A NATURAL ANTIOXIDANTS OF FOOD PLANT - *CHRYSANTHEMUM NANKINGENSE*, BASED ON ITS ANTIOXIDANT ACTIVITY WITH ACID POLYSACCHARIDES

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Abstract. Plant polysaccharides exhibit good antioxidant activity and might represent potential novel antioxidants. Chemical composition, characteristics, and biological activity of plant polysaccharides vary between different species. *Chrysanthemum nankingense*, a vegetable in South China, contains many active chemicals which have been used as materials in medicine to treat various conditions. Here, we extracted crude polysaccharides from *Chrysanthemum nankingense* plant using ultrasonic-assisted extraction with a 1:35 (w/v) ratio of leaf powder to water, extraction time of 1h at 60°C. CNP-1 was isolated, using anion-exchange chromatography on a diethylaminoethyl-cellulose column. We determined molecular weight and chemical composition of the fractions using Fourier transform infrared spectroscopy (FT-IR), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) spectrometry. Monosaccharide composition analysis indicated that glucuronic acid was the most abundant unit in CNP-1 polysaccharide fractions. Superoxide radical scavenging assessment, 2, 2-diphenyl-1- picrylhydrazyl (DPPH) assay, and a ferric reducing ability of plasma (FRAP) indicated that CNP-1 has significant antioxidant activity in a dose-dependent manner. In summary, *C. nankingense* contains acidic polysaccharides that may represent a promising natural source of antioxidants for healthy food.

Keywords: *Chrysanthemum nankingense, polysaccharide, structure characterize, antioxidant activities, in vitro*

Introduction

Polysaccharides derived from natural sources are associated with a wide range of therapeutic effects and health-improvement properties (Wang et al., 2016). Polysaccharides are effective antioxidants, and may have antitumor, anticancer and antiviral capabilities, and regulate the immune system (Yang et al., 2014). The natural polysaccharides attracted more and more attention for their potential benefits and perceived safety. Polysaccharides extracted from plants have antioxidant effects on free

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radicals, which suggests that they may be helpful, for example, in limiting the effects of aging, lowering blood sugar or adjusting lipid metabolism (Qi et al., 2006; Capek and Turjan, 2009; Chen et al., 2012; Lv et al., 2014). Consequently, the study of naturally occurring polysaccharides in plants might lead to the discovery of a novel source of antioxidants beneficial to humans.

Chrysanthemum nankingense is a perennial herb that belongs to the tribe Anthemideae of Asteraceae (*Figure 1*). Its young leaves are used as a vegetable in South China. Moreover, it is used by traditional medicine to treat various conditions, including stomach heat, bitter taste in the mouth, constipation, headache or red eyes (Dai and Wen, 2015; Wu et al., 2015).

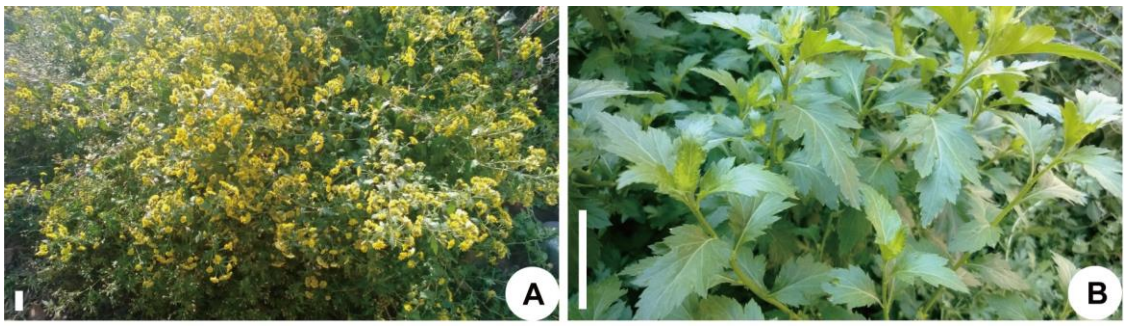

Figure 1. *The Chrysanthemum nankingense plant. A) In flower plant (November). B) Foliage plant (September)*

C. nankingense contains a variety of organic compounds such as flavonoids, polyphenols, alkaloids, organic acids, borneol, and camphor (Yang et al., 2005; Lv et al., 2007; Wu et al., 2015). However, we have little information on the polysaccharides in *C. nankingense*. In this paper, we isolated the polysaccharides present in *C. nankingense* leaves and characterized their properties by Fourier transform infrared (FT-IR) transmission spectroscopy, high performance liquid chromatography (HPLC), and high performance gel permeation chromatography (HPGPC) and nuclear magnetic resonance (NMR) spectrometry. We further explored the polysaccharides antioxidant activity using a variety of in vitro techniques. The objective of this study was to investigate the natural antioxidants fount in *Chrysanthemum nankingense* which may be used as a natural source of antioxidants for healthy food.

Materials and methods

Materials

Chrysanthemum nankingense plants used in this study were collected in the Hubei province, grown in the Nursery Garden of Northeastern University of China. Fresh leaves were collected and washed with distilled water, defatted with two 95% ethanol

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washes, and dried in vacuum for 16 h at 60°C. Dry leaves were crushed into powder and filtered with an 80 mesh sieve. The powder was stored in an airtight container at dry place until needed.

All the chemical reagents used were of analytical grade except 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid (Vc) , 1, 3, 5-tri (2-pyridyl) -2, 4, 6-triazine (TPTZ) and DEAE cellulose DE-52 (DEAE-52) were purchased from the Sinopharm Chemical Reagent Co. (Beijing, China).

Extraction of polysaccharide

About 15 g powder samples were dissolved into deionized water at a 1:35 (w/v) ratio of powder leaves to water, incubated in a water bath at 60°C for 30 min, and subjected to ultrasonic extraction twice for 30 min at a power of 240 W. The mixture was centrifuged at 3000 rpm for 10 min. Then the supernatant was obtained and condensed in a rotating evaporator until a precipitate appeared. The extracts were disposed by activated carbon and Sevag method for decolorizing and removing free proteins. The polysaccharide was precipitate with 80% absolute ethanol overnight at 4°C. The mixture was centrifugated at 3000 rpm for 10 min and lyophilized to obtain raw *C. nankingense* polysaccharides (CNP), which were then stored at −20°C for further use.

Separation and purification of crude CNP

The raw polysaccharides were purified successively by DEAE-52 and Sephadex G-100 chromatography according to the method of Qiao et al. (2009). Five hundred milligrams of crude CNP were dissolved in 10 mL water and applied onto a DEAE-52 column equilibrated with 20 mM phosphate solution (NaH_2PO_4/Na_2HPO_4, pH 6.8). The column was eluted with the 20 mM phosphate solution and sequentially NaCl-phosphate solution gradient (0.05, 0.1, and 0.5 M) with a flow rate of 2 mL/ min. The eluates (5 mL/tube) were collected and monitored by the sulfuric acid anthrone method (Somani et al., 1987). The fraction was collected, concentrated in a rotating evaporator at 50°C, dialyzed in a 21 mm dialysis bag in deionized water for 48 h, and then lyophilized and stored at -20°C till needed.

Characterization of CNPs

FT-IR spectra analysis of CNPs

The infrared spectrum of purified fractions grounded into 1 mm KBr pellets were determined using a FTIR spectrophotometer (Bruker Tensor 27 FT-IR, Bruker Co., Germany) in the frequency range of 4000−400 cm^{-1} (Peng et al., 2012).

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Nuclear magnetic resonance (NMR) spectrometric analysis of CNPs

About 30 mg CNP1 was dissolved in 500 μL 99.9% D_2O and analysized using Bruker Advance III 600 MHz spectrometer (Bruker Co., Germany) to obtain ^1H NMR spectra. Chemical shifts were determined in ppm.

Monosaccharide composition analysis of CNPs

Ten milligram of purified CNP were complete hydrolyzed by applying 3 mL trifluoroacetic acid (2 M) at 120°C for 6 hours, then centrifuged at 12000 rpm for 5 min after cooling. 0.2 M NaOH was added to the supernatant to adjust the pH to 7.0. The hydrolysate eluted was aadded to 10 mL and stored in sealed container.

The derivatization was determined following the method of Honda et al. (1989) with some modification. Samples (10 mg/mL) and monosaccharide standards arabinose, glucuronic acid, glucose, rhamnose, mannose, galactose, and xylose were mixed with 0.2 M NaOH and 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP) solution in 1 mL test tubes. The solutions were thoroughly mixed and incubated at 70°C for 40 min. Then 0.2 M HCl were used to neutralize the NaOH after the samples cooled. One hundred microliters of trichloromethane were added to the mixture and completed mixed, then centrifuged 5000 rpm for 10 min. The supernatant was extracted for three times with chloroform. The final supernatant was filtered with a 0.45 μm membrane and used for high-performance liquid chromatography (HPLC) with a C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was 19% (V/V) ammonium acetate buffer solution (pH 5.5) and the flow rate was 1.0 mL/ min.

Determining the molecular weight of CNPs

Molecular weights were measured by high performance gel permeation chromatography (HPGPC) using a Waters HPLC system equipped with two serially linked TSK-GEL G3000 PWXL (7.8 mm × 300 mm) columns and RI and connected to a UV detector. The mobile phase was 0.71% Na_2SO_4 and the flow rate was 0.6 mL/min and the temperature of column was kept at 35°C. Molecular weights of the samples were obtained by comparison with a calibration curve created using the Dextran T-series standards with known molecular weights (23800, 80900, 147600 and 273000). The linear equation of the standard curve was: $LogM = -0.34974968tR + 9.75252445$, with a correlation coefficient of 0.9981.

Determining the antioxidant activity of CNPs in vitro

Determining CNPs' scavenging activity against oxide anions

The measurement of polysaccharide scavenging activity against superoxide anions were conducted as previously described (Marklund and Marklund, 1974). First, 4.5 mL 0.05 M tris-HCl solution (pH 8.0) was incubated in a water bath at 25°C for 20 min. Then 50, 100, 150, or 200 μL of sample (2 g/L) were added and stirred at 25°C for

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20 min. Finally, the reaction was terminated by adding 100 μL 1, 2, 3-phentriol (3 M). The mixture of tris-HCl and 1, 2, 3-phentriol was used as negative control (A_0). The absorbance of the mixture was measured spectrophotometrically at 325 nm and the scavenging activity against superoxide anions was calculated according to the following equation: scavenging effect (%) = (A_0−Asample)/A_0×100%; rate of oxidation (%) = (A_9−A_1)/3×100%, where A_1 is the absorbance of the first detection and A_9 is the absorbance of the ninth detection.

Scavenging ability of DPPH radical

DPPH radical scavenging activity of CNP was measured following the method reported previously (Jia et al., 2014). Two milliliters of sample at various concentrations (0.3, 0.5, and 0.7 mg/mL) were added to 2 mL DPPH ethanol solution (0.06 mM) in a 5 mL cuvetteand mixed completely. The mixture was kept in the dark for 20 min and the absorbance of the sample was measured at 517 nm using a spectrophotometer (721G, China). Ascorbic acid (Vc) was used as the control. The scavenging rate was obtained according the formula: DPPH radical scavenging activity (%) = (1- As/Ab) × 100%, where As is the absorbance of the sample, and Ab is the absorbance of the blank.

Assessment of iron reducing activity

The ferric reducing activity of plasma (FRAP) was measured following the method reported by Jia et al. (2014), with some modifications. Fresh FRAP reaction solution was prepared by mixing 25 mL acetate buffer (300 mM; pH 3.6) with 2.5 mL 1, 3, 5-tri (2-pyridyl) -2, 4, 6-triazine (TPTZ) solutions (10 mM) and 2.5 mL $FeCl_3$•$6H_2O$ (20 mM). Then, 1000 μL of the sample were put into a cuvette to measure the initial absorbance after the sample was warmed to 37°C. The absorption of the solution was measured at 593 nm while 40 μL sample at different concentration (0.2, 0.4, 0.8 or 1.0 mg/mL) were added to the cuvette. The absorption value was recorded at 10 s intervals until it reached a plateau. Iron reducing activity was calculated referencing to the calibration curve prepared of $FeSO_4$•$7H_2O$ solution with several concentrations (100-1000 M). Three technical replicates were conducted for each analysis mentioned above. Three parallel measurements and technical replicates were conducted for each analysis mentioned above. All the data were processed in Origin 8.5.1.

Results and discussion

Purification and segregation of CNP

We obtained the primary polysaccharides from *C. nankingense* using ultrasonic pretreatment with an isolation time of 5 hours, raw materials to water ratio of 1:35 (w/v), power of 240 W, and a combination of activated carbon decolorization and Sevag method to remove the pigment and proteins. The yield of raw polysaccharide from

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C. nankingense was about 4.32%. Four elution peaks (CNP-1, CNP-2, CNP-3, and CNP-4) were presented from the crude CNP using a DEAE-52 cellulose anion-exchange chromatogram (*Figure 2*). The major fraction CNP-1 was condensed and lyophilized for next analysis.

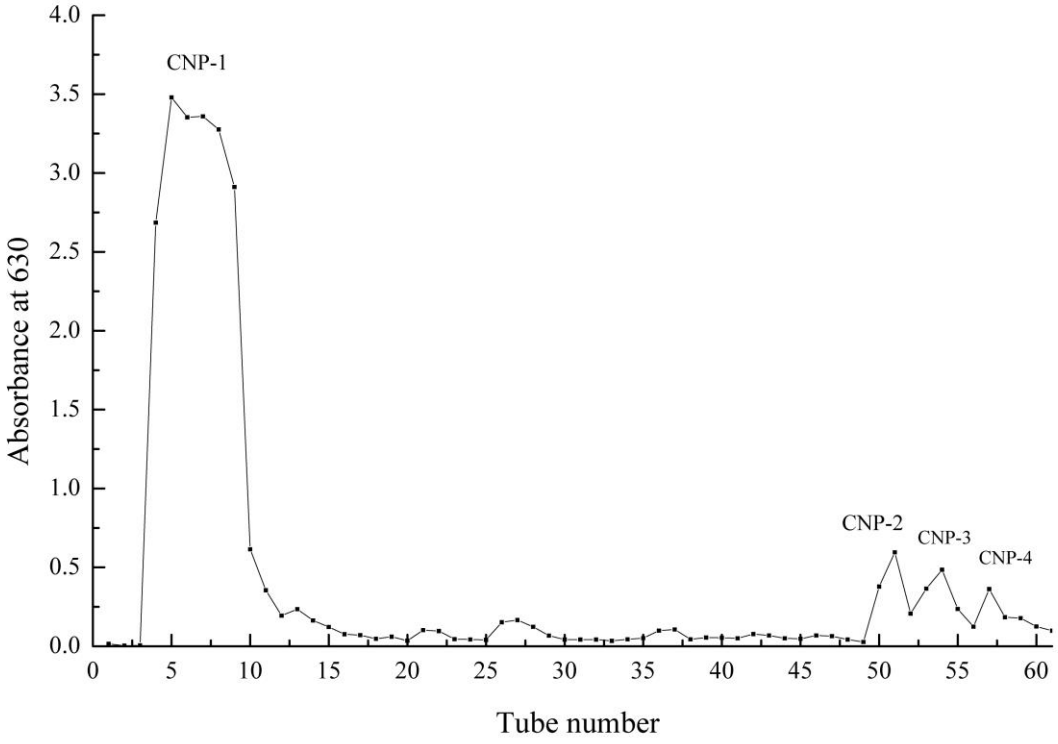

Figure 2. *Elution curve of crude Chrysanthemum nankingense polysaccharide detected by sulfuric acid anthrone method at 490 nm using DEAE52-cellulose column chromatography. CNP-1 was eluted by phosphate solution. CNP-2, CNP-3, and CNP-4 were eluted by 0.5 M NaCl-phosphate solution*

Structure analysis of CNP-1

FT-IR spectra analysis of CNP-1

We characterized the absorption of CNP-1 using FT-IR spectrum (*Figure 3*). This sample showed a wide, intense signals at 3600–3200 cm^{-1}, representing an O-H stretching group, and a weak signal at 3000–2800 cm^{-1}, which we attributed to a C–H stretching vibration (Jia et al., 2014). We also observed peaks in the region of 1630–1510 cm^{-1} and 1420 cm^{-1}, attributed to C=O asymmetric stretching vibration (Cerna et al., 2003), suggesting the carboxyl groups presenting here (Zha et al., 2012). The peaks at 1000–1200 cm^{-1} in the CNP-1 polysaccharide fractions suggests the presence of C–O–C and C–O–H signals, possibly an alduronic acid such as glucuronic acid or mannuronic acid. The CNP-1 fractions presented peaks between 3600 and 3200,

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3000 and 2800, 1250 and 1000 cm^{-1}, the typical signals for polysaccharides (You et al., 2014). A characteristic signal at 891±7 cm^{-1} was attribute to glycosidic linkages. CNP-1 has a characteristic signal at 937.3 cm^{-1} induced by asymmetric stretching vibration of pyran ring (Coimbra et al., 1998). Thus, we propose that CNP-1 might be pyranose.

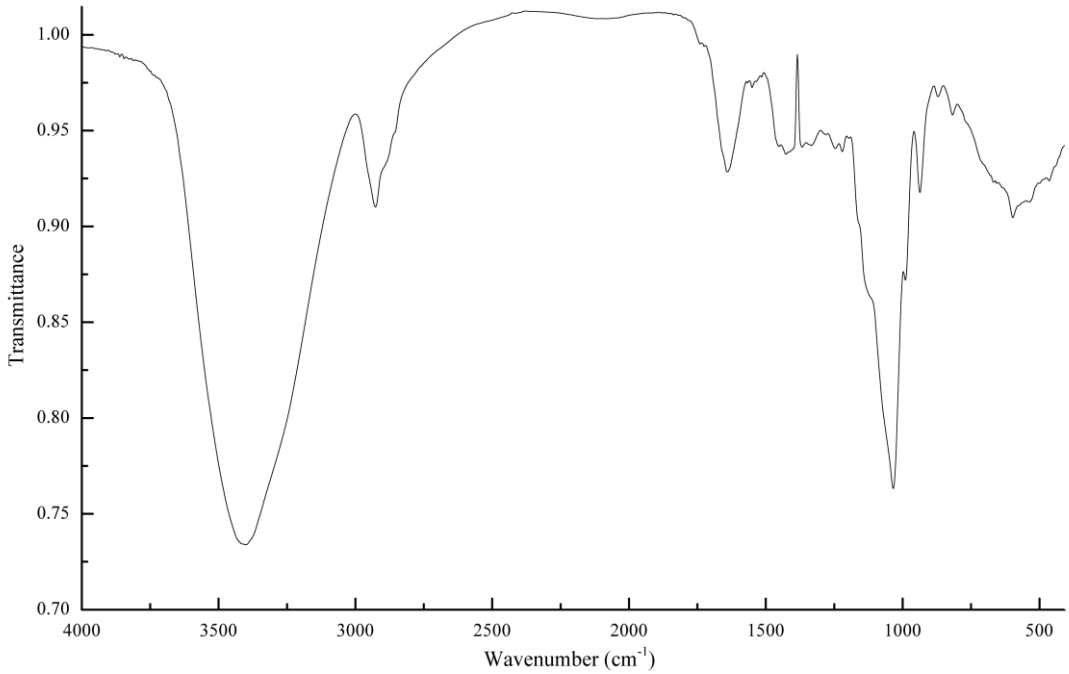

Figure 3. *Fourier transform infrared spectra of CNP-1*

NMR spectra of CNP-1

The structural of CNP-1 was confirmed using ^{1}H-NMR analysis (*Figure 4*). The spectra of CNP was presented between 3.0–5.3 ppm (^{1}H NMR) which were typical signals for polysaccharides (Chen et al., 2016; Wang et al., 2018). The signals induced by protons on sugar rings were present in the region of 3.50–4.50 ppm. We predicted that CNP-1 has a-configuration of saccharide residues for all the resonance signals greater than the shift of 4.8 ppm (Chen et al., 2016). This conclusion further confirmed the results of the infrared spectrum.

Monosaccharide analysis of CNP-1

We characterized the monosaccharide composition of CNP-1 by HPLC (*Figure 5*). Monosaccharide standards of glucuronic acid, mannose, rhamnose, xylose, glucose, galactose, and arabinose produced well separated peaks, with retention times of 16.18, 21.03, 24.47, 26.62, 30.91, 34.12, and 45.69 min. Glucuronic acid was the most abundant monosaccharide in the CNP-1 fraction; mannose and galactose were also

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present in trace amounts. These results indicate that CNP-1 is primarily composed of sugar acids, which suggests that it may have deoxidizing activity.

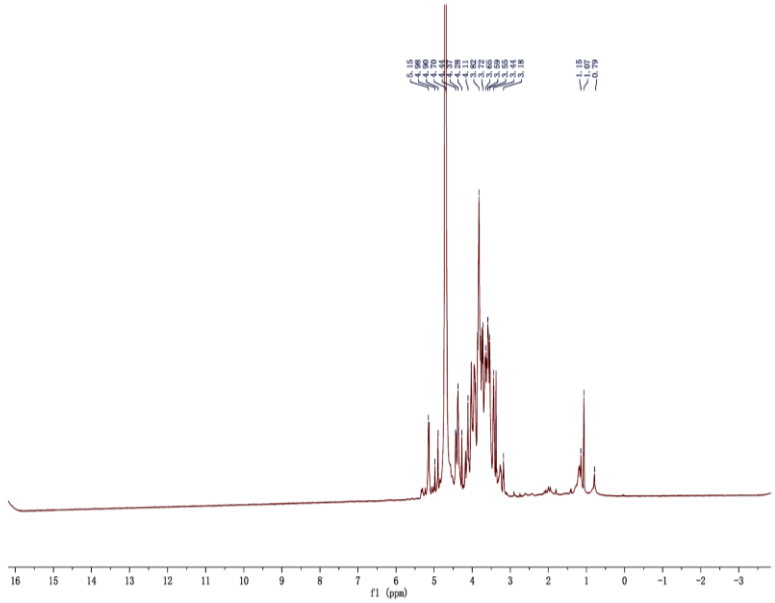

Figure 4. *1H NMR spectra of CNP1*

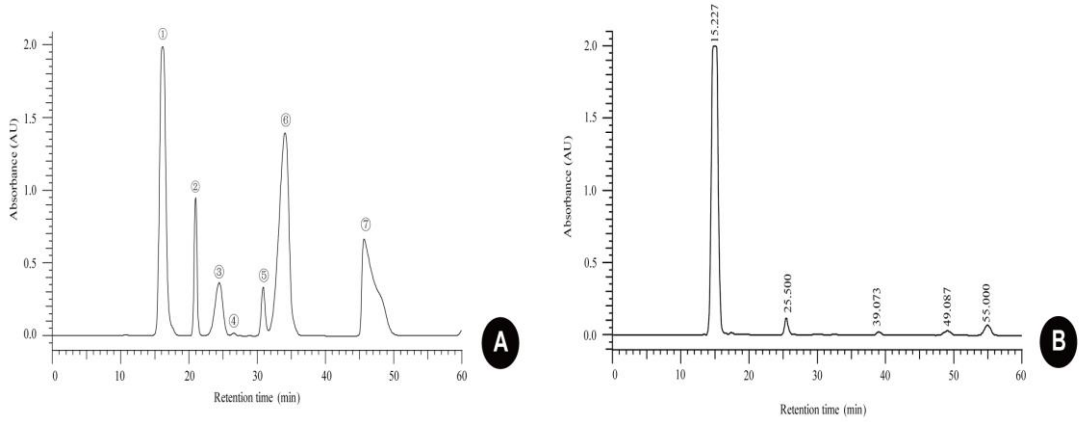

Figure 5. *High-performance liquid chromatograms of three monosaccharide fractions isolated from Chrysanthemum nankingense. Part A shows peaks for the monosaccharide standards glucuronic acid (1), L-mannose (2), D-rhamnose (3), L-xylose (4), L-glucose (5), D-galactose (6), and L-arabinose (7). Parts B shows C. nankingense monosaccharide fractions CNP-1*

Molecular weight of CNP-1

We determined the molecular weight of CNP-1 using the HPGPC method. Based on the reference curve created with dextran standards, we estimated that the average molecular weights of CNP-1 was 4779 Da.

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Antioxidant activity of CNP-1 in vitro

Using the DPPH assay, we found that the activity to scavenge free radical of CNP-1 at 0.7 mg mL^{-1} was 21.39%, while that of Vc was 89.00% (*Figure 6A*). The results suggested that CNP-1 had a radicals scavenging effect on DPPH. The FRAP value of CNP-1 increased with the concentration of this fraction (*Figure 6B*). At 0.8 mg/mL, the FRAP value of CNP-1 was 2.726 mmol. These results demonstrate that CNP-1 possesses strong ferric reducing activity.

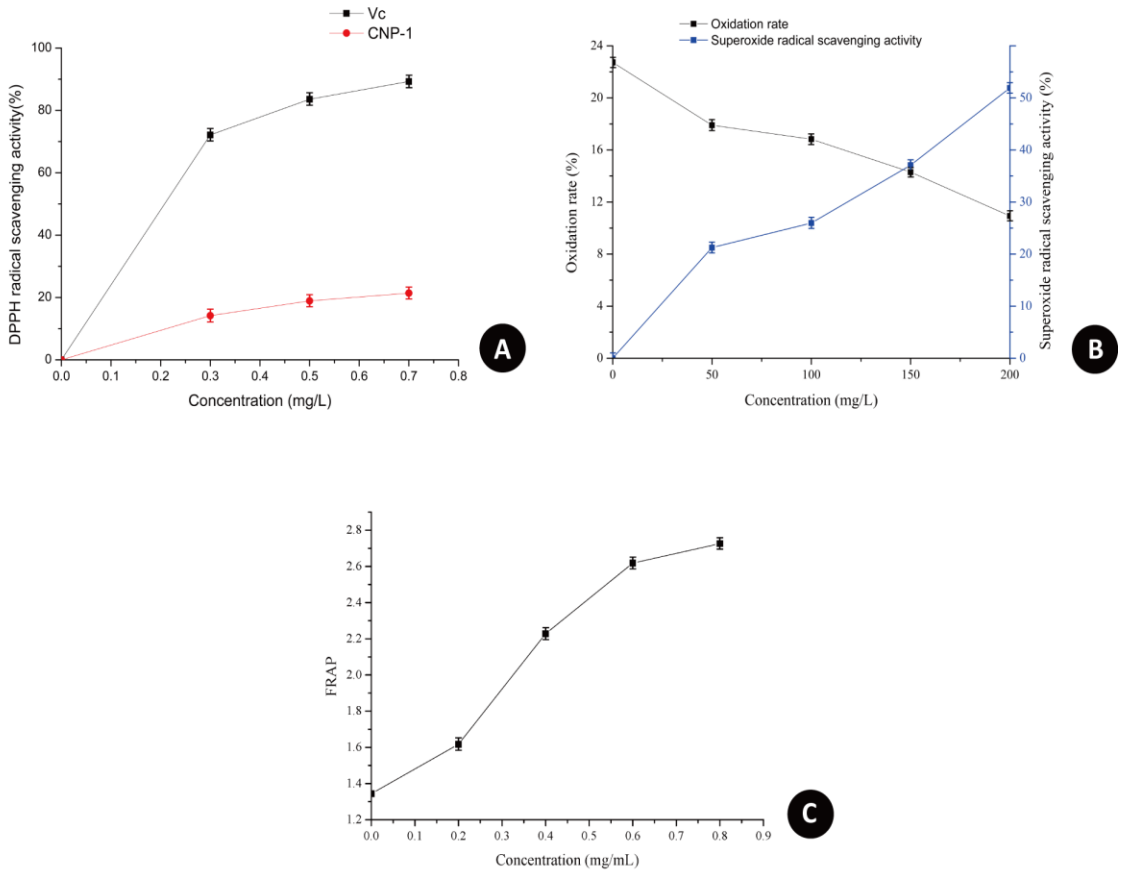

Figure 6. *Antioxidant activity of the purified polysaccharide fraction CNP-1. A. DPPH radical scavenging activity. B. Ferric reducing activity. C. Superoxide radical scavenging activity*

We measured the scavenging activity of CNP-1 on superoxide radical (*Figure 6C*), finding that the oxidation rate of the solution decreased with increases in CNP-1 concentration, while with sample concentration increasing, the scavenging activity of CNP-1 increased also. The scavenging activity of CNP-1 reached 51.91% at the doses of 200 mg L^{-1}. These results demonstrate that CNP-1 is characterized by strong superoxide radical scavenging activity.

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Conclusion

In this study, we isolated polysaccharides from *C. nankingense*, and characterized its structural features. Glucuronic acid was the main component of the polysaccharides of this plant by monosaccharide composition analysis. Antioxidant activity analysis demonstrated that CNP-1 had high ferric reducing ability and superoxide radical scavenging activity. These findings suggest the polysaccharides from *C. nankingense* leaves might be a potential source of natural antioxidants in functional foods or pharmacological applications. In addition, the studies showed that most of metabolities were associated with the plant phenotypes (Schauer et al., 2006; Sulpice et al., 2009; Chen et al., 2014; Dan et al. 2016). *Chrysanthemum* is complex taxa with different habitat and various ploidy levels. The phylogeny of *Chrysanthemum* is still not clear. Polysaccharide, as a widely existed metabolite in plant, might be a new way to explore the relationship between *Chrysanthemum* species.

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