Synthesis of urea-functionalized chitosan derivatives for potential antifungal and antioxidant applications

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1. Introduction

Chitosan, (1→4)-2-amino-2-deoxy-\(β\)-\(\alpha\)-glucan, is a natural cationic linear polymer obtained from partial or complete deacetylation of chitin, which is a natural polysaccharide mostly extracted from marine crustaceans, such as shrimps and crabs (Kurniasih, Purwati Cahyati, & Dewi, 2018; Sajomsang, Gonil, Saesoo, & Ovatlarnporn, 2012; Zhang et al., 2017). Chitosan has attracted extensive attention in the biotechnology, pharmaceutics, wastewater, cosmetics, agriculture, food science, and textiles fields (Wang et al., 2017; Yar et al., 2017) because of its multifaceted properties, such as biocompatibility, biodegradability, nontoxicity, antimicrobial activity, antihypercholesterolic activity, and film-forming property (Duan, Liang, Cao, Wang, & Zhang, 2015; Jena, Mohanty, Mallick, Jacob, & Sonawane, 2012; Nataraj, Sakkara, Meghwal, & Reddy, 2018; Sathiyabama & Parthasarathy, 2016). Especially in agricultural settings, chitosan can effectively inhibit mycelium growth of pathogens and promote plant resistance to abiotic and biotic stresses (Xing et al., 2018). It indicates that chitosan has the potential to control plant diseases in agricultural operations. The research of chitosan in field of antioxidant is also very active. However, its poor solubility hinders its further applications. To overcome this issue, chemical modifications, such as acylation, etherification, and quaternization, can be used on amine (-NH\textsubscript{2}) functional groups, which are abundant along the chitosan chain. In addition, studies have demonstrated that chemically modified chitosan derivatives show enhanced biological activities, including antifungal (Li, Tan, Zhang, Gu, & Guo, 2016), antibacterial (Fardioui, Meftah Kadmiri, Qais, & Bouhid, 2018), and antioxidant (Tan, Zhang, Zhao et al., 2018) activities. Meanwhile, this method overcomes the water insoluble drawback of chitosan and provides a reference of the new agricultural use of the chitosan and derivatives. Therefore, chitosan could be an ideal candidate for use in the preparation of new materials for biomedical applications.

Heterocyclic compounds (compounds that contain at least one heteroatom in addition to the carbon atoms) (Venepally & Reddy Jala, 2017) have been used for many medical and synthetic chemistry applications because of their excellent bioactivities, including antifungal (Cao et al., 2014), antibacterial (Azab, Youssef, & El-Bordany, 2013), antitumor (Chen et al., 2014), anti-inflammatory (El-Sawy, Ebaid, Abo-Salem, Al-Sehami, & Mandour, 2014), and antiviral (Saleem, Sakr, El-
Senousy, & Madkour, 2013) activities. In particular, some nitrogen-containing heterocycles, which show good biological properties, such as thiazoles, thiadiazoles, and triazoles, are used significantly in current commercial agrochemicals. For example, Łączkowski et al. synthesized a series of thiazole derivatives with high antifungal and antioxidant activities. The authors reported that the lipophilic property of compounds had an important role in antifungal activity (Łączkowski et al., 2018). Furthermore, sulfonamide-1,2,4-triazoles and 1,3,4-thiadiazoles derivatives have also been reported to show good antifungal activity, with triazole-3-thiones exhibiting more antifungal activity against all fungal species because of their high dipole movement, increased number of hydrogen bond acceptors, high energy of solvation, increased electron affinity, and low ionization potential values (Zoumpoulakis et al., 2012). In addition, the urea group has been frequently reported as a key structural motif in antifungal agents (Tale, Rodge, Hatnapure, & Keche, 2011). Past studies also demonstrated the crucial role of urea groups in increasing the bioactivity of some poly saccharides (Zhang, Tan, Mi et al., 2018; Zhang, Tan, Zhang et al., 2018). Therefore, as a highly active group, several urea groups containing heterocycles were synthesized and grafted onto chitosan to enhance the antifungal and antioxidant activities of these compounds.

In the current research, the main purpose is to improve the antifungal and antioxidant properties of crude chitosan by using heterocycle-containing urea groups. More importantly, gaining insights into the mechanism of the structure and biological activities of chitosan derivatives is also one of our research targets. Therefore, a series of novel chitosan derivatives structuring with heterocycle-bearing urea groups were synthesized and characterized. The antifungal and antioxidant activities of chitosan derivatives were tested and the results showed that the biological efficiency might be related to the electron-withdrawing capacity of urea groups. Additionally, the cytoxic effects of all samples were also evaluated on L929 cells by CCK-8 assay in vitro.

2. Materials and methods

2.1. Materials

Chitosan with an 83% degree of deacetylation was purchased from Qingdao Baicheng Biochemical Corp. (China). And its average molecular weight was 200 kDa. The materials, such as iodomethane, chloroacetyl chloride, nicotinoyl chloride hydrochloride, 2-aminothiazole, 2-amino-1,3,4-thiadiazole, 2-amino-5-methyl-1,3,4-thiadiazole, 2-amino-5-phenyl-1,3,4-thiadiazole, and 3-amino-1,2,4-triazole, used in this research were purchased from the Sigma-Aldrich Chemical Corp. Sodium azide, sodium hydroxide, N-Methyl pyrrolidone (NMP), N, N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetone, and methylbenzene were supplied by Sinopharm Chemical Reagent Co., Ltd., Shanghai, China and used as received. Four fungitoxicating agents were provided by Qingdao Academy of Agricultural Sciences.

2.2. Chemical synthesis

The synthetic strategy of the chitosan derivatives bearing urea groups is shown in Scheme 1. Firstly, several unsymmetrically substituted urea groups were synthesized via pyridine-3-isocyanate and the biologically active nitrogen-containing heterocycles and pyridine rings were combined in this way. Then, quaternized chitosan with better water solubility was achieved through modifying C2-NH2. Subsequently, chloroacetyl group was attached to C6-OH of quaternized chitosan to obtained CTCS, since pyridine on urea groups could be attacked easily by chloride acetyl group to give N-alkylypyridinium salts. Finally, the target products were prepared through a nucleophilic substitution of CTCS and urea groups. The specific operations are as follows:

2.2.1. Synthesis of the urea of 3-amino-pyridine

As shown in Scheme 1, firstly, nicotinyl chloride hydrochloride (20 mmol) was dispersed in 15 mL of acetone and stirred under the condition of ice bath. The mixture was then added dropwise to the aqueous solution of sodium azide (3.9 g NaN3 dissolving in 12 mL of deionized water) and the mixture was stirred for 3 h at 0 °C. After the reaction was completed, the solution had been stratified and the lower layer was removed with a glass capillary. The remaining solution was poured into 10 mL of methylbenzene solvent at 60 °C and stirred for 2–3 h. Then, the mixture was cooled and some pink crystals were precipitated out. The precipitate was filtered and the intermediate-pyridine-3-isocyanate was obtained.

Finally, pyridine-3-isocyanate (30 mmol) and 2-aminothiazole, 2-amino-1,3,4-thiadiazole, 2-amino-5-methyl-1,3,4-thiadiazole, 2-amino-5-phenyl-1,3,4-thiadiazole, or 3-amino-1,2,4-triazole (30 mmol) were added to methylbenzene (20 mL) in a 50 mL flask. The mixture was stirred for 24 h at 60 °C, then a large amount of solid formed and was filtered, which was further purified by crystallization from the solvent that the ratio of water and ethanol was 1:1. Five different urea groups (N-(2-thiazole)-N'-pyridylurea, N-(2-thiazole)-N'-pyridylurea, N-(5-methyl-2-thiazole)-N'-pyridylurea, N-(5-phenyl-2-thiazole)-N'-pyridylurea, and N-(3-triazole)-N'-pyridylurea) were obtained in this way.

2.2.2. Synthesis of chitosan derivative

In a 50 mL flask, 2 mmol chitosan was dispersed in 20 mL of N-methyl-2-pyrrolidone (NMP) and stirred for 1 h at room temperature (20 °C–25 °C). Then, NaI (0.9 g), 15% NaOH aqueous solution (3 mL), and CH3I (3 mL) were added and the mixture was refluxed at 60 °C for 2 h. Subsequently, the solution was precipitated into excess ethanol and the flavescent precipitant were filtrated to obtain chitosan derivative with quaternary ammonium salt (Elemental analysis: C: 31.43%, N: 4.52%, H: 5.49%, C/N: 6.95, degree of substitution (DS): 0.59). The obtained precipitate was then dissolved in 30 mL of N, N-di-methylformamide (DMF) with chloracetyl chloride (1.5 mL) and stirred at 30 °C for 12 h. Next, the solution was precipitated and filtered with ethanol. Finally, the chitosan derivative (CTCS) was obtained after washing with excess ethanol and freeze-drying under vacuum for 24 h.

The final chitosan derivatives with urea groups (a–e) were synthesized as follows: the solution, containing 1 mmol CTCS and 3 mmol urea groups (N-(2-thiazole)-N'-pyridylurea, N-(2-thiazole)-N'-pyridylurea, N-(5-methyl-2-thiazole)-N'-pyridylurea, N-(5-phenyl-2-thiazole)-N'-pyridylurea, or N-(3-triazole)-N'-pyridylurea) in 20 mL of dimethyl sulfoxide (DMSO), was carried out for 24 h at 60 °C. The solution was poured into acetone and obtained some precipitates. These precipitates were then filtered and washed with ethanol. The unreacted reagents were extracted with ethanol for 48 h in Soxhlet apparatuses. Finally, the corresponding chitosan derivatives, including 2-(N,N,N-trimethyl)-6-O-(3-thiazoleureido-pyridyl)acetoyl chitosan chloride (a), 2-(N,N,N-trimethyl)-6-O-(3-thiazoleureido-pyridyl)acetoyl chitosan chloride (b), 2-(N,N,N-trimethyl)-6-O-(3-5(5-thiazoleureido-pyridyl)acetoyl chitosan chloride (c), 2-(N,N,N-trimethyl)-6-O-(3-5(5-thiazoleureido-pyridyl)acetoyl chitosan chloride (d), and 2-(N,N,N-trimethyl)-6-O-(3-(3-triazoleureido-pyridyl)acetoyl chitosan chloride (e), were obtained after freeze-drying.

2.3. Analytical methods

2.3.1. Fourier transform infrared (FT-IR) spectroscopy

The infrared spectra of the samples were recorded on a Jasco-4100 FT-IR spectrometer (Japan, provided by JASCO Co., Ltd., Shanghai, China) using transmittance modes at a resolution of 4.0 cm−1 in the 4000–400 cm−1 region. The tested samples were mixed with KBr by the ratio 1:100 to form laminae for observation with accumulation of 16 scans at 25 °C.
were calculated by the following equations: 

\[
\text{DS}_1 = \frac{n_1 \times M_C - M_N \times W_{CN}}{n_1 \times M_C} 
\]

\[
\text{DS}_2 = \frac{M_N \times W_{CN} + n_2 \times M_C \times DS_1 - n_1 \times M_C}{n_2 \times M_C} 
\]

\[
\text{DS}_3 = \frac{M_N \times W_{CN} + n_3 \times M_C \times DS_1 - n_1 \times M_C - n_3 \times M_C \times DS_2}{n_3 \times M_C - n_4 \times M_C \times DS_3} 
\]

\[
\text{DS}_4 = \frac{-n_4 \times M_C \times DS_2 - n_4 \times M_C \times DS_3}{n_4 \times M_C - n_5 \times M_N \times W_{CN}} 
\]

where \( DS_1, DS_2, DS_3, \) and \( DS_4 \) represent the deacetylation degree of chitosan, the degrees of substitution of \( N,N,N \)-trimethyl in chitosan derivative with quaternary ammonium salt, chloroacetyl in chitosan derivative (CTCS), and urea groups in chitosan derivatives (a-e); \( M_C \) and \( M_N \) are the molar mass of carbon and nitrogen, \( M_C = 12, M_N = 14; n_1, n_2, n_3, n_4, n_5, n_6, n_7, n_8, n_9, \) and \( n_1, n_2, n_3, n_4, n_5, n_6, n_7, n_8, n_9, \) are the number of carbon of chitin, acetamido group, trimethyl, chloroacetyl group, and urea group, \( n_1 = 8, n_2 = 2, n_3 = 3, n_4 = 2; a: n_5 = 9, b: n_5 = 8, c: n_5 = 9, d: n_5 = 14, e: n_5 = 8; n_1 \) and \( n_2 \) are the number of nitrogen of trimethyl and urea group, \( n_1 = 1, a: n_2 = 4, b: n_2 = 5, c: n_2 = 6, d: n_2 = 5, e: n_2 = 6, W_{CN} \) represents the mass ratio between carbon and nitrogen in chitosan derivatives.

### 2.3.2. Nuclear magnetic resonance (NMR) spectroscopy

The \(^1\)H NMR spectra of chitosan derivatives were measured on a Bruker Avance III 500 NMR Spectrometer (500 MHz, Switzerland, provided by Bruker Tech. and Serv. Co., Ltd., Beijing, China) at 25°C. The samples were analyzed by dissolving in 1.0 mL of D$_2$O or DMSO.

### 2.3.3. Elemental analysis

The elemental analyses by combustion were performed using an element analysis instrument (Vario Micro Elemental Analyzer, Elementar, Germany). The samples should be completely wrapped with tin foil before analysis. The carbon-nitrogen ratios were used to evaluate the degree of substitution (DS) in chitosan derivatives and the DS were calculated by the following equations:

\[
\text{DS}_1 = \frac{n_1 \times M_C - M_N \times W_{CN}}{n_1 \times M_C} 
\]

\[
\text{DS}_2 = \frac{M_N \times W_{CN} + n_2 \times M_C \times DS_1 - n_1 \times M_C}{n_2 \times M_C} 
\]

\[
\text{DS}_3 = \frac{M_N \times W_{CN} + n_3 \times M_C \times DS_1 - n_1 \times M_C - n_3 \times M_C \times DS_2}{n_3 \times M_C - n_4 \times M_C \times DS_3} 
\]

\[
\text{DS}_4 = \frac{-n_4 \times M_C \times DS_2 - n_4 \times M_C \times DS_3}{n_4 \times M_C - n_5 \times M_N \times W_{CN}} 
\]

where \( DS_1, DS_2, DS_3, \) and \( DS_4 \) represent the deacetylation degree of chitosan, the degrees of substitution of \( N,N,N \)-trimethyl in chitosan derivative with quaternary ammonium salt, chloroacetyl in chitosan derivative (CTCS), and urea groups in chitosan derivatives (a-e); \( M_C \) and \( M_N \) are the molar mass of carbon and nitrogen, \( M_C = 12, M_N = 14; n_1, n_2, n_3, n_4, n_5, n_6, n_7, n_8, n_9, \) and \( n_1, n_2, n_3, n_4, n_5, n_6, n_7, n_8, n_9, \) are the number of carbon of chitin, acetamido group, trimethyl, chloroacetyl group, and urea group, \( n_1 = 8, n_2 = 2, n_3 = 3, n_4 = 2; a: n_5 = 9, b: n_5 = 8, c: n_5 = 9, d: n_5 = 14, e: n_5 = 8; n_1 \) and \( n_2 \) are the number of nitrogen of trimethyl and urea group, \( n_1 = 1, a: n_2 = 4, b: n_2 = 5, c: n_2 = 6, d: n_2 = 5, e: n_2 = 6, W_{CN} \) represents the mass ratio between carbon and nitrogen in chitosan derivatives.

### 2.4. Antifungal assay

The antifungal ability was assessed by the model of Tan’s methods (Tan, Zhang, Mi et al., 2018). Briefly, the stock solutions of chitosan and derivatives with a concentration of 6 mg/mL were prepared by dissolving them in distilled water. Then, each sample solution was added to sterilized potato dextrose agar (PDA) medium to obtain final concentrations of 0.1, 0.5 and 1.0 mg/mL. The culture media containing samples were transferred to Petri dishes (7 cm). After solidification, the Petri dishes were inoculated with fungi mycelium and incubated at 27°C for 2–3 days. When the mycelia of fungi reached the edges of the control plate (without the presence of samples), the inhibition indices of all samples were calculated as follows:

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

where \( D_a \) is the diameter of the growth zone in the test plates and \( D_b \) is the diameter of the growth zone in the control plate.

### 2.5. Antioxidant assay

#### 2.5.1. Hydroxyl-radical scavenging activity assay

The test of hydroxyl-radical scavenging ability was carried out according to the following method. Containing testing samples of starch or starch derivatives (10 mg/mL, 0.045, 0.09, 0.18, 0.36, and 0.72 mL), EDTA-Fe$^{2+}$ (220 μM), safranine O (0.23 μM), and H$_2$O$_2$ (60 μM) in potassium phosphate buffer (150 mM, pH 7.4), the reaction mixture was incubated for 30 min at 37°C. The absorbance of the mixture was measured at 520 nm. Three replicates for each sample were tested and the hydroxyl-radical scavenging effect was calculated according to the following equation:

\[
\text{Scavenging effect [%]} = \frac{A_{\text{control} 520 \text{nm}} - A_{\text{blank} 520 \text{nm}}}{A_{\text{control} 520 \text{nm}} - A_{\text{blank} 520 \text{nm}}} \times 100
\]

where \( A_{\text{sample} 520 \text{nm}} \) is the absorbance of the samples, \( A_{\text{control} 520 \text{nm}} \) is the absorbance of the control (potassium phosphate buffer replaced H$_2$O$_2$), and \( A_{\text{blank} 520 \text{nm}} \) is the absorbance of the blank (distilled water replaced samples).

#### 2.5.2. Superoxide-radical scavenging activity assay

The superoxide-radical scavenging ability was assessed following the model of Tan’s methods (Tan, Zhang, Zhao et al., 2018). The reaction mixture, involving test samples (5 mg/mL, 0.06, 0.12, 0.24, 0.48, and 0.96 mL), phenazine methosulfate (PMS, 30 μM), nicotinamide

![Scheme 1](image-url)
adrenaline dinucleotide reduced (NADH, 338 μM), and nitro blue tetra-zolium (NBT, 72 μM) in Tris–HCl buffer (16 mM, pH 8.0), was incubated 5 min at room temperature. The absorbance was measured at 560 nm against blank. Three replicates for each sample were tested and the superoxide-radical scavenging effect was calculated according to the following equation:

\[
\text{Scavenging effect (\%)} = \left(1 - \frac{A_{\text{sample 560 nm}} - A_{\text{control 560 nm}}}{A_{\text{blank 560 nm}}} \right) \times 100
\]

Where \(A_{\text{sample 560 nm}}\) is the absorbance of the samples, \(A_{\text{control 560 nm}}\) is the absorbance of the control (distilled water replaced NADH), and \(A_{\text{blank 560 nm}}\) is the absorbance of the blank (distilled water replaced samples).

2.6. Cytotoxicity assay

The cytotoxicity of chitosan and synthesized chitosan derivatives (a–e) on L929 cells at different concentrations (1.0, 10.0, 100.0, 500.0, and 1000.0 μg/mL) was determined by CCK-8 assay in vitro. L929 cells were cultured in RPMI medium (containing 1% mixture of penicillin & streptomycin and 10% fetal calf serum) at 37 °C. These cells were seeded on 96-well flat-bottom culture plates at a density of \(1.0 \times 10^5\) cells and incubated (37 °C, 5% CO_2). After 24 h of cell attachment, the samples with different final concentrations were introduced to cells, separately. Next, the cells were cultured for 24 h. Afterward, 10 μL of CCK-8 solution was added in each well and incubated for another 4 h at 37 °C. The absorbance at 450 nm was recorded using a microplate reader. Cell viability was recorded using the following formula:

\[
\text{Cell viability (\%)} = \frac{A_{\text{sample}} - A_{\text{negative}}}{A_{\text{negative}} - A_{\text{blank}}} \times 100
\]

Where \(A_{\text{sample}}\) is the absorbance of the samples (containing cells, CCK-8 solution, and sample solution), \(A_{\text{negative}}\) is the absorbance of the blank (containing RPMI medium and CCK-8 solution), and \(A_{\text{blank}}\) is the absorbance of the negative (containing cells and CCK-8 solution).

2.7. Statistical analysis

All the data related to the antifungal activity was illustrated as the means ± standard deviation (SD, n = 3) for triplicates. Significant difference analysis was determined using Scheffe’s multiple range test. The significant differences were defined at \(p < 0.05\).

3. Results and discussion

3.1. Chemical synthesis and characterization

Each step of the synthesis was followed by FT-IR (Fig. 1) and 1H NMR (Fig. 2) spectroscopy. The yields and the degrees of substitution of chitosan derivatives are shown in Table 1.

3.1.1. FT-IR spectra

Chitosan and synthesized chitosan derivatives were analyzed using FT-IR and the results are depicted in Fig. 1. The spectrum of chitosan showed characteristic absorption bands at approximately 3421 cm\(^{-1}\) (O–H and N–H stretching vibrations), 2921 cm\(^{-1}\) and 2881 cm\(^{-1}\) (−CH asymmetric stretching), 1654 cm\(^{-1}\) (axial C=O stretching), 1596 cm\(^{-1}\) (vibration modes of amino group), and 1072 cm\(^{-1}\) (C=O stretching vibration) (Almada et al., 2017; Querghemmi et al., 2018). After modification, a signal at 1470 cm\(^{-1}\) was assigned to the characteristic band of trimethyl group was observed from the FT-IR spectrum of CTCS. (Tang, Zhang, & Yu, 2016). Meanwhile, the peaks at 1749 cm\(^{-1}\) and 785 cm\(^{-1}\) were the vibrations of C=O and C–Cl, which indicated the existence of the chloroacetyl group. (Labafzadeh, Vyawaharkar, Kavakka, King, & Kilpelainen, 2015; Li, Guo, & Jiang, 2010; Zhang, Tan, Zhang et al., 2018). Compared to CTCS, the spectra of chitosan derivatives (a–e) displayed a decrease of the relative intensity of the C=O band at 1749 cm\(^{-1}\) and C–Cl at 790 cm\(^{-1}\) and new peaks appeared at 1698 cm\(^{-1}\), 1555 cm\(^{-1}\), 1510 cm\(^{-1}\), and 820 cm\(^{-1}\), which were assigned to the characteristic peaks of urea groups, showing that the band of C–Cl had been attacked by new active groups. Among these characteristic absorptions, the structure of -NH–CO-NH- belonging to the urea group was evidenced by the peak at 1698 cm\(^{-1}\). Also, the additional peaks at 1555 cm\(^{-1}\), 1510 cm\(^{-1}\), and 820 cm\(^{-1}\) resulted from the typical absorption of nitrogen-containing heterocycles (Li et al., 2013). There were also characteristic peaks of N–CH\(_2\) at 1470 cm\(^{-1}\), which indicated that the quaternary ammonium salts groups remained in the final products. Therefore, these results confirmed that the urea groups were grafted onto CTCS and that the targeted chitosan derivatives had been synthesized successfully.

3.1.2. NMR spectra

Fig. 2 shows the 1H NMR spectra of chitosan and chitosan derivatives. The signal of N-acetyl group protons appeared at 6.8 ppm. The signals at 8.3–5.5 ppm were assigned to the protons of the sugar skeleton of chitosan. In particular, the single peak at 8.30 ppm represented protons on C-2, multiple peaks at 8.36–3.9 ppm were caused by the resonances of protons on C-3–C-6, and the peak at 8.55 ppm was assigned to the hydrogen protons on C-1 (Badawy, Rabea, & Taktak, 2014; Wu et al., 2016). In terms of the 1H NMR spectrum of CTCS, because of the presence of trimethyl and chloroacetyl groups, peaks were observed at 8.31 ppm and 8.44 ppm, which were attributed to proton signals of N’(CH\(_3\)\(_3\)) and –CO\(_2\)HCl, respectively, as reported in the literature (Zhang, Tan, Zhang et al., 2018). After CTCS had been reacted with urea groups, changes in the 1H NMR spectra of chitosan derivatives (a–e) were obvious. For example, the characteristic signal of –CO\(_2\)HCl at 8.44 ppm was weaker and new signals appeared at 86.8–9.7 ppm. Generally, the peaks at 89.6–9.7 ppm were related to
the protons of -NH- on the urea groups. Other assignments in the range of 8.75–9.0 ppm were attributed to the protons on the pyridine ring and the small peaks 8.68–7.5 ppm to the protons of the thiazole, triazole, or benzene ring (Qin et al., 2013; Tan, Li et al., 2018). The specific positions of other signals of the urea groups are indicated in Fig. 2. In brief, the peaks observed indicated the presence of both chitosan and urea groups in the sample and, thus, it was concluded that chitosan derivatives had been synthesized successfully.

![Fig. 2. 1H NMR spectra of chitosan and chitosan derivatives.](image)

### Table 1: Yields and the degrees of substitution of chitosan derivatives.

| Compounds | Yields (%) | Elemental analyses (%) | Degrees of Substitution | Deacetylation |
|-----------|------------|------------------------|-------------------------|---------------|
|           |            | C  | N  | H  | C/N | |
| CS        | 43.42      | 7.98 | 6.30 | 5.44 |
| CTCS      | 85.9       | 35.90 | 4.50 | 6.27 | 7.98 | 0.60 | 0.83 |
| a         | 69.5       | 40.41 | 7.70 | 6.88 | 5.25 | 0.21 |
| b         | 78.1       | 38.64 | 8.35 | 8.74 | 4.52 | 0.22 |
| c         | 68.9       | 38.79 | 8.21 | 6.12 | 4.72 | 0.21 |
| d         | 73.4       | 38.84 | 7.91 | 7.00 | 4.91 | 0.24 |
| e         | 66.9       | 39.94 | 9.42 | 6.76 | 4.24 | 0.20 |
3.1.3. Elemental analysis

The degrees of substitution (DS) for chitosan derivatives were estimated by elemental analysis and the results calculated by formulas 1–4 are shown in Table 1. Among all derivatives, the DS of CTCS is the highest. As to the final products, their degrees of substitution are all around 0.2. In some cases, the antifungal property can be affected directly by the degree of substitution since urea group is a critical factor in improving the bioactivity of chitosan derivatives.

3.2. Antifungal activity

In agriculture, plant pathogenetic fungi can cause devastating diseases and limit the yield of crops worldwide (Fan et al., 2018). At present, chemical fungicides are the main means to control these fungal infections. (Hasheminejad, Khodaiyan, & Safari, 2018; Wang, Sun, Zhang, Zhang, & Feng, 2016). However, the negative effects, such as environmental pollution and accumulation of harmful element, associated with the use of these fungicides have resulted in increased interest in developing effective and environmentally friendly antifungal agents. In this essay, the antifungal activities of chitosan and chitosan derivatives against F. oxysporum f. sp. niveum, P. asparagus, F. oxysporum f. sp. cucumerium Owen, and B. cinerea were estimated and the inhibition rates calculated by formula 5 are shown in Figs. 3–6.

Fig. 3 shows the antifungal activities of chitosan and chitosan derivatives against F. oxysporum f. sp. niveum. In this experiment, the inhibitory rates of samples were concentration dependent. For instance, the inhibitory rates of derivative (a) were 12.65, 44.69, and 70.36% for the corresponding concentrations of 0.1, 0.5, and 1.0 mg/mL. At the concentration of 1.0 mg/mL, the antifungal activities of derivatives (a–e) against F. oxysporum f. sp. niveum were > 70% compared with chitosan with an inhibitory rate of 20%. This suggested that chitosan derivatives bearing urea groups exhibited higher antifungal effects compared with pristine chitosan and confirmed the advantage of adding urea groups. Furthermore, two obvious inhibition rules were found: derivative (d) > derivative (c) > derivative (b) > derivative (CTCS) > chitosan and derivative (e) > derivative (b) > derivative (a) > derivative (CTCS) > chitosan, which were identical to the order of the electronegativity of urea groups in chitosan derivatives. For example, the antifungal indices of derivative (e), derivative (d), derivative (c), derivative (b), derivative (a), derivative (CTCS), and chitosan were 93.08, 92.66, 88.98, 81.97, 70.36, 20.19, and 19.545% at 1.0 mg/mL, respectively. The relationship between the structure and antifungal order is discussed further below.

The antifungal abilities of chitosan and synthesized derivatives against P. asparagus at various concentrations are shown in Fig. 4. A considerable increase in antifungal activity could be observed for chitosan derivatives (a–e), with inhibitory values of 78.65%, 84.02%, 89.05%, 90.98%, and 91.24%, compared with chitosan and CTCS, with inhibitory rates of 11.80% and 15.86% at 1.0 mg/mL, it suggested that the urea groups significantly promoted the antifungal activity of these compounds. Moreover, the dose effect of all samples was still evident. For example, when the corresponding concentrations were 0.1 mg/mL, 0.5 mg/mL, and 1.0 mg/mL, the inhibitory rates of sample (b) were 5.97, 32.27, and 84.02%.

![Fig. 3. The antifungal activity of chitosan and chitosan derivatives against F. oxysporum f. sp. niveum (The sample concentration in the mycelium growth pictures was 1.0 mg/mL).](image_url)
The result of antifungal effect of chitosan and chitosan derivatives against *F. oxysporum* f. sp. *cucumebrium* Owen is given in Fig. 5. Generally, the results of compounds (a–e) against *F. oxysporum* f. sp. *niveum* discussed above were similar for the antifungal activity of these compounds against *F. oxysporum* f. sp. *cucumebrium* Owen. For example, the concentration-dependent bioactivity was distinct for all samples. The fungistatic rules, which were derivative (d) > derivative (c) > derivative (b) > derivative (CTCS) > chitosan and derivative (e) > derivative (b) > derivative (a) > derivative (CTCS) > chitosan, were also consistent with the results described earlier. However, a slight difference was observed. The inhibitory rate of derivative (d) was slightly higher than that of derivative (e) in this experiment, whereas the antifungal activity of derivative (d) against *F. oxysporum* f. sp. *niveum* was similar to that of derivative (e). It might be that the strong electronegativity of the benzene ring and the degree of substitution of the active urea group were all factors involved in inhibiting the growth of *F. oxysporum* f. sp. *cucumebrium* Owen.

As shown in Fig. 6, all samples showed antifungal activity against *B. cinerea* at the tested concentration. The five final products had remarkable antifungal activity against *B. cinerea* and the inhibition rates increased by > 50% compared with chitosan and CTCS at 0.5 mg/mL. This result further confirmed that the introduction of urea groups into chitosan contributed significantly to the antifungal action of the synthesized compounds.

### 3.3. Antioxidant activity

The accumulation of reactive oxygen species (ROS) can lead to oxidative stress and oxidative stress is the cause of various human chronic diseases. Therefore, protecting human body from attacks by ROS is crucial. Although a variety of chemically synthesized antioxidants have been used for many years, their safety has been questioned. It is more and more urgent to exploit innoxiousness natural antioxidants. In this paper, several chitosan derivatives bearing urea groups with high antioxidant activity were synthesized and their hydroxyl-radical scavenging rates calculated by formula 6 and superoxide-radical scavenging rates calculated by formula 7 are shown in Fig. 7.

As shown in Fig. 7, the hydroxyl radical-scavenging activity and superoxide-radical scavenging activity of all samples were concentration-dependent. Chitosan showed relatively weak scavenging activity. The synthesized chitosan derivatives bearing urea groups gave much stronger radical scavenging ability compared with chitosan and CTCS. This result provided information that the urea groups enabled chitosan better radical-scavenging activity and superoxide-radical scavenging activity. In hydroxyl radical-scavenging assay, of all chitosan derivatives bearing urea groups, derivative (e) showed the strongest scavenging capacity (94.99%), followed by derivative (d) (88.05%), derivative (c) (81.73%), derivative (b) (75.37%), and derivative (a) (46.63%) at the concentration of 0.8 mg/mL. Besides, the scavenging activities of chitosan derivatives (a–e) against superoxide radicals attain above 95% compared to chitosan with scavenging rate 34.72% at the concentration of 1.6 mg/mL and the scavenging indices of derivative (e), derivative (d), derivative (c), derivative (b), and derivative (a) were 99.79, 98.83, 98.83, 98.44, and 96.18%, respectively. It seems that the antioxidant rules are consistent with antifungal rules, which are derivative (d) > derivative (c) > derivative (b) > derivative (CTCS) > chitosan and derivative (e) > derivative (b) > derivative (a) > derivative (CTCS) > chitosan. Therefore, the relationship between structure and biological
activities deserves further discussion.

As the results of biological activity test showed, chitosan derivatives (a–e), which contained urea groups in their molecular structure, exhibited enhanced antifungal and antioxidant properties compared with pristine chitosan and derivative (CTCS). Hence, it is reasonably to assume that the improved biological actions of chitosan derivatives (a–e) were the result of the functions of the urea group. Nevertheless, because of the differences in the structure of the active groups on urea, the products presented a certain biological activity regularity. In addition, the bioactive orders of derivative (d) > derivative (c) > derivative (b) > derivative (CTCS) > chitosan and derivative (e) > derivative (b) > derivative (a) > derivative (CTCS) > chitosan could be explained as follows: it is well known that N or S atoms have strong electronegativity and can strengthen the positive charge densities of the synthesized compounds. Hence, the synthesized chitosan derivatives containing N and S atoms could interact with the electronegative charged components on fungal cell walls or cytomembranes through electrostatic attraction. The electronegative groups that adhere to the outer membranes of the fungi could lead to a significant alteration of the structure of the membranes of microbial cells, which might prevent nutrients from entering the cells and cause leakage of intracellular electrolytes and proteinaceous compounds, ultimately leading to fungal death. (Li, Yang, & Yang, 2015; Tejero, Lopez, Lopez-Fabal, Gomez-Garces, & Fernandez-Garcia, 2015). In addition, the electrophilic derivatives could also attract more single electron of free radicals to inhibit the free radical chain reaction. Thus, the antifungal and antioxidant potentials of the test compounds were related to the electronegativity of the substituted groups and the biological activities of the samples increase with the increase of electronegativity. Thus, the orders of the bioactivity, derivative (e) > derivative (b) > derivative (a) and derivative (d) > derivative (c) > derivative (b), were consistent with the electron-withdrawing properties (triazole > thiadiazole > thiazole and phenyl-thiadiazole > methyl-thiadiazole > thiadiazole) of the different substituted groups on urea.

In summary, the above analysis suggested that the electronegativity of the substituted groups was the main factor affecting the antifungal and antioxidant activities of the compounds tested in this study. Thus, future research should focus on the electronegativity of compounds to determine and assess its effects on the control of fungal species.

3.4. Cytotoxicity analysis

To explore the biocompatibility of chitosan and its derivatives at different concentrations, cytotoxicity test was carried out by CCK-8 assay. The growth of 1.929 cells subjected to different degrees of inhibition after 24 h of treatment using chitosan and its derivatives is shown in Fig. 8. The normal cell morphology is spindle or oval and more normal cells means less cytotoxicity. At the tested concentration, the cell viabilities of chitosan were about 100%. It meant that pristine chitosan was not cytotoxic. However, strong cytotoxicity of derivative (CTCS) could be observed. When the tested concentration was 100 μg/mL, the cell viability of CTCS was less than 50%, let alone at concentrations of 1000 μg/mL and 500 μg/mL. After the urea groups were grafted onto CTCS, the cytotoxicities of the obtained products were...
reduced. Among them, derivative (a) and derivative (d) showed weak cytotoxicity. For example, even at the highest test concentration (1000 \( \mu \text{g/mL} \)), the cell viabilities could reach to more than 80%. Viability values of cells treated with derivative (b), derivative (c), and derivative (e), by contrast, were lower and their cell viabilities at concentrations of 1000 \( \mu \text{g/mL} \) were 58.36, 59.59, and 59.89%, respectively. When the concentration was 500 \( \mu \text{g/mL} \), the L929 cells incubated with derivative (b), derivative (c), and derivative (e) had survival rates of nearly 80%. For derivatives (a–e), when the concentration was 10 \( \mu \text{g/mL} \), their cell viabilities were about 100%, which indicated that these derivatives had no cytotoxicity at this concentration. Therefore, chitosan derivatives could be considered to have good biocompatibility because of their slight cytotoxicity.

![Graph showing antifungal index % with different concentrations and derivatives](image1)

**Fig. 6.** The antifungal activity of chitosan and chitosan derivatives against *B. cinerea* (The sample concentration in the mycelium growth pictures was 1.0 mg/mL).

![Images of mycelium growth with different treatments](image2)

**Fig. 7.** Hydroxyl-radical scavenging activity (a) and superoxide-radical scavenging activity (b) of chitosan and chitosan derivatives.
4. Conclusions

In this paper, five urea-functionalized chitosan derivatives were successfully synthesized. FTIR and $^1$H NMR results confirmed the structure characteristics of these derivatives. The antifungal activity and antioxidant activity of chitosan and chitosan derivatives were also investigated. The in vitro assay indicated that the five final products exhibited higher antifungal and antioxidant activities than chitosan. The results of this study confirmed the key role of urea groups in enhancing the bioactivity of chitosan. Moreover, the biological activity could be influenced by the electron-withdrawing capacity of the substituted groups. The chitosan derivatives with stronger electronegative groups would have higher biological activity. Furthermore, the cytotoxicity of chitosan derivatives against L929 cells was assessed by CCK-8 method and the results indicated that the synthetic urea-functionalized chitosan derivatives had low cytotoxic effect. In summary, these new types of urea-functionalized chitosan derivatives represent a novel class of highly effective polymers and may serve as antifungal and antioxidant agents to partially substitute for the harmful fungicides and antioxidants in fields of agriculture, food, and cosmetics. Further studies in improving the performance of these biomaterials as well as understanding the mode of action of these chitosan derivatives will be developed.

Conflict of interest statement

The authors have declared no conflicts of interest.

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