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The evaluation of antioxidant and antifungal properties of 6-amino-6-deoxychitosan in vitro

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Abstract

We synthesized 6-amino-6-deoxychitosan (NCS) through a series of reactions from chitosan (CS). The antioxidant ability of CS and NCS were investigated in vitro, including that of DPPH-radical and hydrogen peroxide, reducing power, chelating abilities and inhibition of lipid peroxidation. As expected, after the introduction of amino groups, antioxidant ability had improved significantly. Especially, scavenging effect against DPPH-radical and hydrogen peroxide of NCS were 97% and 92% at 1.6 mg/mL, respectively. Moreover, inhibition of lipid peroxidation was 57% at 0.1 mg/mL. And the reducing power of NCS was 0.68 at 0.8 mg/mL. Meanwhile, inhibitory effects against four fungi were also tested. Antifungal activity of NCS were better than those
of CS and antifungal activity had improved more than 20% at 0.5 mg/mL against these four kinds of plant pathogens. Based on the above results, it was reasonable to speculate that the obtained antioxidant ability and antifungal activity of NCS might benefit from amino groups on chitosan backbone. These in vitro results suggest the possibility that NCS as antioxidant compound could be effectively alleviate oxidative stress and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

**Keywords:** chitosan, 6-amino-6-deoxychitosan, antioxidant ability, antifungal activity

1. **Introduction**

Chitin is a widely distributed polysaccharide on the earth. It can be extracted from an abundant source of shellfish exoskeletons such as crabs, shrimps, lobsters, prawns, or the cell walls of some microorganisms and some fungi [1-3]. Chitosan (CS) is produced by alkaline deacetylation of chitin. CS with low molecular weight is soluble in water, but CS with high molecular weight is commonly dispersed in 1% (v/w) acetic acid solution because of its insolubility in water.

CS and its derivatives have some beneficial properties, including wound healing, antimicrobial activity, low toxicity, excellent biocompatibility and antioxidant property [4-6]. And their antioxidant property has attracted attention in food and pharmaceutical industries, since free radical and transition metal ions can cause DNA damage, and result in seriously deteriorate the quality of food [7, 8]. And H₂O₂ can damage cells, proteins and DNA, leading to various disease [9, 10]. Therefore, antioxidant can protect human body against many chronic diseases such as Alzheimer’s disease (AD), diabetes, sepsis, arthritis, respiratory distress syndrome, chronic renal failure and cancer [11-13].
The (acetyl) amino groups on the polysaccharide backbone undoubtedly play crucial roles in exhibiting various unique properties. It has been reported that amino groups of aminoglycosides might interact with anionic components of the cell wall, which led to the formation of an impervious layer around the cell and prevent the transport of essential nutrients or change permeation property of the microbial cell envelope, and ultimately leading to cell death [14]. Therefore, more amino groups were incorporated into polysaccharide to enhance its antimicrobial and antioxidant activities [15-17].

And many studies have reported the synthesis of 6-amino-6-deoxychitosan (NCS) [18-20]. In previous studies, Satoh found NCS had more effective transfection efficiency than chitosan and was a good gene carrier. Yang got NCS with low molecular weight (2-14 kDa), and NCS showed higher antibacterial than CS at pH=5.4-7.5. In addition, Yang also got NCS with high weight (48 kDa) and the scavenging capacity of NCS against hydroxyl radical and superoxide radical at low concentration (were 99% at 1.6 mg/mL and 82% at 0.2 mg/mL, respectively). In addition to this, there are few papers about other antioxidant and antifungal activities of NCS, yet.

Antioxidant property has attracted attention in food and pharmaceutical industries due to they are able to prevent chain initiation, bind transition metal ions, decompose peroxides, and so on. Generally, since the antioxidant activity is influenced by many factors, it is evaluated by multiple assays rather than described with one single reaction system. Therefore, in the present study, we modified chitosan at C-6 position with amino groups and synthesized NCS. In short, we prepared our product from N-
phthaloyl-chitosan through C6-selective bromination/azidation to afford 6-azido-6-deoxy-N-phthaloyl-chitosan and then, 6-amino-6-deoxy-chitosan (NCS) was successfully synthesized through reduction by triphenylphosphine and hydrazine monohydrate. Then we evaluated its antioxidant ability and antifungal activity. In our report, scavenging activities (against DPPH-radical and hydrogen peroxide) test, reducing power test, chelating abilities test and inhibition of lipid peroxidation test were employed to test the antioxidant activity. Meanwhile, inhibitory effects against fungi were tested using *Fusarium oxysporum* f. sp. *cucumerium* (*F. oxysporum* f. sp. *cucumerium*), *Fusarium oxysporum* f. *niveum* (*F. oxysporum* f. *niveum*), *Phomopsis asparagi* (*P. asparagi*) and *Gibberella zeae* (*G. zeae*).

2. Experimental

2.1 Materials

Chitosan was supplied by Zhejiang Golden-Shell Pharmaceutical Co. Ltd. (Yuhuan, China), and the molecular weight was 700 kDa and deacetylation degree of 95%. The deacetylation degree (DD) was determined by elemental analysis. The other reagents such as *N,N*-Dimethylformamide (DMF), *N*-bromobutanimide (NBS), triphenylphosphine, *N*-Methyl pyrrolidone (NMP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), etc., were supplied by Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

2.2 Analytical methods

2.2.1 Elemental analysis

The elemental analyses by combustion were used to quantitatively assess the
extent of functionalization (degree of substitution) in chitosan derivative. The analyses of elemental carbon, hydrogen and nitrogen in chitosan derivative was performed on a Vario EL III (Elementar, Germany).

2.2.2 Fourier transform infrared (FTIR) spectroscopy

All spectra were recorded on a Jasco-4100 Fourier Transform Infrared Spectrometer (Japan, provided by JASCO Co., Ltd. Shanghai, China) at 25 °C in the transmittance mode. All spectra were scanned against a blank KBr pellet background in the range of 4000–400 cm⁻¹ with resolution of 4.0 cm⁻¹.

2.2.3 Solid-state ¹³C NMR spectroscopy

¹³C CP/MAS NMR experiments were performed on Bruker AVANCE III 600 spectrometer at a resonance frequency of 150.9 MHz. ¹³C CP/ MAS NMR spectra were recorded using a 4mm MAS probe and a spinning rate of 12 kHz. A contact time of 2 ms and a recycle delay of 3 s were used for the ¹H-¹³C CP/MAS measurement.

2.2.4 Thermogravimetric analysis (TGA)

The thermogravimetric analysis (TGA) was performed using the TGA/DSC1/1100 (Mettler-Toledo). The samples were heated from 25 °C to 600 °C with a temperature rate of change of 10 °C per min. The thermogravimetric analysis was performed in N₂.

2.2.5 X-ray diffraction (XRD)

The X-ray diffraction patterns of samples were recorded at room temperature on an X-ray diffractometer (D8 advance, Bruker, Germany). The data was collected in the 2 Theta range 3–60° with a step size of 0.02° and a counting time of 5 s per step.

2.3 Synthesis
The reaction showed in Scheme 1 was carried out according to literature with modification [18].

2.3.1 6-deoxy-N-phthaloyl-chitosan (2)

15.1 g of phthalic anhydride was dissolved in 300 mL DMF containing 5% (v/v) water. Then 5.1 g of CS was added to the solution, and the mixture was heated at 120 °C. After 9.5 h of reaction, the resulting mixture was cooled to room temperature and gradually poured into ice water. The precipitate was collected on a filter, washed two times with distilled water and ethanol, and dried at 60 °C. The product was obtained as flesh-colored powdery material. Found: C, 52.92; H, 4.45; N, 4.68%. The degree of substitution was 0.9, as determined by the C/N ratio of elemental analysis.

2.3.2 Preparation of 6-Bromo-6-deoxy-N-phthaloyl-chitosan (3)

3.5 g phthaloylated chitosan (2) was dissolved in 330 mL DMF, then 21.4 g NBS and 31.4 g triphenylphosphine were added slowly in an ice bath. The mixture was stirred at 80 °C for 2 h. The reaction mixture was poured into ethanol, and the resulting precipitate was collected by filtration. Found: C, 45.49; H, 4.14; N, 4.31%.

2.3.3 Preparation of 6-azido-6-deoxy-N-phthaloyl-chitosan (4)

A mixture of 1.6 g 6-bromo-6-deoxy-N-phthaloyl-chitosan (2) and 50 mL DMF was treated with sodium azide (2.0 g) at 100 °C for 7 h. The resulting mixture was cooled to room temperature and stirred overnight. The solution was then poured into 400 mL ice water. The precipitate was collected, washed with distilled water, ethanol and then dried at 60 °C to give the product. Found: C, 51.50; H, 4.17; N, 15.96%.

2.3.4 Preparation of NCS
0.95 g 6-bromo-6-deoxy-N-phthaloyl-chitosan (4) was suspended in 100 mL DMF. Then 2.67 g triphenylphosphine was added. After 20 h of reaction at room temperature, 95 mL hydrazine monohydrate was then added to the mixture and the mixture was heated to 100 °C. After 8 h of reaction, the resulting solid was washed with 200 mL of ethanol and dried to give 6-amino-6-deoxychitosan. Found: C, 42.54; H, 7.83; N, 15.01%. The degree of substitution was 0.81, as determined by the C/N ratio of elemental analysis.

2.4 Investigation of the antioxidant ability

2.4.1 DPPH-radical scavenging ability assay

The DPPH scavenging properties of the products were evaluated by the following method [21]. Different concentrations of samples (in 2% acetic acid) and 2 mL of DPPH ethanol solution (180 μmol/L) was incubated for 30 min at room temperature. Then, the absorbance of the remained DPPH radical was measured at 517 nm against a blank. Three replicates for each sample were tested and the scavenging effect was calculated according to the following equation:

Scavenging effect (%) = \[1 - \frac{A_{\text{sample 517 nm}} - A_{\text{control 517 nm}}}{A_{\text{blank 517 nm}}}\] \times 100

where \(A_{\text{sample 517 nm}}\) is the absorbance of the sample (with DPPH) at 517 nm; \(A_{\text{control 517 nm}}\) is the absorbance of the control (without DPPH) at 517 nm; and \(A_{\text{blank 517 nm}}\) is the absorbance of the blank (without samples) at 517 nm.

2.4.2 Hydrogen peroxide scavenging ability assay

The \(\text{H}_2\text{O}_2\) scavenging activity was measured according to the literature method [22]. \(\text{H}_2\text{O}_2\) concentration was determined spectrophotometrically by measuring
absorption at 230 nm in a spectrophotometer. Samples (in 2% acetic acid) were added to a H₂O₂ solution. The absorbance of solution at 230 nm was determined after 10 min. The percentage scavenging of H₂O₂ by melatonin and standard compounds was determined as follows:

\[
\text{Scavenging effect (\%)} = \left[1 - \frac{A_{\text{sample \ 230 \ nm}} - A_{\text{control \ 230 \ nm}}}{A_{\text{black \ 230 \ nm}}} \right] \times 100
\]

where \(A_{\text{sample \ 230 \ nm}}\) is the absorbance of the sample (with H₂O₂) at 230 nm; \(A_{\text{control \ 230 \ nm}}\) is the absorbance of the control (without H₂O₂) at 230 nm; and \(A_{\text{blank \ 230 \ nm}}\) is the absorbance of the blank (without samples).

2.4.3 Reducing power assay

The reducing power was determined according to the method of Zhong [23]. 1.5 mL of testing sample (in 3% acetic acid) was mixed with 1.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50 °C for 20 min. Thereafter, 1.5 mL of 10% trichloroacetic acid was added. The upper layer (2.0 mL) was mixed with 2.0 mL of water and 0.2 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. A higher absorbance indicates a stronger reducing power [24].

2.4.4 Metal ion chelating assay

The ferrous ion-chelating potential of sample was investigated according to the method of Decker [25]. Briefly, 1 mL of sample (in 3% acetic acid) was mixed with 3.7 mL of methanol and 0.1 mL of ferrous chloride solution (2 mM). The reaction was initiated by the addition of 0.2 mL ferrozine (5 mM). After 10 min reaction at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance indicates a stronger chelating ability. The ability to chelate ferrous
ion was calculated using the following equation:

\[
\text{Chelating effect (\%)} = \left( \frac{A_{\text{black} \ 562 \text{ nm}} - A_{\text{sample} \ 562 \text{ nm}}}{A_{\text{blank} \ 562 \text{ nm}}} \right) \times 100
\]

where \(A_{\text{blank} \ 230 \text{ nm}}\) is the absorbance of the blank (without samples) at 562 nm and \(A_{\text{blank} \ 230 \text{ nm}}\) is the absorbance of the blank (with samples) at 562 nm.

2.4.5 Linoleic acid peroxidation with TBARS assay

The Linoleic acid peroxidation was measured according to the literature method [26]. Briefly, 1.6 mL of sample (in 3% acetic acid) was mixed with 0.5 mL Tris-HCl solution (100 mM, pH 7.5), 0.5 mL linoleic acid solution (20 mM), 0.1 mL FeSO\(_4\) (4 mM), and 0.1 mL ascorbic acid (2 mM). The mixture was incubated for 30 min at 37 °C and then 2 mL of trichloroacetic acid (10%) was added. After that, 2 mL of mixture was removed and added with 2 mL thiobarbituric acid (1%), followed by heating for 10 min at 95 °C. The absorbance of thiobarbituric acid-reacting substances (TBARS) in the supernatant was measured at 532 nm. The percentage of antioxidant activity was determined using the following equation:

\[
\text{Linoleic acid peroxidation inhibition (\%)} = \left( \frac{A_{\text{control} \ 532 \text{ nm}} - A_{\text{sample} \ 532 \text{ nm}}}{A_{\text{control} \ 532 \text{ nm}} - A_{\text{blank} \ 532 \text{ nm}}} \right) \times 100
\]

where \(A_{\text{sample} \ 532 \text{ nm}}\) is the absorbance of the control (with samples) at 532 nm; \(A_{\text{control} \ 532 \text{ nm}}\) is the absorbance of the control (without samples) at 532 nm; and \(A_{\text{blank} \ 532 \text{ nm}}\) is the absorbance of the blank (without FeSO\(_4\)) at 532 nm.

2.5 Evaluation of antifungal activity in vitro

Antifungal assays were performed by following the plate growth rate method described by Guo et al [27]. Briefly, the compounds were dissolved in 0.35% acetic
acid at a concentration of 5.0 mg/mL. Then, each sample (CS and NCS) solution was added to Fungi Medium to give final concentrations of 0.1, 0.5 and 1.0 mg/mL. Carbendazim solution with the same concentration was used as a positive control. After the mixture was cooled in the plate, 5.0 mm diameter of fungi mycelium was transferred to the test plate and incubated at 27 °C for 3 days. When fungi mycelium in control plate (without samples) reached edges, the antifungal index was calculated as follows:

\[
\text{Antifungal index (\%)} = \left[1 - \frac{D_a}{D_b}\right] \times 100
\]

where \(D_a\) is the diameter of growth zone in test plate and \(D_b\) is the diameter of growth zone in control plate. Each experiment was performed in three replicates.

2.6 Statistical analysis

All data were expressed as means ± standard deviation. The Scheffe method was used to evaluate the differences in antioxidant index in the antioxidant assays. Data were analyzed by the analysis of variance to guarantee statistical significance.

3. Results and discussion

3.1 Chitosan derivatives preparation and characterization

3.1.1. Synthesis of NCS

Up to now, there are two synthetic schemes to prepare NCS. One is to prepare it from 6-halo-6-deoxy-N-phthaloyl-chitosan via 6-azidation. The other is from 6-tosyl-6-deoxy-N-phthaloyl-chitosan via 6-azidation. CS was degraded severely during the tosylation reaction in the latter scheme[19]. Therefore, we chose the former method in the experiment.

3.1.2 FT-IR analysis
The FT-IR spectroscopy has been demonstrated to be a powerful tool for study the physicochemical properties of polysaccharide. The syntheses were confirmed by the FT-IR showed in Fig 1.

For chitosan, absorption at around 3400 cm\(^{-1}\) was assigned to stretching vibration of –OH and –NH\(_2\). The band at around 1600 cm\(^{-1}\) was assigned to bending vibration of –NH\(_2\). The twin strong absorptions at around 1080 and 1030 cm\(^{-1}\) were related to C–O stretching vibration of second hydroxyl group and primary hydroxyl group, respectively [28]. The spectra of N-phthaloyl-chitosan showed two strong characteristic absorptions at 1774 cm\(^{-1}\) and 1709 cm\(^{-1}\) which were assigned to stretching vibration of C=O. The peak at 721 cm\(^{-1}\) was due to the bending vibration of C–H in the aromatic ring. Although there was no obvious peak at around 530 cm\(^{-1}\) in spectra of compound 3, the C–O stretching band at 1030 cm\(^{-1}\) corresponding to the primary hydroxyl group disappeared, which verified bromination of the C\(_6\)–OH. For compound 4, a new peak at around 2106 cm\(^{-1}\) could be attributed to stretching vibration of azide group. It also confirmed the successful synthesis of the previous step. Finally, for NCS, the peak of 2106 cm\(^{-1}\) disappeared. At the same time, absorptions at 1774 cm\(^{-1}\) and 1709 cm\(^{-1}\) also disappeared, which seemed that azide group and N–phthaloyl groups were removed. Meanwhile, the peak at around 1600 cm\(^{-1}\) was assigned to the –NH\(_2\) group. Compared with compound 1, the C–O stretching band at 1030 cm\(^{-1}\) related to the primary hydroxyl group disappeared, which also confirmed the synthesis of desired compound.

3.1.2 Solid-state $^{13}$C NMR analysis

Solid-state NMR spectroscopy has been identified as the most useful and accurate
tool for structurally analyzing insoluble complex biomaterial. The solid-state $^{13}$C NMR spectra of CS and NCS were showed in Fig. 2. In the $^{13}$C NMR spectrum of CS, the signal at 105 ppm was attributed to C-1; the signal at 83 ppm was assigned to C-4; the signal at 75 ppm was attributed to C-3 and C-5. And the signals at 61 ppm and 57 ppm were assigned to C-6 and C-2, respectively. In the $^{13}$C NMR spectrum of NCS, the peak at 61 ppm about -CH$_2$OH was disappeared, a new peak at 42 ppm was attributed to C-6 at the same time. It was shifted to higher field compared with the signal of C-6 of CS, which suggested hydroxyl groups had been substituted with amino groups and this carbon was linked to the amino group.

3.1.3 Thermogravimetric and derivative thermogravimetric analysis (TGA/DTG)

Thermal gravimetric analysis of CS and derivative has been used to investigate the thermal degradation and crystallization of the polymers. Fig. 3 shows the TGA curves and the corresponding derivate-grams (DTG) of CS and NCS. The CS (Fig. 3A) shows two major peaks on the TG and DTG curves. CS underwent a 4.5% loss of mass between 30 and 117°C, which resulted from evaporation of water already within the polymer structure [29]. In the second step, between 256 and 600°C (DTG$_{max}$ at 297°C), 52.1% mass loss was observed. This mass loss could be attributed to the degradation of the polysaccharide structure of the molecule and decomposition of the deacetylated units of CS [30, 31]. While presented in Fig. 3B, two decomposition steps were observed. NCS showed a 8.3% loss of mass between 30 and 140°C during the first step, In the second step, between 224 and 600°C (DTG$_{max}$ at 286°C), 46.3% mass loss was observed.
In this study, the total mass loss was 61.8% for CS and 58.6% for NCS. The DTG$_{\text{max}}$ was observed at 297°C for CS and 286°C for NCS. Interestingly, for CS and NCS, the thermal degradation temperatures and thermally stable were similar to one another.

3.1.4 X-ray diffraction (XRD) analysis

Due to large number of hydrogen bonds exist in chitosan molecules, so that the main chain of chitosan has a strong rigidity, while chitosan molecules also have a certain internal crystallinity. The crystalline structure of CS was analyzed by X-Ray diffraction. And the strongest 2θ reflection was observed around 12° and 20° which corresponded to crystal form I and form II. The X-ray diffraction pattern for CS and NCS were given between 3° and 60° of 2θ in Fig. 4. In case of CS spectrum, chitosan showed two crystalline peaks at 2θ = 12.88° and 20.12° with the height of 850 and 1250, respectively. Hence, a typical CS spectrum was observed. And chitosan in this study was observed to be similar to those commercial chitosan from shrimp and crab [30, 31].

In case of NCS, there are two sharp peak, 2θ = 11.22° and 20.63° with the height of 600 and 850, respectively. The crystal diffraction intensity decreased, indicating that the crystal structure of chitosan changed. Compared with CS which showed a broad peak at 12.88°, NCS showed a narrow peak at 11.22°, this suggest that the degree of crystallinity of NCS decrease and the content of the amorphous region increased. And NCS in the early research showed two crystalline peaks at 2θ = 11.8° and 23.9° with the height of 1000 and 1600, respectively [20]. Compared to Yang’ result, our product had smaller angle and lower diffraction intensity, suggest a decrease in crystalline
perfection.

From spectrum, we conclude that the introduction of the amino group did not lead to the destruction of hydrogen bonding between intra- and intermolecular. However, the substitution might have led to the decrease of crystallinity which can be seen in the form of relatively weaker reflection in the spectra of NCS.

3.2 Antioxidant activities
3.2.1 Scavenging activity against DPPH radicals

DPPH is a useful reagent for investigating the free radical-scavenging activity of compounds. This method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant and form stable molecule of DPPH–H. [32]. The scavenging property of CS and NCS against DPPH-radical at various concentrations was showed in Fig. 5A. The scavenging rate increases with concentration. NCS has stronger antioxidant activity against the DPPH-radical than CS in the texted concentration range. This is attributed to -NH2 is easier to release of hydrogen, and pair up through with the free electron on DPPH radical and form stable molecule of DPPH–H. Results showed that NCS exhibited remarkable improvement on DPPH-radical scavenging activity, scavenging effect were 87% at 0.8 mg/mL and 97% at 1.6 mg/mL, respectively.

With the increase of concentration, NCS, which had more free amino groups compared to CS, and showed more pronounced antioxidant activity against the DPPH radical than CS. It is reasonable to presume that the amino groups grafted on C-6 shall be an important factor that influences the scavenging activity against DPPH radicals, which is in accord with the conclusion that the introduction of the amino group can
improve the DPPH radical scavenging activity [15].

3.2.2 Scavenging activity against hydrogen peroxide

Although H$_2$O$_2$ itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals, especially at the presence of transition metal ions [33]. Therefore, removing H$_2$O$_2$ is an important protection of both foodstuffs and living organisms [34]. According to the data showed in Fig. 5B, we could conclude as follows. Firstly, H$_2$O$_2$ scavenging activity increased with the increase of sample concentration. Secondly, NCS had an evident scavenging activity against H$_2$O$_2$ compared with CS. At 0.1 mg/mL, NCS and CS exhibited 68% and 39% scavenging activity against H$_2$O$_2$, respectively. And with the increase of concentration, NCS showed high scavenging activity of 92%; in contrast, CS scavenging activity was only 60%. NCS shows superior antioxidant activity against H$_2$O$_2$ as compared to CS.

The results were similar to those of scavenging ability against DPPH-radicals. CS showed weaker scavenging effect against hydrogen peroxide compare to NCS. According to the results, we speculate that the introduction of amino groups and the increase in concentration of NCS resulted in the increase of total amine groups which then scavenged more hydrogen peroxide. This result is in accord with the conclusion that aminated derivatives of saccharide are more potent than natural saccharide [16].

3.2.3 Reducing power assay

The ferric ion reducing power assay tests reducing power based on an electron transfer reaction. In the assay, the presence of reductants (antioxidants) cause the reduction of the ferric ion/ferricyanide complex to the ferrous form by donating an
electron, with a characteristic formation of Perl’s Prussian blue under acidic condition, which can be measured spectrophotometrically based on the procedure reported [35]. From Fig. 5C, we can clearly see that: the reducing power of NCS increases with concentration. By contrast, reducing power of CS is not affected by different concentrations in our condition. NCS exhibited stronger reducing power than CS and showed reducing power of 0.67 at 0.8 mg/mL. However, in Yang’s study [20], NCS showed reducing power of 0.29 at 0.8 mg/mL. And this can be explained by high deacetylation degree of our raw material.

In the reducing power assay, the reducing power of NCS increases obviously. This might be due to -NH₂ is easier to release of hydrogen, which could break free radical chain by donating a hydrogen atom or react with certain precursors of peroxide, thus preventing peroxide formation. The results, again, suggest that NCS can be considered as efficient antioxidant polymers and amine groups play a role in reducing activities. The more amine groups, the higher reducing power. Other authors [36] has observed a direct correlation between antioxidant activity and reducing power. Our results on the reducing power suggest that NCS likely contribute significantly toward the antioxidant effect.

3.2.4 Metal ion chelating assay

Chelating agents may serve as the secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of metal ions [37]. In Fig. 5D, chelating abilities of NCS and CS are only 17% and 7% at 1.0 mg/mL, respectively.

NCS has a better ability to chelate the metal ions because the atomic
electronegativity of nitrogen is smaller than that of oxygen and nitrogen is easier to give the electron. Moreover, CS showed low chelating abilities and no significant difference with concentration, which differed from other investigators [38-40]. We speculate that one important aspect that can cause the difference is the molecular weight. Chelating ability of chitosan is mainly related to the content of active amino and hydroxyl groups on the polysaccharide backbone. Chitosan with high molecular weight has higher viscosity and stronger intramolecular hydrogen bonds. Hence, the chance of exposure of its amino and hydroxyl groups may be restricted which would account for less activity. And with the decrease of molecular weights, the antioxidant activity of chitosan and its derivatives will be enhanced due to the partially elimination of molecular hydrogen bonds [41]. The results obtained by Xing [42] and Chien [43] are also consistent with the inference.

3.2.5 Linoleic acid peroxidation with TBARS assay

Linoleic acid is usually used as a model compound in lipid oxidation and antioxidation-related assays in which peroxyl radicals and hydroperoxides, etc., are involved in the oxidation process. These peroxidic products interact with thiobarbituric acid to form colored compounds, which show absorption at about 530 nm. As shown in Fig. 5E, inhibition of lipid peroxidation of NCS is 57% at 0.1 mg/mL, whereas, CS has no inhibition of lipid peroxidation at the same concentration. When CS concentration changes from 0 to 0.8 mg/mL, its antioxidant capacity is in the range of 0-32%. However, antioxidant capacity of NCS shows no obvious enhancement and changes only from 49% to 61%. Obviously, besides the amino group and the concentration,
antioxidant capacity of NCS should also be affected by other factors. As reported before, factors affecting antioxidant abilities were complex [20]. The relationship between the antioxidant activities and properties of polysaccharides needs to be further investigated.

Based on the results mentioned above, it is easy to draw conclusions that compared with CS, NCS had more amino groups to inhibit the oxidation of linoleic acid and had evident ability to reduce lipid oxidation, especially at low concentrations. The antioxidant capacity observed in this experiment is consistent with the conclusion that amino groups can inhibit the oxidation of linoleic acid, therefore, increasing antioxidant activities [1]. This consequence is also accord with the speculated result that NCS had good antioxidant activity in previous reduction experiments.

3.2.6 Correlations between antioxidant activities

Inclusion of different assays is important to make precise estimates and comparisons of antioxidant activities. Collectively, these studies add to the body of evidence that NCS has antioxidant capacities and can be used as a promising candidate material in pharmaceutical and food industries. And these antioxidant assays are interrelated. And combining these antioxidant properties, will lead to stacking effects. For example, we can remove ferric iron by the reducing activity and ion chelating ability of NCS. Reducing activity of NCS results in the reduction of ferric ion to ferrous ion which can be removed by chelation. Iron is known as the most important pro-oxidant due to its high reactivity. It can initiate lipid peroxidation, then start a chain reaction and seriously deteriorate the quality of food [4]. Therefore, the elimination of iron is very important in food industries.
Another example is the removal of hydroxyl groups by the scavenging of hydrogen peroxide and chelating ions. Hydroxyl radicals is the most reactive free radical and can be formed from H$_2$O$_2$ in the presence of metal ions such as Fe$^{2+}$ and Cu$^{2+}$. Our product, NCS, has a good scavenging effect on H$_2$O$_2$ (68-92% at 0.1-1.6 mg/mL) and certain capacity of ion chelating, we speculate that it can scavenge hydroxyl radicals. The scavenging activity is not due to the direct scavenge but inhibition of hydroxyl radical generation by scavenging hydrogen peroxide and chelating ions.

In addition, our results on the reducing power suggested that NCS might contribute significantly to the antioxidant effect. The results obtained for linoleic acid peroxidation assay is also in accord with the conclusion that NCS has good antioxidant activity. Meanwhile, inhibition of lipid peroxidation and reduction activity confirmed each other. Accordingly, the reducing capacity of a compound is a useful indicator of its potential antioxidant activity. All the results indicated that amino group grafted on chitosan could increase its antioxidant activity obviously and NCS might be used in food and pharmaceutical industries if permitted.

3.3 Antifungal activity

The action of fungi causes the most economically important seed or plant diseases. Control of these plant-threatening fungi can benefit vegetables and fruits production. Chitosan has poor solubility in water, so we used 0.4% acetic acid to dissolve CS and NCS in antifungal activity test. We tested the antifungal activity of the CS and NCS against common plant-threatening fungi *F. oxysporum* f. sp. *cucumerium*, *F. oxysporum* f. *niveum*, *P. asparagi* and *G. zeae*. The antifungal activity assayed herein was showed
As illustrated by the data, we found that NCS obviously inhibited the selected fungi compared with CS. The NCS inhibitory index was enhanced with concentration increase. Compared to CS, inhibitory indices of NCS were improved by 20%, 30%, 25% and 26% at 0.5 mg/mL, respectively. That is to say, amino groups play a major role in the antifungal activity and NCS has the potential as antifungal agents.

The antifungal activity of chitosan depends on its positive charge number [44]. For this reason, sample with higher positive charge was expected to have a more potent antifungal activity. The positive charge density of chitosan was strengthened by amino groups grafted on chitosan, so, NCS should have better antifungal activity than CS. The observation is also in agreement with the results obtained by Ren [17], who found that the antifungal activity of carbohydrate derivatives against the plant pathogenic fungi increased with an increase in active amino group substituent.

**Conclusion**

In summary, the antioxidant activity of CS and NCS were determined using different assays. As expected, we obtained several satisfactory results as follows. Firstly, comparing with CS, NCS had higher antioxidant abilities. Secondly, NCS also showed better inhibitory activity against fungi compared to CS. In additional, these antioxidant experiments were interrelated. The results above suggest that amino group is the major factor of antioxidant ability and antifungal activity. NCS has good antioxidant capacities and can be effectively used as an antioxidant to protect the human body from free radicals and retard the progress of many chronic diseases. Therefore, NCS is
endowed with antioxidant and can be used as a candidate material in food and pharmaceutical industries.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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Scheme 1. Synthetic route for the preparation of 6-amino-6-deoxychitosan (NCS).

Fig 1. IR spectra of the chitosan (CS), phthaloylation of chitosan (2), 6-bromo-6-deoxy-N-phthaloyl-chitosan (3), 6-azido-6-deoxy-N-phthaloyl-chitosan (4) and 6-amino-6-deoxychitosan (NCS).

Fig 2. The solid-state $^{13}$C NMR spectra of CS and NCS.

Fig 3. TGA thermogram and DTG curves. (A) CS, (B) NCS.

Fig 4. XRD spectra of CS (A) and NCS (B).

Fig 5. Antioxidant effect and antifungal activity of samples: (A) DPPH-radical scavenging ability; (B) Hydrogen peroxide scavenging ability; (C) Reducing power; (D) Chelating ability; (E) Inhibition of the linoleic acid peroxidation.

Table 1. Antifungal activity against various fungi at 0.1, 0.5 and 1.0 mg/mL (from 1 to 4 were represented *F. oxysporum* f. sp. *cucumerium*, *F. oxysporum* f. *niveum*, *P. asparagi* and *G. zeae*, respectively).
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| Sample | Concentration (mg/mL) | Various Fungi |   |   |   |
|--------|------------------------|---------------|---|---|---|
|        |                        | 1              | 2 | 3 | 4 |
| CS     | 0.1                    | 7.3±0.7        | 6.4±0.7 | 4.2±0.1 | 0.1±0.1 |
|        | 0.5                    | 59.5±4.9       | 60.5±2.1 | 59.5±0.7 | 52.5±0.7 |
|        | 1.0                    | 72.5±0.6       | 71.4±0.7 | 69.0±1.4 | 82.3±0.7 |
| NCS    | 0.1                    | 9.5±0.6        | 7.3±1.2 | 4.4±0.2 | 4.3±0.2 |
|        | 0.5                    | 79.5±2.1       | 81.0±2.8 | 85.3±0.4 | 79.0±1.4 |
|        | 1.0                    | 89.0±1.6       | 96.6±0.7 | 90.5±0.7 | 96.2±0.2 |