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Purification, composition analysis and antioxidant activity of the polysaccharides from *Dendrobium nobile* Lindl.

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ABSTRACT

Crude water-soluble polysaccharides were extracted from the stem of *Dendrobium nobile* Lindl. using boiling-water. The polysaccharides were successively purified by chromatography on DEAE-cellulose and Sephadex G-200 column, giving four major polysaccharide fractions termed DNP1-1, DNP2-1, DNP3-1, DNP4-2. The gel permeation chromatography (GPC) analysis showed that the average molecular weight (Mw) of polysaccharides (DNP1-1, DNP2-1, DNP3-1, DNP4-2) were approximately 136 kDa, 27.7 kDa, 11.8 kDa and 11.4 kDa, respectively. Monosaccharides analysis revealed the dominance of mannose, glucose, galactose, and smaller amounts of rhamnose, arabinose and xylose in the four polysaccharides. The evaluation of antioxidant activity suggested that DNP4-2 had good potential for scavenging activity of ABTS radical, and higher scavenging activity of hydroxyl radical and DPPH radical than the other polysaccharides, and should be explored as a novel potential antioxidant.

1. Introduction

Dendrobium nobile Lindl., Orchidaceae, is a precious herbal plant in Chinese traditional medicine (Shu, Guo, Chen, Wang, & Yang, 2004), and it is one of the three *Dendrobium* species which were specified in the Chinese Phamacopeia (2005). There are many published reports on *Dendrobium* constituents (Chen & Guo, 2001), and the water extractable fraction from its stem has been used for the treatment of cataracts and digestive system disorders. In recent years, studies on *Dendrobium* have attracted more attention due to the potential inhibition and prevention of cancer functions, immunostimulatory and antioxidant activity. Polysaccharides have been isolated from some *Dendrobium* species (Fan et al., 2009; Hua, Zhang, Fu, Chen, & Chan, 2004; Wang, Zheng, & He, 1988; Zha, Luo, Luo, & Jiang, 2007; Zhao, 1994). However, the polysaccharides from *D. nobile* have not been investigated. In our prevenient work, we obtain a crude water-soluble polysaccharide (DNP) from the stem of *D. nobile* by boiling-water and precipitated with ethanol, and we found that DNP is a potential antioxidant. While, a great number of studies showed that isolated pure polysaccharides from crude polysaccharides were more effective antioxidants *in vitro* than crude polysaccharides (Xu et al., 2009). Therefore, the aim of present study is to characterize the compositions of the isolated pure polysaccharides from *D. nobile* and evaluate the antioxidant activities of these polysaccharides *in vitro*.

2. Materials and methods

2.1. Materials and chemicals

DEAE-cellulose, Vitamin C and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were purchased from Sigma Co. Sephadex G-200 and Dextrans of different molecular weights were purchased from Pharmacia Co. The standard monosaccharides (D-glucose, D-mannose, L-rhamnose, D-galactose, D-xylose and L-arabinose) were purchased from Chinese Institute for the Control of Pharmaceutical and Biological Products. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) radicals were purchased from Merck. Trifluoroacetic acid (TFA), pyridine, methanol and acetic acid, ethanol, acetic anhydride and all other chemicals and reagents were of grade AR.

2.2. Extraction of polysaccharide

The powder of *D. nobile* was extracted successively with petroleum ether and ethanol, and then extracted with double-distilled water at 100 °C for 2 h thrice. All water-extracts were combined, filtrated, concentrated. The proteins in the product of condensation were deproteinized using the Sevag reagent (Navarini et al., 1999). After removal of the Sevag reagent, the aqueous fraction was dia-

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lyzed against deionized water and precipitated by ethanol. The precipitate was collected by centrifugation, washed successively with ethyl acetate and acetone, and then dissolved in water and lyophilized to yield the crude polysaccharide (DNP). The extract yield of crude polysaccharide is 5.36%.

2.3. Isolation and purification

The freeze-dried sample (DNP) was redissolved in deionized water and forced through a filter (0.45 μm), then applied to a column (300 × 26 mm) of DEAE-cellulose and eluted successively with deionized water, 0.05 M, 0.1 M, 0.2 M, 0.4 M and 0.8 M NaCl solutions for 200 min, respectively, at a flow rate of 1.0 ml/min. Fractions (8 ml) were collected by a fraction collector. All of these fractions were analyzed for the carbohydrate content by the phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

Five peaks were collected, and four fractions of which were further purified on a Sephadex G-200 gel filtration column (600 × 16 mm), and eluted with deionized water, at a flow rate of 0.3 ml/min. Fractions (3 ml) were collected and analyzed for the carbohydrate content.

2.4. Molecular weight determination

The molecular weight of fractions were evaluated and determined by the gel permeation chromatography (GPC) with a Waters HPLC apparatus (Waters 515, Waters Co. Ltd., USA) equipped with an ultrahydrogel column (300 × 7.8 mm), a model 2410 refractive index detector (RID). The detailed operation conditions were mobile phase: 0.2 M phosphate buffer (pH 7.0); flow rate: 0.7 ml/min; column temperature: room temperature; injection volume: 20 μl; running time: 20 min.

The calibration curve for molecular weight determination was made using a series of Dextran T standards, following the method described by Alosp and Vlachogiannis (1982). Empower software was used for the calculation of average molecular weights.

2.5. Analysis of monosaccharide composition

Monosaccharide compositions of polysaccharides were determined by gas chromatography–mass spectrometry (GC–MS) (QP2010, Shimadzu, Japan). Ten milligrams of sample was hydrolyzed with 2 ml of 2 M trifluoroacetic acid (TFA) at 120 °C for 6 h in a sealed glass tube according to the method of Erbing, Jansson, Widmalm, and Nimmich (1995), with some modifications. The resulting solution was concentrated in vacuo and the excess of acid was removed by repeated co-distillations with anhydrous ethanol.

Then the hydrolyzed products were prepared for acetylation. The acetylation was carried out with 10 mg of hydroxylamine hydrochloride and with 0.5 ml of pyridine by getting heated in a water bath for 30 min at 90 °C. After incubation, the mixture was cooled at room temperature, and then 0.5 ml of acetic anhydride was added and mixed thoroughly by vortexing. The tube was sealed and incubated in a water bath for another 30 min at 90 °C. After cooling, approximately 1 μl of clear supernatant was loaded onto an Rtx-5SilMS column (30 m × 0.32 mm × 0.25 μm) of the GC–MS. Alditol acetates of authentic standards (glucose, mannose, rhamnose, galactose, xylose, and arabinose) with myo-inositol (2 mg) as the internal standard were prepared and subjected to GC–MS analysis separately in the same way.

The operation was performed in the following conditions: N2: 1.0 ml/min; injection temperature: 240 °C; detector temperature: 240 °C; column temperature programmed: 160 °C for 2 min, then increased to 240 °C at 5 °C/min and finally holding for 5 min at 240 °C.

2.6. Infrared spectral analysis

The IR spectra of the polysaccharides were determined using a Fourier transform IR spectrophotometer (FTIR) (PerkinElmer, USA). The purified polysaccharides were ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4000–500 cm^{-1} (Kumar, Joo, Choi, Koo, & Chang, 2004).

2.7. ABTS radical scavenging assay

The radicals scavenging activity of the polysaccharides against radical cation ($ABTS^+$) were measured using the methods of Re et al. (1999) with some modifications. $ABTS^+$ was produced by reacting 7 mmol/L of $ABTS^+$ solution with 2.45 mmol/L of potassium persulphate, and the mixture would be kept in the dark at room temperature for 16 h. In the moment of use, the $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. Each sample (0.2 ml) with various concentrations (0.01–2.0 mg/ml) were added to 2 ml of $ABTS^+$ solution and mixed vigorously. After reaction at room temperature for 6 min, the absorbance at 734 nm was measured. The $ABTS^+$ scavenging effect was calculated by the following formula:

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

where A_{control} is the absorbance of control without sample, A_{sample} is the test sample without $ABTS^+$.

2.8. DPPH radical scavenging assay

The DPPH radicals scavenging activity of the purified polysaccharides were measured according to the method of Braca and Shimada (Braca et al., 2001; Shimada et al., 1992), with some modifications. Vitamin C was used as reference material. Three milliliters of sample (0.01–2.0 mg/ml) was added to 1 ml of 0.1 mmol/L methanol solution of DPPH. The absorbance at 517 nm was measured after the solution was kept at room temperature for 30 min. The DPPH radical scavenging effect was calculated as Section 2.7.

2.9. Hydroxyl radical scavenging assay

The hydroxyl radicals scavenging activity of the purified polysaccharides were measured according to the method of Wang, Zhang, Zhang, and Li (2008), with some modifications. Different concentrations (0.01–2.0 mg/ml) samples were incubated with 2 mmol/L EDTA–Fe (0.5 ml), 3% H_2O_2 (1 ml) and 0.36 mg/ml crocus in 4.5 ml sodium phosphate buffer (150 mM, pH 7.4) for 30 min at 37 °C and hydroxyl radical was detected by monitoring absorbance at 520 nm. The hydroxyl radical scavenging effect was calculated as Section 2.7.

3. Results and discussion

3.1. Isolation and purification

Ion-exchange chromatography on a DEAE-cellulose column obtained five peaks from deionized water and NaCl elution, and were named as DNP1 (eluted with deionized water), DNP2 (eluted with 0.05 M NaCl), DNP3 (eluted with 0.1 M NaCl), DNP4 (eluted with 0.2 M NaCl) and DNP5 (eluted with 0.4 M NaCl) (Fig. 1).

The polysaccharides were further separated and sequentially purified through Sephadex G-200 column. Fractions (3 ml) were collected. Four major polysaccharide peaks, DNP1-1, DNP2-1, DNP3-1, and DNP4-2 (Fig. 2), were collected and lyophilized.

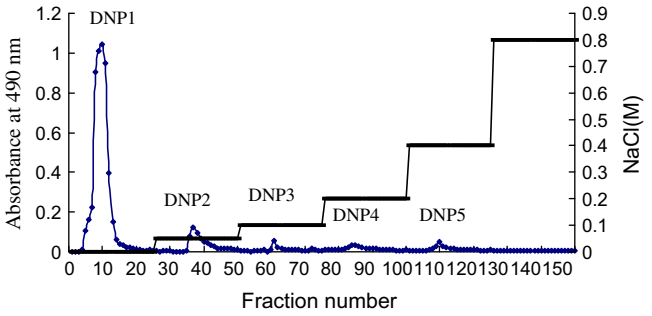

Fig. 1. Chromatography of eluted crude polysaccharide (DNP) on DEAE-cellulose column (26 × 300 mm). DNP1 eluted with deionized water; DNP2 eluted with 0.05 M NaCl; DNP3 eluted with 0.1 M NaCl; DNP4 eluted with 0.2 M NaCl; DNP5 eluted with 0.4 M NaCl.

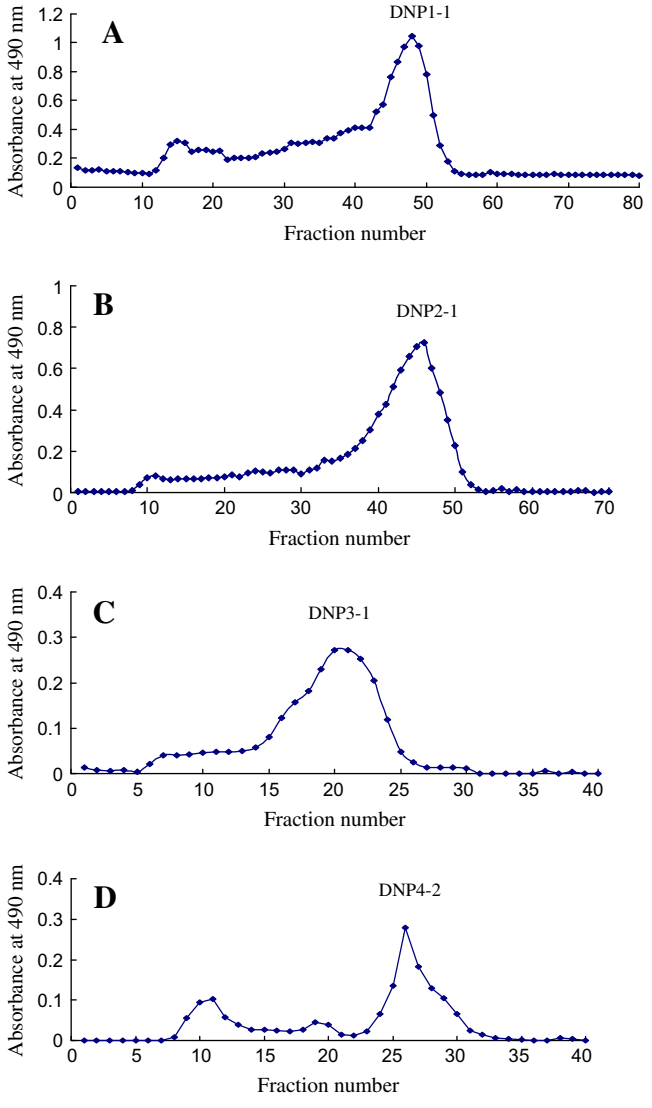

Fig. 2. Sephadex G-200 column chromatogram of DNP1 (A), DNP2 (B), DNP3 (C) and DNP4 (D) (eluted with deionized water).

3.2. Molecular weight and monosaccharide compositions of purified fractions

GPC has been shown to be an effective method for the polysaccharide molecular weight determination (Dreher, Hawthorne, & Grant, 1979). The GPC analysis showed that the average molecular weight (Mw) of DNP1-1, DNP2-1, DNP3-1, and DNP4-2 were approximately 136 kDa, 27.7 kDa, 11.8 kDa, and 11.4 kDa, respectively.

GC–MS analysis showed in Table 1, the monosaccharide compositions of the four fractions were similar. The total contents of mannose, glucose, and galactose residues were 12.97%, 44.65%, 35.85% in DNP1-1 according to GC–MS analytic data, respectively. The results implied the dominance of mannose, glucose and galactose in DNP1-1. DNP2-1 was composed of only three monosaccharides, namely mannose, glucose and galactose. DNP3-1 was composed of five monosaccharides, and the content of galactose was higher than other monosaccharides. As is shown in Table 1, the content of rhamnose was obvious higher in DNP4-2 than it in other fractions.

3.3. IR spectra of purified fractions

As is shown in Fig. 3, the FTIR spectra of four fractions were found to be similar. The band between 3600 and 3200 cm^{-1} (DNP1-1: 3421 cm^{-1}, DNP2-1: 3421 cm^{-1}, DNP3-1: 3429 cm^{-1}, DNP4-2: 3413 cm^{-1}), represented the stretching of the hydroxyl groups. The small band at around 2923 cm^{-1} (DNP1-1: 2926 cm^{-1}, DNP2-1: 2928 cm^{-1}, DNP3-1: 2928 cm^{-1}, DNP4-2: 2929 cm^{-1}) was attributed to the C–H stretching and bending vibrations. The bound at 1629 cm^{-1} (DNP1-1), 1638 cm^{-1} (DNP2-1), 1650 cm^{-1} (DNP3-1) and 1640 cm^{-1} (DNP4-2) were due to the bound water (Yuhong & Fengshan, 2007; Park, 1971). Each particular polysaccharide has a specific band in the 1200–1000 cm^{-1} region. This region is dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic band vibration. The absorptions at 1027, 1080 and 1154 cm^{-1} (DNP1-1), at 1022, 1080 and 1157 cm^{-1} (DNP2-1), at 1022, 1080 and 1156 cm^{-1} (DNP3-1), at 1030, 1078 and 1150 cm^{-1} (DNP4-2) indicated a pyranose form of sugars (Zhao, Kan, Li, & Chen, 2005).

Absorptions at 872 cm^{-1} (DNP1-1), 855 cm^{-1} (DNP2-1) and 856 cm^{-1} (DNP3-1) were typical for α-dominating configuration in pyranose form (Barker, Bourne, Stacey, & Whiffen, 1954). A characteristic peak at around 894 cm^{-1} was found in DNP4-1, indicating the β-configuration of the sugar units (Coimbra, Gonçalves, Barros, & Delgadillo, 2002).

On the basis of the aforementioned results, it can be concluded that DNP1-1, DNP2-1 and DNP3-1 were composed of α-dominating configuration in pyranose form sugars. DNP4-2 was composed of β-configuration in pyranose form sugars.

3.4. Scavenging effect on ABTS radical

ABTS assay is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants (Huang et al., 2008; Katalinic, Milos, Kulisic, & Jukic, 2006). Specific absorbance at 734 nm can be used in both organic and aqueous

Table 1
Monosaccharide compositions of the polysaccharide fractions from *Dendrobium nobile* Lindl.

Fragments	Weight (%)					
	Rhamnose	Arabinose	Xylose	Mannose	Glucose	Galactose
DNP1-1	2.11	3.54	0.89	12.97	44.65	35.85
DNP2-1	–[a]	–	–	16.99	53.26	29.74
DNP3-1	3.76	8.48		6.55	12.58	68.63
DNP4-2	12.59	4.20		11.64	23.47	48.10

[a] Undetectable.

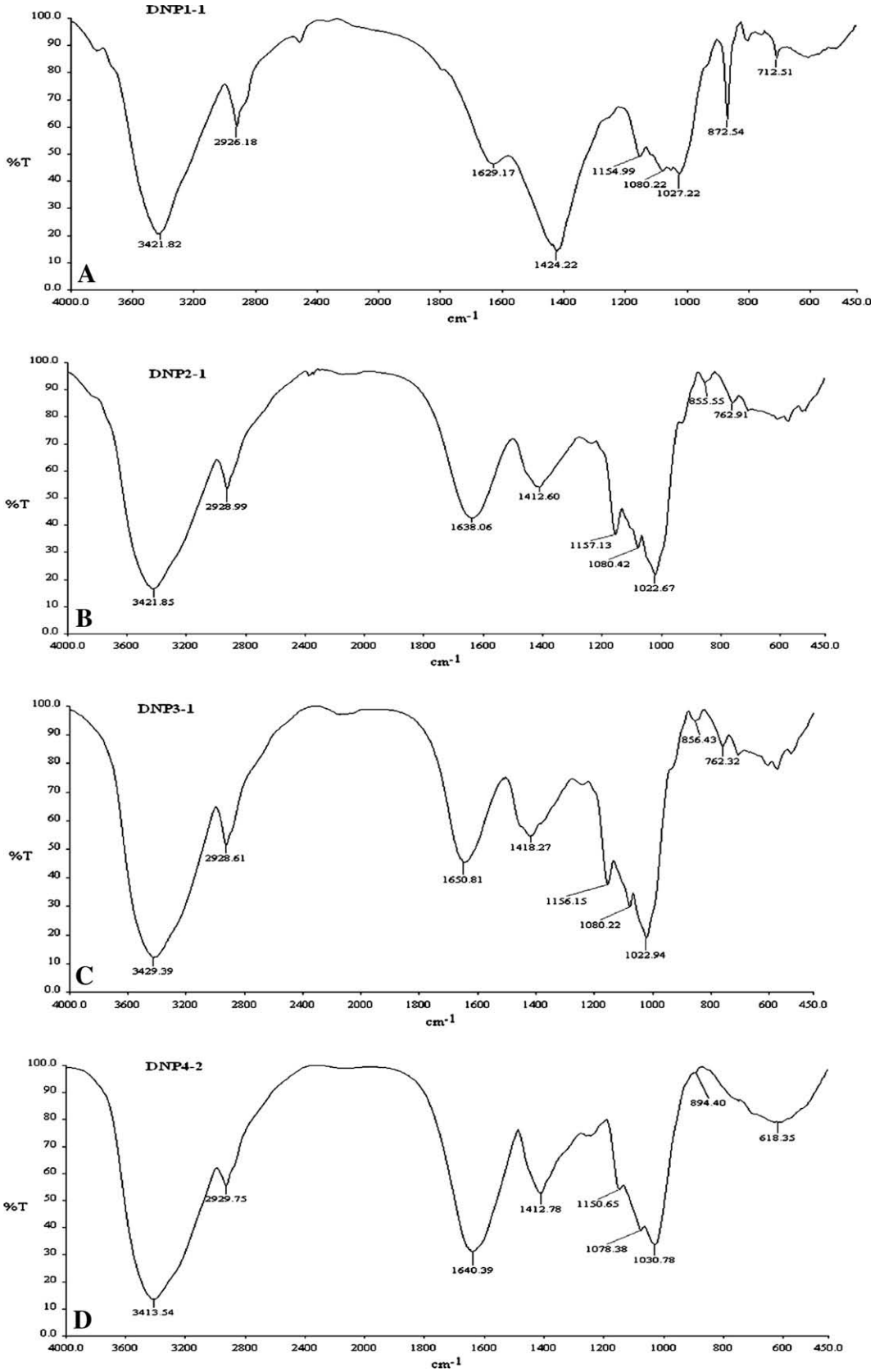

Fig. 3. FTIR spectra of the polysaccharides of DNP1-1 (A), DNP2-1 (B), DNP3-1 (C) and DNP4-2 (D).

solvents as an index reflecting the antioxidant activity of the extracted polysaccharides (Han, Weng, & Bi, 2008; Wu et al., 2006). In the experiment, the scavenging ability of purified polysaccharides on ABTS free radical was shown in Fig. 4. Their scavenging powers correlated well with increasing concentrations. Moreover, DNP4-2 showed pronounced high radical scavenging activity,

which was close to that of Vitamin C. The scavenging ability of DNP4-2 was 82.6% at the concentration 2.0 mg/ml, and it was the strongest among all the others ($P < 0.05$). The ABTS scavenging ability decreased in the order of Vitamin C > DNP4-2 > DNP2-1 > DNP3-1 > DNP1-1. These results indicated that DNP4-2 had strong scavenging power for ABTS radical and should be explored as potential antioxidants.

3.5. Hydroxyl radical scavenging activity of capsule polysaccharides

Hydroxyl radical is mainly responsible for the oxidative injury of biomolecules (Ke et al., 2009). Fig. 5 depicted the scavenging power of hydroxyl radical of the four polysaccharides and Vitamin C. The scavenging power of DNP4-2 was stronger than DNP1-1, DNP2-1 and DNP3-1 ($P < 0.05$), but was significantly lower than Vitamin C at the range of 0.1–2.0 mg/ml.

3.6. Scavenging effect on DPPH radical

The DPPH free radical is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant. So it has been widely accepted as a tool for evaluating the free radical scavenging activities of natural compounds (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004; Leong & Shui, 2002). Fig. 6 shows the DPPH radical scavenging activity of the purified polysaccharides. The results indicated that DNP1-1, DNP2-1 and DNP3-1 exhibited very low radical scavenging activity at every concentration point. Compared to these fractions, the effect of DNP4-2 was strong (38% at the concentration 1.0 mg/ml), but lower than Vitamin C (95% at the concentration 1.0 mg/ml).

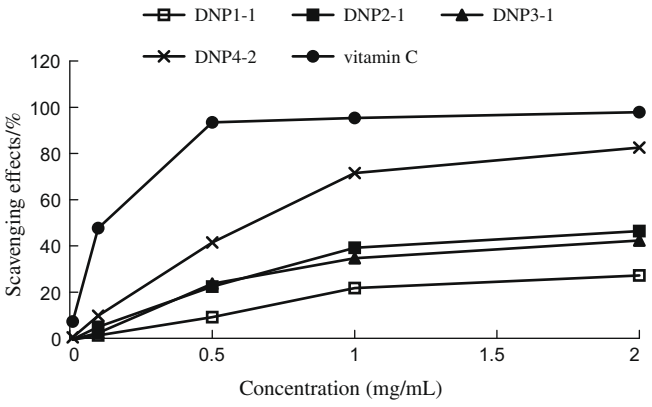

Fig. 4. The scavenging effect of different polysaccharides fractions on ABTS radicals. Results are presented as means ± standard deviations ($n = 3$). Differences are considered to be statistically significant if $P < 0.05$ when compared to control.

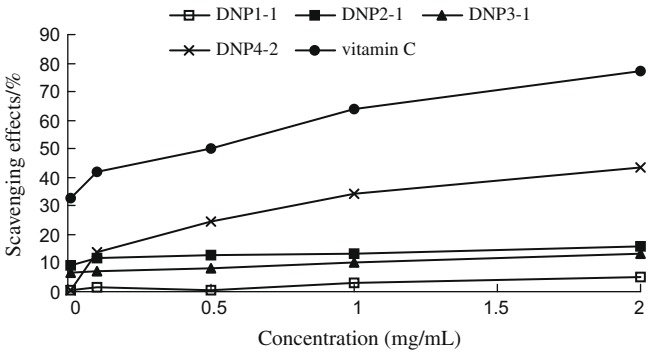

Fig. 5. The scavenging effect of different polysaccharides fractions on hydroxyl radicals. Results are presented as means ± standard deviations ($n = 3$). Differences are considered to be statistically significant if $P < 0.05$ when compared to control.

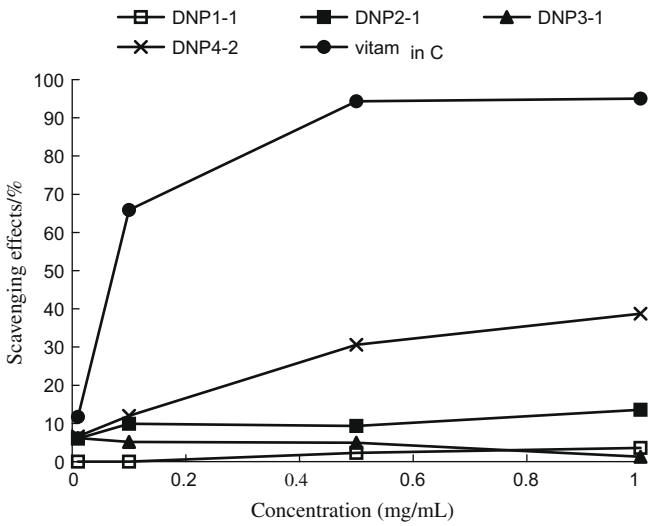

Fig. 6. The scavenging effect of different polysaccharides fractions on DPPH radicals. Results are presented as means ± standard deviations ($n = 3$). Differences are considered to be statistically significant if $P < 0.05$ when compared to control.

In this experiment, four polysaccharides showed different degree antioxidant effects. A great number of studies showed that the biological activity of polysaccharides were supposed to relate to the structural characteristic of the polysaccharides, including molecular weights, monosaccharide compositions and configuration.

From the GPC analysis, the average molecular weights (Mw) of the polysaccharide fractions were different. The Mw of DNP1-1 was the maximal among the four samples, approximately 136 kDa, but the antioxidant effect was lowest. Although they own small molecular weights, the other fractions (DNP2-1, DNP3-1, DNP4-2) exhibited stronger antioxidant activity.

In the Infrared spectra analysis, we found DNP4-2 was composed of β-configuration in pyranose form sugars, on the contrary, DNP1-1, DNP2-1 and DNP3-1 were composed of α-dominating configuration in pyranose form sugars. In our antioxidant effects test, DNP4-2 with β-configuration in pyranose form sugars exhibited stronger biological activity.

Monosaccharide compositions analysis tests shown glucose and galactose residues were major compositions of the four fractions, but there were obviously differences between DNP4-2 and the others. There was obvious high content of rhamnose (12.59%) in DNP4-2, it was far higher than the other fractions, which was supposed to relate to the strong antioxidant effects of DNP4-2.

4. Conclusions

In this work, it was concluded that *D. nobile* contained four major polysaccharide fractions (DNP1-1, DNP2-1, DNP3-1 and DNP4-2) purified by DEAE-cellulose and Sephadex G-200 column chromatography. In addition, DNP4-2 exhibited valuable high scavenging activity for ABTS radical, and the antioxidant effect of DNP4-2 is the strongest among the four polysaccharides from *D. nobile*. Sure, in order to corroborate the antioxidant ability of DNP4-2, further investigation of its antioxidant activities in vivo will be carried out in our later work.

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