Synthesis and Biological Evaluation of Three New Chitosan Schiff Base Derivatives

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ABSTRACT: Recently, chemical modifications of chitosan (CS) have attracted the attention of scientific researchers due to its wide range of applications. In this research, chitin (CH) was extracted from the scales of Cyprinus carpio fish and converted to CS by three chemical steps: (i) demineralization, (ii) deprotonation, and (iii) deacetylation. The degree (measured as a percentage) of deacetylation (DD %) was calculated utilizing the acid-base titration method. The structure of CS was characterized by Fourier transform infrared (FT-IR) spectroscopy and thermogravimetric analysis (TGA). Three new CS Schiff bases (CSSBs) (CS-P1, CS-P2, and CS-P3) were synthesized via coupling of CS with 2-chloroquinoline-3-carbaldehyde, quinazoline-6-carbaldehyde, and oxazole-4-carbaldehyde, respectively. The newly prepared derivatives were verified, structurally, by nuclear magnetic resonance (1H and 13C NMR) and FT-IR spectroscopy. Antimicrobial activity was evaluated for the prepared compounds against both “Gram-negative” and “Gram-positive” bacteria, namely, Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, and Streptococcus mutans, in addition to two kinds of fungi, Candida albicans and Aspergillus fumigates. Cytotoxicity of the synthesized CSSBs was evaluated via a MTT screening test. The results indicated a critical activity increase of the synthesized compound rather than CS generally tested bacteria and fungi and the absence of cytotoxic activity. These findings suggested that these new CSSBs are novel biomaterial candidates with enhanced antibacterial and nontoxic characteristics for applications in areas of both biology and medicine.

KEYWORDS: Amino Polysaccharide, Chitin, Chitosan, Chitosan Schiff base, Antimicrobial polymer, Cytotoxicity test

1. INTRODUCTION

CS has a chemical structure of α(1→4)-2-amino-2-deoxy-β-D-glucopyranose derived from the N-deacetylation of CH, a typical biopolymer found in the exoskeletons of roaches and crustaceans and fungi cell walls (Figure 1). The main source of CS is shellfish, for example, crabs, shrimp, and lobsters, and fish scales. It has a few receptive amino groups, which offer further chemical modifications for the development of an incredible assorted variety of valuable derivatives that are cost-effective. CS, deacetylated CH, is an extremely useful, readily available bioactive polymer, which is a renewable, natural, nontoxic, edible, and biodegradable polymer characterized by biocompatibility. CS shows several advantageous biological properties, such as antitumor, antimicrobial, and hemostatic activities, and promotes wound healing. It has versatile applications ranging from biomedical designing, pharmaceuticals, drug transport, restorative materials, metal particle chelation, and absorptivity to water treatment and plant security. CS derivatives, especially those synthesized via a Schiff base reaction, are the most important due to their organic application characteristics. Recently, the reaction of CS with the rings of aromatic and heterocyclic aldehydes resulted in the efficient production of stable Schiff bases (SBs), which are exceptional compounds in many application areas, particularly in pharmacology and medicine, e.g., as antimicrobial and cancer prevention agents. CSSBs are characteristically prepared by the superficial reaction of CS amine sites with aldehydes or ketones by the elimination of water particles.

Furthermore, quinoline and quinazoline compounds are present in several natural products and in manufactured pharmacologically significant heterocyclic materials. Quinoline and quinazoline derivatives are called antimalarial, antiviral, antibacterial, analgesic, antitumor, and anti-inflammatory agents. Also oxazole derivatives are known as one of the most essential kinds of heterocyclic compounds, which are very significant for medicinal chemistry.

In this study, based on the above facts, first, CH is extracted from the local Cyprinus carpio fish scales by demineralization and deprotonation followed by deacetylation to produce CS using fresh reagents, thereby reducing the time for the overall procedure to obtain CS with a degree of deacetylation (DD)
percentage of at least 60. Second, this study aims to determine the DD % of the products; samples of CS are obtained in different steps of the procedure by the acid–base titration method. Third, this study also aims to characterize and verify the physicochemical properties of the products employing FT-IR and TGA. Finally, this study attempts to develop a synthesis method for three new CSSB derivatives CS-P1, CS-P2, and CS-P3 through coupling CS with 2-chloroquinoline-3-carbaldehyde, quinazoline-6-carbaldehyde, and oxazole-4-carbaldehyde, respectively. The structures of the prepared derivatives were confirmed using 1H NMR, 13C NMR, and FT-IR techniques.

2. RESULTS AND DISCUSSION

Drug resistance of microorganisms to antibiotics has encouraged researchers to search for new antibiotics to challenge this dangerous phenomenon. Many CS derivatives were synthesized to improve the antimicrobial effectiveness of CS, for example, SBs, via methylation, amination, etc.24,25 In the present study, CS was extracted and used to prepare three new SBs of CS by reacting CS with 2-chloroquinoline-3-carbaldehyde, quinazoline-6-carbaldehyde, and oxazole-4-carbaldehyde. The structures of the prepared CSSBs were confirmed using 1H NMR, 13C NMR, and FT-IR techniques.

2.1. CS Extraction Description.

The details of the treatment of C1, C2, C3, and C4 samples are presented in Table S1 in the Supporting Information (SI).

2.1.1. Acid–Base Titration Method.

The DD % for sample C4 was determined by the acid–base titration method. The DD % was calculated using the endpoint of the acid–base titration when the indicator changed into blue-green colors.

Figure 1. Structure of CH and Cyprinus carpio fish.

Scheme 1. Preparation of CSSB Derivatives

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**Table 1. Solubility Characteristics of CSSBs in a Variety of Solvents**

| comp. codes | solvents | CH$_3$COOH | CF$_3$COOH | DMSO | HCl | NaOH | H$_2$O | KOH | DMSO + CF$_3$COOH |
|-------------|----------|------------|------------|-------|-----|------|-------|-----|------------------|
|             | 25 °C    | 70 °C      | 25 °C      | 70 °C | 25 °C | 25 °C | 25 °C | 25 °C | 25 °C             |
| CS-P1       | S        | S*         | S**        | S**  | S   | S*   | S    | S*   | S*               |
| CS-P2       | S        | S*         | S**        | S**  | S   | S    | S*   | S    | S*               |
| CS-P3       | S        | S*         | S**        | S**  | S   | S    | S*   | S*   | S               |

*S = soluble, S+ = insoluble, S* = partially soluble and swelling, S** = partially soluble.

The DD % values of the deacetylated products were calculated according to the following equations$^{25,27}$

$-(\text{NH}_2 \%) = \frac{(C(V_1) - (C(V_2))}{W} \times 100 \quad (1)$

$\text{(DD \%)} = \frac{203(\text{NH}_2 \%/ (16 + 42(-\text{NH}_2 \%))}{100} \quad (2)$

where $C_1$ and $V_1$ are the concentration and the volume of HCl used, respectively, $C_2$ and $V_2$ are the concentration and the amount of NaOH used for titration, respectively, $W$ is the weight of samples used for acid–base titration. The calculated DD for each sample is tabulated in Table S2 and Figure S1 in the SI.

2.1.2. FT-IR Spectroscopic Study. FT-IR spectroscopy was used to study the structures of CH and the related derivatives. The FT-IR spectra of CS, CH, C3, and C4 are shown in Figures S2–S5 in the SI, respectively. By comparing the spectra for sample C3 and CH, and sample C4 and CS, several bands in the range of 4000–400 cm$^{-1}$ were noted in the spectra. As shown in Table S3 in the SI, bands of the synthesized CSSBs match with those of CH. The primary amines showed various bands at 1655 cm$^{-1}$ corresponding to the methyl group in pyranose. The vibrations at 1400 cm$^{-1}$ were related to the presence of the carbonyl group, which in turn indicated that no residue of free aldehydes remained. The vibration bands at 2921 and 2883 cm$^{-1}$ were related to the C–H stretching of methyl and methylene groups, respectively.$^{28}$ The glycosidic bonds showed bands at 1155 and 900 cm$^{-1}$. The vibration bands at 1205–975 cm$^{-1}$ were related to the C–O, C–C, and C–O–C stretching of glycosidic bonds and the pyranose ring.$^{35}$

The structure of the prepared CSSBs was confirmed by $^1$H and $^{13}$C NMR. The $^1$H NMR spectra of the synthesized derivatives CS-P1, CS-P2, and CS-P3 are shown in Figures S13–S15 in the SI, respectively. The $^{13}$C NMR spectra for CS-P1, CS-P2, and CS-P3 are shown in Figures S16–S18 in the SI, respectively. The $^1$H and $^{13}$C NMR data of all SBs are shown below.

2.2. Preparation of CS Schiiff Base Derivatives. Three CSSBs were prepared, as shown in the schematic diagram in Scheme 1. Extracted CS was dissolved in 2.0% aqueous acetic acid, followed by addition of an equimolar amount of the carbonyl compounds dissolved in ethanol to obtain CSSBs. The resultant compounds obtained from the reaction of CS with 2-chloroquinoline-3-carbaldeyde, quinazoline-6-carbaldeyde, and oxazole-4-carbaldehyde were labeled CS-P1, CS-P2, and CS-P3, respectively.

2.2.1. Characterization of CSSBs. 2.2.1.1. FT-IR Spectroscopy and $^1$H NMR and $^{13}$C NMR Analyses. The FT-IR spectra of CSSBs (CS-P1, CS-P2, and CS-P3) are shown in Figures S10–S12 in the SI, respectively. All spectra of the new derivatives displayed a vibration band at 1633–1655 cm$^{-1}$ corresponding to the (–C=–N) group. The aromatic ring showed a stretching vibration band ranging from 1400 to 1500 cm$^{-1}$ related to the C–C bond, while the absorption band at 1057 cm$^{-1}$ corresponded to the aromatic in-plane C–H bending.$^{34}$ No band was observed in the region 1660–1730 cm$^{-1}$, which proved the absence of the carbonyl group, which in turn indicated that no residue of free aldehydes remained. The vibration bands at 2921 and 2883 cm$^{-1}$ were related to the C–H stretching of methyl and methylene groups, respectively.$^{34}$ The glycosidic bonds showed bands at 1155 and 900 cm$^{-1}$. The vibration bands at 1205–975 cm$^{-1}$ were related to the C–O, C–C, and C–O–C stretching of glycosidic bonds and the pyranose ring.$^{35}$

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The synthesized new CSSBs presented typical peaks of the Cs and SB parts.

2.2.1.5. Solubility Study. Different organic solvents were used to test the solubility of the synthesized compounds. Table 1 shows the results. The prepared compounds dissolve in dimethyl sulfoxide and mixtures of equal proportions of dimethyl sulfoxide and trifluoroacetic acid. Partial dissolution or swelling was observed in some solvents such as dilute hydrochloric acid and acetic acid at 70 °C. In contrast, the products are not soluble in most inorganic solvents.

2.2.2. In Vitro Cytotoxicity Study. The cytotoxicity assay for the synthesized compounds was carried out based on MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. MTT assay is a colorimetric assay used for assessing cell viability and to measure cytotoxicity. As illustrated in Table 2, the results of the tested compounds CS-P1, CS-P2, and CS-P3 show a small variation between samples in comparison with the control. Several earlier research studies have demonstrated that CS and CSSB derivatives have little cellular toxicity. Consequently, CS has many applications in the medical field. The assay revealed that using a higher amount of the sample (200 mg) showed a cell viability of 89, 90, 90.1, and 91% for CS, CS-P1, CS-P2, and CS-P3, respectively. Conversely, a smaller amount (25 mg) of the tested compound showed a slight cytotoxicity of up to 2.5% compared with high concentrations, which is extremely suitable in medical applications. According to reported studies, compounds with higher than 75% cell viability are considered noncytotoxic.

The cell viability assessment proved that the selection of these compounds was carried out based on MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Several earlier research studies have demonstrated that CS and CSSB derivatives have little cellular toxicity. Consequently, CS has many applications in the medical field. The experiment was repeated three times and the mean was calculated.

Table 2. Cytotoxicity Test of CS and Their SB Derivatives on the Viability of Mouse Fibroblast Cell Lines

| comp. conc. (mg) | viable cells in the presence of CS | viable cells in the presence of CS-P1 | viable cells in the presence of CS-P2 | viable cells in the presence of CS-P3 |
|------------------|-----------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
| 25               | 99 ± 0.83                         | 99 ± 0.73                            | 99 ± 0.60                            | 99 ± 0.75                            |
| 50               | 97 ± 0.73                         | 99 ± 0.91                            | 98.1 ± 1.3                           | 99 ± 0.89                            |
| 100              | 94 ± 0.63                         | 98 ± 0.74                            | 98 ± 1.2                             | 98 ± 0.50                            |
| 150              | 93 ± 0.88                         | 97 ± 0.65                            | 98 ± 0.62                            | 96 ± 1.2                             |
| 200              | 89 ± 0.53                         | 90 ± 0.72                            | 90.1 ± 0.74                          | 91 ± 1.2                             |

Table 3. Antimicrobial and Antifungal Activity Results of CS and CSSB Derivatives

| comp. codes | Gram-negative bacteria | Gram-positive bacteria | fungi |
|-------------|------------------------|------------------------|-------|
|             | E. coli                | K. pneumonia           | S. aureus | S. mutans | C. albicans | A. fumigatus |
| CS          | 24 ± 0.63              | 26 ± 0.73              | NA       | NA       | 26 ± 0.79   | 16 ± 0.83    |
| CS-P1       | 22 ± 0.73              | 28 ± 0.91              | 22 ± 0.3 | 15 ± 0.89 | 34 ± 0.99   | 26 ± 0.91    |
| CS-P2       | 27 ± 0.83              | 27 ± 0.72              | 20 ± 1.2 | 17 ± 0.50 | 31 ± 1.29   | 25 ± 0.72    |
| CS-P3       | 22 ± 0.98              | 26 ± 0.65              | 19 ± 0.62 | 18 ± 1.20 | 26 ± 0.49   | 21 ± 0.65    |

*NA means not detected.

Based on reported research, many mechanisms are proposed to clarify the action path of CS on microorganisms, which differs depending on the metabolic procedure and the structure of the cell wall. The first suggestion is the interruption of the cell wall of the organism because of the electrostatic attraction between the positively charged amine groups in CS and the negative residue group in the bacterial cell wall, such as −COO− or PO4−3. The second mechanism suggested is the interaction of bacterial DNA with CS, which causes the inhibition of protein synthesis and mRNA by permeation of CS into the bacterial cell and then the nuclei. Another suggestion is based on the ability of CS to form a complex with metals, for instance, Zn2+, Mg2+, and Ca2+; these metals are important for bacterial metabolic processes and growth.

3. CONCLUSIONS

The search for new antibiotics has increased parallelly to the increase in the number of antibiotic-resistant microbes known as superbugs. CS might be a promising material in this field. CS with DD = 89% was extracted from the scales of Cyprinus carpio fish obtained from the local market. The structure of CS was characterized, and the DD % was determined by the acid–base titration method. Three novel CSSB derivatives with different parts as branches were synthesized, and their configurations were proved by FT-IR and 1H and 13C NMR spectroscopy. The new SB structure showed antibacterial activity against most microorganisms and fungi that were tested. The prepared CSSBs demonstrated, almost, no cytotoxic effect on mouse fibroblast cell lines. In light of the above findings, it is safe to say that the prepared CSSBs might be used in different biomedical fields with a high degree of safety and efficacy.
4. MATERIALS AND METHODS

4.1. Materials. 4.1.1. Chemicals and Raw Resources. *Cyprinus carpio* fish scales were collected from the local market in Kirkuk city, Iraq. Its DD was 89%, which was confirmed via a titration procedure. Ethanol (99.7%), hydrochloric acid (37%), 2-chloroquinoline-3-carbaldehyde, methyl orange, quinazoline-6-carbaldehyde, oxazole-4-carbaldehyde, and acetic acid were obtained from Merck (Germany). Sodium hydroxide pellets, sodium bicarbonate, hexamethylenetetramine, magnesium sulfate, aqueous ammonia, sodium borohydride, tetrachloromethane, and trifluoroacetic were purchased from R&M Chemicals Pvt. Ltd., India. Aniline blue was purchased from Alpha Chemika, India. All reagents were of analytical grade and directly used without any further purification.

4.1.2. Devices. FT-IR spectra were obtained using a PerkinElmer System 2000 FT-IR spectrometer. A PerkinElmer TGA 7 thermogravimetric analyzer was applied. A PerkinElmer 2400 Series II analyzer was used to determine the percentage of N in the samples. A Bruker AC-400 NMR spectrometer was used to record the $^1$H and $^{13}$C NMR spectra. Deuterated dimethyl sulfoxide ($d_6$-DMSO) was used as the solvent.

4.2. Microorganisms. Two eukaryote and four bacterial strains were used for assessing the antimicrobial effectiveness of CS and the newly prepared derivatives. The examined microorganisms included four species of bacteria, *E. coli*, *K. pneumonia*, *S. aureus*, and *S. mutans*, in addition to two species of fungi, *C. albicans* and *A. fumigates*. These organisms were collected from the biology department of Kirkuk University from patients who had a liver transplant. Nutrient agar was collected from the biology department of Kirkuk University of fungi, *C. albicans* strains were used for assessing the antimicrobial efectiveness of the samples. A suitable fungus suspension (100 CFU/mL) was added to a beaker, separately. The mixture was stirred and heated in a vacuum oven at 60 °C overnight. The schematic diagram shown in Scheme 1 illustrates the synthesis routes to the new CSSBs.

4.3. Cytotoxicity Evaluation. The cytotoxicity evaluation of CS and the new SB derivatives was carried out using MTT assay. A change in the reagent color to yellow was an indication of cell viability. Laminar flow cabinet biosafety class III was used to carry out all tests. A mouse fibroblast cell line (NIH3T3) was used in the test, which was grown in Dulbecco's modified Eagle's medium (DMEM), completed with trypsin/EDTA (100 μg/mL), and enriched with 10% FBS at 37 °C. Different amounts (25, 50, 100, 150, and 200 μg/mL) of CS and the SB derivatives were used to assess their cytotoxicity. For the assessment of cytotoxicity of the newly prepared compounds, a microtiter plastic plate with 96 wells was coated with cells at a concentration of 10 $\times$ 10$^4$ cells per well and incubated overnight. The cells were incubated for 48 h with a Schiff base sample concentration of 90 μg/mL and alone as a negative control. At the end of the incubation time MTT (40 μL, 3 μg/mL) was added and kept at 37 °C for another 4 h. After the formation of crystals, sodium dodecyl sulfate (SDS) in distilled water (15%, 250 μL) was added to end the reaction. Doxorubicin (100 μg/mL) was used as a positive control under the same conditions. The absorbance was recorded at 595 and 620 nm as standard wavelengths. The tests were repeated three times ($n$ = 3), and the mean value with mean ± SD was calculated. The SPSS 11 program was used to calculate the statistical significance between samples and the negative control. The below equation was used to calculate the percentage of change in cell viability:

\[
\frac{R_{\text{Sample}}}{R_{\text{Negative control}}} - 1 \times 100
\]

4.4. Extraction of CS from Fish Scales. Approximately 100 g of fish scales was weighed and then washed and sun-dried for 3 days. The fish scales were crushed using a high-speed Hamilton beach blender. The scales were then immersed in hot ethanol (50–60 °C) for an hour to kill the germs and remove the pungent odor. The resulting sample (designated as $C_1$) was washed with an ample amount of water and subjected to freeze-drying. $C_1$ was treated with 5% HCl at 25 °C for 24 h to eliminate the CaCO$_3$ component through a demineralization process. Then, 10.0 g of the sample was collected and labeled $C_2$. The remaining sample was processed with a deproteinization step; 10% NaOH was used to remove the protein component at 60 °C. Then, 10.0 g of the sample was collected and labeled $C_3$. The remaining sample was subjected to a deacetylation process by treating with NaOH (50%) under refilling conditions to produce CS. Then, 10.0 g of the sample was collected and labeled $C_4$.

4.5. Synthesis of CSSB Derivatives. The new CSSB derivatives were synthesized according to a reported method. A gram of CS in 50 mL of glacial acetic acid (2%) was stirred at 25 °C for 7 h. A mixture of the aldehyde was dissolved in ethanol (1:1, molar ratio aldehyde to CS) that was added to the mixture gradually. The mixture was stirred and heated in a water bath at 50 °C for 10 h. Aqueous sodium hydroxide 6% was added to the reaction mixture until precipitation of the desired compound. The precipitate was collected and washed several times with distilled water and ethanol to remove any remaining materials. The products were filtered and dried in a vacuum oven at 60 °C overnight.

4.6. Characterization of Extracted CS. 4.6.1. Acid–Base Titration. 4.6.1.1. Preparation of the Indicators. A solution of methyl orange and aniline blue (0.10 g of each, 100 mL of distilled water) was added to a beaker, separately. The mixture was then transferred into a volumetric flask (100 mL).

4.6.1.2. Titration Methods. In four clean and dry conical flasks, 0.30 g of each sample, $C_3$, $C_4$, CH, and CS, was added. Then, 30 mL of 0.1 M HC was added to each flask. Equivalent amounts of both methyl orange and aniline blue solutions were added to each set; the mixture was mixed with a glass rod until the color of the mixture became stable. The titration method was carried out for each sample set against NaOH (0.1 M) until the indicator color changed.

4.6.2. FT-IR Analysis. FT-IR spectra of CH, CS, $C_3$, and $C_4$ and the CSSB derivatives (CS-P1, CS-P2, and CS-P3) were recorded via a FT-IR spectrooscope (Model 8400, Shimadzu). KBf was mixed with 2 mg of the sample and pressed to form a homogeneous disc with $\approx$0.5 mm thickness. The spectral region between 4000 and 400 cm$^{-1}$ was scanned.

4.6.3. Thermogravimetric Investigation (TGA). The thermal stability of deacetylated CS was evaluated; approximately 5 mg of CS was analyzed using a thermogravimetric analyzer (model 50/50H, Shimadzu). The heating was done as described from 60 to 750 °C at a rate of 5 °C/min under 30 mL/min flow rate of nitrogen.
ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01342.

General procedure and details of the treatment of CS and CH$_2$ tables show the results of the acid–base titration method and the characteristic absorption bands in the FT-IR spectra of standard and experimentally prepared CS$_2$; figures display the DD % of samples; TG and DTG plots; and $^1$H NMR, $^{13}$C NMR, and FT-IR spectra for all prepared compounds (PDF)

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All authors contributed to the writing of the manuscript. All authors have approved the final version of the manuscript. The photo in Figure 1 was taken by the authors.

Notes
The authors declare no competing financial interest.

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