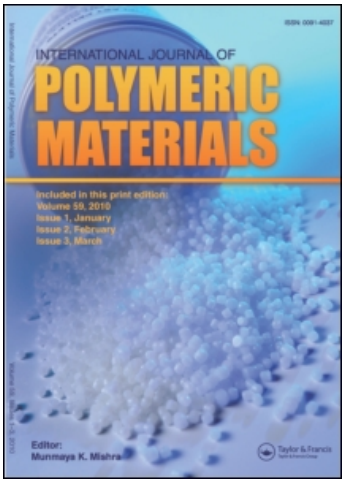

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Preparation, Antibacterial and Physicochemical Behavior of Chitosan/Ofloxacin Complexes

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Preparation, Antibacterial and Physicochemical Behavior of Chitosan/ Ofloxacin Complexes

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A novel chitosan derivative with ofloxacin (OFX) has been successfully prepared. The IR and 1H-NMR results revealed that the chitosan/ofloxacin (CH-OFX) complex exhibited an electrostatic interaction. The crystalline and surface morphology were analyzed by X-ray diffraction (XRD) and scanning electron microscopy (SEM). The antimicrobial activity of the complexes against various micro-organisms viz. *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* was tested. It was established that their antibacterial activity is up to four times greater than that of free quinolone drug and chitosan, probably due to the conjunction of favorable pharmacokinetics, excellent bacterial susceptibility and good stability towards metabolic degradation.

Keywords antibacterial activity, chitosan, ofloxacin, SEM

INTRODUCTION

Ofloxacin, a fluoroquinolone antibiotic, is an anti-infective agent under extensive investigation for the treatment of ocular infections [1]. The drug is bactericidal against a broad spectrum of bacterial organisms, including various species of *Staphylococcus*, *Streptococcus*, *Pseudomonas* and other

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species isolated from nonocular and ocular sources [2,3]. It exhibits potent in vitro activity against a wide array of gram-positive and gram-negative species, chlamydial organisms, certain anaerobes, and other less common pathogenic organisms [4]. While widely used to treat infections, such as those affecting the gastrointestinal and respiratory tracts, it is also used to treat urinary tract infections [5–8]. This is because ofloxacin can achieve high concentrations in the urinary tract in unchanged form after oral administration [9]. Ofloxacin, ciprofloxacin and other fluoroquinolones inhibit bacterial DNA gyrase, an enzyme active in the process of DNA supercoiling, which allows the DNA strands within the bacterial cell to be compacted in an orderly fashion. Inhibition of DNA gyrase permits the DNA strands to become entangled, thus preventing DNA replication, transcription, recombination, and repair, and resulting in bacterial death [10,11]. The appearance of ofloxacin-resistant bacterial strains is uncommon, and when resistant strains do appear they generally have a lower probability of being effective pathogens. Because ofloxacin is a member of a structurally distinct class of drugs, the fluoroquinolones, there is little likelihood of cross-resistance to other classes of antibiotics. Ofloxacin is commonly used in clinics but its bioavailability and pharmacokinetic profile needs to be described in local population and environments. Novel *N*-carboxyethylated and *N*-carboxymethyl chitosan derivatives revealed an antigenotoxic activity and intraocular penetration effect toward acridine orange and ofloxacin, and that different mechanisms of protection are involved in elicitation of this effect [12,13].

It is well-known that chitosan from ocean resources has wide application in various areas as a result of its biocompatibility and nontoxicity, especially in the pharmaceutical and biomedical fields, such as drug delivery, wound dressing, food packing and antimicrobial agents [14–18]. It is well-known that microspheres and microcapsules prepared from chitosan matrices have found wide application in controlled drug delivery [19–22], including hirudin [23], bovine serum albumin [24], and the nerve growth factor [25]. Chitosan has one primary amino and two secondary alcoholic groups for each anhydroglycosidic unit. Due to the easy availability of free amino groups in chitosan, it carries a positive charge and thus in turn reacts with many negatively charged surfaces/polymers and also undergoes chelation with metal ions [26] like cobalt [27]. Chitosan is a weak base and is insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution (pH 6.5), which can convert the glucosamine units into a soluble form $R\text{-}NH_3^+$ [28–31]. It gets precipitated in alkaline solution or with polyanions and forms a gel at lower pH.

Chitosan, a well-known cationic polymer of natural origin, has shown excellent ocular compatibility, and also the ability to interact with the negatively charged conjunctiva [32]. N-methylated chitosan microspheres encapsulated with ofloxacin and chitosan/polyethylene glycol blend films encapsulated with ciprofloxacin hydrochloride are used in controlled drug release [33,34]. Felt et al. [35] reported that co-administration of ofloxacin

and chitosan in eyedrops resulted in increased antibiotic bioavailability and time of efficacy in tear fluid compared to commercial eyedrops and ascribed this effect to the high viscosity of the chitosan solution.

In this paper, a simple, sensitive and specific agar diffusion bioassay method is described for the determination of physiochemical and antibacterial activity of CH-OFX pro-drug complex.

EXPERIMENTAL

Materials

Chitosan powder was purchased from Sigma Aldrich and its average molecular weight was about <5000 g/mole (*Mv*) and a degree of deacetylation (DD) of 85%. Ofloxacin was received as a gift sample from Ranbaxy India. The test strains, *E. coli* MTCC 1303, *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 10145 and *S. aureus* ATCC 6538, were used by IMTECH, Chandigarh. All solvents and solutions were used as such, without further purification. The purity of all the synthesized compounds has been checked by thin layer chromatography using silica gel with different nonaqueous solvent systems.

Measurements

Fourier transform infrared spectroscopy (FTIR) measurements were recorded on a Perkin Elmer RX1 FTIR spectrophotometer using KBr discs. The morphology was studied by using scanning electron microscopy (SEM) with JEOL JSM-5200 at 15 kV. The ^{1}H NMR spectra have been recorded on a 400 MHz Jeol FX90Q FTNMR spectrometer using TMS as an internal standard in DMSO d_6. Electronic absorbance spectra (ultraviolet spectra) were recorded on a 1650PC UV spectrometer using a 1.0 cm quartz cell at ambient temperature, and XRD measurements on powder samples were performed with the XPERT-PRO (PW3050/60) using Cu-K_α radiation ($\lambda = 1.54060$ Å), operated at 30 kV and 30 mA. The powder samples were placed in 0.8 mm diameter Lindemann glass capillaries. The sample-detector distance was 10 cm. The intensity vs. scattering angle (2θ) was plotted.

Preparation of CH-OFX Complexes

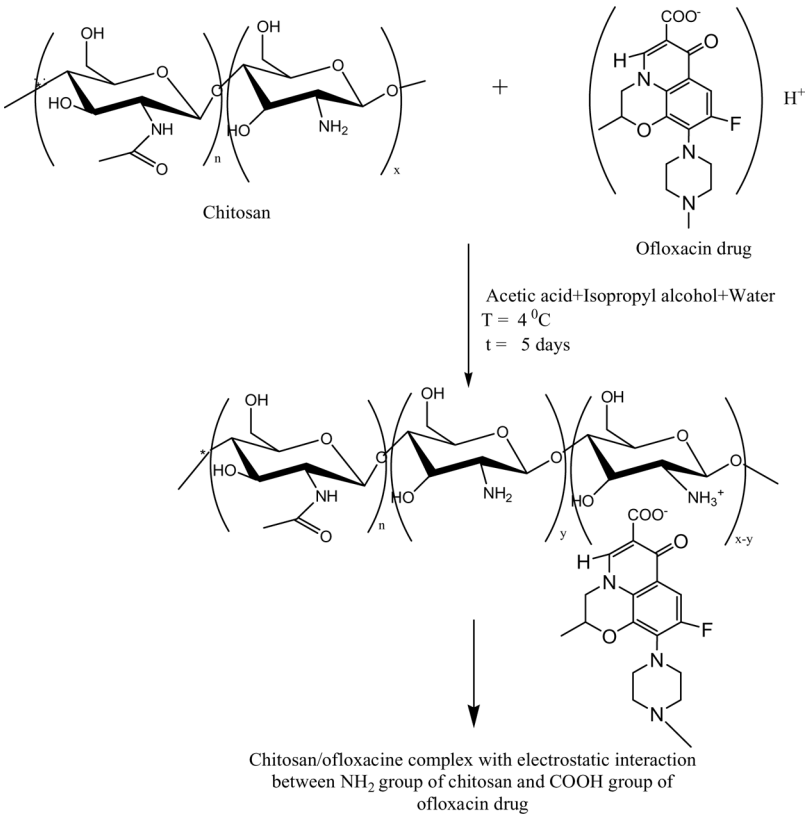
To synthesize CH-OFX complexes, 50 mg of chitosan powder was dispersed into 10:20:2 mL of glacial acetic acid (1% w/v), isopropyl alcohol and water to dissolve the chitosan. Water is added to raise the dielectric constant of the isopropyl alcohol and the mixture was stirred until the polymer was completely dissolved. Then, ofloxacin drug (0.355 g) was gradually added to the above

solution over 30 min under cooling with ice water. The ratio of chitosan and drug (OFX) encapsulation is 1:7 in solution. The mixture was then stirred for 3 h at room temperature (20°C) and subsequently kept at 5°C for 5 days (Scheme 1).

Each mixture was successively washed with isopropyl alcohol and water mixture (5:1, v/v) and dried in air. The product was recrystallized from chloroform and isopropyl alcohol solution and washed several times with an isopropyl alcohol/water mix-solvent. The final product was dried, and kept in a desiccator until the analysis was performed.

Biological Activity: Antimicrobial Assay

The antimicrobial activity test of the CH-OFX complex derivative was carried out using the agar plate diffusion method. The solution (0.1 and 0.01% in DMSO) of the CH-OFX was absorbed in sterilized discs (approximately 60 μL of solution) and the antimicrobial activity was evaluates against two gram-positive (*B. subtilis* and *S. aureus*) and two gram-negative (*E. coli*



Scheme 1: Synthesis of CH-OFX complex derivative.

and *P. aeruginosa*) bacterial test cultures. The sterilized discs were placed on nutrient agar plates making lawns of the above test cultures. The plates were then incubated at 37°C for 24 h. The diameters of the inhibitory zone surrounding the discs were then measured.

RESULTS AND DISCUSSION

Ultraviolet Spectroscopy

Chitosan itself is transparent in the UV and visible region, and so its structure is hard to characterize by spectroscopy methods. However, we can overcome this natural handicap by borrowing chromophores from foreign compounds. The acid group moieties play the role of reporter molecules for the spectroscopy study, from which the structural and antibacterial activity is deduced. Figure 1(a) shows the UV spectra of OFX in aqueous acid (225, 226, 256 and 362 nm) and aqueous base (294 and 330 nm). Ofloxacin in aqueous solution has two peaks, a strong peak at 294 nm and a weak peak at 330 nm. The observed strong peak corresponds to the chromophore involving the N-1 position to the carboxylic chromophore involving the nitrogen of the piperazinyl group to the carbonyl group, while the weak absorption peak corresponds to the chromophore involving the nitrogen of the piperazinyl group to the carbonyl group. The UV spectrum band of CH-OFX (Figure 1(b)) complex showed red-shifted emission at 307 and 338 nm because the attachment of the group to the backbone will enlarge the degree of delocalization π-bond on the polymer chain due to complexion of chitosan with OFX.

Fourier Transform Infrared Spectroscopy (FTIR)

The infrared spectra of chitosan and CH-OFX complex derivative are shown in Figure 1. For the chitosan spectrum (Figure 2(a)): 3422 cm^{-1} (which is assigned to the N–H and hydrogen bonded O–H stretch vibrational frequencies), while a sharp (shoulder) peak at 3610 cm^{-1} is that of free O–H bond stretch of glucopyranose units [36], 2921 and 2867 cm^{-1} (C–H stretch), 2364 cm^{-1} (C–N asymmetric band stretching), 1653 cm^{-1} (amide II band, C–O stretch of acetyl group), 1592 cm^{-1} (amide II band, N–H stretch) 1375 cm^{-1} (asymmetric C–H bending of CH_2 group) and 1071 cm^{-1} (skeletal vibration involving the bridge C–O stretch) of glucosamine residue. CH-OFX complex (Figure 2(b)): the spectral band appear at 3404 cm^{-1} (axial O–H group of chitosan), 3037 and 2918 (symmetric or asymmetric CH_3 stretching vibration attributed to OFX and pyranose ring of chitosan) 2777 cm^{-1} (NH_2^+ and NH^+ stretch), 2355 cm^{-1} (C–N asymmetric band stretching), 1714 cm^{-1} (carbonyl group vibration), 1530 cm^{-1} (symmetrical bending stretching of amine salt NH_3^+), 1621 cm^{-1} (C=O asymmetrical band stretching of COO^- anion),

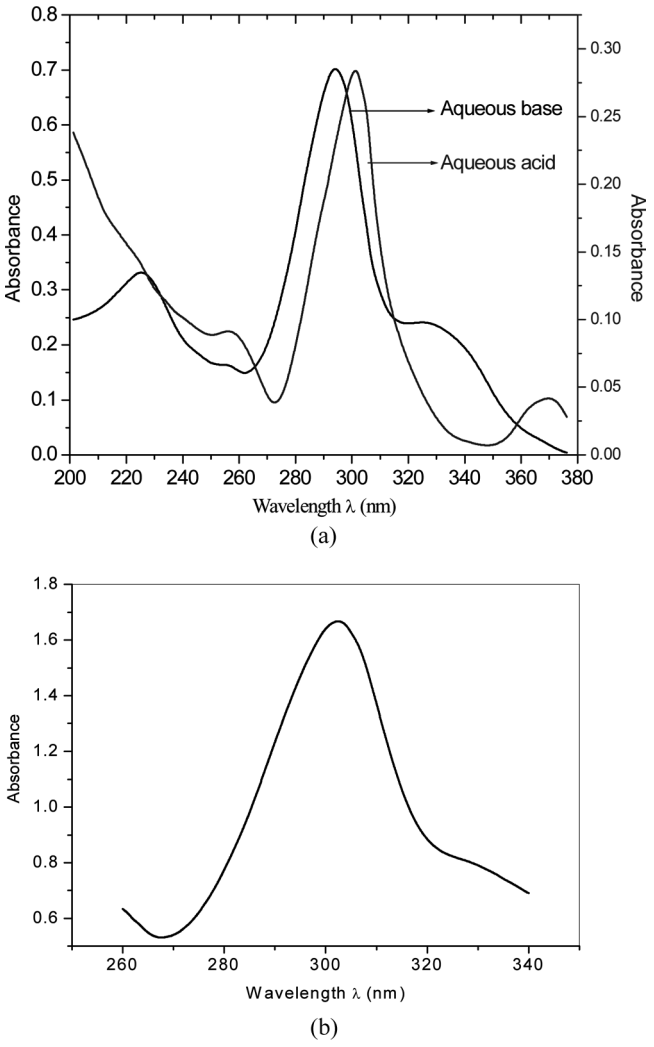
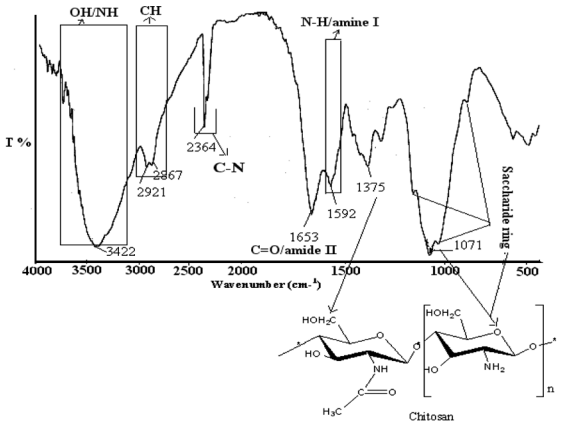


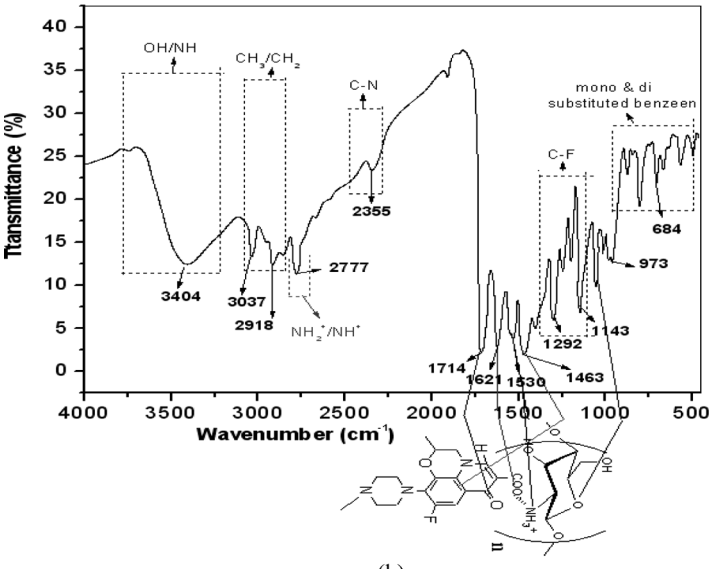
Figure 1: UV spectra of (a) OFX in aqueous acid and aqueous base and (b) CH-OFX complex derivative in DMSO solution with concentration 50 mg/L.

1463 cm^{-1} (C=C band stretching of OFX), 1292–1199 cm^{-1} (C–F band stretching of OFX), 1143–1008 cm^{-1} (superimposed C–O–C band stretching of chitosan and OFX) and 973–684 cm^{-1} (mono and di-substituted benzene ring).

Compared with that of chitosan, the IR spectra of CH-OFX complex showed that the purely electrostatic nature of the interaction between chitosan (NH_3^+) and OFX (COO^-) is the new signal at 1530 cm^{-1}, and should be assigned to the absorption band characteristic of the symmetrical bending-stretching of amine salt [28]. This result suggested that the NH_2 group on chitosan chains was protonated by the H^+ supplied by the acid group [37,38] by the OFX. The intensity



(a)



(b)

Figure 2: FTIR spectra of (a) chitosan and (b) CH-OFX complex derivative.

of these bands depends on the amount, type and bulkiness of the acid. Degree of substitution (DS) also affects the intensity band, and OH stretching which becomes broader and moves to a lower frequency with increasing DD up to ~50%, indicating an increase in the disordered structure.

X-ray Diffraction (XRD)

The X-ray diffraction pattern (XRDP) for chitosan and the CH-OFX complex derivative is shown in Figure 3. Chitosan (Figure 3(a)) exhibits three

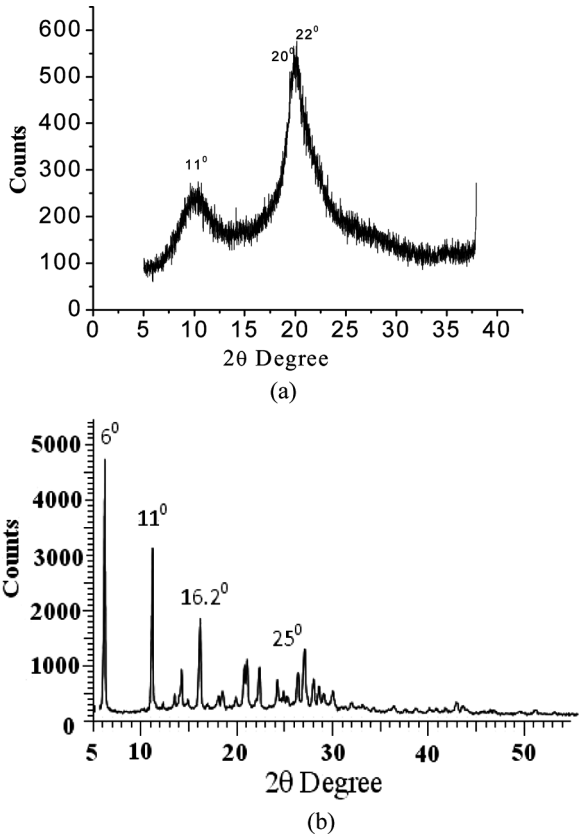


Figure 3: XRD spectra of (a) chitosan and (b) CH-OFX complex derivative.

reflection falls at $2\theta = 11^\circ$, 20° and 22°. Chitosan shows very broad lines especially for the smaller diffraction angles, thereby indicating that long-range disorder. The intense small angle peaks in chitosan acid complexes exhibit higher long-range order. The main diffractive region in the CH-OFX complex (Figure 3(b)) is at $2\theta = 6^\circ$, 11° and 16.2°, with the highest peak intensity of about 4600, 3200, and 1855 counts, respectively. This indicates that the long-range disorder increases towards the higher angle. This long-range amorphous structure in the CH-OFX complex gives way to greater short-range order for diffracting atom pairs at 2θ, which is less than 25°.

Multiple scanned angles at lower intensity of CH-OFX complex could be due to impurities. Since sample size and experimental XRPD setup are the same, the lattice arrangement and the intensity of diffractive light, especially within polymer derivatives may be varied. Intensity is not a routine predictor of crystal structure, but can be obtained from XRDP and reflects the unit cell dimensions. Intensity is therefore a means of obtaining structural information

from powder diffraction. In addition, the lattice dimension is also related to the interplaner distance, (d) can easily be described for the particle size and geometry of the unit cell [39,40]. XRDP proves here that the crystal lattice has transformed from an amorphous structure into a relatively more crystalline structure in chitosan to a CH-OFX complex derivative.

1H-NMR Study of CH-OFX Complex

The ^{1}H-NMR spectra of chitosan and CH-OFX complex derivative are shown in Figure 4. Proton assignment of chitosan (Figure 4(a)), $\delta = 4.89$ ppm (s) appears for chemical shift of the internal standard, $\delta = 4.37$ ppm (s) is due to chemical shift of the acetal proton (C−H) of the glucosamine overlaps the chemical shift of the internal standard, $\delta = 3.27$ ppm (s) for $-CH-NH_2$ protons (H2), $\delta = 3.92$–3.72 ppm (m) for (H3, H4, H5 and H6) protons of glucosamine ring, $\delta = 3.27$ ppm appear for chemical shifts of (H2) protons and upfield $\delta = 2.04$ ppm (s) for ($-NHCO-CH_3$) acetamido protons. For ^{1}H-NMR (DMSO-d_6 + $CDCl_3$) spectra of CH-OFX complex (Figure 4(a)), $\delta = 8.93$ ppm (s) due to NH_3^+ protons of amine salt, $\delta = 8.24$ ppm (s) O−H protons, $\delta = 7.56$ ppm (s) aromatic proton, $\delta = 4.8$ ppm (s) CH−F proton of ofloxacin, 4.55–4.31 ppm (m) is due to CH_2 proton of ofloxacin, $\delta = 3.9$ ppm (s) N−CH_3 proton of ofloxacin $\delta = 3.2$ ppm (s) CH_2OH proton of chitosan, $\delta = 2.49$–2.23 ppm (q and d) is due to CH−CH_3 unit of ofloxacin and $\delta = 1.45$–1.19 ppm (m) is due to glucosamine residue (H2 H3 H4 H5) of chitosan.

Compared with chitosan, the CH-OFX complex derivative shows a new proton signal at $\delta = 8.93$ which confirms the complex formation between the NH_3^+ group of chitosan and the COO^- group of OFX under mild conditions.

Scanning Electron Micrographs (SEM)

The scanning electron micrograph (Figure 5(a) and (b)) has been employed for the observation of the surface morphology of the chitosan and CH-OFX complex derivative. The SEMs of the native chitosan are shown in Figure 5(a)(i) and (ii). It exhibited nonporous, smooth membranous phases consisting of dome-shaped orifices, microfibrils and crystallites. It also exhibited flat lamellar phases on which a large number of protruding microfibrils are evident.

The microstructure images of the CH-OFX pro-drug complex derivative {Figure 5(b)(i), (ii), (iii) and (iv)}, prepared by mixing, showed a polyphasic, rough surface and microporous structure in which OFX particles (with irregular forms) are relatively well-dispersed in the chitosan matrix. The result shows acid was successfully integrated into the matrix with no visible agglomerate formation at low particle amounts.

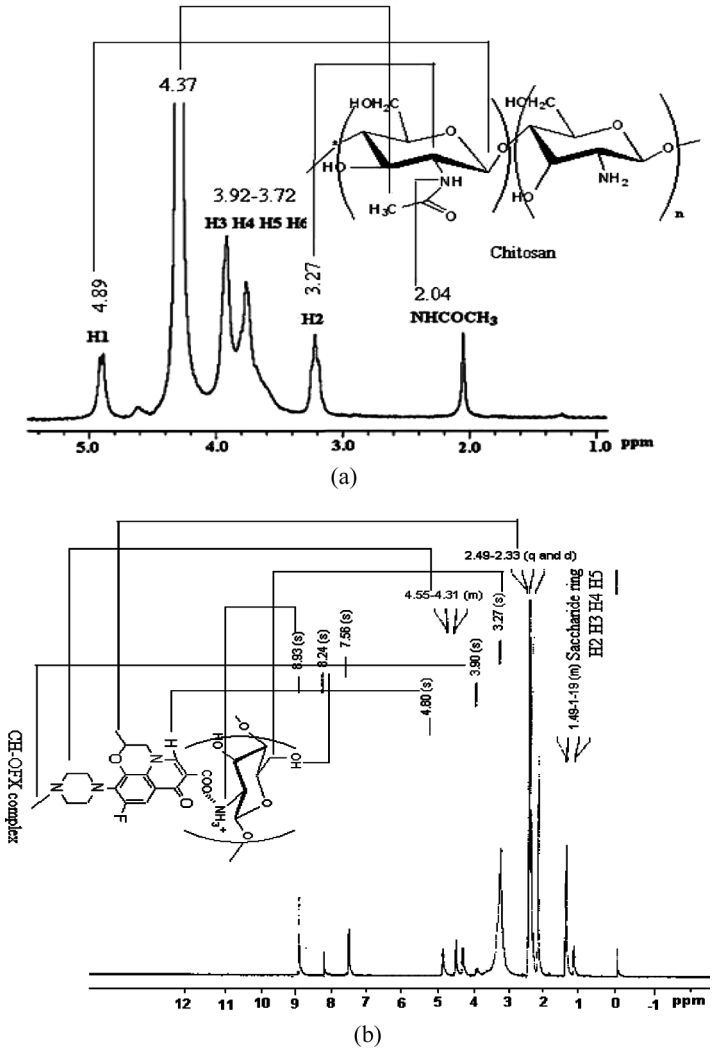


Figure 4: ^{1}H-NMR spectra of (a) chitosan and (b) CH-OFX complex derivative.

Antimicrobial Activity Tests and Biological Relevance of CH-OFX Complexes

Figure 6 shows the inhibitory effect of CH-OFX complexes against *E. coli* (a and a′), *B. subtilis* (b and b′), *P. aeruginosa* (c and c′) and *S. aureus* (d and d′) in 0.1% and 0.01% DMSO solution. The inhibitory activity was measured based on the average diameter of the clear inhibition zone. If there was no clear zone surrounding as revealed in Figure 6 (P and P′), it was seen that there was no inhibitory effect and furthermore, the diameter was valued as zero. The control aqueous acetic acid (0.1%) solution is shown to have a very

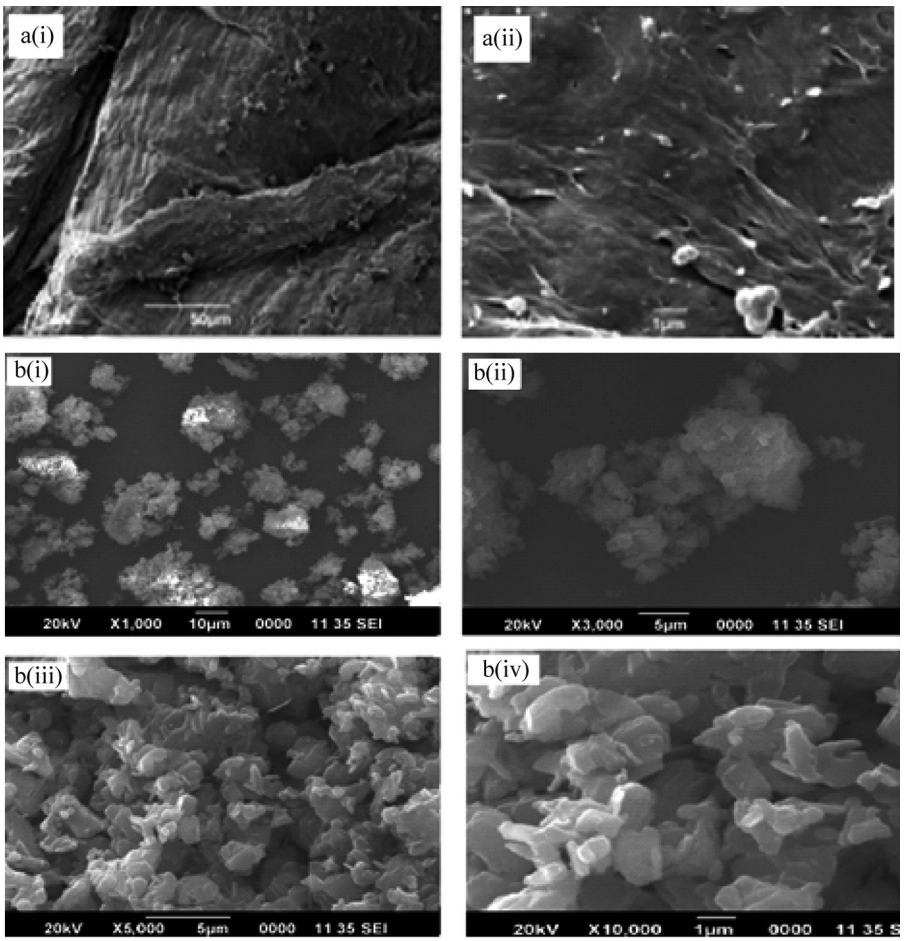


Figure 5: Scanning electron micrographs of (a) (i) and (ii) chitosan and (b) (i, ii, iii and iv) CH-OFX complex derivatives.

small inhibitory effect (16 mm) against *B. subtilis*. The details of antimicrobial activity of the CH-OFX complex derivative are shown in Table 1.

In terms of the surrounding clearing zone, CH-OFX complexes showed a very clear inhibitory zone of both gram-positive and gram-negative bacteria. However, decreasing levels of OFX at a lower concentration did not reveal a significant increased inhibitory effect. It was generally caused by the maximum capability of chitosan polymer to carry active agents beside the occurrence of functional groups interaction phenomenon. The antimicrobial effect of the CH-OFX complex occurred without migration of active agents [41]. As CH-OFX complex is in a solid form, therefore, only organisms in direct contact with the active sites of CH-OFX complexes is inhibited. CH-OFX complexes are incapable of diffusing through the adjacent agar media [42].

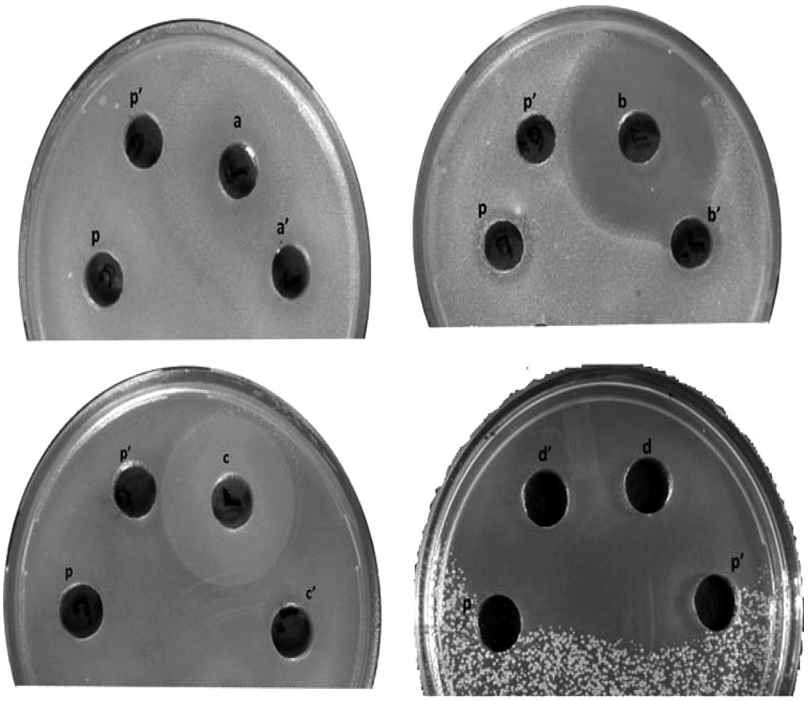


Figure 6: Inhibitory effect of CH-OFX complex derivative against (a and a') *E. coli*, (b and b') *B. Subtilis*, (c and c') *P. aeruginosa* and (d and d') *S. aureus* in 0.1% and 0.01% DMSO solution, respectively, and (P and P') shows the inhibitory effect of control 0.1% and 0.01% solution made in aqueous acetic acid.

The agar diffusion test is a method commonly used to examine antimicrobial activity regarding the diffusion of the compound tested through a water-containing agar plate. The diffusion itself is dependent on the size, shape and polarity of the diffusion material.

Table 1: Diameter (mm) of inhibitory zone of the control and CH-OFX solution against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*.

	Diameter (mm) of inhibitory zone in aq. acetic acid solution of the control		**Diameter (mm) of inhibitory zone in DMSO solution (mm) of the sample**	
Test culture	**0.1%**	**0.01%**	**0.1%**	**0.01%**
Gram positive				
B. subtilis	16	Nil	42	16
S. aureus	Nil	Nil	60	50
Gram negative				
E. coli	Nil	Nil	25	19
P. aeruginosa	Nil	Nil	34	Nil

In fact, one of the reasons for the antimicrobial character of chitosan is that it is a positively charged amino group which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms [43]. In the gram-positive bacteria, the major constituent of its cell wall is peptidoglycan and there is very little protein. The cell wall of gram-negative bacteria also has an outer membrane, which constitutes the outer surface of the wall [44]. Jiang et al. [45] observed that electron micrographs for gram-positive and gram-negative bacteria in the presence of chitosan show the cell membrane of gram-positive bacteria was weakened or even broken, while the cytoplasma of gram-negative bacteria was concentrated and the interstices of the cell were clearly enlarged. There is a parallel relationship between antibacterial activity of ofloxacin and its inhibitory action against DNA gyrases from ofloxacin-susceptible and ofloxacin-resistant clinical isolates of microorganism. This study indicated that the mechanisms of the antimicrobial activity of chitosan were different between gram-positive and gram-negative bacteria.

CONCLUSION

Chitosan is a natural biodegradable polymer where OFX is a synthetic fluoroquinolone antibiotic. The electrostatic interaction between the polycationic nature of chitosan and the anionic form of OFX in pro-drug is confirmed by means of FTIR and ^{1}H-NMR spectroscopy. The morphology as well as the compatibility of the CH-OFX complex has been studied using SEM and XRD methods. From these studies the heterogeneity of the complexes has been predicted. Thermal stability of the CH-OFX complex has resulted in a crystalline structure and a drop in thermal stability due to disruption of the intermolecular hydrogen bonds in the polymer chain. OFX and chitosan alone have an antimicrobial effect against both gram-positive and gram-negative bacteria, but the complexation (CH-OFX) is better enhanced by the antimicrobial activity. These results indicate that the high antibacterial activity of CH-OFX complexes and the related new quinolone derivative can be explained by their potent inhibitory activities against DNA gyrase in bacterial cells. The above findings open new prospects for promising materials, which undoubtedly widens the scope of applications of chitosan-based material in pharmaceutical applications.

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