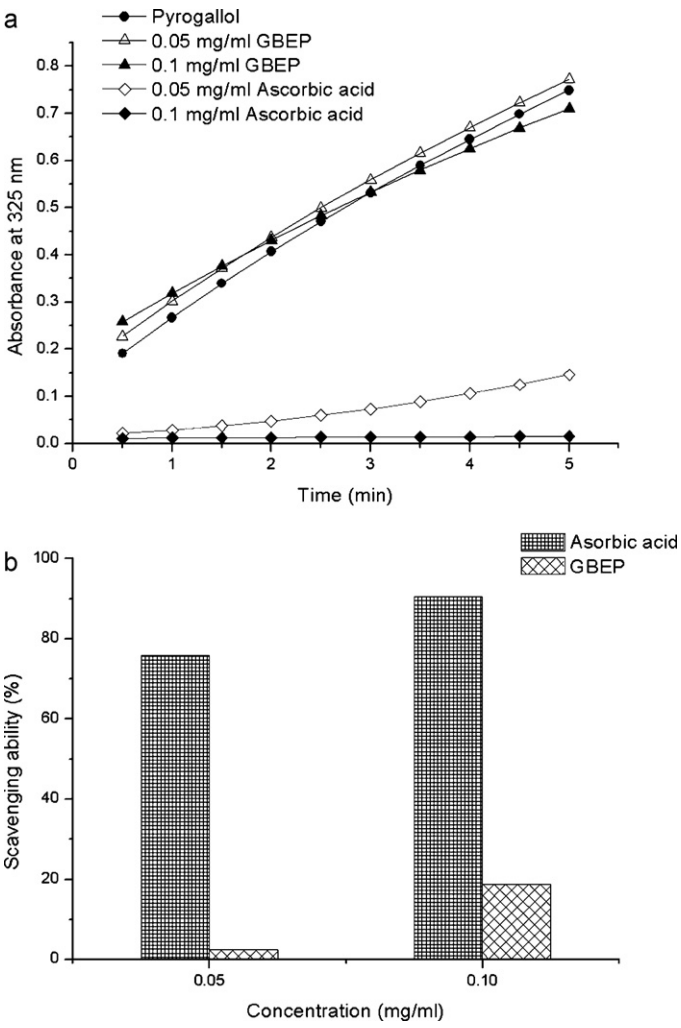

Fig. 5. (a) Auto oxidation rate of pyrogallol; (b) superoxide anion-scavenging activity of GBEP.

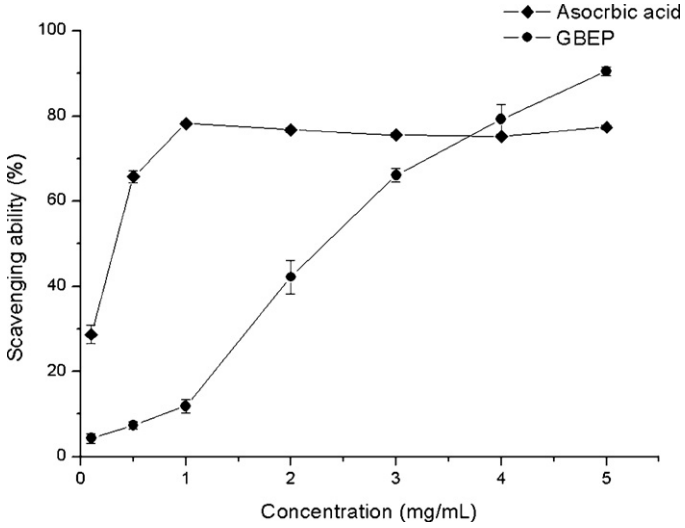

Fig. 4. Hydroxyl radical scavenging activity of GBEP.

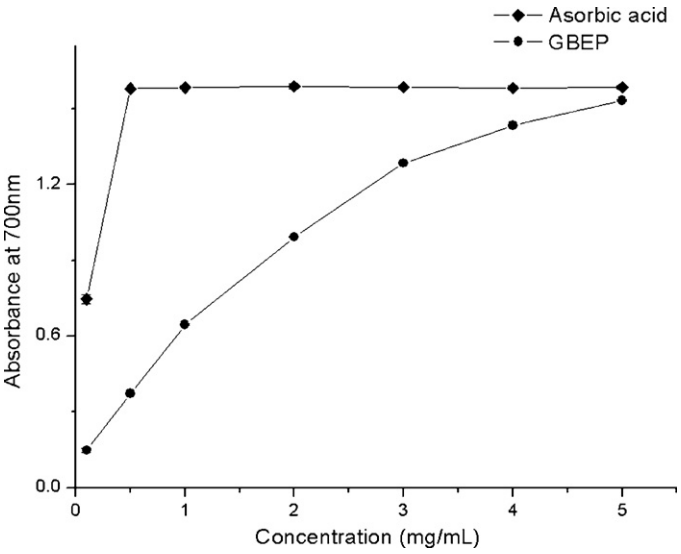

Fig. 6. Reducing power of GBEP.

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