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Research Article

Synthesis and Antimicrobial Activity of *N*-(6-Carboxyl Cyclohex-3-ene Carbonyl) Chitosan with Different Degrees of Substitution

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Five products of *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan as antimicrobial agents were prepared by reaction of chitosan with tetrahydrophthalic anhydride (THPA) at different degrees of substitution (DS). The antimicrobial activity was evaluated against four plant bacteria and eight fungi. The results proved that the inhibitory property and water solubility of the synthesized chitosan derivatives, with increase of the DS, exhibited a remarkable improvement over chitosan. The product with a DS of 0.40 was the most active one with MIC of 510, 735, 240, and 385 mg/L against *Erwinia carotovora*, *Ralstonia solanacearum*, *Rhodococcus fascians*, and *Rhizobium radiobacter*, respectively, and also in mycelial growth inhibition against *Alternaria alternata* (EC_{50} = 683 mg/L), *Botrytis cinerea* (EC_{50} = 774 mg/L), *Botryodiplodia theobromae* (EC_{50} = 501 mg/L), *Fusarium oxysporum* (EC_{50} = 500 mg/L), *F. solani* (EC_{50} = 260 mg/L), *Penicillium digitatum* (EC_{50} = 417 mg/L), *Phytophthora infestans* (EC_{50} = 298 mg/L), and *Sclerotinia sclerotiorum* (EC_{50} = 763 mg/L). These compounds based on a biodegradable and biocompatible chitosan could be used as potentially antimicrobial agents in crop protection instead of hazardous synthetic pesticides.

1. Introduction

Chitosan is a linear biopolymer consists of higher than 70% of β-(1-4)-2-deoxy-β-D-glucopyranose (GlcN) and lower than 30% of β-(1-4)-2-acetamido-2-deoxy-β-D-glucose (GlcNAc) units linked by β-1,4-glucosidic bonds. It can be obtained through a deacetylation process of purified chitin, a naturally abundant polysaccharide, and the supporting material of crustaceans, insects, and fungi [1–3]. It has unique characteristics such as biocompatibility, biodegradability, and low toxicity to mammals and possesses reactive functional groups that make it useful in different areas of application related to agriculture [4–6], food industry and nutrition [7], biotechnology [8, 9], and biomedicine [10].

In agriculture, pre- and postharvest protection from different pathogens rely mainly on synthetic fertilizers and hazardous pesticides. Therefore, alternative solutions are needed due to concerns for public health, environmental protection, and development of resistant pests. Chitosan is widely used as a biocide either alone or blended with other natural products against plant pathogens that induce decay and harmful effects of the agricultural crops during the growing season and postharvest phase [5, 6, 11–13]. The antimicrobial activity of chitosan is coming from its polycationic nature and with chemical modification [5, 11, 12, 14, 15]. However, its low solubility at the pH higher than of 6.5 is the major limiting factor in its utilization, that is, its application in biology, since many enzyme assays are performed in neutral media. Therefore, the preparation of water-soluble chitosans could enhance its biological and physiological potential [5, 11, 15, 16]. From the synthetic point, all of the chemical modifications have been performed on the primary amino group (at C-2) or the two hydroxyl groups (at C-3 and C-6) of the chitosan molecule such as *N*-reductive amination, quaternization, hydroxyalkylation, *N*,*O*-carboxymethylation,

N,*O*-acylation, phosphorylation, *N*,*O*-phthaloylation, *N*,*O*-succinylation, chitosan-amino acid and chitosan-peptide conjugates, and graft copolymerization [17–23]. Acylation of chitosan was the typical method involves reacting chitosan with either an acid chloride or acid anhydride forming *N*-(acyl) chitosan, *O*-(acyl) chitosan, *N*,*O*-(acyl), *N*-(hydroxyacyl) chitosan, or *N*-(carboxyacyl) chitosan derivatives, but the reactions are not regioselective partly because of the heterogeneous reaction conditions [24–26].

N-(Carboxyacyl) chitosans were also prepared by reactions with intramolecular carboxylic anhydrides including maleic, glutaric, phthalic [27], and succinic [27, 28] anhydrides. On the other hand, *N*-(carboxyacyl) chitosans filaments were obtained by reaction of chitosan with carboxylic anhydrides in methanol at room temperature overnight [29–31]. The products could be used in many kinds of application due to their solubility in wide range of the pH [32]. Badawy and Rabea synthesized *N*-carboxyacyl chitosans at DS 0.09–0.86 by treatment of chitosan with glutaric anhydride at different mol ratios in a solution of 2% aqueous acetic acid-methanol (1 : 1, v/v) and evaluated their antimicrobial activity against plant pathogens. As a result, *N*-(4-carboxybutyroyl) chitosan derivatives (DS = 0.09, 0.26, 0.45, 0.52, and 0.86) were isolated at 80–93% yields [33].

Based on the current state of research and progress in corresponding areas, this article aims at preparing water-soluble *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan derivatives to enhance the antimicrobial activity of chitosan molecule against plant pathogenic bacteria *Erwinia carotovora*, *Ralstonia solanacearum*, *Rhodococcus fascians*, and *Rhizobium radiobacter*. In addition, the antifungal activity was tested against *Alternaria alternata*, *Botrytis cinerea*, *Botryodiplodia theobromae*, *Fusarium oxysporum*, *Fusarium solani*, *Penicillium digitatum*, *Phytophthora infestans*, and *Sclerotinia sclerotiorum*. The antimicrobial activities as MICs for bacteria and EC_{50}s for fungi were investigated and discussed in detail.

2. Materials and Methods

2.1. Chemicals and Reagents. Low molecular weight chitosan, tetrahydrophthalic anhydride (THPA), deuterium oxide, deuterated acetic acid, and 2,3,5,-triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich Co. (USA). Potato Dextrose Agar (PDA), Nutrient Broth (NB), and Nutrient Agar (NA) media were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK). NA was used to reactivate and propagate the tested bacteria. All materials were used without further purification.

2.2. Tested Microorganisms. Four plant pathogenic bacteria *Erwinia carotovora*, *Ralstonia solanacearum*, *Rhodococcus fascians*, and *Rhizobium radiobacter* were obtained from Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt. Bacteria were maintained on NA medium at 37°C. Eight plant pathogenic fungal strains *Alternaria alternata* (Family: Pleosporaceae, Class: Dothideomycetes), *Botrytis cinerea* (Family: Moniliaceae, Class: Deuteromycetes), *Botryodiplodia theobromae* (Family: Botryosphaeriaceae, Class: Dothideomycetes), *Fusarium oxysporum* (Family: Nectriaceae, Class: Sordariomycetes), *F. solani* (Family: Nectriaceae, Class: Sordariomycetes), *Penicillium digitatum* (Family: Trichocomaceae, Class: Eurotiomycetes), *Phytophthora infestans* (Family: Pythiaceae, Class: Oomycota), and *Sclerotinia sclerotiorum* (Family: Sclerotiniaceae, Class: Leotiomycetes) were provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt, and kept during the experiments on PDA medium at 27 ± 2°C.

2.3. Synthesis of N-(6-Carboxyl Cyclohex-3-ene Carbonyl) Chitosans. *N*-(6-Carboxyl cyclohex-3-ene carbonyl) chitosan derivatives were synthesized according to the method of Hirano and Moriyasu [31] and the modification by Badawy and Rabea [33] as follows: A portion (1.7 g) of chitosan was dissolved in 50 mL of 2% aqueous acetic acid (1%, v/v), and the solution was diluted with methanol (50 mL). To this solution, THPA was added (0.1, 0.3, 0.5, 0.8, and 1.0 mol/glucose amine (GlcN) unit). The mixture was stirred at 50°C for 5 min and kept at room temperature overnight. The mixture, which became in gel or solidified form, was washed with acetone to give pale yellow solid of *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosans (Figure 1).

2.4. NMR Spectroscopy. ^{1}H- and ^{13}C-NMR measurements were performed on a JEOL A-500 NMR spectrometer (Faculty of Science, Alexandria University, Alexandria, Egypt) under a static magnetic field of 500 MHz at 25°C. Sample was introduced into 5 mm Φ NMR tube, to which 0.5 mL of 1% CF_3COOD/D_2O solution was added, and finally the tube was kept at room temperature to dissolve the product.

2.5. ^{1}H- and ^{13}C-NMR Spectral Data

2.5.1. Spectral Data for Chitosan

^{1}H-NMR (25°C). δ 2.09–2.12 ppm (br s, $NHCO\underline{CH_3}$), 3.15–3.30 ppm (br m, H-2 of GlcN residue), 3.57–4.10 ppm (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.88–5.00 ppm (m, H-1 of GlcN and GlcNAc units).

^{13}C-NMR (25°C). δ 22.08 ppm ($NH(CO)\underline{C}H_3$), 56.59 ppm (C-2), 61.02–61.28 ppm (C-6), 70.59–70.76 ppm (C-3), 75.15–75.44 ppm (C-5), 77.78 ppm (C-4), 98.24 ppm (C-1), 174.79 ppm ($\underline{C}(O)CH_3$).

2.5.2. Spectral Data for N-(6-Carboxyl Cyclohex-3-ene Carbonyl) Chitosan Derivatives (Compounds 1–5)

^{1}H-NMR. 2.01–2.11 ppm (br s, NHAc), 2.34–2.55 ppm (br, $2CH_2$ of cyclohex-3-enecarboxylic acid), 3.09–3.28 ppm (br s, H-2 of GlcN residue), 3.45–4.20 ppm (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.60–4.65 ppm (br s, H-1 of GlcNAc residue), 4.85–4.95 ppm (br s, H-1 of GlcN residue), 5.75–5.77 ppm (s, 2H of cyclohex-3-enecarboxylic acid), and 11.50 ppm (s, H of COOH of cyclohex-3-enecarboxylic acid).

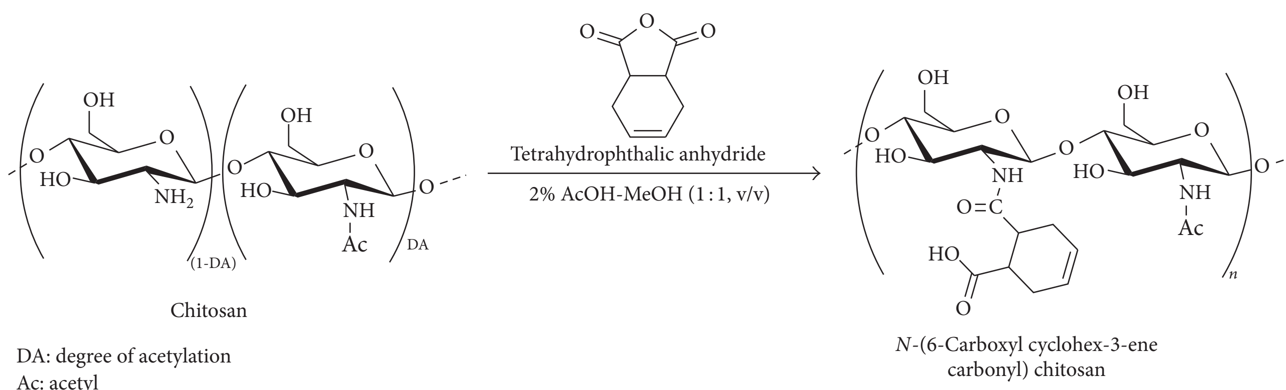


Figure 1: Reaction scheme of chitosan with tetrahydrophthalic anhydride at different mol ratios.

^{13}C-NMR. δ 20.51 ppm (CH_2 (β)), 22.20 ($NH(CO)CH_3$), 25.35 ppm (CH_2-CH_2 of cyclohex-3-ene), 39.50 ppm (CH-CH of cyclohex-3-ene), 55.92 ppm (C-6), 60.10 (C-2), 70.19 ppm (C-3), 74.88 ppm (C-5), 76.54 ppm (C-4), 97.67 ppm (C-1), 125.28 ppm (HC=CH of cyclohex-3-ene), 176.23 ppm ($C(O)CH_3$), 177.89 ppm (COOH), 178.23 ppm (O=C-NH).

2.6. Average Molecular Weight (MW). The average MWs of chitosan products were obtained from Mark-Houwink equation ($\eta = KM^a$) [34, 35], where η is the intrinsic viscosity and K and a are viscometric parameters depending on the solvent. For a chitosan dissolved in 0.5 M CH_3COOH/0.2 M CH_3COONa buffer, K and a are found to be 3.5×10^{-4} and 0.76, respectively [36, 37]. The viscosity of chitosan solution of different concentrations in acetate buffer (pH = 5) was measured by using Ubbelohde Viscometer (capillary section size 0.7 mm) immersed in a constant temperature bath at 25°C and having flow time for buffer solution, $T_0 = 53.65$ seconds. During preparation, all the solutions were magnetically stirred for 1 h to ensure proper dissolution of chitosan and were filtered using Whatman filter paper number 4. The flow times of chitosan solutions and solvent were recorded in triplicate and the average value was calculated. The intrinsic viscosity $[\eta]$ was calculated graphically by extrapolating the curve of reduced viscosity versus chitosan concentration (g/dL) to zero concentration. The MW was then calculated by using Mark-Houwink equation.

2.7. Solubility Test. The solubility of chitosan and N-(6-carboxyl cyclohex-3-ene carbonyl) chitosan products was performed in distilled water, aqueous acetic acid (0.1, 0.5, and 1%, v/v), and 1% NaOH (w/v). A sample was soaked in each solvent at the concentration of 10 mg/mL and the solubility was checked after standing for 24 h at room temperature [38].

2.8. Antimicrobial Assay

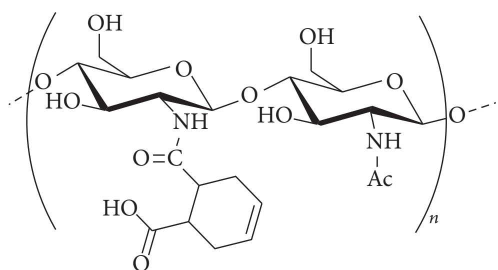
2.8.1. Antibacterial Activity. Nutrient Broth (NB) medium was used to grow the bacterial strains to a final inoculum size of 5×10^5 cfu/mL calculated as a number of colonies × dilution factor/volume of culture plate using haemocytometer. Chitosan and its derivatives were dissolved in 0.5% aqueous acetic acid and added to the wells of a sterile 96-well microtitre plate, followed by the addition of 140 μL of NB medium and then 20 μL of bacterial suspension. The final volume in each well was 200 μL and the concentrations of 75, 100, 150, 200, 300, 400, 600, 800, 1000, 1200, and 1600 mg/L were tested for each compound. Control wells were prepared with culture medium, bacterial suspension only, and solvent. The contents of each well were mixed on a microplate shaker at 200 rpm for 1 min prior to incubation for 24 h at 37°C. To indicate respiratory activity the presence of color was determined after adding 10 μL/well of TTC dissolved in water (0.01%, w/v) as a chromogenic marker and incubated under appropriate cultivation conditions for 30 min in the dark [39, 40]. The absorbance was measured at 492 nm in an Ultra Microplate Reader (Robonik, PVT, LTD). Positive controls were wells with a medium and the compounds. Negative controls were wells with the growth medium, bacterial suspension, and the TTC reagent. The minimum inhibitory concentration (MIC) was determined as the lowest concentration where no viability was observed after 24 h based on metabolic activity. All measurements of MIC values were repeated in triplicate.

2.8.2. Antifungal Activity. The activity was tested using mycelia radial growth technique [41]. The compounds were dissolved as described in the antibacterial assay and serial concentrations of 250, 500, 1000, 1500, 2000, 2500, and 3000 mg/L were tested. The aliquots of the stock solutions were added to the PDA medium and then transferred to Petri dishes. After solidification, the mixtures were inoculated with a 5 mm in diameter mycelium fungi at the center of Petri dishes and these were incubated in the dark at 27 ± 2°C. The fungal growth was measured when the control had grown to the edge of the plate. The inhibition of fungal growth was calculated as the percentage of inhibition of radial growth compared to the control. The effective concentration that inhibits 50% of mycelial growth (EC_{50}) for each compound was estimated by probit analysis [42] using of SPSS 21.0 software.

Table 1: Chemical structure and properties of chitosan and its derivatives with tetrahydrophthalic anhydride [*N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan] at different mol ratios (see Scheme 1).

Compound	THPA[a]/mol GlcN[b]	DA[c]	DDA[d]	DS[e]	FW[f]	Yield[h] (%)	Intrinsic viscosity (η)	Average MW[g] (Da) $\times 10^5$
Chitosan	—	0.09	0.91	—	164.78	—	4.77	2.76
1	0.1	0.07	0.84	0.09	177.27	84.79	5.09	3.01
2	0.3	0.08	0.74	0.18	191.58	86.01	5.59	3.39
3	0.5	0.06	0.68	0.26	203.63	94.86	6.18	3.87
4	0.8	0.07	0.62	0.30	210.14	96.43	6.47	4.11
5	1.0	0.06	0.54	0.40	224.70	97.11	7.20	4.74

[a]THPA = tetrahydrophthalic anhydride. [b]GlcN = glucosamine. [c]DA is a degree of acetylation (calculated as 1 − DDA). [d]DDA is a degree of deacetylation. [e]DS is a degree of substitution. [f]FW is a formula weight. [g]MW is the molecular weight in g/mol and it was determined for chitosan by measuring its intrinsic viscosity according to the Mark-Houwink-Sakurada equation and it was calculated for compounds 1–5 as follows: [h]yield was determined by weight recovery in accordance with the change in FW.



Scheme 1

2.9. Statistical Analysis. Statistical analysis was performed using SPSS 21.0 software (Statistical Package for Social Sciences, USA). The log dose-response curves allowed determination of the EC_{50} values for the fungal bioassay according to the probit analysis [42]. The 95% confidence limits for the range of EC_{50} values were determined by the least-square regression analysis of the relative growth rate (% control) against the logarithm of the compound concentration.

3. Results and Discussion

3.1. Characterization of N-(6-Carboxyl Cyclohex-3-ene Carbonyl) Chitosans. The chemical structure, degree of deacetylation (DDA), degree of substitution (DS), and formula weights (FW) were estimated by ^{1}H-NMR spectra [17, 43] and the data are presented in Table 1. Chitosan products were obtained at a range of DS 0.09–0.40 when five mole ratios (0.1, 0.3, 0.5, 0.8, and 1.0 mole) of THPA per glucose amine were tested. A hydrogel was produced in the reaction mixture with each product. The products of *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosans were obtained with 84.79, 86.01, 94.86, 96.43, and 97.11% yield at DS of 0.09, 0.18, 0.26, 0.30, and 0.40, respectively. Figure 2 shows the ^{1}H- and ^{13}C-NMR spectra of chitosan and compound 5. The peak at δ 2.07–2.12 ppm is assigned to the proton of residual CH_3 in acetyl group (Figures 2(a) and 2(b)). The peak at δ 3.09–3.30 ppm is attributed to H-2 of GlcN residue. The broad multiple peak at δ 3.49–4.15 ppm is assigned for H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit. The intense band at 4.8–5.30 ppm is related to OH groups and HDO (solvent). In this region, as observed more clearly from an extended spectrum, some different anomeric protons (H-1 of GlcN and GlcNAc units) appeared at 4.83–5.00 ppm [24, 44]. Regarding product 5, the new peak at δ 2.35–2.50 ppm represents the resonance of the two methylene groups of cyclohex-3-enecarboxylic acid; however the singlet peak at δ 5.74–5.82 represents the two hydrogen protons of cyclohex-3-enecarboxylic acid and the new peak at δ 11.50 ppm originated from the proton of COOH of cyclohex-3-enecarboxylic acid substituent (Figure 2(b)) confirming again the successful grafting of this substituent onto chitosan molecule at the amino group.

The DDA was calculated to be 91% in chitosan (Table 1) from the integral ratio between the proton on the C-2 and the GlcN unit protons. However, DDA of *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosans ranged from 0.54 to 0.84. Determination of the DS value was based on the ratio between the areas of the protons in the substituent and the protons of the pyranose unit. The result proved that increasing the DS resulting in a decrease of the DDA that confirms the reaction mainly occurred on the amino group on C-2 of the GlcN. FW was calculated as follows: FW = 161 × DDA + 203 × DA + MW of *N*-(6-carboxyl cyclohex-3-ene carbonyl) glucosamine unit × DS, where 161 and 203 are the FW of GlcN unit and GlcNAc, respectively. The results indicate that the FW was increased with the mol ratio increase. FW for chitosan, 1, 2, 3, 4, and 5 were 177.27, 191.58, 203.63, 210.14, and 224.70, respectively, and the average molecular weight of the compounds ranged from 3.87 to 4.91×10^5 g/mol (Table 1). The derivatives were reported to have average MW 3.01, 3.39, 3.87, 4.11, and 4.77×10^5 Da compared to 2.76×10^5 for chitosan indicating that the MW is correlated with the intrinsic viscosity (η).

Further evidence for confirmation the chemical structure was obtained from ^{13}C-NMR spectroscopy. The carbon peaks due to C=O and CH_3 were found at 174.97 and 22.08 ppm in chitosan, respectively. When we grafted THPA into the amino group of chitosan, new chemical shifts at 176.23, 177.89, and 178.23 ppm appeared due to the resonance of the carbonyl group (C=O) present in the carboxyl group (COOH) and

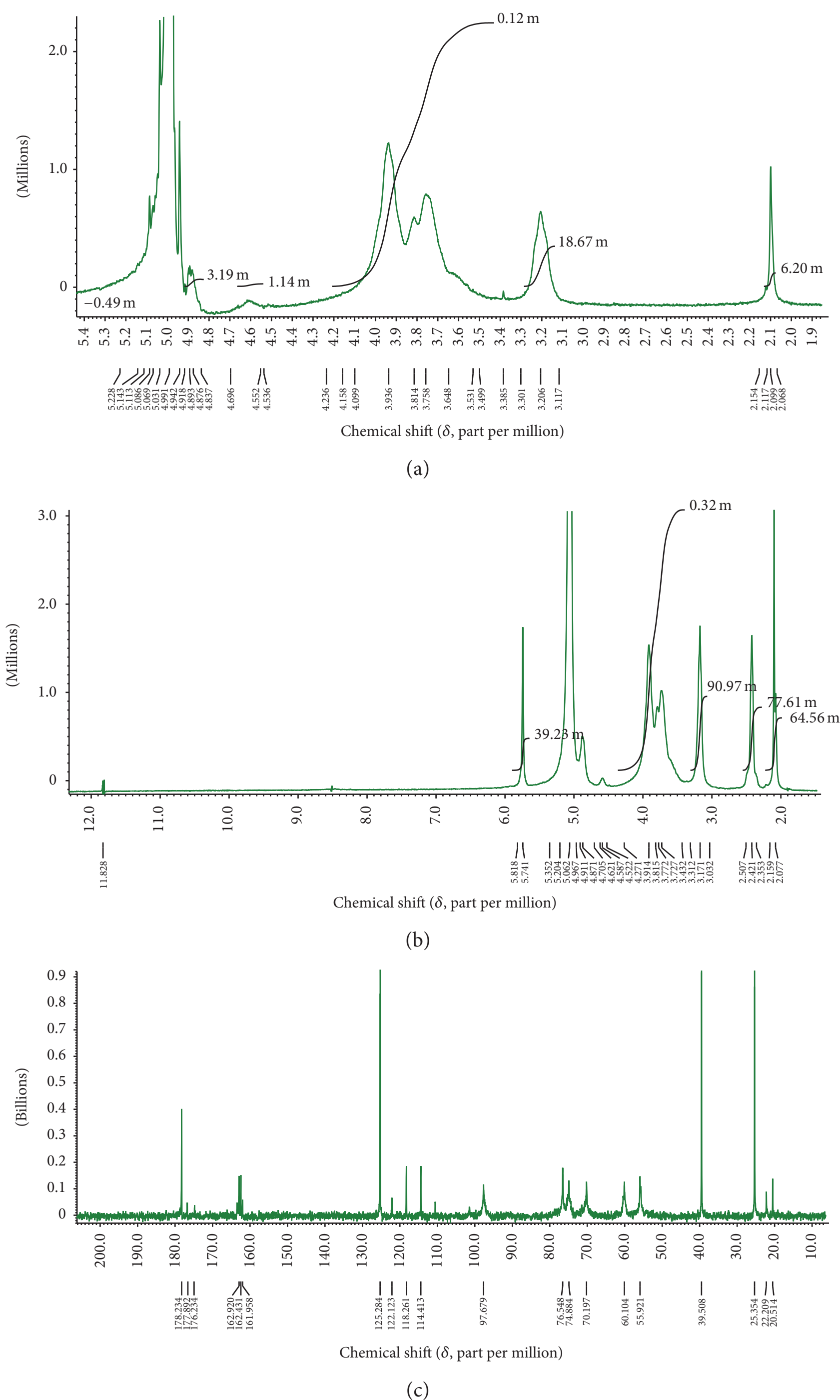


Figure 2: ^{1}H-NMR spectra of chitosan (a) and *N-(6-carboxyl cyclohex-3-ene carbonyl) chitosan* (5) and ^{13}C-NMR of product 5 (c) in 1% CF_3COOD/D_2O at 25°C.

acyl group of *N*-(6-carboxyl cyclohex-3-ene carbonyl) moiety and that was found in the acetyl group of chitosan structure (Figure 2(c)). Strong and intense peaks at 25.35, 39.50, and 125.28 ppm were obtained due to the presence of carbon atoms in the *N*-(6-carboxyl cyclohex-3-ene carbonyl) moiety.

The result of solubility of the chitosan and *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosans (1–5) is shown in Table 2. The data shows that the derivatives were more soluble than chitosan in water and diluted aqueous acetic acid solutions. In addition, the derivatives became soluble also in alkaline basic medium (sodium hydroxide solution) indicating the presence of the carboxyl group in the product [33].

3.2. Antibacterial Activity of Chitosan Derivatives. The *in vitro* antibacterial activities of chitosan and *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosans against *E. carotovora*, *R. solanacearum*, *R. fascians*, and *R. radiobacter* are presented in Table 3 as MIC. The results proved that the

Table 2: Solubility of chitosan and *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan derivatives.

Compound	Solubility				
	Distilled water	Aqueous acetic acid (0.1%)	Aqueous acetic acid (0.5%)	Aqueous acetic acid (1%)	Aqueous sodium hydroxide (1%)
Chitosan	Insoluble	Gel	Soluble	Soluble	Insoluble
1	Swelling	Gel	Soluble	Soluble	Soluble
2	Swelling	Gel	Soluble	Soluble	Soluble
3	Gel	Soluble	Soluble	Soluble	Soluble
4	Gel	Soluble	Soluble	Soluble	Soluble
5	Gel	Soluble	Soluble	Soluble	Soluble

Table 3: Antibacterial activity of chitosan and *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan derivatives against *E. carotovora*, *R. solanacearum*, *R. fascians*, and *R. radiobacter* by broth microdilution technique.

Chitosan product	MIC[a] (mg/L)			
	E. carotovora	*R. solanacearum*	*R. fascians*	*R. radiobacter*
Chitosan	1050	1880	1100	1160
1	755	1125	585	615
2	690	1045	480	510
3	640	945	330	460
4	590	810	255	410
5	510	735	240	385

[a]MIC is a minimum inhibitory concentration. MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

derivatives showed higher inhibition (MIC ranged from 240 to 1125 mg/L) than chitosan (MIC ranged from 1050 to 1880 mg/L) against all the tested bacteria. The data indicated that the inhibitory effects increased with the DS increase and the most inhibition effect was observed with compound 5 (DS = 0.40) with MIC of 510, 735, 240, and 385 mg/L against *E. carotovora*, *R. solanacearum*, *R. fascians*, and *R. radiobacter*, respectively.

Recently, the antibacterial activity of *N*-(4-carboxybutyroyl) chitosan derivatives at different DS values (0.10, 0.25, 0.48 and 0.53) was evaluated against *Agrobacterium tumefaciens* and *E. carotovora*. They reported that the compound of DS 0.53 was the most active one (MIC = 725 and 800 mg/L against *E. carotovora* and *A. tumefaciens*, resp.) [33]. Previous studies on the antibacterial activity of *N,N,N*-dimethylalkyl chitosans as water-soluble derivatives and *N*-(benzyl) chitosan derivatives was done under the same conditions as the present study against *E. carotovora* and *A. tumefaciens* [41, 45].

3.3. Antifungal Activity of Chitosan Derivatives. The antifungal activity of chitosan and *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan derivatives against *A. alternata*, *B. cinerea*, *Bd. theobromae*, *F. oxysporum*, *F. solani*, *P. digitatum*, *Ph. infestans*, and *S. sclerotiorum* by using mycelia radial growth technique is presented in Table 4. The native chitosan molecule in the present study has low activity against the tested pathogens. Conversely, *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan derivatives appeared to be synergistic, and a marked increase in antifungal activity was noted. Unmodified chitosan showed EC_{50} higher than 3000 mg/L against *B. cinerea*, *Bd. theobromae*, *P. digitatum*, and *S. sclerotiorum*. However, a notable effect was exerted against *A. alternata*, *F. oxysporum*, *F. solani*, and *P. infestans* with EC_{50} of 2849, 2697, 1848, and 1600 mg/L, respectively. The derivatives were more active than chitosan and the inhibition effect was increased significantly with the DS increase. Compound 5 with a DS 0.40 exerted the prominent antifungal activity with EC_{50} of 683, 774, 501, 500, 260, 417, 298, and 763 mg/L against *A. alternata*, *B. cinerea*, *Bd. theobromae*, *F. oxysporum*, *F. solani*, *P. digitatum*, *Ph. infestans*, and *S. sclerotiorum*, respectively. However, compound 1 with the lowest DS (0.09) was the lowest active one with EC_{50} of 1390, 1560, 971, 1338, 641, 1645, 511, and 1746 mg/L, respectively. In regard to the susceptibility of the eight tested fungi, it can be noticed that the fungi of *F. solani* and *Ph. infestans* were more susceptible (EC_{50} ranged from 260 to 641 mg/L) to these derivatives than the other tested fungi.

The present study noticed that the antifungal activity was increased dramatically with an increase in DS and MW values. This phenomenon is in agreement with Zhong and others who reported that the amount of grafted acetyl phenyl-thiosemicarbazone in the acetyl and benzoyl phenyl-thiosemicarbazone derivatives of chitosan correlated with the antifungal activity of the derivatives [46, 47]. Jia and coauthors found that pyridine chitosan exhibited enhanced antifungal activity by comparison with pristine chitosan [48]. The values of the MIC and the minimal fungicidal concentration of pyridine chitosan against *B. cinerea* were 130 and 4000 mg/L, respectively. Severe morphological changes of pyridine chitosan-treated *B. cinerea* were observed, indicative of the fact that pyridine chitosan could damage and deform

Table 4: The *in vitro* antifungal activity of chitosan and *N*-(6-carboxyl cyclohex-3-ene carbonyl) chitosan derivatives against *A. alternata*, *B. cinerea*, *B. theobromae*, *F. oxysporum*, *F. solani*, *P. digitatum*, *P. infestans*, and *S. sclerotiorum* by using mycelia radial growth technique.

Chitosan product	EC_{50}[a] (mg/L) with 95% confidence limits							
	A. alternata	*B. cinerea*	*B. theobromae*	*F. oxysporum*	*F. solani*	*P. digitatum*	*P. infestans*	*S. sclerotiorum*
Chitosan	2849 (2112–4711)	>3000	>3000	2697 (1921–4952)	1848 (1323–3407)	>3000	1600 (1247–2330)	>3000
1	1390 (1155–1718)	1560 (1259–1994)	971 (452–2129)	1338 (823–2394)	641 (305–995)	1645 (1056–3512)	511 (214–834)	1746 (1491–2088)
2	1288 (822–2327)	1284 (856–1965)	866 (398–1835)	1097 (640–1888)	418 (32–780)	1120 (880–1459)	428 (156–695)	1145 (605–2364)
3	942 (509–1612)	1037 (642–1559)	746 (442–1206)	938 (579–1418)	369 (22–697)	548 (144–988)	337 (147–503)	1038 (643–1619)
4	892 (213–2313)	872 (484–1389)	649 (314–1205)	703 (358–1090)	290 (17–560)	454 (63–865)	329 (53–569)	840 (400–1431)
5	683 (280–1127)	774 (453–1163)	501 (303–728)	500 (252–742)	260 (34–477)	417 (83–747)	298 (69–488)	763 (408–1183)

[a]The concentration causing 50% mycelialgrowth inhibition.

the structure of fungal hyphae and subsequently inhibit strain growth. The antifungal activities of chitosan derivatives (1,2-diaminoethaneanhydrous, butylamine, and pyridine) were significantly increased against *Rhizoctonia cerealis*, *F. oxysporum*, and *B. cinerea* and the inhibition rate ranged from 22.48 to 63.56% [20]. In the bioassay of *F. oxysporum* and *P. debaryanum*, N-(benzyl) chitosan derivatives exhibited high inhibition percentage of spore germination at 1000 mg/L [45].

Previous study with water-soluble N-(4-carboxybutyroyl) chitosan derivatives with different DS reported that the highest DS 0.53 exerted the highest antifungal activity against *B. cinerea*, *P. debaryanum,* and *R. solani* (EC_{50} = 899, 467, and 1413 mg/L, resp.) [33]. However, compound 1 with a MW of 3.78×10^5 g/mol and DS 0.10 was the lowest active one with EC_{50} of 2170, 814, and 2395 mg/L against *B. cinerea*, *P. debaryanum,* and *R. solani*, respectively. Other derivatives include N-(cinnamyl), N-(cuminyl), and N-(p-dimethylaminobenzyl) chitosan proved moderately antifungal activity, and the EC_{50} values were 1520, 1673, and 1786 mg/L, respectively, against *B. cinerea* [17]. O-(Phenoxyacetic) chitosans were more potent as antifungal activity than chitosan and O-(o,p-dichlorophenoxyacetic) chitosan was the highest in its mycelial growth inhibition with EC_{50} of 1084, 715, 1216, 1417, 638, and 1285 mg/L against *A. alternata*, *Bd. theobromae*, *F. oxysporum*, *F. solani*, *P. infestans,* and *P. debaryanum*, respectively [49]. N,O-(p-Chlorobutyryl) chitosan, N,O-decanoyl chitosan, N,O-cinnamoyl chitosan, and N,O-(p-methoxybenzoyl) chitosan were the most potent ones within eighteen derivatives of N,O-acyl chitosans [24] against *B. cinerea* (EC_{50} = 430, 440, 450, and 500 mg/L, resp.) and were 12- to 13-fold more active than chitosan. However, N-(benzo[d][1, 3]dioxol-5-ylmethyl) chitosan and N-(methyl-4H-chromen-4-one) chitosan were the most active within five heterocyclic chitosan derivatives against *P. debaryanum* and *F. oxysporum* [18].

The most antifungal mechanism of chitosan is that the positively charged chitosan gives this one great physiological and biological polymer property. It consumes electronegative charges on the surface of the microbial cells resulting in change in cell wall permeability, so that the interaction results in leakage of electrolytes, intracellular protein components, and the disturbance of the plasma membrane [21, 50, 51]. In addition, chitosan caused a decrease in the H^+-ATPase activity on plasma membrane of *R. stolonifer*; this effect could provoke the accumulation of protons inside the cell, which would result in the inhibition of the chemiosmotic driven transport that allows the H^+/K^+ exchange [52]. Another important mechanism on fungi stated that the chitosan enters fungal cells and then adsorbs essential nutrients, which inhibit or retard the synthesis of mRNA and protein [53]. Chitosan also acts as a chelating agent that selectively binds trace metals and thus inhibits toxin production and microbial growth [54].

4. Conclusion

The antimicrobial activity is one of the most important bioactivities of chitosan and it will be improved in some of the derivatives, which is determined by the groups grafted to chitosan. In the present study, N-(6-carboxyl cyclohex-3-ene carbonyl) chitosans with five DS values were synthesized and tested against some plant pathogens. The products at the tested concentrations exhibited a wide range of the antibacterial and antifungal activity *in vitro*. It was observed that the highest DS value is more active than the lowest one. Therefore, we can suggest that such products could be used in plant protection program for controlling the plant pathogens that cause destruction of the crops and vegetables. However, formulating of such compounds is essential for commercial uses of the pesticidal chitosan products with further *in vivo* studies being essentially needed. Such formulations can be used in organic and conventional agricultural systems if the formulations are improved for foliar application.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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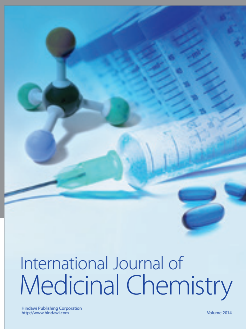
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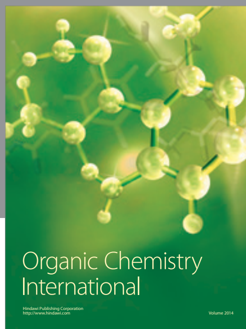
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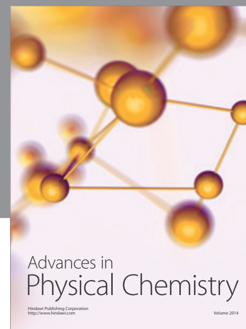
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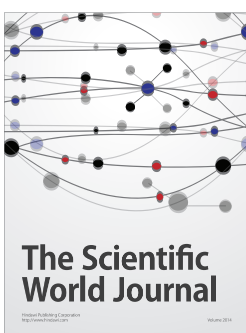
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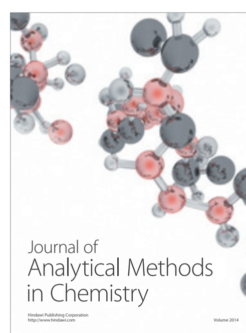
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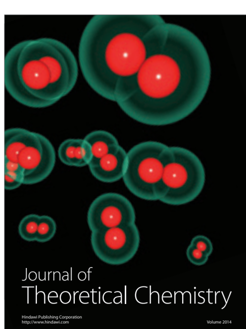
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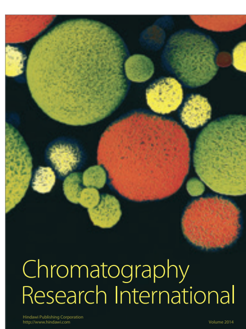
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