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Low Molecular Weight Chitosan-based Schiff Bases: Synthesis, Characterization and Antibacterial Activity

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ABSTRACT

In the present study, Chitosan Schiff Bases (CSBs) were synthesized via condensation reaction of Low Molecular Weight Chitosan (LMWC) with aromatic aldehydes including either benzaldehyde, p-chlorobenzaldehyde, p-N,N-dimethylaminobenzaldehyde or p-methoxybenzaldehyde (anisaldehyde, naturally occurring aldehyde) to enhance the antibacterial activity of chitosan. The chemical structure of obtained Schiff bases were characterized using UV, FT-IR and ^{1}H NMR spectroscopy. Moreover, the structural features were examined by Scanning Electron Microscopy (SEM). FT-IR spectra revealed that the characteristic bands of Schiff base owing to C-N stretching vibrations appeared at ~1645, 1634, 1632 and 1610 cm^{-1}. The chemical substitutions were also confirmed in the four types of synthesized Schiff bases by ^{1}H NMR spectroscopy, in which new signals were appeared at 7-12 ppm that are belonging to the aromatic moieties of the Schiff bases. The Degree of Substitution (DS) ranged from 20.5-22.9% depending on the type of reacted aromatic aldehydes. The contact angle measurements for the obtained chitosan Schiff bases-based films indicated that surface of these films are more hydrophobic than that of chitosan one. The antibacterial activity of CSBs were measured against two types of gram positive bacteria (*Streptococcus pyogenes* and *Staphylococcus aureus*) and two of gram negative bacteria (*Escherichia coli* and *Shigella dysenteriae*). The bacterial growth was assessed by measuring the Optical Density (OD) of culture media at 610 nm. These results exhibited that all prepared CSBs have higher inhibition efficiency against gram-positive bacteria than gram-negative ones. However, Schiff base of anisaldehyde exhibited the highest antibacterial activity.

Key words: Chitosan, benzaldehyde, p-chlorobenzaldehyde, p-N,N-dimethylaminobenzaldehyde, p-methoxybenzaldehyde, Schiff bases, bioactive biodegradable materials

INTRODUCTION

Chitosan, a versatile biopolymer derivative, is obtained by alkaline deacetylation of chitin. The distribution of the precursor, chitin, in nature is ubiquitous among the shells of crustaceans such as crabs, shrimps and lobsters as well as in the exoskeleton of marine zoo-plankton including coral, jellyfish and squid pens (Lertsutthiwong *et al*., 2002). Chemically, chitosan is a linear heteropolysaccharide composed of β-1,4-linked-D-glucoseamine (GlcN) and N-acetyl-D-glucosamine (GlcNAc) at varying ratios (Kumar, 2000). The proportion of GlcNAc in relation to GlcN is defined as the Degree of Acetylation (DA), where chitin can be differentiated from chitosan by that the

latter have DA≤0.5 (Cabrera and Cutsem, 2005). This differentiation can be also practically defined by chitosan solubility and chitin insolubility in aqueous acidic medium such as organic acids as acetic , formic , citric , as well as inorganic acids, diluted hydrochloric acid (Rinaudo *et al.*, 1999). Chitosan exhibits several valuable inherent properties such as antibacterial, antifungal, antiviral, antacid, nontoxic, total biodegradable as well as film , fiber and hydrogel formation properties (Guinesi and Cavalheiro, 2006). By virtue of these properties, chitosan has prospective applications in many fields such as medical, waste water treatment, cosmetics, dentifrices, food, agriculture, pulp and paper and textile industries (Kumar, 2000; Li *et al.*, 2010; Guo *et al.*, 2006, 2007; Jeon *et al.*, 2001; Shahidi *et al.*, 1999). However, the performance of this biopolymer in certain applications depends on the viscosity of its solution which in turn is a function of its molecular weight. Furthermore, degree of deacetylation and molecular weight are reported to be the main factors affecting antibacterial activity of chitosan. Where, it has been reported that the higher DA (85-95%) gives stronger antibacterial activity (Chatelet *et al.*, 2001; Jung *et al.*, 2010). This have been explained on the basis that an increase of deacetylation degree leads to increase the charge density on chitosan chains (Chatelet *et al.*, 2001), whereas even at a pH less than 7, sufficient amounts of cationic sites (NH_3^+ group) remain at C_2 of GlcN residues, creating polycationic structure that leads to electrostatic interactions with the negative charges of anionic components of the surface of cell membranes of the bacteria (-COO^- or PO_4^{3-} groups). So, interactions between positively charged chitosan molecules and negatively charged residues on the bacterial cell surface are considered to play an important role in the antibacterial activity of chitosan. The difference in cell surface electronegativity might lead to the difference in the susceptibility of microbial cells toward chitosan (Xia *et al.*, 2011). Thus, leads to variation in the Relative Adsorption Ratio (RAR) of chitosan resulting in a variation in the susceptibility of test (Kong *et al.*, 2008). On the other hand, It has been reported that chitosan of lower molecular weight (LMWC) (50 kDa) exhibit better biological activities than chitosan of higher molecular weight (100 kDa) (Liu *et al.*, 2006). Two mechanisms have been suggested to explain this antimicrobial action of LMWC, the mostly accepted mechanism based on that these substances can alter the permeability of microbial cell membrane and subsequently prevent the entry of nutrients or cause leakage of cell constituents that finally leads to death of bacteria (Ramesh *et al.*, 2012). Another mechanism suggests that these permeable substances can block RNA transcription via its adsorption on bacterial DNA (Serag and Edrees, 2011).

GlcN residues of chitosan bearing free amino groups at C-2 position allow chemical substitution producing several chitosan derivatives with a large spectrum of applications (Rinaudo *et al.*, 1999; Mourya *et al.*, 2010). Among these derivatives, Schiff bases which obtained by reacting these free amino groups of chitosan with active carbonyl compounds such as aldehyde or ketone (Tirkistani, 1998; Dos Santos *et al.*, 2005). The characteristic imine groups (-RC = N-) of these Schiff bases offer several potential analytical and environmental applications by enhancing the adsorption / complexation properties (Varma *et al.*, 2004). Moreover, some Schiff bases of chitosan are reported to have antimicrobial activity. It has been found to be stronger than that of chitosan (Guo *et al.*, 2007; Jin *et al.*, 2009).

Up to now, although researchers have reported some Schiff bases derivatives of chitosan, there are few reports on synthesis of these derivatives from chitosan of lower molecular weight. Therefore, the design and synthesis of four chitosan-based Schiff base compounds have been investigated in this study by reacting low molecular weight chitosan with different aromatic aldehydes including benzaldehyde and three of its derivatives, p-chlorobenzaldehyde, p-N,N-dimethylaminobenzaldehyde and p-methoxybenzaldehyde (anisaldehyde, naturally

occurring aldehyde) in this study. Chemical and structural properties of these compounds have been also characterized by various methods, such as UV, FT-IR and ^{1}H NMR spectroscopy and Scanning Electron Microscopy (SEM). The work is also extended to evaluate the antibacterial activity of these LMWC-based Schiff base compounds against two species of gram positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) and two species of gram negative bacteria (*Escherichia coli* and *Shigella dysenteriae*). The present work may give some key information to the design of new functional chitosan derivatives having potential use as food additives and antimicrobials.

MATERIALS AND METHODS

Materials: Low Molecular Weight Chitosan (LMWC) with deacetylation degree of 95% was purchased from Oxford laboratory reagent, Mumbai, India. Benzaldehyde was purchased from Alpha laboratory reagent, Mumbai, India. P-chlorobenzaldehyde and P-N,N-dimethylaminobenzaldehyde with purity of ≥98 were purchased from Acros Organics, New Jersey, USA, P-methoxybenzaldehyde with purity 98% was purchased from Alfa Aesar A Johnson Matthey Company, Karlsruhe, Germany. Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pyogenes*) and gram negative bacteria (*Escherichia coli* and *Shigella dysenteriae*) were supplied by Microbiology Laboratory of the Ain Shams University of Egypt.

Synthesis of Schiff bases: LMWC (16.7 mmole) was dissolved in 200 mL of an aqueous acetic acid solution (1%) at ambient temperature. Then predetermined amounts of aldehydes (benzaldehyde, p-chlorobenzaldehyde, p-N,N-dimethylaminobenzaldehyde or p-methoxybenzaldehyde) dissolved in ethanol were added to the chitosan solution. These reaction mixtures were let to react at temperature of 55°C with stirring for 3 h. The resulting deep yellow gels of Schiff bases were filtered, washed several times with ethanol to remove any unreacted aldehydes and dried in vacuum oven at 70°C. The obtained yellow powder was kept in a dissector over silica gel for further analyses. Schiff bases of chitosan were synthesized according to the scheme presented in Fig. 1.

Characterization

Infrared spectral analysis: The unmodified and chemically modified chitosan were examined after their preparation in form of KBr pellets using Shimadzu 8400S spectrometer (Shimadzu, Japan). About 4-8 mg of the crushed sample was mixed with 200 mg of potassium bromide and was further ground in an agate mortar with pestle. The mixture was then transferred to a die and pressed into a disc in a vacuum press at 80 kN. The spectra (128 scans at 2 cm^{-1} resolution) were collected with the frequency range of 4000-400 cm^{-1}. The FTIR spectra were Fourier-deconvoluted with a resolution enhancement factor of 1.5 and a bandwidth of 15 cm^{-1} (George *et al*., 2011).

Ultraviolet spectral analysis: All samples were stored in a desiccator containing silica gel for at least 48 h at room temperature to ensure minimal moisture content before spectroscopic analysis. UV spectra were obtained using double-beam UV/Vis spectrophotometer (Hitachi U-3900 spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan) with an accuracy of ±1%was used for the determination of UV absorbance and the A_{max} (the optimal wavelength for measuring absorbance) of unmodified chitosan and Schiff bases samples (0.5 in 1% acetic acid aqueous solution) (Kumirska *et al*., 2010).

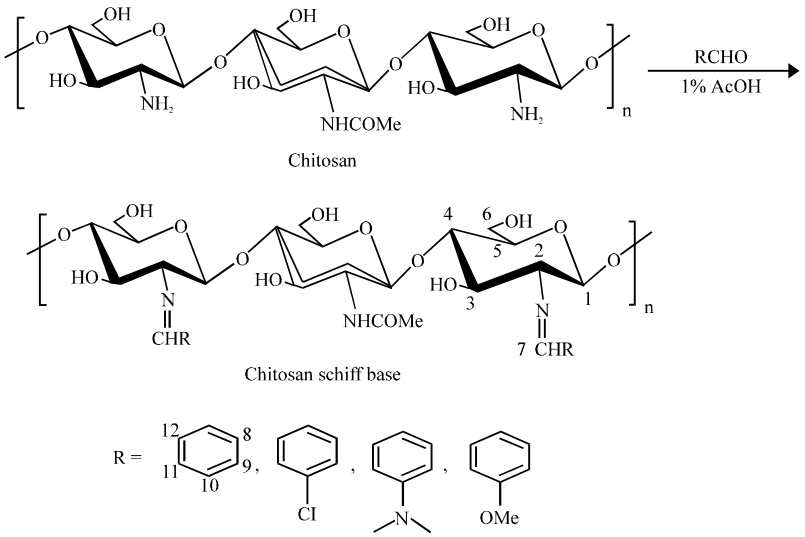

Fig. 1: Synthesis scheme of chitosan-based Schiff base compounds

NMR spectral analysis: ^{1}H NMR spectra were obtained using JEOL 500 MH NMR spectrometer, Japan at 500.2 MHZ. Samples dissolved in DMSO/TFA were analyzed by a 5 mm ^{13}C-^{1}H dual probe head at 25°C. The spectra were accumulated into 32 K data points and processed using exponential multiplication with 2 Hz line broadening into 128 K spectra. For the resulting spectra 25000-35000 scans were accumulated. All spectra were accumulated under identical conditions using power gated Waltz decoupling with 25 degree measurement pulse and 1 sec prepulse delay (Lavertu *et al*., 2003).

Microstructure examination: The structural characteristics of powders of Schiff bases were examined by a Joel 6360LA scanning electron microscope (JEOL Ltd., Tokyo, Japan) operated at an acceleration voltage of 15 kV. Samples were mounted on stainless steel stubs with double sided tape, 10-20 nm thick layer of gold was sputtered on the samples by JFC-1100E sputter (JOEL Ltd., Tokyo, Japan) (Bhuvaneshwari *et al*., 2007).

Contact angle measurement: Unmodified and chemically modified chitosan (Schiff bases) (1 g) were dissolved in 100 mL 1% acetic acid . The resultant film forming solutions were cast in a Petri dish and left to dry at ambient temperature for 48 h. Afterwards, the resultant dried films were immersed in 4% NaOH, then washed with distilled water and dried at ambient temperature for 24 h. The film thickness was measured by a hand-held electronic digital micrometer (Mitutoyo, Japan) and the films with mean thickness value of 100 μm were used in these measurements. Contact angle measurements were carried out using a goniometer (Rame´-hart, model 500-F1, France). Four micro liters droplet of de-ionized water was placed on the surface of the pre-prepared membrane was an automatic piston syringe and photographed. An image analyzer was used to measure the angle formed between the base, constituted of the surface of the film in contact with the droplet of water and the tangent to the droplet of water. For each film specimen, at least five measurements on different ten positions of film surface were taken place, the average was calculated (Correlo *et al*., 2007).

***In vitro* antibacterial assays:** Antibacterial activity of LMWC and its synthesized Schiff bases were assessed against four bacterial strains, two gram-positive bacteria (*Streptococcus pyogenus* and *Staphylococcus aureus*) and two gram-negative bacteria (*Shigella dysenteriae* and *Escherichia coli*) by measuring the growth of these bacterial strains in the presence of chitosan and its Schiff bases that done by measuring OD of culture media at 610 nm as described by (Jeon *et al*., 2001). Bacterial strains were grown in a nutrient agar culture medium (trypton 1%, yeast 0.5%, NaCl 1%, in distilled water, pH = 7.4) and incubated at 37°C overnight. One milliliter of cultured bacterial suspension was added to mixture of 4 mL of the chitosan or Schiff bases solution (0.5% in an aqueous acetic solution 0.5%) and 15 mL of nutrient broth medium sterilized by autoclaving at 121°C for 15 min. Then, the tubes were incubated with shaking at 37°C for 24 h. The antibacterial activity was estimated periodically by measuring the optical density of the culture medium at 610 nm with UV-vis spectrophotometer (Hitachi U-3900 spectrophotometer, Hitachi High Technologies Corporation, Tokyo, Japan). Acetic acid with concentration of 0.5% (v/v) was used in place of chitosan or its Schiff bases in a control. Each batch experiment was carried out in triplicate and results were reported as an average of three replicates.

RESULTS AND DISCUSSION

Infrared spectral analysis: IR spectrum of LMWC was represented in Fig. 2a. This spectrum exhibited main characteristic broad band at 3200-3600 cm^{-1} corresponding the stretching vibration of N-H and O-H groups of chitosan. Further peaks appeared at 1062.70, 1419.51 and 1577.66 cm^{-1} are due to stretching vibration of C-O-C, C-N and C = O, respectively. However, the IR spectra of Schiff bases of chitosan (Fig. 2b-e) showed strong characteristic absorption peaks at 1610.45, 1645.17, 1631.67 and 1633.59 cm^{-1} are attributed to vibration of C = N of imines formed in chitosan Schiff bases with benzaldehyde, p-chlorobenzaldehyde, p-N,N-dimethylaminobenzaldehyde and p-methoxybenzaldehyde, respectively. Moreover, peaks appeared at 1568.02 and 1577.66 cm^{-1} in spectra of chitosan Schiff bases with p-chlorobenzaldehyde and p-N,N-dimethylaminobenzaldehyde, respectively (Fig. 2c-d) are attributed to deformation of C = C of the aromatic ring, However, the peaks appeared at 810.05 and 827.41 cm^{-1} in spectra of chitosan Schiff bases with p-N,N-dimethylaminobenzaldehyde and p-methoxybenzaldehyde, respectively (Fig. 2d-e) are attributed to vibration of -CH of the aromatic ring. The characteristic peaks due to stretching of C-O pairing in β (1→4) glycosidic bonds of polysaccharide appeared at 1074.28, 1060.78, 1049.20, 1128.28 and 1037.63 cm^{-1} in IR spectra of chitosan Schiff bases with benzaldehyde, p-chlorobenzaldehyde, p-N,N-dimethylaminobenzaldehyde and p-methoxybenzaldehyde, respectively (Fig. 2b-e) (Brugnerotto *et al*., 2001; Dos Santos *et al*., 2005). However, peaks appeared in these spectra (Fig. 2b-e) at 1392.51, 1411.80, 1352.01 and 1371.29 cm^{-1} are attributed to C-N axial deformation. On the other hand, no evidence for the presence of the free aldehydes that can be characterized by appearing peaks around 1665 cm^{-1} due to free aromatic aldehyde groups. These results confirm formation of the Schiff bases through reacting LMWC with aldehydes with no traceable residues of free aldehydes.

Ultraviolet spectral analysis: UV spectra of chitosan and its Schiff bases recorded in range of 100-600 nm were shown in Fig. 3 (Kumirska *et al*., 2010). The adsorption bands of chitosan were noticeably shown in the UV spectrum at 225, 250 and 350 nm. However, the adsorption bands of chitosan Schiff bases exhibited higher intensity than those of chitosan with a red shift differed based on the type of aldehydes bearing various substitutions, where, adsorption bands appeared

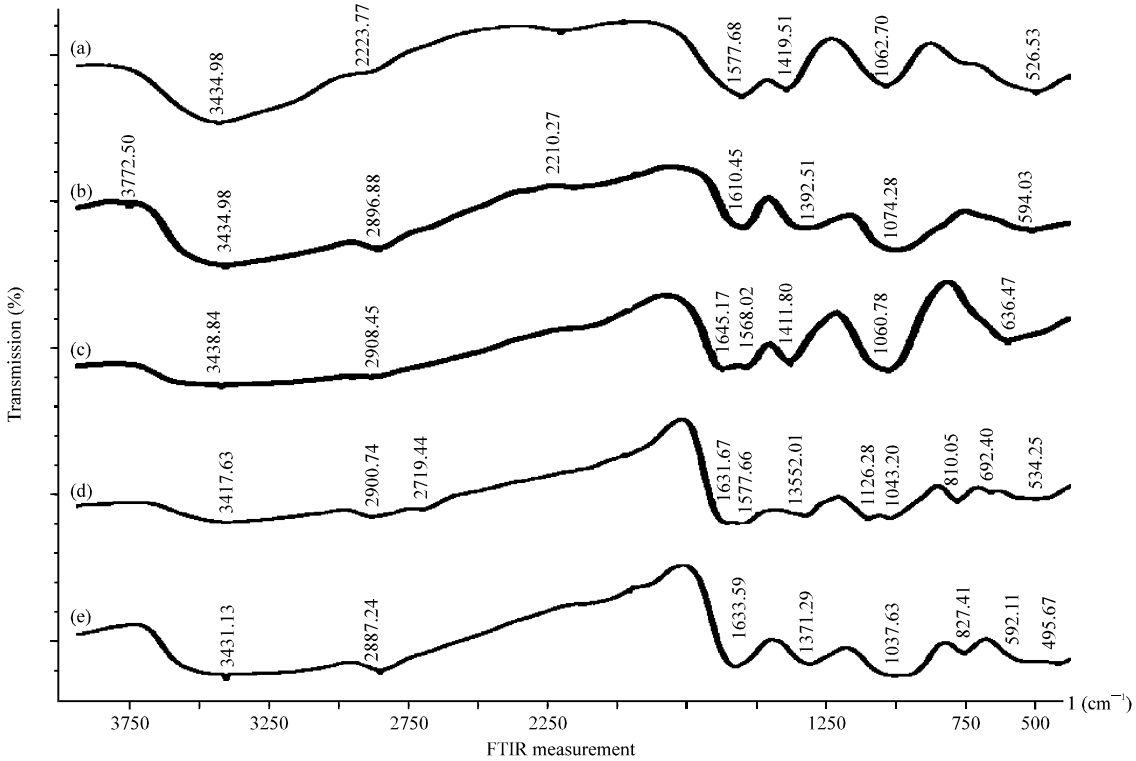

Fig. 2(a-e): (a) FT-IR spectra of chitosan and its Schiff bases with (b) benzaldehyde, (c) p-chlorobenzaldehyde, (d) p-N,N-dimethylaminobenzaldehyde and (e) p-methoxybenzaldehyde

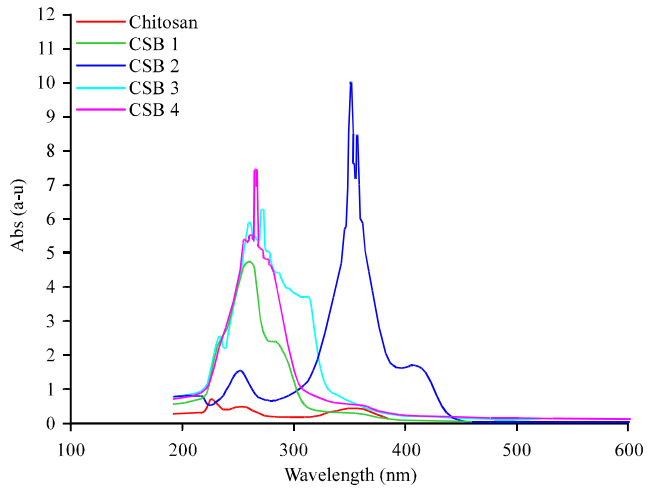

Fig. 3: UV spectra of chitosan and its Schiff bases with benzaldehyde (CSB 1), p-chlorobenzaldehyde (CSB 2), p-N,N-dimethylaminobenzaldehyde (CSB 3) and p-methoxybenzaldehyde (CSB 4)

in range of 250-300 nm and other near 360 nm. The adsorption peaks of chitosan with low intensity that appeared at 225, 250 and 350 nm were attributed to n-σ* transition. By substitution

of aldehydes the amine groups of chitosan are coupled with conjugated phenolic group of the aldehyde which donate the nitrogen atom, steam of electrons and subsequently the energy level of σ^* was decreased. However, coupling such transition with acquired $n\text{-}\pi^*$ transition leads to shift peaks to appear at 250 and 275 nm in case of Schiff base of benzaldehyde, at 250 and 350 nm for Schiff base of p-chlorobenzaldehyde, at 275 and 310 nm for Schiff base of p-N,N-dimethylaminobenzaldehyde, at 250 and 360 nm (with high intensity) for Schiff base of p-methoxybenzaldehyde.

NMR spectral analysis: LMWC and its Schiff bases were chemically characterized by ^{1}H NMR spectroscopy as described in many researches (Dos Santos *et al*., 2005). The chemical substitution on chitosan can be obviously confirmed by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra of Schiff bases presented in Fig. 4 exhibited significant alterations comparing with chitosan. Whereas, the assignments of the ^{1}H NMR signals of the synthesized chitosan Schiff bases were recorded and tabulated in Table 1. These informative spectra impelled us to propose a method to determine the degree of substitution. This determination is based on the ratio of the areas of the proton in imine groups (H-7) to the proton of the pyranose ring (H-2), as presented in the following equation:

$$DS\ (\%) = \frac{A_{imine}}{A_{H\text{-}2}} \times 100$$

Where:

DS = Degree of substitution
A_{imine} = Area of imine proton peak
$A_{H\text{-}2}$ = Area of H-2 proton peak

DS values were calculated using the aforementioned equation. These results that presented in Table 2 indicated that DS values ranged from 21.2-22.9%. Moreover, it was indicated that degrees of substitution for all types of chitosan Schiff bases were close to each other. However, the yield of this process was calculated using the following equation:

$$\text{Process yield}\ (\%) = \frac{\text{Product (Schiff base) weight}}{\text{Reactants (chitosan + aldehyde) weight}} \times 100$$

The calculated yield values were tabulated in Table 2. These values ranged from 73.5 to 84.6% based on type of the reacted aldehyde and degree of substitution.

Table 1: Assignments of the ^{1}H NMR spectra signals of chitosan and chitosan-based Schiff base compounds

	Chemical shift (δ) ppm				
Compound	H-1	H-2	H-Ac	H-7	H-8, 9, 11, 12
Chitosan	4.6928	3.5411	2.4704	Absent	Absent
CSB 1	4.7050	3.5495	2.4704	9.9201	7-8
CSB 2	4.6958	3.5411	2.4704	9.8971	7-8
CSB 3	4.6913	3.5396	2.4704	9.9140	7-8
CSB 4	4.6897	3.6127	2.4689	9.7474	7-8

CSB 1: Chitosan Schiff base with benzaldehyde, CSB 2: p-chlorobenzaldehyde, CSB 3: p-N,N-dimethylaminobenzaldehyde and CSB 4: p-methoxybenzaldehyde

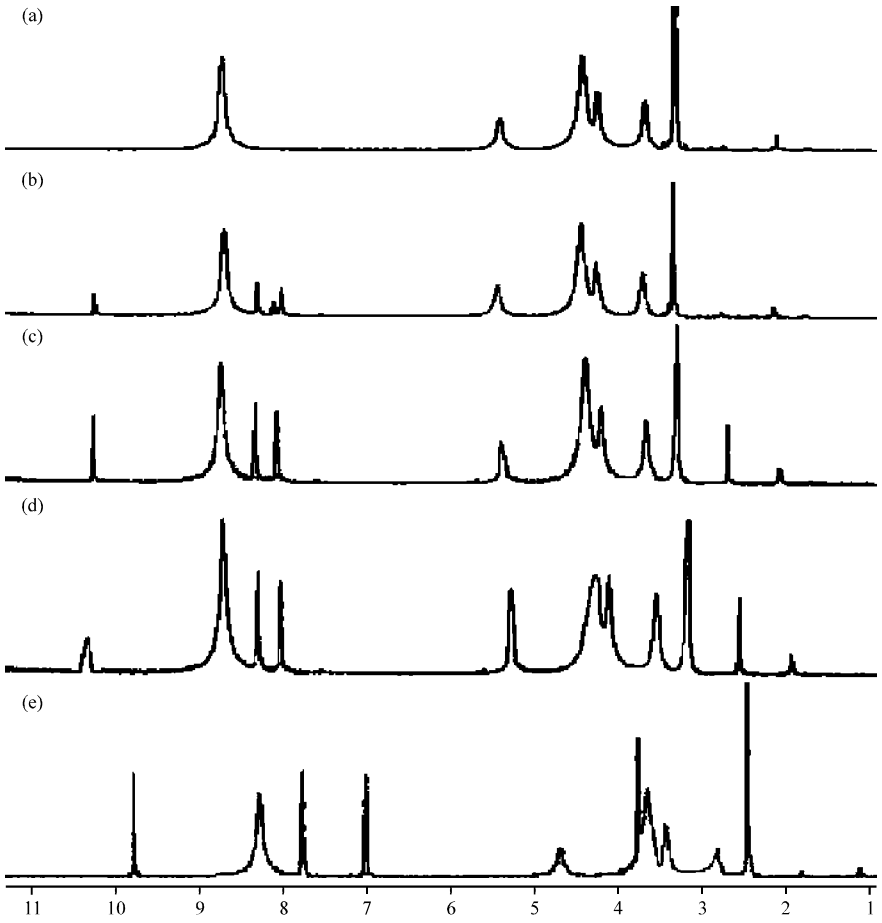

Fig. 4(a-e): (a) ^{1}H NMR spectra of chitosan and its Schiff bases with (b) benzaldehyde, (c) p-chlorobenzaldehyde, (d) p-N,N-dimethylaminobenzaldehyde and (e) p-methoxybenzaldehyde

Table 2: Substitution degree and yield of chitosan-based Schiff base compounds

Chitosan-based Schiff base	DS (%)	Yield (%)
CSB 1	21.2	72.9
CSB 2	22.7	74.1
CSB 3	20.5	72.7
CSB 4	22.9	83.0

CSB 1: Chitosan Schiff base with benzaldehyde, CSB 2: p-chlorobenzaldehyde, CSB 3: p-N,N-dimethylaminobenzaldehyde and CSB 4: p-methoxybenzaldehyde

Microstructure: The topographical features of chitosan and its Schiff bases samples was examined using Scanning Electron Microscopy (SEM). SEM micrographs were presented in Fig. 5. The topography of Schiff bases samples showed noticeable wrinkles. These topographical changes can be attributed to the alteration of inter- and intra-molecular interactions. Introducing phenolic moieties of aldehyde molecules between chitosan polymeric chains leads to weaken the internal forces between polysaccharide chains including hydrogen bonding and subsequently its alignment within the polymeric matrix. On the other hand, hydrophobic interactions can occur

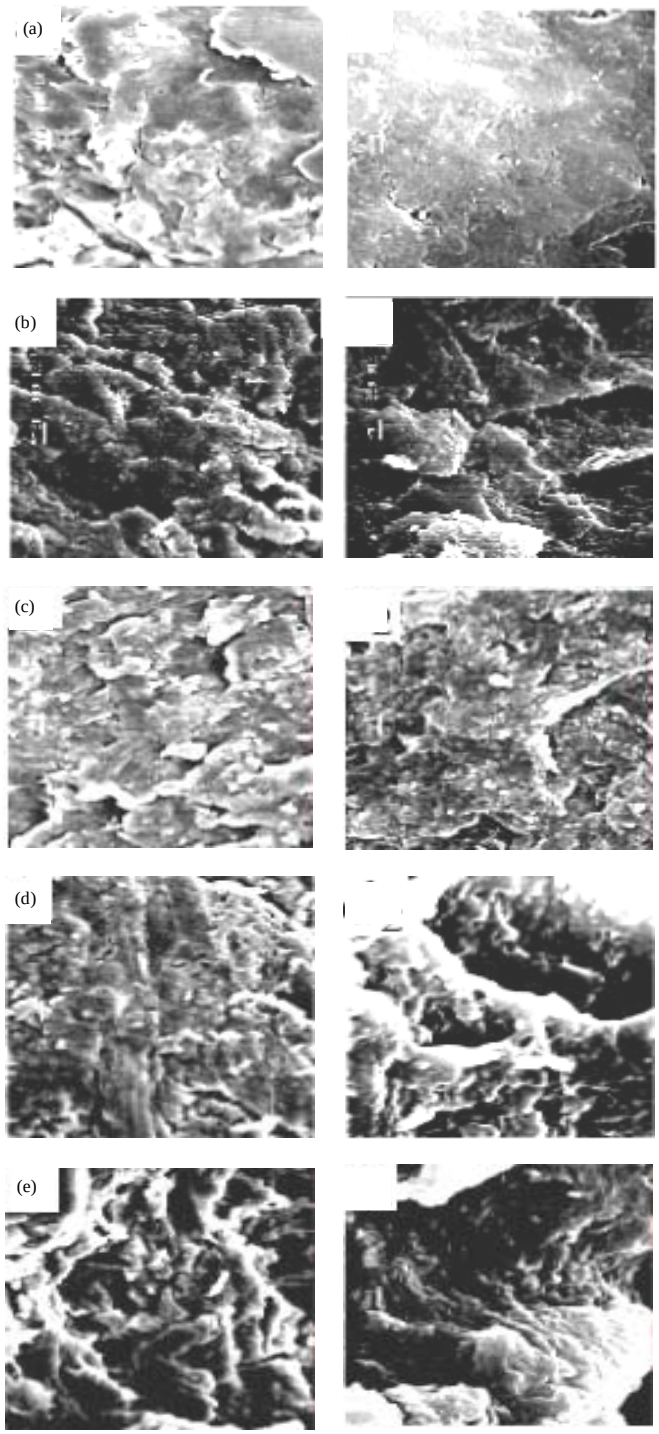

Fig. 5(a-e): (a) Scanning electron micrographs of chitosan and its schiff bases with (b) benzaldehyde, (c) p-chlorobenzaldehyde, (d) p-N,N-dimethylaminobenzaldehyde and (e) p-methoxybenzaldehyde at a magnification power of 1000×(1) and 10000×(2)

between these phenolic moieties that can lie in the internal structures and/or on the surface giving the Schiff base matrices hydrophobic nature. This explanation can be supported with the results of contact angle measurements (Bhuvaneshwari *et al*., 2007).

Contact angle measurement: When a drop of liquid is placed on a solid surface, the liquid spread across the surface to form a thin, approximately uniform membrane (duplex membrane) or spread to a limited extent but remain as a discrete drop on the surface. The contact angle (θ) is defined as the angle formed by the intersection of the two tangent lines to the liquid and solid surfaces at the perimeter of contact between the two phases and the third surrounding phase mostly air or vapor (Wong *et al*., 1992). This contact angle is taken as an indication of superficial hydrophilicity (hydrophobicity) of the membrane. The contact angles of chitosan and chitosan Schiff bases-based films were tabulated in Table 3. These results showed that the contact angles of chitosan Schiff bases-based films were higher than those of chitosan films. The contact angles can be ordered ascending as contact angle (θ) of Schiff bases films based on p-methoxybenzaldehyde (CSB4) benzaldehyde (CSB1) p-chlorobenzaldehyde (CSB3) p-N,N-dimethylaminobenzaldehyde. These results indicate that the chitosan Schiff bases-based films are more hydrophobic than the chitosan film.

Antibacterial activity: The antibacterial activities of chitosan and its Schiff bases against two of the gram-positive bacteria (*Streptococcus pyogens* and *Staphylococcus aureus*) and two of the gram-negative bacteria (*Shigella dysenteriae* and *Escherichia coli*) were shown in Table 4. From these results, it can be generally indicated that the inhibition efficiency of both of chitosan and its Schiff bases is higher on gram positive bacteria comparing with that on gram negative ones. Moreover, the viable population of the different species of bacteria was much less on treating their

Table 3: Contact angles of chitosan and chitosan-based Schiff bases films

Schiff base-based film	θ_R	θ_L	Mean
Chitosan	74.55±0.22^{d}	80.05±0.17^{e}	77.3±0.19^{e}
CSB1	80.75±0.18^{c}	82.40±0.34^{d}	81.5±0.26^{c}
CSB2	81.50±0.15^{b}	90.10±0.17^{b}	85.8±0.11^{b}
CSB3	85.70±0.24^{a}	91.30±0.21^{a}	88.5±0.23^{a}
CSB4	70.82±0.33^{e}	88.48±0.24^{c}	79.7±0.29^{d}

CSB 1: Chitosan Schiff base with benzaldehyde, CSB 2: p-chlorobenzaldehyde, CSB 3: p-N,N-dimethylaminobenzaldehyde and CSB 4: p-methoxybenzaldehyde, n = 5, Values not followed by the same letters are significantly different at p≤0.05

Table 4: Effect of various aromatic substituent groups on antibacterial activity of Schiff bases

	Inhibition efficiency (%)				
	Chitosan and its Schiff bases				
Bacterial species	Chitosan	CSB1	CSB2	CSB3	CSB4
Streptococcus pyogens	2.10±1.1^{lm}	48.5±0.9^{cd}	57.1±0.6^{c}	57.8±1.1^{bc}	69.0±0.4^{a}
Staphylococcus aureus	38.0±2.1^{ef}	47.0±0.8^{d}	16.0±2.3^{j}	45.1±1.3^{de}	58.6±1.1^{b}
Shigella dysenteriae	2.30±0.2^{l}	2.5±0.1^{l}	13.3±2.4^{jk}	8.6±2.2^{k}	31.8±1.6^{g}
Escherichia coli	21.4±1.2^{i}	30.0±1.1^{g}	37.0±1.3^{f}	41.2±1.9^{e}	23.7±1.7^{h}

CSB 1: Chitosan Schiff base with benzaldehyde, CSB 2: p-chlorobenzaldehyde, CSB 3: p-N,N-dimethylaminobenzaldehyde and CSB 4: p-methoxybenzaldehyde, n = 5, Values not followed by the same letters are significantly different at p≤0.05

culture media with the chitosan Schiff bases than that treated with chitosan, i.e., chitosan Schiff bases have higher inhibition efficiency than non-modified chitosan. On the other hand, Schiff base formed by condensation of chitosan with p-methoxybenzaldehyde (anisaldehyde, naturally occurring aldehyde) among all synthesized Schiff bases exhibited highest antibacterial activity.

As indicated from this study, Schiff base of chitosan exhibited a synergetic inhibitory effect against bacteria with that owing to the electrostatic interaction between cationic components of chitosan (NH_3^+ groups) and anionic residue of bacteria (-COO^- or PO_4^{-3} groups). This can be explained on the basis that the π-electrons of imine groups (C = N) of Schiff bases enhance the lipophilicity of chitosan which can facilitate penetration of the chitosan Schiff base molecules into the microbial cell membranes and then can disturb the respiration process of the cell and block the synthesis of proteins which hinder the further growth of bacteria (Imran *et al*., 2007).

On the other hand, substitutions on the phenyl ring of the aromatic aldehyde play a key role in change of the physicochemical properties and subsequently biological properties (Soliman *et al*., 2012). Whereas, Schiff base which has been synthesized by condensing chitosan with p-methoxybenzaldehyde (anisaldehyde, naturally occurring aldehyde) exhibited the highest antibacterial activity. These results are in agreement with those reported previously in many studies on the inhibitory efficiency of anise oil (Parasa *et al*., 2012; Singh *et al*., 2007) and anisaldehyde, the major component of anise oil on bacteria. Therefore, high antibacterial activity of Schiff base of chitosan with p-methoxybenzaldehyde shown in this study may be due to the structural characteristics of Schiff base of anisaldehyde in which anisaldehyde contains methoxy group at the Para position of phenolic ring that is a good electron donating group. By condensation amine group of chitosan and carbonyl group of anisaldehyde, the phenyl ring withdraw electrons from this methoxy group. These structural properties lead to originate higher electron density from the π-electrons of imine groups (C = N) of Schiff base than the other Schiff bases produced from the other three types of aldehydes. Furthermore, this π-electrons enhance the hydrophilicity of the resultant Schiff base (low contact angle).

The effect of molar ratio of anisaldehyde to chitosan on the inhibition efficiency of the resultant Schiff bases were presented in Fig. 6. These results indicated that this ratio had a significant effect

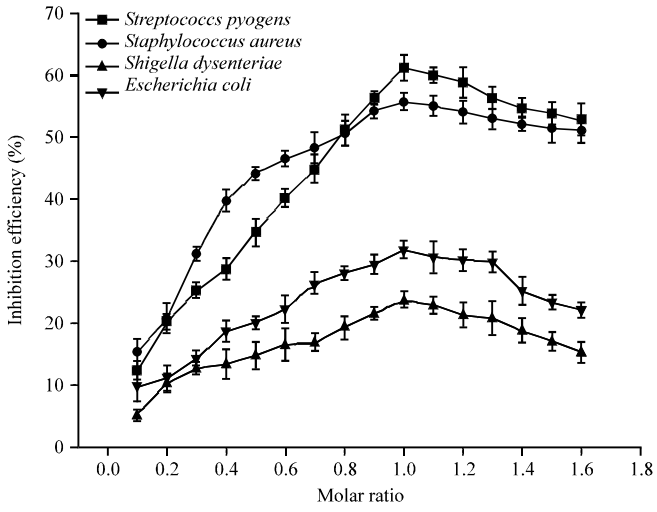

Fig. 6: Effect of molar ratio of anisaldehyde/chitosan on inhibition efficiency of Schiff bases

on the antibacterial activity, where, increasing the molar ratio of anisaldehyde/chitosan led to a gradual increase in the inhibition efficiency of the resultant Schiff bases up to molar ratio of 1:1, then this efficiency was re-decreased with the further increase of the molar ratio of anisaldehyde/chitosan. These results can be attributed to the alterations of hydrophilic/hydrophobic balance occurring with the further increase of the molar ratio of anisaldehyde/chitosan leading to reduce the product solubility and suppress the electrostatic interactions between the protonated amino groups of the synthesized Schiff bases and the anionic residues of the microbial cell membranes and subsequently decrease the inhibitory efficiency of chitosan Schiff bases. By another words, these chemical substitutions can lead to change the Relative Adsorption Ratio (RAR) and permeability of chitosan resulting in a variation in the susceptibility of bacterial cells.

From the results presented in Fig. 6 indicated also that the resultant Schiff bases based on p-methoxybenzaldehyde at different molar ratio of aldehyde/chitosan exhibited higher antibacterial activity against gram positive bacteria than gram negative ones. Besides, Such Schiff base had higher inhibitory efficiency against *Streptococcus pyogens* than that exhibit against *Staphylococcus aureus*.

CONCLUSION

Four chitosan-based Schiff base compounds were successfully synthesized by condensation of low molecular weight chitosan with benzaldehyde and its p-Cl, p-N(CH_3) and p-MeO derivatives under mild acidic conditions. These poly Schiff bases have been chemically and structurally characterized by UV, FTIR and ^{1}H NMR spectral analyses and scanning electron microscopy. The degree of substitution was determined by a proposed ^{1}H NMR spectrometric procedure. It has been found that the substituents noticeably affect on the substitution degree. The formation of Schiff base groups led to the difference of crystallinity that can be attributed to the spatial hindrance and hydrophobic forces in the aromatic substituent groups. Moreover, bioactivities of chitosan-based Schiff bases was evaluated against four bacterial strains, two gram-positive bacteria (*Streptococcus pyogenes, staphylococcus aureus*) and two gram-negative bacteria (*Shigella dysenteriae, Escherichia coli*). These Schiff bases exhibited stronger anti-bacterial activities comparing with that of chitosan of similar molecular weight. Schiff base of anisaldehyde (naturally occurring compound) had higher antibacterial activity than other Schiff base compounds. This may be due to improve hydrophilic/hydrophobic balance required for enhancing the bioavailability of chitosan derivatives. On basis of these results, bioactivities of low molecular weight of chitosan and chito-oligomer-based derivatives may provide novel insights into the functionality of chitosan derivatives.

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