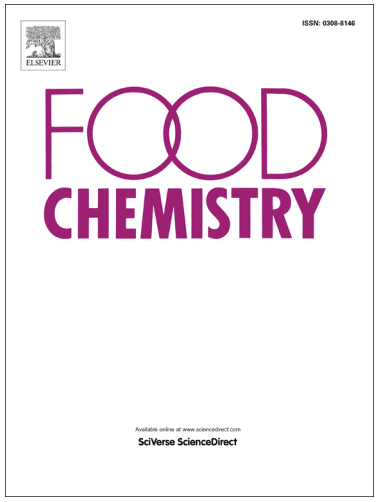

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Preparation, Antioxidant and Antimicrobial Evaluation of Hydroxamated Degraded Polysaccharides from *Enteromorpha prolifera*

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Abstract: In order to improve the antioxidant and antimicrobial abilities, hydroxamated degraded polysaccharides from *Enteromorpha prolifera* (HCDPE) were prepared from the corresponding carboxymethylated degraded polysaccharides (CDPE). HCDPE was characterized by FT-IR. The weight-average molecular weight of HCDPE was determined as 55.4 kDa. The *in vitro* antioxidant activity of HCDPE was evaluated by determining the radical (1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and superoxide anion radicals) scavenging abilities and total antioxidant activity. It was found that DPPH radical scavenging ability and total antioxidant activity of HCDPE were significantly improved compared to those of CDPE. The inhibitory effects of polysaccharides against the five bacterial strains (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella spp.*) were also evaluated by bacterial inhibition zone and minimum inhibitory concentration (MIC) assays. The results indicated that CDPE and HCDPE possess marked antimicrobial ability, while such an effect was not observed for the crude polysaccharides (PE) and the degraded polysaccharides (DPE).

Keywords: Enteromorpha Prolifera; Polysaccharide; Hydroxamate; Antioxidant activity; Antimicrobial activity

1. Introduction

Polysaccharides are widely distributed in animals, plants and microorganisms. They form an important component of most organisms and possess many key biological roles. Natural polysaccharides have been used in the food industry and in medicine for many years (Fan, Li, Deng, & Ai, 2012). Some natural polysaccharides have been demonstrated to possess multiple functions, such as anti-hyperlipidemic (Mao, Li, Gu, Fang, & Xing, 2004), antitumor (Chen et al., 2012), antimicrobial (Ghazala et al., 2015), and immune activities (Huang, Zhou, & Zhang, 2006). Therefore, polysaccharides have many potential applications in the food and pharmaceutical industries. However, their limited water-solubility and relatively low biological activities hinder their application. Chemical modification of polysaccharide chains, such as phosphorylation (Song, Ni, Hu, & Li, 2015), sulphation (Jin, Wang, Huang, Lu, & Wang, 2014), selenylation (Zhao et al., 2013), or the introduction of hydroxamates (Aiedeh & Taha, 2001), leads to changes in the physicochemical and biological properties of polysaccharides.

In the food industry, with the exception of functional food ingredients, polysaccharides could play an important role in controlling food quality due to their antioxidant and antimicrobial activities. Oxidation of lipids and microbiological spoilage in food products are the main causes for the decrease of food quality, which can be delayed or prevented by the incorporation of polysaccharides with antioxidant and antimicrobial activities. For instance, a polysaccharide isolated from garlic straw was reported to possess a strong antioxidant activity and marked antimicrobial activity, and

this polysaccharide was able to effectively extend the shelf-life of beef meat during refrigerated storage (Kallel et al., 2015). The xylan-chitooligomer-zinc complex, which possesses antioxidant and antimicrobial activity, was explored as a novel food preservative (Wu, Du, Hu, Shi, & Zhang, 2013). Anionic polysaccharides such as alginate and some dietary fibers (xanthan gum and pectin) have been reported to improve the oxidative stability of polyunsaturated lipids in fish oil (Salvia-Trujillo, Decker, & Mcclements, 2016; Qiu, Zhao, Decker, & McClements, 2015). Chitosan has attracted much attention as a natural preservative due to its antimicrobial activity against a range of foodborne filamentous fungi, yeast, and bacteria (Brasil, Gomes, Puerta-Gomez, Castell-Perez, & Moreira, 2012). In addition, the application of polysaccharide-based edible coating/film in food packaging has been widely investigated (Cazón, Velazquez, Ramírez, & Vázquez, 2017; Valencia-Chamorro, Palou, Del Río, & Pérez-Gago, 2011). Thus, there is considerable interest in developing polysaccharides with potent antioxidant and antimicrobial activities (Dong et al., 2017).

In our previous attempts to improve the antioxidant activity of polysaccharides from *Enteromorpha prolifera* (PE), PE was degraded, followed by chemical modification to produce carboxymethylated polysaccharide (CDPE). The radical scavenging activities and total antioxidant ability of CDPE were demonstrated to be greatly improved in comparison with PE and the degraded polysaccharide (DPE) (Shi et al., 2017). In order to further improve the biological activities of the polysaccharide, in the present study CDPE was modified to prepare hydroxamated polysaccharide (HCDPE). The antioxidant

activities of HCDPE are compared with those of CDPE, and the antibacterial activities of both HCDPE and CDPE are also reported.

2. Materials and methods

2.1. Materials and reagents

E. prolifera was harvested in February 2013 in Zhoushan, Zhejiang, China. The raw sample was rinsed carefully with fresh water and air-dried. The dried seaweed was milled with a blender, sieved (0.125 mm) and stored at 20 ^{o}C before use.

1,1-Diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid and iron standard solution (1000 mg/l) were purchased from Aladdin Chemical Reagents Co. (Shanghai, China). Riboflavin, nitroblue tetrazolium and DL-methionine were purchased from Sinopharm Chemical Reagents Co. (Hongkong, China). The solvents and other chemicals used in this work were of analytically pure reagent grade.

2.2. Preparation of hydroxamated polysaccharide (HCDPE)

Carboxymethylated degraded polysaccharides from *E. prolifera* (CDPE) were prepared according to our recent report (Shi et al., 2017). Briefly, crude polysaccharides (PE), extracted from *E. prolifera* with hot water, were degraded by ascorbic acid in combination with hydrogen peroxide. In the presence of 9 mM of ascorbic acid and hydrogen peroxide, DPE was obtained after incubation for 2 h. Next, a solution of DPE (0.3 g) in DMSO (12.5 ml) and NaOH (20%, 5 ml) was stirred at 40 ^{o}C for 3 h, then

chloroacetic acid solution in DMSO (12.5 ml) and NaOH (20%, 5 ml) was added (the final concentration of chloroacetic acid was 2.1 M). The resulting solution was heated to 55 ^{o}C for 4 h. The reaction mixture was cooled to room temperature and adjusted to pH 7 with 0.5 M HCl, diluted with water, and dialyzed (MW cut off 3500) in distilled water for 48 h, then lyophilised, yielding carboxymethylated extract (CDPE). The resulting CDPE had a degree of carboxymethyl substitution (DS) of 0.849, which was determined using a colorimetric method as described in our previous report (Shi et al., 2017).

CDPE was further modified to prepare hydroxamated polysaccharide derivatives (HCDPE) based on a previously published method with some modifications (Aiedeh & Taha, 2001). To a solution of CDPE (0.3 g in 16 ml water), which was adjusted to pH 4.0-4.5 with HCl (1.0 M), was added *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC) hydrochloride (0.1 g, 0.52 mmol). The mixture was stirred at room temperature for 2h. Hydroxylamine hydrochloride (0.2 g, 2.89 mmol) was added, followed by the addition of 4-dimethylaminopyridine (DMAP, 0.01 g) as a catalyst. The reaction mixture was stirred for 2 h, then adjusted to pH 6.0 with NaOH (1.0 M). Stirring was continued for 2 h. Thereafter, the pH of the reaction mixture was raised to 9.0 with NaOH (1.0 M), and the resulting reaction mixture was stirred at room temperature for 24 h. After completion of the reaction, the solution was concentrated on a rotary evaporator. The residue was mixed with ethanol (95%, 20 ml) and allowed to stand at 4 ^{o}C overnight. The resulting precipitate was collected by centrifugation (8000 rpm, 20 min), and washed with ethanol 4 times. The precipitate was redissolved in water, and dialyzed (MW cut off

3500) in distilled water for 48 h, then lyophilized, yielding the hydroxamated derivative (HCDPE).

2.3 Characterization of HCDPE

2.3.1. Fourier transform infrared spectra (FT-IR) analysis

FT-IR spectra were determined on Nicolet 380 infrared spectrometer with KBr pallet.

2.3.2. Molecular weight analysis

Molecular weight of polysaccharides was measured using high-performance gel permeation chromatography (HP-GPC) on a Waters 1000 HPLC system, with a Grace 3300 Evaporative light Scattering Detector, ELSD. Samples (10.0 mg) were dissolved in distilled water (10.0 ml), passed through a 0.45 μm filter and applied to a gel-filtration chromatographic column of Ultrahydrogel^{TM} Linear (300 mm×7.8 mm. Waters., USA). Deionized water was used as the flow phase at a flow rate of 0.5 ml/min. The temperature of the column was maintained at 30 ^{o}C and the injection volume was 10 μl. Preliminary calibration of the column was carried out using Dextran standards with different molecular weights.

2.3.3. Determination of iron binding capacity

Determination of iron(III) chelating capacity of the HCDPE was carried out using a previously published method with some modifications (Zhou et al., 2015a). HCDPE

solution (15 mg/ml) was prepared by dissolving HCDPE (300 mg) in ammonium bicarbonate solution (20 ml, 50 mM, pH 7.7). A range of solutions with different ratios of HCDPE to iron were prepared by adding iron standards (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml; 5 mg/ml) to the HCDPE solution (1.0 ml). The total volume of the mixed solutions was adjusted to 3.5 ml with ammonium bicarbonate solution (50 mM). All samples were equilibrated at room temperature for 40 min before spectral acquisition. The spectra of the solutions were recorded on a UV spectrophotometer scanning from 280 to 700 nm. The iron-binding capacity of HCDEP is expressed as mmol iron/g HCDPE.

2.4. Assays for antioxidant activity

2.4.1. DPPH radical scavenging activity assay

The effect of scavenging DPPH radicals was measured according to a reported method with minor modification (You, Zhao, Regenstein, & Ren, 2011). DPPH radical solution (0.1 mM) was prepared by dissolving in methanol. A polysaccharide solution (2 ml; 1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml) in distilled water was added to DPPH solution (2 ml, 0.1 mM). The resulting solution was shaken rigorously and incubated in the dark at room temperature for 1 h. The absorbance of the solution was determined at 517 nm. Ascorbic acid was used as a positive control. The control was made in the same manner, except methanol replaced DPPH methanol solution. Distilled water replaced sample solution for the blank. The DPPH radical scavenging activity of the sample was calculated as:

DPPH scavenging rate (%) = [1- (A_{sample} - $A_{control}$) / A_{blank}] × 100%

2.4.2. Hydroxyl radical (·OH) scavenging activity assay

The hydroxyl radical scavenging activity was measured by Deng's method (Deng et al., 2012). A polysaccharide aqueous solution (2 ml) with different concentrations (1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml) was mixed with a H_2O_2 solution (0.7 ml, 3%), $FeSO_4$ solution (1 ml, 1.5 mM) and salicylic acid solution (0.3 ml, 20 mM in ethanol). The resulting mixture was mixed and incubated at 37 ^{o}C for 30 min. The hydroxyl radical was detected by monitoring absorbance at 517 nm. Distilled water replaced salicylic acid-ethanol for the control, while distilled water replaced the sample solution for the blank. Ascorbic acid was used as a positive control. The hydroxyl radical scavenging activity was calculated as:

·OH scavenging rate (%) = [1 - (A_{sample} - $A_{control}$) / A_{blank}] × 100%

2.4.3. Superoxide anion radical (·O_2^-) scavenging activity assay

The effect of scavenging superoxide anion radicals was determined according to Prasad's method with minor modification (Prasad et al., 2009). To a polysaccharide solution (1.5 ml) with different concentrations (1.0, 2.0, 3.0, 4.0 and 5.0 mg/ml) was added to a methionine solution (0.9 ml, 26 mM) in PBS buffer (pH 7.8, 0.1 M), nitroblue tetrazolium (0.3 ml, 0.75 mM) and riboflavin solution (0.3 ml, 20 μM) containing 1.0 μM EDTA. The reaction solution was exposed to a fluorescent lamp under the condition of light 3500 Lx for 30 min and the absorbance was measured at 560 nm. Distilled water replaced reaction liquid (riboflavin, methionine and nitroblue tetrazolium) for the control,

while distilled water replaced sample solution for the blank. Ascorbic acid was used as a positive control. The superoxide anion radical scavenging activity of the sample was calculated as:

$\cdot O_2^-$ scavenging rate (%) = [1- (A_{sample} - $A_{control}$)/A_{blank}] × 100%

2.4.4. Determination of total antioxidant activity

Ferric reducing ability of plasma (FRAP) method (Luo, Li, & Kong, 2012) was employed to measure the total antioxidant capacity of polysaccharide samples by a test kit. Stock solutions included detective buffer, 2,4,6-tripyridyl-s-triazine (TPTZ) solution, TPTZ dilution, 0.5 ml 10 mM $FeSO_4$ solution and 0.1 ml 10 mM Trolox (an analogue of vitamin E) solution. A working solution was prepared freshly by mixing TPTZ dilution, detective buffer and TPTZ solution in a ratio of 10:1:1 (v/v), respectively. The working solution was warmed to 37 ^oC before use. Polysaccharide sample (5 μl, 1–5 mg/ml) was mixed with 180 μl of FRAP working solution and incubated for 5 min at 37 ^oC. The absorbance of the reaction mixture was then recorded at 593 nm. The standard curve was prepared using $FeSO_4$ ranging from 0.15 to 1.50 mM. The absorbance of 1 mM ferrous salt at 593 nm is defined as 1 mM equivalent antioxidant capacity. The activity was expressed by $FeSO_4$ values, which were calculated using standard curves.

2.5. Antibacterial assays

2.5.1. Microbial strains and culture conditions

Two Gram-positive bacteria (*Bacillus subtilis* CGMCC 1.0108 and *Staphylococcus aureus* CGMCC 1.0089) and three Gram-negative bacteria (*Escherichia coli* CGMCC 1.0907, *Pseudomonas aeruginosa* CGMCC 1.2342 and *Salmonella spp.* CGMCC 1.1552) were purchased from China General Microbiological Culture Collection Center (CGMCC). The media used in this study were nutrient agar medium (NA) and tryptone soybean broth (TSB), which were purchased from Beijing Land Bridge Technology Co. Ltd. All bacteria were inoculated on NA and cultured at 37 °C for 16-18 h. Single colonies were inoculated into 5 ml of TSB and incubated at 37 °C for 18-24 h. The cultures were then transferred into 50 ml of fresh TSB and incubated at 37 °C to an OD value corresponding to a bacterial number of approximately 10^8 cfu/ml. Serial decimal dilution of the test bacteria cultures was performed to select the optimum bacteria concentration for antibacterial assays (Chen et al., 2016).

2.5.2. Determination of bacterial inhibition zone

The Oxford cup method was employed to determine the bacterial inhibition zone according to the literature with slight modification (Xu et al., 2013). Melted agar medium was added to each sterile Petri dish and allowed to solidify. One millilitre of suitable diluted inoculum (approximately 10^4 cfu/ml) was added to agar medium and mixed well. Ten millilitre of this culture was spread on the surface of the solidified media in the above mentioned Petri dish. The mixture was immediately mixed and allowed to solidify, labeling was done at the bottom of the dish. The sterile Oxford cups (Φ 6 mm) were placed on the surface of the seeded agar medium and filled with sample solutions (200 μl,

16 mg/ml). Sterile water was used as control. The plates were incubated at 37 °C for 20-24 h. The antimicrobial activity was evaluated by measuring the diameter of transparent inhibition zone against the test bacteria. All the antibacterial assays were performed in triplicate.

2.5.3. Determination of minimum inhibitory concentrations (MICs)

MIC values of polysaccharide samples against five bacterial species were determined by the serial dilution method (Siddiqui, Farooq, Musthafa, Ahmad, & Khan, 2013) with some modifications. The incubation medium was TSB. The polysaccharide solution was serial diluted by TSB. All tubes (13×100 mm) contained 1.0 ml polysaccharide solution and 1.0 ml of diluted bacterial inoculum (approximately 10^4 cfu/ml), with a final concentration of polysaccharide solution of 0, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml. After incubation at 37 ^{o}C for 24 h, MICs were measured by visual inspection of the turbidity of broth in tubes (Xie et al., 2013). All assays were carried out in triplicate.

2.6. Statistical analysis

All experiments were performed in triplicate. The data were statistically analyzed using analysis of variance test (SPSS Statistics 19 software). Significant differences between the treatments were examined by Duncan's new multiple range test (DMRT). $P < 0.05$ was considered as statistically significant difference.

3. Results and discussion

3.1. Preparation and characterization of HCDPE

In the presence of EDC and DMAP, hydroxylamine was coupled to the carboxyl group in CDPE, providing the hydroxamate derivative HCDPE (Fig. 1). Hydroxylamine hydrochloride was added in 5-fold excess (compared to EDC) to ensure efficient derivatisation, particularly under the sterically hindering environment of the polysaccharide. The successful introduction of hydroxamic acid groups was confirmed by FT-IR analysis of HCDPE, and UV-visible spectra of HCDPE-iron(III) complex.

FT-IR analysis. FT-IR spectra of HCDPE are presented in Fig. 2. The IR spectra of CDPE was previously reported (Shi et al., 2017) and also included in Fig. 2 for comparison. The broad band at 3376 cm^{-1} in the spectra of HCDPE is assigned to the stretching vibration of O-H and N-H groups, which is at lower wave number when compared to the stretching vibration of O-H in CDPE appearing at 3456 cm^{-1}. The peak at 1607 cm^{-1} in the spectra of CDPE is assigned to asymmetrical COO^- stretching vibrations, which is shifted to 1651 cm^{-1} in the spectra of HCDPE, confirming the formation of hydroxamic carbonyl groups. The bands at 1541 cm^{-1} and 1041 cm^{-1} were assigned to N-H and C-N , respectively (Hoidy, Ahmad, Al-Mulla, Yunus, & Ibrahim, 2010; Rha et al., 2011).

Iron(III)-chelating capacity of HCDPE. Complexation of HCDPE with iron(III) were undertaken at room temperature for at least 40 minute in order to ensure the binding

process was complete. The maximum absorbance wavelength of iron(III)-HCDPE complex was found to be 452 nm (Fig. 3a), which is in accordance with the maximum wavelength of bis(hydroxamate)-iron(III) complex (Galvez, Ruiz, Cuesta, Colacio, & Dominguez-Vera, 2005). The difficulty in the formation of tris(hydroxamate)-iron(III) complex could be attributed to hindrance of polysaccharide, resulting in failure to form an ideal octahedral iron(III) coordination site for each of the bidentate ligands (Zhou, Kong, Liu, Liu, & Hider, 2008). The intensity of absorbance at 452 nm increased with increasing the amount of iron until iron addition reached 0.7 ml, after which the absorbance remained essentially constant, indicating no further formation of HCDPE-iron(III) complex (Fig. 3b). The iron chelating capacity of HCDEP was calculated to be 0.417 mmol/g. Thus, the content of hydroxamate group in HCDEP was estimated to be 0.834 mmol/g.

Determination of molecular weight. The molecular weight distribution of the polysaccharide derivatives was measured by HPGPC. According to the standard curve obtained from Dextran standards with different molecular weights ($\log Mw = -0.3463Rt + 12.4796$, $R^2 = 0.9991$) and retention time (Rt) of polysaccharide peak, the weight-average molecular weight (Mw) of HCDPE was calculated to be 55.4 kDa, which was higher than that of CDPE (53.7 kDa).

3.3. Antioxidant activity analysis of HCDPE

In our previous report (Shi et al., 2017), the antioxidant ability of CDPE was demonstrated to be greatly improved when compared to PE and DPE. In this study, the antioxidant ability of HCDPE was investigated (Fig. 4). For comparison, the data for CDPE are also presented in Fig.4.

3.3.1. Scavenging effects on DPPH radicals

DPPH radical scavenging activity is a general method for the determination of the anti-radical effects of substances (Zoete et al., 2000). When DPPH free radicals are scavenged by samples, a color change from red to yellow is observed, showing anti-radical activity (Rha et al., 2011). Thus, the absorption at 517 nm increases in a concentration-dependent manner. The DPPH free radical scavenging effects of CDPE and HCDPE were monitored (Fig. 4a). The DPPH radical scavenging ability of HCDPE was found to be significant higher than that of CDPE ($P < 0.05$). For instance, at 5 mg/ml the removal of DPPH radical by HCDPE reached 68.1%, while the data for CDPE was 39.6%. The IC_{50} values of HCDPE and CDPE for scavenging DPPH were calculated to be 2.8 and 5.7 mg/ml, respectively. Both are weaker than vitamin C (IC_{50} 0.03mg/ml). It was reported that the DPPH scavenging activity of low-viscosity alginic acid hydroxamates (LVA-NHOH) and medium-viscosity alginic acid hydroxamates (MVA-NHOH) were greatly increased, with IC_{50} values of 0.0298 and 0.0245 mg/ml, respectively (Liu, Wu, Liang, & Hou, 2007), while little or no DPPH scavenging activity of low-viscosity alginic acid or medium-viscosity alginic acid (original material) was found at the same concentrations. Improved DPPH scavenging activity of a

hydroxamate derivative of pectin was also reported (Yang, Cheng, Lin, Liu, & Hou, 2004). The scavenging of DPPH radicals is dependent on hydrogen-donating ability of the hydroxyl groups. The hydroxyl group on hydroxamate moiety is likely to be a good hydrogen donor for the reduction of DPPH radicals.

3.3.2. Hydroxyl radical scavenging effect

One of the reactive oxygen species generated in cells is the hydroxyl radical, which can react easily with biomolecules, such as amino acids, proteins and DNA. This can lead to physiological disorders (Cacciuttolo, Trinh, Lumpkin, & Rao, 1993). Avoiding the influence of the hydroxyl radical is important for antioxidant defense in cell and food systems. In this study, the Fenton-type reaction was used to generate hydroxyl radicals, and the hydroxyl radical scavenging activity of the polysaccharide was determined using salicylic acid as a molecular probe. As shown in Fig. 4b, with increasing concentration, the scavenging activity of CDPE and HCDPE increased slightly ranging from 1 to 5 mg/ml. At a concentration of 2 mg/ml, the inhibition rates of HCDPE and CDPE on hydroxyl radical were determined to be 84.6% and 89.4%, respectively. The IC_{50} values of HCDPE and CDPE for scavenging hydroxyl radical were calculated to be 0.73 and 0.70 mg/ml, respectively, which were higher than that resulting from Vitamin C (IC_{50} 0.40 mg/ml), but were lower than that of degraded polysaccharide (DPE, IC_{50} 2.5 mg/ml)) and the original polysaccharide (PE, IC_{50} 6.2 mg/ml) (Shi et al., 2017). The enhanced hydroxyl radical scavenging activity of HCDPE compared to DPE and PE could be attributed to the iron-binding ability of hydroxamate moieties. The formation of

HCDPE-Fe^{3+} complex inhibited the occurrence of Fenton reaction, thereby reducing hydroxyl radical generation. However, the hydroxyl radical scavenging activity of HCDPE was found to be slightly lower than that of CDPE, which possessed a lower iron(III) affinity than HCDPE. Thus, another mechanism of OH• elimination by a polysaccharide, so-called hydrogen atom transfer (HAT) reaction, could also be involved (Blanksby & Ellison, 2003). It was speculated that methylene hydrogens in carboxymethyl groups are removed more easily by reactive hydroxyl radicals (Machová, Čížová , & Bystrický, 2014).

3.3.3. Scavenging effects on superoxide anion radical

The superoxide radical can be generated by numerous biological and photochemical reactions (Fan, Li, Deng, & Ai, 2012), for instance, using reduced flavins, it can reduce nitroblue tetrazolium to form blue formazan, measured as a rise in absorbance at 560 nm, which represents the superoxide radical content. The scavenging effects of the two polysaccharides on the superoxide radical are shown in Fig. 4c. The superoxide radical scavenging activity of CDPE reached 65.4% at 5 mg/ml, while HCDPE showed 62.9% scavenging activity at the same concentration. The IC_{50} values of HCDPE and CDPE for scavenging superoxide anion radical were calculated to be 3.3 and 3.0 mg/ml, respectively, indicating that the superoxide anion radical scavenging ability of HCDPE was slightly weaker than that of CDPE (P>0.05). Liu (Liu, Lin, Lee, & Hou, 2008) reported that the galacturonyl hydroxamic acid exhibited dose-dependently scavenging activity against superoxide radicals and the IC_{50} was calculated to be 0.823 mM, while

the galacturonic acid had no effects on superoxide radical scavenging activity up to 2.5 mM.

3.3.4. Total antioxidant activity

The FRAP method has been widely used for the evaluation of total antioxidant activity. This simple and reliable test measures the potential of an antioxidant reducing TPTZ-Fe(III) complex to produce a TPTZ-Fe(II) complex by electron donation. The corresponding $FeSO_4$ values were calculated using standard curves and regression equations. A high $FeSO_4$ value indicates a higher ferric reducing power. As shown in Fig.4d, the total antioxidant activity of HCDPE is appreciably enhanced, when compared to that of CDPE ($P < 0.05$). At 5 mg/ml, the total antioxidant activity of HCDPE was determined to be equivalent to 2.12 mM $FeSO_4$, while that of CDPE was 1.16 mM $FeSO_4$. The superior ferric reducing power of HCDPE to CDPE is likely to be associated with the iron-binding ability and electron-donating property of hydroxamic acid moieties in HCDPE (Jia et al., 2014).

3.4. Antimicrobial activity

The antimicrobial activity of polysaccharide derivatives against the five tested strains (two Gram-positive bacteria *S. aureus* and *B. subtilis,* and three Gram-negative bacteria *E. coli*, *Salmonella spp.* and *P. aeruginosa*) were evaluated by inhibition zone assay and determination of MICs assay using kojic acid, a natural metal chelator, as a positive control.

3.4.1. Bacterial inhibition zone assay

As shown in Table 1, both CDPE and HCDPE exhibited marked inhibitory effects against all five strains, while PE and DPE had no inhibitory activity at the same concentration (16 mg/ml). The inhibitory influence of polysaccharides against bacteria has rarely been reported. The inhibition of the iron absorption by the bacteria by scavenging iron in the environment is a possible antibacterial mechanism of modified polysaccharides, as iron is an essential element for bacterial growth. Chelators with high iron(III) affinities have been demonstrated to possess antibacterial activity (Xu et al., 2011; Zhou et al., 2015b; Bergan, Llaveness, & Aasen, 2001). HCDPE showed a stronger antibacterial activity than CDPE against most tested strains with the exception of *E. coli*. Bidentate hydroxamates can bind iron(III) with a relatively high affinity ($\log\beta_3$ is about 28) (Zhou, Winkelmann, Dai, & Hider, 2011). CDPE, binds iron using the carboxyl and hydroxyl groups, and therefore is assumed to possess a lower iron(III) binding affinity. As for the exception in the case of *E. coli*, this is probably due to the fact that enterobactin secreted by *E. coli* possesses extremely high iron affinity ($\log K_1$ is about 48) (Hider, & Kong, 2010), and thus can compete with other chelators for iron binding. In such cases, the iron(III) binding affinity of chelators may be not the dominating factor in the bacterial inhibition. Although HCDPE was found to exhibit slightly better inhibitory effect than that of kojic acid against *P. aeruginosa* and *Salmonella spp.*, kojic acid exhibited a superior inhibitory effect to that of HCDPE in the cases of *B. subtilis, S. aureus* and *E. coli*. The sensitivity of five strains to HCDPE follows the order: *P. aeruginosa* > *B.*

subtilis > *Salmonella spp.* > *S. aureus* > *E. coli*. It was previously reported that the antibacterial effects of degraded polysaccharide from *E. prolifera* were not obvious under the experimental concentrations of 20, 40 and 80 mg/ml (Lü, Gao, Shan, & Lin, 2014). However, degraded polysaccharide selenide (Se-LEP) showed some antibacterial effect at the above concentrations, the inhibition zone diameters being 10.88, 12.39 and 14.50 mm for *E. coli*, and 8.90, 9.20 and 12.13 mm for *S. aureus*, respectively.

3.4.2. MICs assay

The minimum inhibitory concentration (MIC) assay also demonstrated that CDPE and HCDPE exhibited antibacterial activity (Table 2). MICs of CDPE against *E. coli*, *S. aureus*, *B. subtilis*, *Salmonella spp.*, *P. aeruginosa* were determined to be 2, 4, 4, 2 and 2 mg/ml, respectively, the corresponding data for HCDPE were 4, 2, 1, 2 and 1 mg/ml, respectively. Thus, the MIC assay also demonstrated that HCDPE possessed superior antibacterial effect against most of the tested bacterial strains, which is in agreement with the results of inhibition zone assay. Among the tested strains, *P. aeruginosa* and *B. subtilis* were found to be more sensitive to HCDPE. The results regarding the sensitivity order of these bacterial strains to polysaccharides derivatives are in good agreement with those of inhibition zone test.

4. Conclusions

In this study, hydroxamated degraded polysaccharide originating from *E. Prolifera* (HCDPE) was prepared by coupling of carboxylmethylated polysaccharide (CDPE) with

hydroxylamine. DPPH radical scavenging activity and total antioxidant ability of HCDPE have been greatly improved when compared to those of CDPE. HCDPE also exhibits superior inhibitory effect against most test bacterial strains, and so could find application in the food and pharmaceutical industries.

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Figure captions

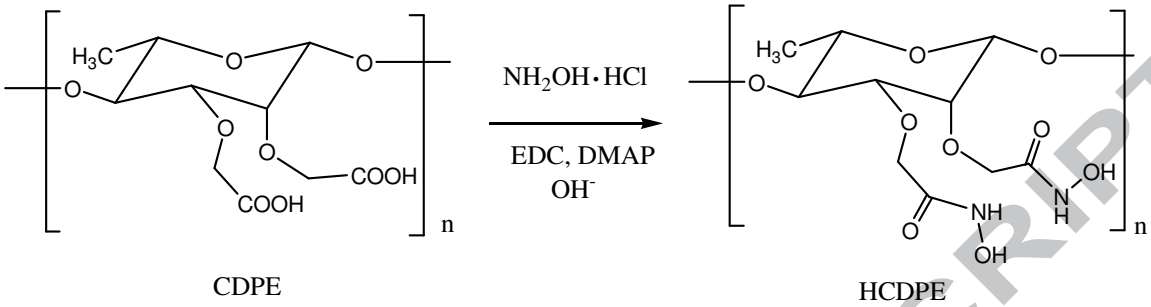
Fig. 1. Preparation of hydroxamated degraded polysaccharides (HCDPE).

Fig. 2. Fourier transform infrared spectra of polysaccharides. Upper: HCDPE; lower: CDPE.

Fig. 3. (a) UV-Vis spectra of HCDPE-iron(III) spectra; (b) Plots absorbance (452 nm) of sample versus amounts of added iron(III) solution.

Fig. 4. Antioxidant assays of CDPE and HCDPE. (a) DPPH radical scavenging activity; (b) hydroxyl radical scavenging activity; (c) superoxide anion radical scavenging activity; (d) total antioxidant ability.

Fig. 1



CDPE HCDPE

Fig. 2

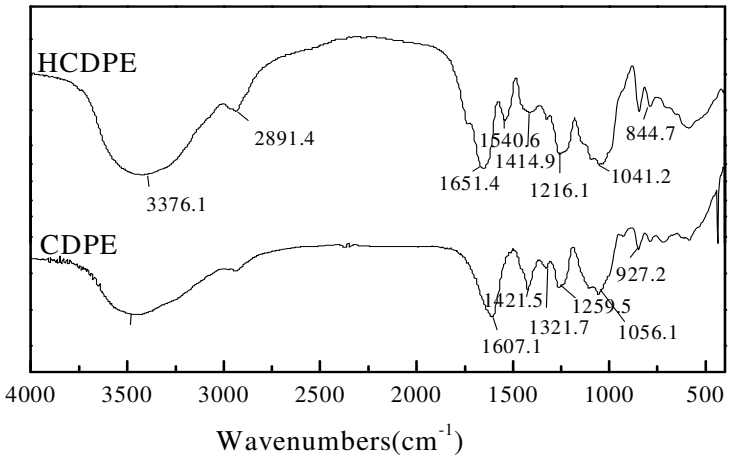


Fig. 3

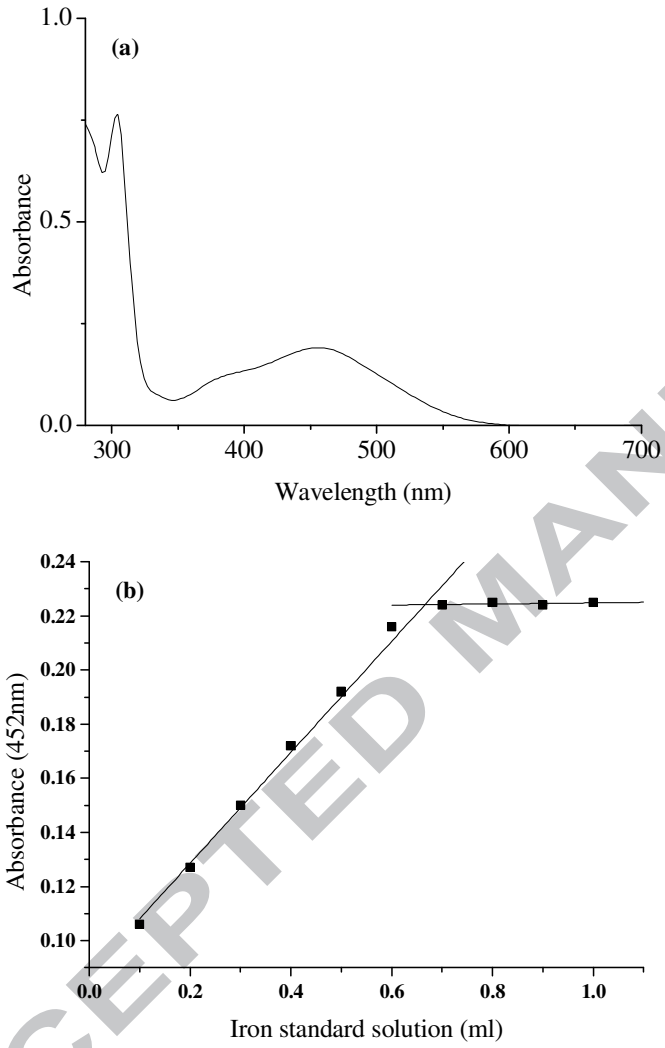
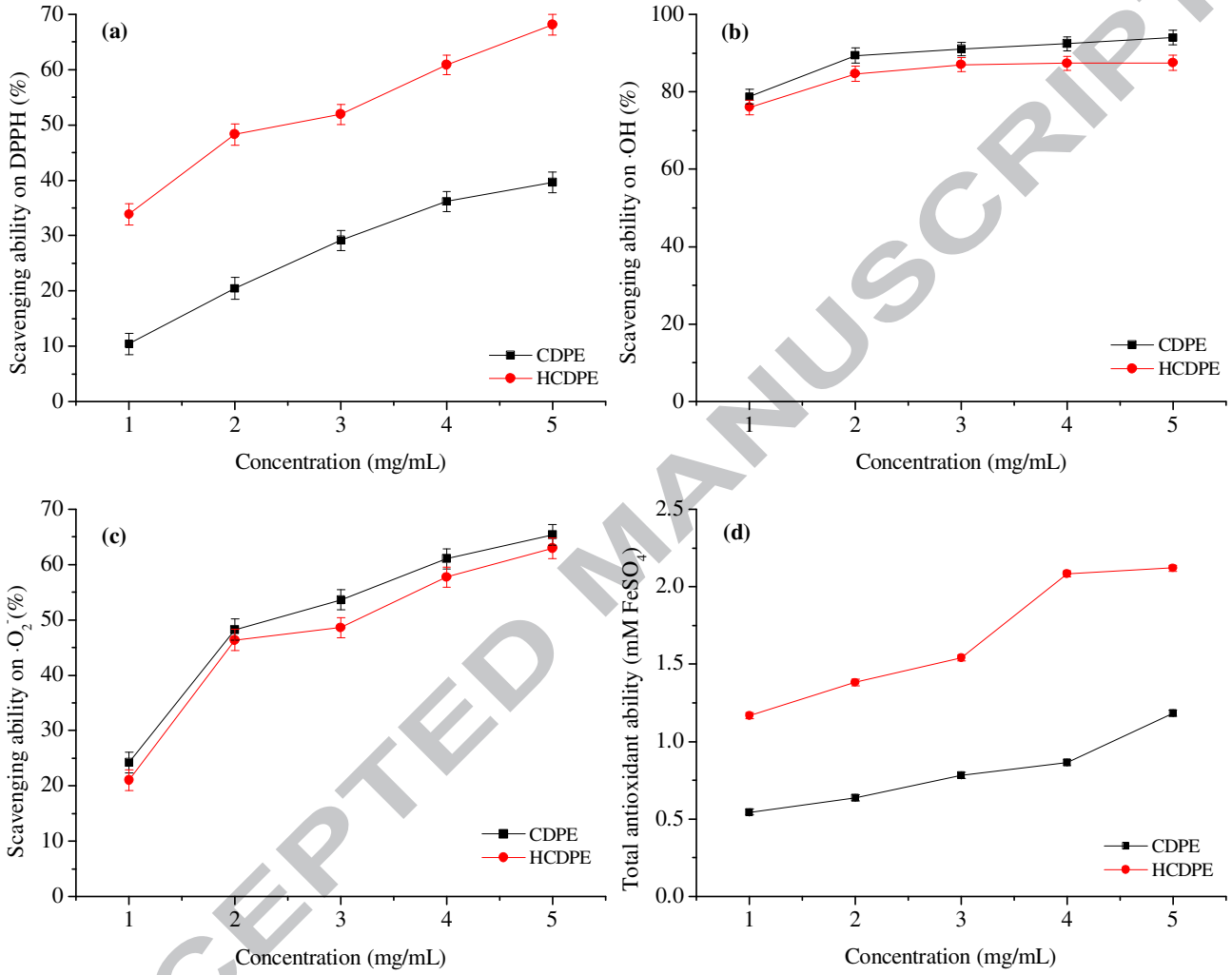


Fig. 4



Tables

Table 1. Inhibition zone diameters against five bacterial strains (mm)

Samples	Gram-negative bacteria			Gram-positive bacteria	
	E. coli	*Salmonella spp.*	*P. aeruginosa*	*B. subtilis*	*S. aureus*
Sterile water	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Kojic acid	16.28^{c}±0.05	15.08^{a}±0.02	15.96^{b}±0.02	17.13^{c}±0.03	16.70^{c}±0.03
PE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
DPE	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CDPE	15.06^{b}±0.02	15.06^{a}±0.03	15.01^{a}±0.03	12.16^{a}±0.06	13.60^{a}±0.04
HCDPE	12.08^{a}±0.03	15.44^{b}±0.02	16.24^{c}±0.03	15.57^{b}±0.04	15.03^{b}±0.04

Note: The different letters within each group indicate significant differences ($P < 0.05$).

Table 2. The MICs of polysaccharides against five bacterial strains (mg/ml)

Bacterial strains	CDPE	HCDPE
E. coli	2	4
S. aureus	4	2
B. subtilis	4	1
Salmonella spp	2	2
P. aeruginosa	2	1

Highlights

- Hydroxamated derivative of degraded polysaccharides from *E. prolifera* was prepared.
- The antioxidant activity of the hydroxamated polysaccharide was greatly improved.
- Both hydroxamated and carboxylmethylated polysaccharide possess antibacterial activity.