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Preparation of Isatin/chitosan Schiff base as Novel Antibacterial Biomaterials



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THE research on biopolymers and its derivatives has received much attention from the researchers toward preparing a novel material. The biopolymers and its derivatives have broad applications due to its biodegradability, nontoxicity and biocompatibility. The present study aimed to develop and characterizes a unique chitosan Schiff base by coupling chitosan with isatin under acidic conditions to form isatin/chitosan Schiff base. The changing in chitosan chemical structure was proved by FT-IR, electronic spectra. The physico-chemical study shows a decrease of samples water uptake and solubility in aqueous acidic solution by increased isatin content as a result of an increase in hydrophobicity character of chitosan by modification. Antibacterial activity was tested against four different bacterial strains onegram-positive: (Staphylococcus aureus) and three gram negative (Pseudomonas aeruginosa, Salmonella and Proteus vulgaris). The results showed increases in the antibacterial activity of substituted chitosan against both gram-negative and gram-positive bacteria by the rise in isatin content.

Keywords: Chitosan, Isatin, Schiff base, Antibacterial activity.

Introduction

Chitosan as a biopolymer can isolate from walls of Mucorrouxiifrm[1] and can be prepared by deacetylation of chitin. Chitin can be obtained commercially from the skeleton of crustaceans. The presence of both hydroxyl and amino groups along chitosan backbone provide the sites for numerous attractive chemical modifications. Chitosanderivatives exhibited various physicochemical and biochemical properties, such as good biocompatibility, biodegradability, antibacterial and antifungal activities, moisture absorption, blood clotting, etc. Therefore, it has considered applications in many fields such as agriculture, pharmaceuticals, cosmetics, foods industries, environmental protection, and biotechnology and many other fields [2-11].

Chitosan and its derivatives are broadly studied as antimicrobial agents against a wide range of bacteria including both grams positive and negative bacteria.

The antimicrobial characteristics of chitosan haven standardized, and, accordingly, it is very hard to explain results according to one study [12, 13]. Continuous mutation of bacteria is driving the scientists to improve the antibacterial activity of chitosan via chemical modification of its structures. Chitosan holds three reactive groups, i.e., primary (C-6), secondary (C-3) hydroxyl groups, and the amino groups (C-2) on each deacetylated repeating unit. The reactivity of chitosan amine groups Therefore, these reactive groups of chitosan is readily subjected to chemical modifications to alter its

mechanical and physical properties. Also, the coupling of free amine groups on chitosan with the carbonyl group in aldehydes and ketones was a very easy and common reaction to produce Schiff bases along polymer backbone (RC= N). Several chitosan Schiff bases have been prepared and published as chelating agents, antimicrobial and antioxidant materials, etc. [14–18].

The indole nucleus is found to be the very active nucleus in pharmacy field, as several natural alkaloids having indole as their basic ring are found to be therapeutically active agents. Isatin (indole-2, 3-dione) is an indole derivative, an endogenous compound, widely distributed in mammalian tissues and body fluids [19]. Isatins are synthetically versatile substances that are employed for the synthesis of a very large variety of heterocyclic compounds and possess broad spectrum of biological activities like antibacterial [20], antiviral [20], antifungal [20], anti-inflammatory [21], analgesic [22], antitubercular[23], antidepressant[24]. In view of these facts, we contemplated to synthesize some new Schiff bases of Isatin and planned to screen for their in vitro antibacterial activities.

Materials and Methods

Materials

Shrimp shells were collected from wastes of seafood restaurants in Alexandria (Egypt). Isatin was kindly donated by Lab of organic synthesis, Chemistry Department, Faculty of Science, Al-Azhar University. Sodium hydroxide (99%), Sulfuric acid (98%), ethanol (99%), hydrochloric acid (purity 37%), and acetic acid (98%) were brought from El-Nasr Company (Alexandria).

Methods

Extraction of chitin from shrimp shells

According to the published procedure [25], the de-mineralization of shells was the main process for chitin preparation. In this step, the shells were scattered in 5% (w/v) HCl at ambient temperature in the ratio of 1:14 (w/v) overnight. Then, the shells were quite squishy and rinsed using water to remove acid and calcium chloride. The de-mineralized shells were treated with 5% (w/v) NaOH at room temperature for 24 h in the ratio of 12:1 (v/w). The residues were collected and washed to neutrality many times in running tap water and then; distilled water to obtain pure chitin.

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Preparation of chitosan from chitin

Preparation of chitosan is naturally deacetylation of chitin in alkaline medium [26]. Removal of acetyl groups from the chitin was achieved using 50% (w/v) NaOH with a solid to solution ratio of 1:50 (w/v) at $100-120^{\circ}$ C for 12 h. The resultant chitosan washed to neutrality with distilled water.

Chitosan purification

According to the previous method [26], chitosan sample was dissolved in 2% (w/v) acetic acid and was left overnight. Then, the chitosan solution was filtrated using cheese cloth to remove contaminants and undissolved particles. Finally, chitosan was precipitated with 5% (w/v) NaOH, collected and washed with distilled water to remove the excess of alkali.

Preparation of Isatin/Chitosan schiff base
Previously purified chitosan (1g) was dissolved
in 50 ml of 2% acetic acid and stirred at room
temperature for 1 hr. To resulting viscous, 10 ml
of THF containing a definite amount of isatin
were added to the solution under stirring to have a
homogenous solution. This mixture was stirred for
3 h at 70°C. The formation of a deep yellow color
refers to the formation of the chitosan Schiff base.
The resulting product was added to an excess of
5% sodium hydroxide solution. The precipitate
was filtered and washed with distilled water and
THF several times to remove un-reacted isatin;
the product was separated and dried in a vacuum
oven at 60°C overnight.

Five different weight ratio of chitosan/isatin were prepared and coded as (1:0.0913) for CH-IS (0.1), (1: 0.114) for CH-IS(0.125), (1:0.183) for CH-IS(0.2), (1:0.228) for CH-IS(0.25), and the native chitosan CH-IS(0).

Physico-chemical characterization

Water uptake

Water uptake (%) was measured by placing a weighed dry sample in distilled water for six hours. After reaching the equilibrium swelling, the sample was filtered off and weighed. The water uptake was calculated as follows:

Water uptake (%) = $\{[M-M0]/M0\} \times 100$

where M is the weight of the swollen sample and M0 is the weight of the dry sample.

Determination of ion exchange capacity

Chitosan or chitosan derivatives (0.1 g) were dissolved in 20 ml of 0.1 M H₂SO₄ on shaking for 3 h. The mixture was than filtered and an aliquot

was titrated against a standard solution of sodium hydroxide. Similarly, a control titration without the addition of chitosan was also performed. The ionic capacity of chitosan samples were calculated using the following equation:

Ion exchange capacity =
$$\{(V_2 - V_1) \times a\}/w$$

(meg/g)

where V_2 and V_1 are the volumes of NaOH solutions required for complete neutralization of H_2SO_4 in the absence and presence of the polymer, respectively, and a is the normality of NaOH and w is the weight of sample taken for analysis[27].

Solubility test.

Solubility test was performed by placing a weighed sampling in acetate buffer at certain pH and stirring well at Room temperature for 6 hrs. The residue was then filtered, dried and weighed [28]. The solubility was determined by the following equation

Solubility% =1-
$$[W1/W0] X 100$$
here

W1 and W0 are weight of insoluble part and Total weight of sample respectively

UV-Visible spectroscopic analysis

The electronic absorbance of the prepared chitosan and its derivatives were investigated using spectrophotometer scanned from 200 to 600

nm.

Fourier transfer infrared spectroscopy (FT-IR) Functional groups in the chemical structure of chitosan and its derivatives were identified using a FT-IR spectrophotometer (Shimadzu FTIR-8400S, Japan) and the data were analyzed using the IR Solution software, version 1.21. The polymer sample (1–2 mg) was added to KBr (200 mg) and scanned between 4000 and 400 cm⁻¹ using 30 scans at a resolution of 4 cm⁻¹.

Broth evaluation method

Antimicrobial activity of chitosan and its derivatives were measured according to the reported method [29]. Briefly, the bacteria were incubated in Luria-Bertani medium (LB medium) (1 % peptone, 0.5 % yeast extract, and 1 % NaCl). The inoculation was conducted at 37 °C for 24 h while shaking. The obtained bacterial suspension was diluted with the previous peptone medium solution. Then, 0.1 ml of diluted bacteria suspension was cultured in 10-ml liquid peptone medium, and dissolved in various amounts of the tested polymer (10, 20, 40, and 50 mg). The inoculated medium remained shaking at 37 °C for 24 h. After incubation, the optical density of each well was determined (TF). Bacterial growth inhibition of chitosan and the chitosan derivative were reported as inhibition percentage (%) by the following equation:

$$Inhibition~(\%) = 1 - \frac{\left(T_{Fsample} - T_{0sample}\right) - \left(T_{Fblank} - T_{0blank}\right)}{\left(T_{Fgrowth} - T_{0growth}\right) - \left(T_{Fblank} - T_{0blank}\right)} \times 100$$

where T0 sample and TF sample are the optical densities at 620 nm of the strain growth in the presence of pure chitosan or grafted chitosan before (T0) and after (TF) incubation, respectively; T0 blank and TF blank corresponded to the medium with pure chitosan or grafted chitosan before and after incubation, respectively; and T0 growth and TF growth correspond to the strain growth in the presence of medium (positive control) before and after incubation, respectively. The number of bacteria was counted by using the ultraviolet absorbance of culture medium at 620 nm.

Results and Discussion

Chitosan is as natural antimicrobial polymer has a wide range of activity against microorganism. In order to improve the antimicrobial activity of chitosan new derivatives of chitosan was prepared.

In the current study, we utilized the presence of amine groups along amine glucose repeating unite to prepare chitosan-Isatin Schiff base derivative as illustrated in Fig. 1. Four different degree of substituted derivatives were prepared by interact different molar ratio of chitosan and Isatin.

Physicochemical characterization

Figure 1 shows the water uptake of chitosan and chitosan isatin schiff base with different substitution content. There is a significant decrease in water uptake and moisture content by coupling isatin with amine groups that can be explained by replacement of terminal hydrophilic groups (i.e., -NH₂) with hydrophobic groups (i.e., isatin). Adsorption of water molecules on polymer are affected by hydroxyl and amine groups that distributed along polymer backbone.

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Fig. 1. Schematic preparation of Chitosan/ Isatin schiff base starting from shrimp shells.

Ion exchange capacity of chitosan based on its amine groups can be taken as indicator for schiff base formation. Used chitosan has ion exchange capacity equivalent to 25.76 meq/g, this value was dropdown to 17.4 meq/g by reaction of chitosan with isatin. These can explain by consumption of chitosan amine groups in Schiff base formation. Same behavior was observed with our similar chitosan derivatives [30].

Solubility

The solubility of chitosan in acidic medium is depending on its amine groups. The protonation of amine to cationic form created a positive charge along polymer backbone.

Solubility of chitosan and prepared chitosan Schiff bases were investigated over wide range of pH stating from pH 3 to pH 8. It was found that there is a general decrease of solubility by increasing the degree of substitution. All tested samples are completely soluble in acidic pH (pH3-pH5) (See Table 1). Starting from pH 6, there is a decline polymer solubility.

Isatin/chitosan schiff base

Electronic spectra characterization

Chitosan and chitosan/isatin schiff base absorption of lights was tested starting from 200 to 600nm (Fig. 3). Increase of intensity and shift of bands at 300 of chitosan to red shift was attributed to lowering of energy of n- σ * transition by coupling chitosan with isatin nucleus[31,32]. The prepared chitosan/Isatin Schiff base show dramatic increase in absorbance intensity at 300nm from 0.5783 for CH-IS(0), 1.143 for CH-IS(0.1), 1.2896 for CH-IS(0.125), 1.3817 for CH-IS(0.2) and CH-IS(0.25) 1.9226, that can be explained by formation of Schiff base boned between chitosan amine groups and Isatin carbonyl groups.

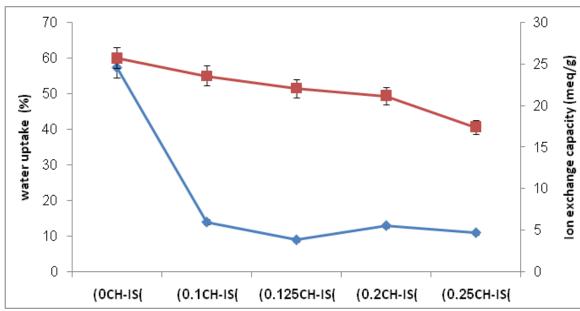


Fig. 2. Water uptake (♦) and ion exchange capacity (■) of chitosan & chitosan IsatinSchiff base.

TABLE 1. Solubility percent of chitosan and chitosan/isatin schiff base.

	CH-IS(0)	CH-IS(0.1)	CH-IS(0.125)	CH-IS(0.2)	CH-IS(0.25)
PH3	100	100	100	100	100
PH4	100	100	100	100	100
PH5	100	100	100	100	100
PH6	12.3	10.7	9.18	7.9	7.7
PH7	7.5	9.14	7	6	5.6
PH8	7.3	6.4	5.16	3.9	3.2

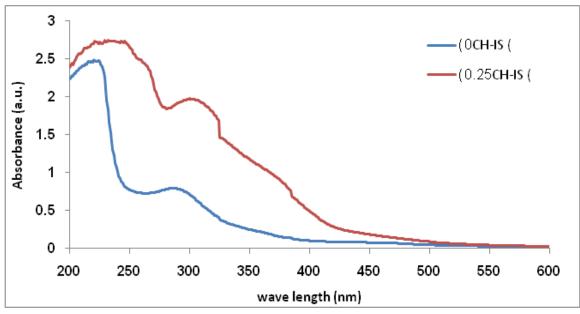


Fig. 3. UV-vis spectrum of chitosan and chitosan/isatin Schiff base.

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Fourier transfer infrared spectroscopy (FT-IR)

Figure 4 shows the regular bands of chitosan and chitosan/ Isatin schiff base function groups.A broad band between 3200 - 3600 cm⁻¹ was observed which corresponds to thestretching vibration of NH and OH groups. Bands between 2835 – 2950 cm⁻¹ is a combination of C-H stretching of methyland methylene groups, bands at 1620 cm⁻¹ point out stretching vibration of C=O and NH-C-O functional groups. Bands between 1066 - 1059 cm⁻¹correspond to C-O-H group stretching. A newband at 1642 cm⁻¹was generated that is attributed to C=N vibrations characteristic of imines [33,34]. This band is not observed in chitosan. On the other hand, there is no evidence ofbands characteristic of free aromatic aldehydes near to 1665 cm⁻¹. The bands at 1580 cm⁻¹ were a result of C=C stretching in thearomatic aldehyde ring.

Antibacterial activity

Figure 5 illustrates the antimicrobial activity of chitosan and chitosan/isatin schiff base against four different bacteria *Staphylococcus aureus*, *Salmonella*, *Proteus* and, *pseudomonas aeruginosa*. From the figure, it can observed a general increase of antibacterial activities of the chitosan/Isatin Schiff base compare to chitosanagainst tested bacteria compared to chitosan.

The significant increase of the antibacterial activity of new chitosan derivative can be attributed to generation of new Schiff base bond on the

other hand immobilized of hydrophobic groups along chitosan backbone by coupling isatin with chitosan amine groups. The incorporation of isatin increases the hydrophobicity of chitosan that can improve the interaction with peptidoglycan of the cell wall and lipoprotein in the outermembrane specifically of gram-negative bacteria. Hence, this interaction results in a block of the channels that are responsible for exchange of electrolytes and nutrients. This chemical interaction gives the chitosan/Isatin Schiff base significant activities against gram-negative and gram-positive bacteria. It is worth noting that the molecular weight of chitosanin this study is a medium molecular weight, thus it can penetrate the cell and bind to DNA. Obtained results were agreedwith that achieved by Krajewska [35-36]. Krajewska concluded that hydrophobic character of chitosan has a role on disturbance ofmicroorganisms cell wall membranes. This effect was maximized by raise hydrophobic character via modification. Therefore, the chitosan/Isantin Schiff base can inhibitthe bacterial propagation using the two proposed mechanisms, butthe most acceptable is the first mechanism because of a high precipitation of the prepared chitosan Schiff base as mentioned previously. It should also be noted that theantibacterial activity of these materials may least in part consist ofdisturbing cell membrane structures [36]. Formore details on the influence of pH and molecular weight on Chitosan interactions with membrane lipids the reader is referred to Krajewska et al., 2011[37].

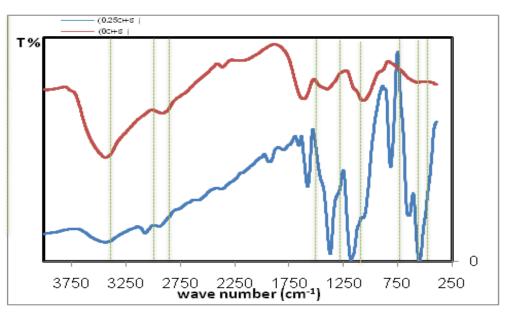


Fig. 4. FT-IR spectrum of chitosan and chitosan/isatin Schiff base.

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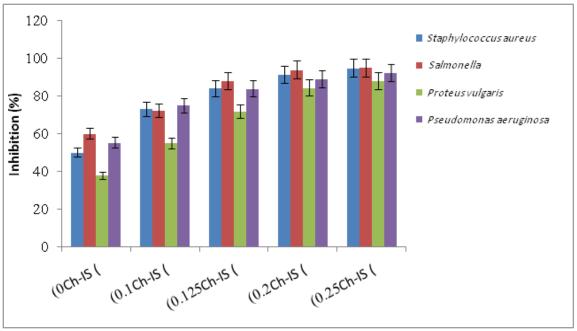


Fig. 5. Antibacterial activity of chitosan and chitosan/isatin schiff base against different bacterial strains.

Conclusion

Chitosan/isatin Schiff base was successfully prepared via coupling chitosan amine groups with isatin carbonyl group. The structure of the modified chitosan was analyzed and confirmed using physical characterization, FT-IR spectra and Uv-vis spectrum. The antimicrobial activity of the chitosan/isatin Schiff base derivative was carried out throughout compared with chitosan alone. The prepared materials showed higher activity against both Gram-negative and Grampositive bacteria. The results concluded that the chitosan/isatin Schiff base could be used as antimicrobial materials in medical applications such as wound dressing after conducting them in vivo on animals.

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تحضير مشتق الكيتوزان/ الإيزاتين كمواد حيوية مضادة للبكتريا

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حظي البحث عن البوليمرات الحيوية ومشتقاتها باهتمام كبير من الباحثين نحو إعداد مادة جديدة. البوليمرات الحيوية ومشتقاتها لها تطبيقات واسعة بسبب قابليتها للتحلل الحيوي، عدم السمية والتوافق الحيوي. هدفت الدراسة الحالية إلى تطوير وتوصيف قاعدة فريدة من نوعها من خلال اقتران الكيتوزان مع الايزاتين تحت الظروف الحمضية لتشكيل قاعدة شيف من الكيتوزان و الايزاتين. وقد أثبت FT-IR، الأطياف الإلكترونية، التغيير في التركيب الكيميائي للكيتوزان. تظهر الدراسة الفيزيائية الكيميائية انخفاضا في عينات امتصاص الماء والقابلية للذوبان في المحلول الحمضي المائي عن طريق زيادة محتوى الايزاتين نتيجة لزيادة في طبيعة المحبة الدهنية من الكيتوزان عن طريق التعديل. تم اختبار النشاط المضاد للبكتيريا ضد أربع سلالات بكتيرية مختلفة واحدة إيجابية الغرام: (Staphylococcus aureus) وثلاثة سالبة سلبية (شاط المضاد للجراثيم من الكيتوزان المستبدلة ضد كل من البكتيريا سالبة الجرام والإيجابية الجرام من خلال ارتفاع محتوى الايزاتين.