The antioxidant and antifungal activity of chitosan derivatives bearing Schiff bases and quaternary ammonium salts

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Abstract

In order to improve the antioxidant and antifungal activity of chitosan, eight chitosan derivatives containing Schiff bases and quaternary ammonium salts were synthesized via an intermediate 6-O-chloroacetyl-2-N,N,N-trimethyl quaternary ammonium salt chitosan. Detailed characterization was carried out using FTIR and ¹H NMR spectroscopy, and elemental analysis. The antifungal activity against F. oxysporum f. sp. cucumerium, B. cinerea, and F. oxysporum f. sp. niveum was evaluated using a mycelium growth rate test. The results indicated that the chitosan derivatives exhibited enhanced antifungal activity when compared to chitosan, especially at 1.0 mg/mL. 6-[4-(2,3-dihydroxybenzimidazole) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (2.3HPATC), 6-[4-(2,3,4-trihydroxybenzimidazole) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (2.3.4HPATC), 6-[4-(2-fluorine-benzimidazole) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (FBPATC), 6-[4-(2-chlorine-benzimidazole) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (CBPATC), 6-[4-(2-bromine-benzimidazole) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (BBPATC), and 6-[4-(2-hydroxy-4-chlorine-benzimidazole) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (HCBPATC) showed inhibitory indices > 90.0% at 1.0 mg/mL against F. oxysporum f. sp. cucumerium and B. cinerea. Furthermore, the chitosan derivatives showed stronger antioxidant activity than chitosan, especially 2.3HPATC and 2.3.4HPATC with inhibitory indices of 100.0% at 1.6 mg/mL against DPPH and superoxide radicals. Based on these data, it is reasonable to suggest that the introduction of phenolic hydroxyl and halogen groups enhances the antifungal and antioxidant activity of chitosan.

1. Introduction

Chitosan, the second most abundant polysaccharide next to cellulose, is the deacetylated derivative of chitin, which is mainly found in the skeletal materials of crustaceans and insects, and the cell walls of bacteria and fungi (Anraku et al., 2018; Chokradjaroen, Theeramunkong, Yui, Saito, & Rujiravanit, 2018; Xia, Liu, Zhang, & Chen, 2011). As a non-toxic, biodegradable, and biocompatible resource, chitosan has attracted the attention of researchers due to its unique physicochemical structure and bioactivity (Anraku et al., 2018; Mohammadzadeh Pakdel & Peighambardoust, 2015). Therefore, chitosan has been widely applied in agriculture, pharmaceuticals, cosmetics, foods, packaging, textiles, biotechnology and many other fields (Kashyap, Xiang, & Heiden, 2015; Libio, Demori, Ferrão, Lionzo, & da Silveira, 2016; Liu, Liu, Zhang, Kan, & Jin, 2019; Mujtaba et al., 2019; Shahid ul & Butola, 2019; Shariatinia, 2019; Smets & Rüdelsheim, 2018). At typical neutral pH conditions above pH 6.5, chitosan (> 10 kDa) is insoluble in dilute aqueous solution and has reduced cationic charge with reduced bioactivity. In order to improve its water solubility and bioactivity, a number of chitosan derivatives have been synthesized, including Schiff base chitosan, quaternized chitosan, and chitosan sulfate derivatives (Doncel-Pérez et al., 2018; Liu et al., 2018; Saranya, Rajan, Biswas, Jayakumar, & Sathianarayanan, 2018; Verlee, Mincke, & Stevens, 2017). Among the various chitosan derivatives reported to data, quaternized chitosan, especially N-quaternized chitosan derivatives, show excellent bioactivities, including antioxidant, antifungal, and antibacterial activity. Jia, Shen, and Xu (2001)) has recently reported that the antibacterial activity of N,N,N-trimethyl chitosan (TMC), N-propyl-N,N-dimethyl chitosan, and N-furfuryl-N,N-dimethyl chitosan are superior to chitosan. Besides, it has been reported by Guo et al. (2007) that N-phenyl-N,N-dimethyl chitosan and N-2-hydroxyphenyl-N,N-dimethyl chitosan have improved antifungal...
activity against Botrytis cinerea Pers. and Colletotrichum lagenarium (Pass) ElLet halst when compared to chitosan. In addition, quaternized chitosan has enhanced hydroxyl radical scavenging activity when compared to chitosan and N-substituted chitosan (Guo, Liu, Chen, Ji, & Li, 2006).

In the structure of chitosan, there are both amino and hydroxyl groups, which can be modified using reactive compounds (Hoven, Tangpasuthadol, Angkitikul, Vallapa, & Kiatsakjamwor, 2007). For N-quaternized chitosan, the amino groups are substituted. However, there were still free hydroxyl groups, which can take part in other reactions to give further derivatives of N-quaternized chitosan. It has been reported that the antifungal activity against Colletotrichum lagenarium, Watermelon fusarium, and Fusarium oxysporum was observed to increase after quaternary phosphonium salts were grafted onto TMC (Tan, Li, Dong, Chen, & Guo, 2017; Tan, Zhang et al., 2017). At the same time, further chemical modification of TMC with 1,2,3-triazolium and pyridinium groups could improve the antifungal activity against Botrytis cinerea, Phomopsis asparagi, Colletotrichum lagenarium, Watermelon fusarium, and Fusarium oxysporum (Tan, Li et al., 2018; Tan, Zhang et al., 2018). Furthermore, the antioxidant and antifungal activity of chitosan got enhanced after urea groups and imidazol groups were grafted onto TMC (Wei et al., 2019; Zhang et al., 2018).

Quaternized chitosan can be treated as a semifinished material to prepare further derivatives which included both quaternary ammonium salts and other active groups. Besides, it has been reported that the Schiff bases of chitosan show excellent antifungal activity (Anush, Vishalakshi, Kalluraya, & Manju, 2018; Guo et al., 2014; Xu, Aotegen, & Zhong, 2017). It is reasonable to combine quaternary ammonium salts and Schiff base groups to synthesize chitosan derivatives with good bioactivity. Subsequently, 6-(4-benzimide pyridine) acetyl-2-N,N,N,N-trimethyl-chitosan chloride (BPATC), 6-(4-hydroxyl-benzimide benzyl) pyridine) acetyl-2-N,N,N,N-trimethyl-chitosan chloride (HPATC), 6-(4-(2,3-dihydroxyl-benzimide) pyridine) acetyl-2-N,N,N,N-trimethyl-chitosan chloride (2.3HPATC), 6-[4-(2,3,4-trihydroxyl benzimide) pyridine] acetyl-2-N,N,N,N-trimethyl-chitosan chloride (2.3.4HPATC), 6-(4-(2-fluorine-benzimide) pyridine) acetyl-2-N,N,N,N-trimethyl-chitosan chloride (FBPATC), 6-[4-(2-chlorine-benzimide) pyridine] acetyl-2-N,N,N,N-trimethyl-chitosan chloride (CBPATC), 6-[4-(2-bromine-benzimide) pyridine] acetyl-2-N,N,N,N-trimethyl-chitosan chloride (2.3HPATC), and 6-[4-(2-hydroxyl-4-chlorine-benzimide) pyridine] acetyl-2-N,N,N,N-trimethyl-chitosan chloride (HCBPATC) were successfully synthesized. Meanwhile, the antifungal activity against Botrytis cinerea (B. cinerea), Fusarium oxysporum f. sp. cucumerum (F. oxysporum f. sp. cucumerum), and Fusarium oxysporum f. sp. niveum (F. oxysporum f. sp. niveum) and the antioxidant activity against hydroxyl, superoxide, and DPPH radicals were measured in vitro.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Qingdao Yunzhou Biochemistry Co., LTD (China). Its degree of deacetylation was 90.8% and the viscosity-average molecular weight was 100.0–200.0 kDa. Low-weight (8.0 kDa) chitosan (the degree of deacetylation 97.0%) was purchased from Introduction of Jinhu Crust Product CO., LTD (China). Iodomethane, chloroacetyl chloride, and 4-arylmethylene were purchased from the Sigma-Aldrich Chemical Corp (Shanghai, China). The other reagents were analytical grade and used without further purification.

2.2. Structural characterization of chitosan derivatives

2.2.1. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of all the samples were recorded using a Jasco-4100 Fourier Transform Infrared Spectrometer (Tokyo, Japan, provided by JASCO China (Shanghai) Co. Ltd., Shanghai, China) with the resolution of 4.0 cm⁻¹ at 25°C. The mixture (sample: potassium bromide = 1:100) was pressed into the disk and scanned using the transmission mode with the accumulation of 16 scans in the mid-infrared range (from 4000 cm⁻¹ to 400 cm⁻¹).

2.2.2. H Nuclear magnetic resonance (¹H NMR) spectroscopy

¹H NMR spectra were recorded on a Bruker AVIII500 spectrometer (Fällanden, Switzerland, provided by Bruker Biospin CN / Bruker (Beijing) Tech. and Serv. Co., Ltd., Beijing, China), using 99.9% Deuterium Oxide (D₂O) and Acetic acid-D as solvents with tetramethylsilane (TMS) as an internal standard at 25°C. Chemical shift values were given in δ (ppm).

2.2.3. Elemental analysis

The elemental analysis (C, H, and N) was performed on a Vario Micro Elemental Analyzer (Elementar Trading (shanghai) Co. Ltd., Shanghai, China). Based on elemental analysis results, the degree of substitution (DS) was calculated according to the following equations:

\[
DS_1 = \frac{n_1 \times M_C - W_1 \times M_N}{n_1 \times M_C} \tag{1}
\]

\[
DS_2 = \frac{(W_2 - W_1) \times M_C}{n_2 \times M_C} \tag{2}
\]

\[
DS_3 = \frac{(W_3 - W_2) \times M_N}{n_3 \times M_C} \tag{3}
\]

\[
DS_4 = \frac{(W_4 - W_3) \times M_N}{n_4 \times M_C - n_3 \times W_2 \times M_N} \tag{4}
\]

Where, DS₁, DS₂, DS₃, and DS₄ represent the deacetylation degree of chitosan, the degrees of substitution of N,N,N,N-trimethyl in chitosan derivatives, chloroacetyl in chitosan derivative CTMC, and Schiff bases of 4-aminopyridine in chitosan derivatives BPATC, HBPATC, 2.3HPATC, 2.3.4HPATC, FBPATC, CBPATC, BHPATC, and HCBPATC. Mₐ and Mₙ are the molar mass of carbon and nitrogen, M_C = 12, M_N = 14; n₁, n₂, n₃, n₄, n₅, and n₆ are the number of chitin, carbon of acetamido group, carbon of methyl, carbon of chloroacetyl, carbon of Schiff bases of 4-aminopyridine, and nitrogen of 4-aminopyridine, respectively. n₁ = 8, n₂ = 2, n₃ = 3, n₄ = 2, n₅ = 12, and n₆ = 2; W₁, W₂, W₃, and W₄ represent the mass ratios between carbon and nitrogen in chitosan derivatives.

2.3. Synthesis of the chitosan derivatives

2.3.1. Synthesis of the Schiff bases of 4-aminopyridine

As shown in Scheme 1, the Schiff bases of 4-aminopyridine were synthesized as follows: 30.0 mmol 4-aminopyridine were dissolved into 100.0 mL ethanol, and 30.0 mmol various aldehydes were added, respectively. The mixture was refluxed with stirring for 4 h, and the solvent was concentrated under reduced pressure to give the crude products of the Schiff bases of 4-aminopyridine.

2.3.2. Synthesis of 6-O-chloroacetyl-2,N,N,N-trimethyl chitosan

Based on the methods of Guo et al. (2014) and Wei et al. (2019), 6-O-chloroacetyl-2-N,N,N-trimethyl chitosan (CTMC) was synthesized as follows: 1.6 g chitosan was dispersed into 40.0 mL N-methyl pyroli-done (NMP) at room temperature, and 0.1 mL NaOH (1 M), 1.5 g NaI, and 4.0 mL CH₃I were added in proper order. The reaction was carried out with stirring at 60°C for 1 h. Then the solution was precipitated by excess acetone to give the crude product of TMC. TMC: yield: 86.2%, DS: 97.2% (Table 1). Next, the above obtained crude TMC were dissolved into 100.0 mL N,N-dimethylformamide (DMF), and 2.4 mL chloroacetyl chloride were wisely added. The reaction was carried out at 30°C for 24 h, and then the temperature was raised to 60°C. After 6 h, the solution was precipitated by excess acetone, and the precipitates were filtrated and washed with enough acetone. After being...
extracted in a Soxhlet apparatus with acetone for 48 h, CTMC was obtained by drying at 60 °C for 24 h. CTMC: yield: 84.6%, DS: 58.3% (Table 1).

2.3.3. The synthesis of 2-N,N,N-trimethyl-6-substituted benzimide pyridine acetyl chitosan

A mixture of the Schiff bases of 4-amino-pyridine and CTMC was dissolved into 20.0 mL DMF, and the reaction was carried out at 60 °C for 24 h. 2-N,N,N-trimethyl-6-substituted benzimide pyridine acetyl chitosan was precipitated in the excess diethyl ether and washed with enough diethyl ether and acetone. The unreacted reagents were extracted in the Soxhlet apparatus with a mixture of ethanol and acetone for 48 h. The products were obtained by freeze-drying overnight in vacuum.

6-(4-benzimide pyridine) acetyl-2-N,N,N-trimethyl-chitosan chloride (BPATC): yield: 78.2%, DS: 43.4%; 6-[4-(2,3-dihydroxyl-benzimide) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (HBPATC): yield: 76.2%, DS: 31.0%; 6-[4-(2,3,4-trihydroxyl-benzimide) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (2.3.4HBPATC): yield: 68.2%, DS: 31.0%; 6-[4-(fluorine-benzimide) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (FBPATC): yield: 74.3%, DS: 38.9%; 6-[4-(2-chlorine-benzimide) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (CBPATC): yield: 69.1%, DS: 27.6%; 6-[4-(2-bromine-benzimide) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (BBPATC): yield: 75.5%, DS: 21.5%; 6-[4-(2-hydroxyl-4-chlorine-benzimide) pyridine] acetyl-2-N,N,N-trimethyl-chitosan chloride (HCBPATC): yield: 74.6%, DS: 27.6% (Table 1).

2.4. Antifungal assays

The antifungal assays were assessed against F. oxysporum f. sp. cucumerium, B. cinerea, and F. oxysporum f. sp. niveum in vitro by mycelium growth rate test (Jasso de Rodríguez et al., 2007). Briefly, 36.0 mg chitosan or the chitosan derivatives were dissolved into 6.0 mL distilled water. Then, each sample solution was added to the sterilized potato dextrose agar (PDA) medium to get a final concentration of 0.1, 0.5, and 1.0 mg/mL. Then the solutions were poured into the sterilized Petri dishes (6.5 cm). Identical volume distilled water substituting samples were poured into control plates. After the mixture was cooled in the plate, the fungi mycelia disk with a diameter of 5.0 mm was placed in the center of the PDA Petri dishes and incubated at 28 °C. When the mycelium of fungi reached the edges of the control plate (without the presence of samples), the antifungal index was calculated by the formula:

\[
\text{Inhibitory index} (%) = \left[1 - \frac{D_a - 5}{D_b - 5}\right] \times 100
\]

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 plates.

### Table 1

The elemental analysis, yields, and the degrees of substitution of chitosan derivatives, and the degree of deacetylation of chitosan.

| Compounds          | Yields (%) | Elemental analysis (%) | Degrees of Substitution(%) | Water-solubility (mg/mL) |
|--------------------|------------|------------------------|---------------------------|--------------------------|
|                    |            | C     | N     | C/N |                  |                        |                          |
| chitosan           | 30.1       | 5.7   | 5.3   | 0.5 | 97.2             | 4.4                    |
| TMC                | 86.2       | 31.2  | 4.0   | 7.8 | 58.3             | 4.3                    |
| CTMC               | 84.6       | 35.2  | 4.0   | 8.8 | 43.4             | 4.9                    |
| CTMC               | 78.2       | 48.0  | 6.8   | 7.1 | 27.6             | 3.8                    |
| CTMC               | 76.2       | 57.5  | 7.8   | 7.4 | 27.6             | 3.8                    |
| 2.3HBPATC          | 78.1       | 53.3  | 7.1   | 7.5 | 27.6             | 3.8                    |
| 2.3.4HBPATC        | 68.2       | 48.9  | 6.6   | 7.4 | 31.0             | 3.4                    |
| FBATC              | 74.3       | 50.0  | 6.9   | 7.2 | 38.9             | 4.8                    |
| CBPATC             | 69.1       | 44.7  | 6.0   | 7.5 | 27.6             | 4.2                    |
| BBPATC             | 75.5       | 44.5  | 5.8   | 7.7 | 21.5             | 4.6                    |
| HCBPATC            | 74.6       | 44.2  | 5.9   | 7.5 | 27.6             | 4.0                    |
the diameter of the growth zone in the control plate.

2.5. Statistical analysis

Each experiment was performed three times, and the data were shown with mean ± standard deviation (SD, n = 3). The Scheffe method was used to evaluate the differences between the samples at the same concentration in the antifungal index. The results were processed by computer programs Excel (Microsoft, Redmond, Washington, USA), OriginPro 8 (OriginLab, Northampton, Massachusetts, USA), and Statistical Product and Service Solutions (SPSS, IBM, Armonk, New York, USA). Results with P < 0.05 were considered statistically significant (Jasso de Rodríguez et al., 2015).

2.6. Water solubility

The chitosan and its derivatives (1.0 g) were added into distilled water 1.0 mL. The mixture was stirred at the room temperature until the resolution reached dissolution equilibrium. The undissolved solids were collected by gravity filtration, washed with acetone, and then obtained by drying at 60 °C for 24 h. The water solubility of chitosan and its derivatives was calculated by the formula:

\[ \text{The water solubility} = \frac{(1000-m_1)}{1} \]  
\[ \text{Where} \ m_1 \text{ (mg) is the weight of the undissolved solids.} \]

2.7. Antioxidant assays

2.7.1. DPPH radicals scavenging ability assay

The DPPH radicals scavenging ability of chitosan and all the chitosan derivatives was measured according to Hu et al. (2014). The procedure was listed as follows: 20.0 mg of chitosan and the chitosan derivatives were dissolved into 2.0 mL distilled water. Ascorbic acid (Vc) was used as the positive control. Sample solutions of different concentrations (0.03, 0.06, 0.12, 0.24, and 0.48 mL, respectively) and 2.0 mL ethanol solution of DPPH (180.0 μmol/L) were incubated for 30 min at 25 °C. Then the absorbance of the residual DPPH radical was measured at 517 nm against a blank. The scavenging effect was obtained according to the following equation:

\[ \text{Scavenging effect} \% = \left( \frac{A_{\text{sample} \ 517 \ nm} - A_{\text{blank} \ 517 \ nm}}{A_{\text{control} \ 517 \ nm} - A_{\text{blank} \ 517 \ nm}} \right) \times 100 \]  
\[ \text{Where} \ A_{\text{control} \ 517 \ nm} \text{ is the absorbance of the control (ethanol instead of DPPH for each concentration) and} \ A_{\text{blank} \ 517 \ nm} \text{ is the absorbance of the blank (distilled water instead of the samples).} \]

2.7.2. Superoxide radicals scavenging ability assay

The superoxide radicals scavenging ability was assessed by the method of Nishikimi, Appaji Rao, and Yagi (1972). 10.0 mg chitosan and the chitosan derivatives were dissolved into 2.0 mL distilled water. The solution including chitosan or the chitosan derivatives (0.06, 0.12, 0.24, and 0.48 mL, respectively), 30.0 μM phenazine methosulfate (PMS), 338.0 μM nicotinamide adenine dinucleotide reduced (NADH), and 72.0 μM nitro blue tetrazolium (NBT) in Tris–HCl buffer (16.0 mM, pH 8.0) was incubated at 25 °C for 5 min. Then the absorbance was read at 560 nm against a blank. The capability of scavenging superoxide radical was calculated using the following equation:

\[ \text{Scavenging effect} \% = \left( \frac{A_{\text{sample} \ 560 \ nm} - A_{\text{control} \ 560 \ nm}}{A_{\text{blank} \ 560 \ nm}} \right) \times 100 \]  
\[ \text{Where} \ A_{\text{control} \ 560 \ nm} \text{ is the absorbance of the control (distilled water instead of NADH for each concentration) and} \ A_{\text{blank} \ 560 \ nm} \text{ is the absorbance of the blank (distilled water instead of the samples).} \]

2.7.3. Hydroxyl radicals scavenging activity assay

The hydroxyl radicals scavenging ability was measured by the method of Liu et al. (2009) with little modification. 20.0 mg chitosan and the chitosan derivatives were dissolved into 2.0 mL distilled water. The total volume of the reaction mixture was 4.5 mL and the solution, containing the samples of chitosan or the chitosan derivatives (0.045, 0.09, 0.18, 0.36, and 0.72 mL, respectively), was incubated with EDTA-Fe²⁺ (220.0 μM), potassium phosphate buffer (150.0 mM, pH 7.4), safranine T (0.23 μM), and H₂O₂ (60.0 μM) for 30 min at 25 °C. Then the absorbance of the mixture was measured at 520 nm. The OH-bleached the safranine T, so decreased absorbance of the mixture indicated decreased ·OH scavenging ability. The capability of scavenging OH was calculated using the following equation:

\[ \text{Scavenging effect} \% = \left( \frac{A_{\text{sample} \ 520 nm} - A_{\text{blank} \ 520 nm}}{A_{\text{control} \ 520 nm} - A_{\text{blank} \ 520 nm}} \right) \times 100 \]  
\[ \text{Where} \ A_{\text{blank} \ 520 nm} \text{ was the absorbance of the blank (distilled water instead of the samples),} \ A_{\text{control} \ 520 nm} \text{ was the absorbance of the control (distilled water instead of H₂O₂).} \]

3. Results and discussion

3.1. Structures of chitosan derivatives

The FTIR spectra of chitosan and synthesized chitosan derivatives are shown in Fig. 1. The spectrum of chitosan shows that the saccharide mainly contains the following characteristic bands: ν (O-H) and ν (N-H) at 3428 cm⁻¹, ν (C-H) at 2919 cm⁻¹, ν (amide I band) at 1643 cm⁻¹, δ (NH₂) at 1601 cm⁻¹, δ (C-H) at 1427 and 1380 cm⁻¹, ν (amide III band) at 1322 cm⁻¹, δ (O-H) at 1261 cm⁻¹, ν (C-O) at 1068 cm⁻¹, and the β glycosidic bond at 989 cm⁻¹. There were new peaks at 1747, 1475, and 795 cm⁻¹ in CTMC, which were assigned to C=O, N-CH₃, and C-Cl, respectively. After Schiff bases of 4-aminopyridine grafted to CTMC, except for the absorption peaks of C=O and N-CH₃, the peak of C-Cl was weakened or disappeared, and new peaks appeared at about 3170, 1541, and 845 cm⁻¹, which were assigned to the characteristic absorption of the benzene and pyridine ring. Furthermore, the strong peaks of C=N of the Schiff bases appeared at 1648–1659 cm⁻¹. These data preliminarily demonstrated the successful synthesis of the aimed chitosan derivatives.

Fig. 2 shows the ¹H NMR spectra of chitosan and the chitosan derivatives, and the absorption peaks of each group are marked. The characteristic signals of the protons of the glucosamine unit skeleton appeared at 3.1–5.0 ppm, and the weak peak at 2.0 ppm was assigned to the residual methyl protons of the N-acetyl group of chitosan. There were new peaks at 3.3, 4.3, and 4.5 ppm in the molecule of CTMC, which were assigned to N⁺(CH₃)₃, CH₂-O, and CH₂-Cl, respectively. After Schiff bases of 4-aminopyridine were grafted, the signal of CH₂-Cl at 4.5 ppm was weakened and a new peak appeared at 2.7 ppm, which was assigned to the group of CH₂-N⁺ (3 in the spectrum). And for BPATC, HBPATC, 2.3HBATC, 2.3.4HBATC, and CBPATC, there were new peaks at 6.5–7.0 ppm and 7.2–8.0 ppm, which were assigned to the protons of phenyl (d–g in the spectrum) and pyridine group (a–e in the spectrum), respectively. The above-mentioned analyses demonstrated the successful synthesis of the chitosan derivatives bearing the Schiff bases and quaternary ammonium salts.

3.2. Solubility and antifungal activity

Chitosan with a molecular weight 100.0–200.0 kDa had poor water solubility. The water solubility of all the chitosan derivatives was improved when compared to chitosan because the quaternary ammonium salts and hydroxy groups can act as hydrophilic moieties to increase the water solubility of chitosan. As shown in Table 1, 2.3HBATC and...
2.3.4HBPATC showed slightly weaker water solubility than others because the new hydrogen bonds could be formed after the introduction of more than one hydroxyl groups. Besides, the low-weight (8.0 kDa) chitosan was used to study the bioactivity in this paper.

Fig. 3 shows the mycelial growth pictures at samples' concentration of 1.0 mg/mL in the PDA plates against F. oxysporum f. sp. cucumerium, B. cinerea, and F. oxysporum f. sp. niveum. The antifungal activity of chitosan and its derivatives against F. oxysporum f. sp. cucumerium is shown in Fig. 4. The inhibitory index of chitosan was 7.6% at 1.0 mg/mL. After the Schiff bases and quaternary ammonium groups were grafted onto chitosan, the resulting chitosan derivatives showed higher inhibitory indices than chitosan, especially for 2.3HBPCATC, 2.3.4HBPATC, FBPATC, CBPATC, BBPATC, and HCBPATC with >91.0% inhibitory indices at 1.0 mg/mL, which was better than that of chitosan derivatives with pyridine, 4-(5-chloro-2-hydroxybenzylideneamino)-pyridine, and 4-(5-bromo-2-hydroxybenzylideneamino)-pyridine showed <77.2% inhibitory indices at 1.0 mg/mL (Li, Guo, & Jiang, 2010). For one thing, the higher density of positive charge would contribute to the antifungal activity. It has been reported that the positive charge could interact with glucan, mannan, proteins, and lipids (anionic substances) to form an impervious layer around the cell, which can prevent nutrient exchange in the cells or damage the cell membrane to cause the leakage of the cell constituents (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). As illustrated in the data, the inhibitory indices of the chitosan derivatives bearing halogen or more than one phenolic hydroxyl groups were higher than...
that of BPATC (inhibitory index: 61.0%) at 1.0 mg/mL. It was reasonable to speculate that the increased antifungal activity of HBPATC, 2.3HBPATC, 2.3.4HBPATC, FBPATC, CBPATC, BBPATC, HCBPATC might be attributed to the phenolic hydroxyl or halogen groups. It has been reported that the introduction of halogen, which possess a strong electron withdrawing ability, was an important strategy to improve the antifungal activity of heterocyclic compounds (Li et al., 2014). Christ-Ribeiro, Graça, Kupski, Badiale-Furlong, and de Souza-Soares (2019) has reported that phenolic compounds could suppress the biosynthesis of cell wall components (such as glucan, chitin, and mannanproteins),
cell membrane components (such as ergosterol), protein, amino acids, and sphingolipids, which can destroy the cell wall and cell membrane as well as preventing electron transport and the maintenance of cell integrity. In addition, the antifungal activity of 2.3HBPATC and 2.3.4HBPATC were enhanced when compared to HBPATC and BPATC, which indicated that the inhibitory index increased with an increase in the number of phenolic hydroxyl groups.

Fig. 5 shows the antifungal activity of all the samples against *B. cinereal*. The inhibitory indices of chitosan, BPATC, HBPATC, 2.3HBPATC, 2.3.4HBPATC, FBPATC, CBPATC, BBPATC and HCBPATC were 23.8%, 68.5%, 77.6%, 99.0%, 100.0%, 100.0%, 99.6%, 99.4%, and 97.7% at 1.0 mg/mL. All the synthesized chitosan derivatives showed much stronger antifungal activity than chitosan, which was accordant with that observed for the antifungal activity against *F. oxysporum f. sp. cucumerium*. Compared with FBPATC, CBPATC, and BBPATC, there was no obvious difference in the antifungal test and it was reasonable to presume that CBPATC and BBPATC could totally inhibit the growth of fungi with the increasing DS. Moreover, it was apparent that the inhibitory index increased with an increase in the number of phenolic hydroxyl groups. 2.3.4HBPATC with three phenolic hydroxyl groups even could totally inhibit the fungi at 1.0 mg/mL. Besides, HCBPATC showed better antifungal activity than HBPATC, which was attributed to the presence of chloride with stronger electron withdrawing ability. It has been reported that the synthesized 6-O-imidazole-based quaternary ammonium chitosan derivatives also showed good antifungal activity against *B. cinereal* (Wei et al., 2019). However, compared with the inhibitory indices of 6-O-imidazole-based quaternary ammonium chitosan derivatives against *B. cinereal*, the antifungal activity of the synthesized chitosan derivatives bearing Schiff bases and quaternary ammonium salts showed better antifungal
activity, which might be attributed to the presence of Schiff bases or the higher DS.

Fig. 6 shows the antifungal activity of chitosan and the chitosan derivatives against \textit{F. oxysporum f. sp. niveum}. All the samples inhibited the growth of \textit{F. oxysporum f. sp. niveum}. Chitosan with an inhibitory index of 16.9\% at 1.0 mg/mL showed weaker antifungal activity. Besides, the same conclusions could be reached that chitosan derivatives with Schiff bases and quaternary ammonium salts groups had much better antifungal activity when compared to chitosan. There was no significant difference between BPATC and HBPATC since BPATC had higher DS. The chitosan derivatives with halogen groups (FBPATC, CBPATC, and BBPATC) showed > 84.0\% inhibitory indices at 1.0 mg/mL since the halogen had stronger electron withdrawing ability in the heterocyclics ring. The compounds such as 2.3HBPATC and

**Fig. 4.** The antifungal activity of chitosan and the chitosan derivatives against \textit{F. oxysporum f. sp. cucumerium}. **(p < 0.01)** represents the differences between samples are extremely significant.

**Fig. 5.** The antifungal activity of chitosan and the chitosan derivatives against \textit{B. cinereal}. **(p < 0.01)** represents the differences between samples are extremely significant.
2.3.4HBPATC with more than one phenolic hydroxyl groups exhibited >86.0% inhibitory indices at 1.0 mg/mL. The chitosan derivative HCBPATC bearing one phenolic hydroxyl group and one chlorine atom showed 88.6% inhibitory index at 1.0 mg/mL. The higher positive charge density and the active groups such as halogen and phenolic hydroxyl groups would contribute to improving the antifungal activity against *F. oxysporum f. sp. niveum*. Furthermore, the inhibitory indices of all the chitosan derivatives enhanced with the increasing concentration. In addition, the inhibitory index showed extremely significant differences between chitosan, BPATC, HBPATC, 2.3HBPATC, 2.3.4HBPATC, FBPATC, CBPATC, BBPATC, and HCBPATC, and carbendazim. The differences between samples were significant (p < 0.01), which was shown in Figs. 4–6.

3.3. Antioxidant activity

The antioxidant activity of chitosan and its derivatives against DPPH, superoxide, and hydroxyl radicals was investigated and shown in Figs. 7–9. Fig. 7 shows the scavenging ability of chitosan and its derivatives against DPPH radicals. All the samples exhibited scavenging activity against DPPH radicals at concentrations of 0.1–1.6 mg/mL. The positive control ascorbic acid (Vc) with a scavenging value of 97.0% at 1.6 mg/mL showed excellent scavenging ability against DPPH radicals. The scavenging values of chitosan, BPATC, HBPATC, 2.3HBPATC, 2.3.4HBPATC, FBPATC, CBPATC, BBPATC, and HCBPATC were 16.9%, 37.1%, 57.0%, 100%, 100%, 46.6%, 46.5%, 42.3%, and 65.3% at 1.6 mg/mL, respectively. It was obvious that all the quaternary ammonium salts chitosan derivatives showed better scavenging ability than chitosan, especially at 1.6 mg/mL. The higher density of positive charge would contribute to the antioxidant activity (Liu et al., 2009). In addition, especially for high molecular weight chitosan, stronger intramolecular and intermolecular hydrogen bonds weakened their activity (Wan, Xu, Sun, & Li, 2013). The introduction of a heterocyclic ring onto chitosan could partially destroy the hydrogen bonds, but at the same time, there would be new hydrogen bonds formed after the introduction of hydroxyl groups and the unreacted hydroxyl groups (Wan et al., 2013). The conjugated double bonds in the chitosan derivatives could contribute to stabilizing the radicals because there was electron delocalization across the molecule. Furthermore, the antioxidant activities of all the chitosan derivatives at 1.6 mg/mL were observed in the following order: (1) The scavenging values of the chitosan derivatives bearing halogen (FBPATC, CBPATC, and BBPATC) were higher than BPATC, especially at 1.6 mg/mL, but the difference was not obvious. These results were similar to the antioxidant effects of halogen to 8-hydroxyquinoline (Cherdtrakulkit et al., 2016); (2) Chitosan derivatives bearing phenolic hydroxyl groups (HBPATC, 2.3HBPATC, 2.3.4HBPATC, and HCBPATC) showed enhanced antioxidant activity when compared to BPATC. It has been reported that phenolic hydroxyl groups, which were potent hydrogen donors, played a significant role in the antioxidant activity (Omidi & Kakanejadifard, 2019; Ren, Li, Dong, Feng, & Guo, 2013). Moreover, 2.3.4HBPATC,
which contained three phenolic hydroxyl groups, could totally scavenge DPPH radicals at 0.4 mg/mL. The scavenging ability increased with increasing the number of phenolic hydroxyl groups. The scavenging values of chitosan and its derivatives against superoxide and hydroxyl radicals are shown in Figs. 8 and 9, respectively. As shown in Fig. 8, all the chitosan derivatives showed scavenging ability against superoxide radicals under the test conditions and the scavenging values were observed to be concentration-dependent. The positive control ascorbic acid (Vc) with a scavenging value of 95.7% at 1.6 mg/mL exhibited higher scavenging ability against superoxide radicals. Similar to the antioxidant activity observed against DPPH radicals, the chitosan derivatives with a scavenging value of > 72.0% at 0.4 mg/mL. The introduction of Schiff bases and quaternary ammonium salts were favorable factors to enhance the scavenging ability against superoxide radicals. Quaternary ammonium salts increase the positive density of the chitosan derivatives, which contributed to the antioxidant activity. In addition, functional groups such as the pyridine and benzene rings, phenolic hydroxyl groups, and halogen were introduced into chitosan via the Schiff base.

The scavenging ability of HBPATC was higher than observed for FPATC, CBPATC, and BBPATC at 1.6 mg/mL under similar DS, which indicated that the phenolic hydroxyl groups were more effective when compared with halogen groups in the superoxide radicals scavenging test. Moreover, the scavenging ability of the chitosan derivatives increased with an increasing number of phenolic hydroxyl groups. 2.3HBPATC and 2.3.4HBPATC with multiple phenolic hydroxyl groups could almost totally scavenge superoxide radicals even at 0.1 mg/mL, which was better than the positive control, ascorbic acid (Vc). However, there was no significant difference between FPATC, CBPATC, and BBPATC. Fig. 9 shows the scavenging ability of chitosan and all the chitosan derivatives against hydroxyl radicals. The scavenging value of ascorbic acid (Vc) against hydroxyl radicals was 27.6% at 1.6 mg/mL. As shown in Fig. 9, all the samples exhibited scavenging activity against hydroxyl radicals. Similar to the scavenging activity observed against DPPH radicals and superoxide radicals, the scavenging values of all the samples increased upon increasing the concentration. The scavenging ability of chitosan against hydroxyl radicals was 58.9% at 1.6 mg/mL. All the chitosan derivatives had an enhanced scavenging ability when compared to chitosan, especially at 1.6 mg/mL, which was attributed to the higher density of positive charge and the active groups such as phenolic hydroxyl groups. Besides, the order of scavenging ability against hydroxyl radicals for all the samples was 2.3.4HBPATC > 2.3HBPATC > HCBPATC > HBPATC > BBPATC ≈ CBPATC ≈ FPATC > BPATC > chitosan. The antioxidant activity was affected by the number of phenolic hydroxyl groups. 2.3.4HBPATC, which contains three phenolic hydroxyl groups, could totally scavenge hydroxyl radicals even at 0.4 mg/mL. 2.3HBPATC and HCBPATC with a scavenging value of 100.0% also showed significant antioxidant capacity at > 0.8 mg/mL. The scavenging values of BBPATC, CBPATC, and FPATC were > 90.0%, which was slightly better than observed for BPATC that has a scavenging value of 86.7% at 1.6 mg/mL. When compared to the products containing halogen, the compounds containing phenolic hydroxyl groups were more effective towards scavenging hydroxyl radicals.

4. Conclusions

The bioactivity of chitosan can be improved upon appropriate chemical modification. The 6-O-chloroacetyl-2-N,N,N-trimethyl quaternary ammonium salt of chitosan was chosen as a reactive intermediate for further reaction to give eight novel chitosan derivatives containing both Schiff bases and quaternary ammonium salts. The antifungal and antioxidant activity was investigated in vitro. All the chitosan derivatives exhibited enhanced antifungal activity against F. oxysporum f. sp. cucumerium, B. cinerea, and F. oxysporum f. sp. niveum when compared to chitosan, especially at 1.0 mg/mL. The introduction of phenolic hydroxyl and halogen groups had a positive influence on the antifungal activity. Furthermore, the inhibitory index increased upon increasing the number of active groups such as phenolic hydroxyl groups. The antioxidant results showed that all the chitosan derivatives exhibited enhanced antioxidant activity when compared to chitosan, especially for 2.3HBPATC and 2.3.4HBPATC, which contains more than one phenolic hydroxyl groups. In addition to the active groups, a higher density of positive charge was also a favorable factor to contribute to the bioactivity of chitosan. These results provided a possible method to obtain chitosan derivatives with higher bioactivity and further research should be focused on their structure-activity relationship.

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