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RESEARCH ARTICLE

Preparation, characterization and antimicrobial evaluation of novel cinnamyl chitosan Schiff base.

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Abstract

Cinnamyl chitosan Schiff base was prepared by coupling chitosan with trans-Cinnamaldehyde -the mean component of cinnamon oil- under acidic conditions to form cinnamyl chitosan Schiff base. Parameters affecting coupling process such as chitosan/cinnamaldehyde molar ratio, reaction time and reaction temperature were studied to optimize reaction conditions. The progress of Schiff base formation was monitored by increasing of absorption of polymer solution at certain wave length 310 nm and decline in ion exchange capacity of chitosan derivatives from 5.78 to 4.16 meq/g. The changing in chitosan chemical structure was proved by FT-IR, electronic spectra, and Scan electron microscope. Moreover, thermal characterization using TGA and DSC analysis show that the prepared derivatives exhibit more thermal stability than chitosan itself. The results show decrease of samples water uptake and solubility in aqueous acidic solution by increased substitution degree as a result of increase in hydrophobicity character by modification. Antimicrobial activity of cinnamyl chitosan Schiff base against five different bacteria strains and three fungal species was investigated. Antibacterial activity was tested against five different bacterial strains (two gram positive: *Staphylococcus aureus and Streptococcus pyogenes* & three gram negative: *Pseudomonas aeruginosa, Proteus vulgaris and Shigella*). Also, antifungal activity was done against three different fungal species (*Fusserium, Aspergillus Niger and Penicillium*). The results showed increasing in antibacterial activity of substituted chitosan against both gram negative and gram positive bacteria by increase the degree of substitutions, also, it showed directly dose dependant. Antibacterial activity of chitosan Schiff base was declined at high pH as a result of decreasing the solubility especially for high substituted polymer. As the same, antifungal test shows increase of antifungal activity of Schiff bases rather than chitosan itself. Antifungal activity is dose dependant and increased by increased degree of substitution.

INTRODUCTION

Chitosan is biocompatible, biodegradable, nontoxic renewable biopolymer produced by alkali treatment of Chitin - the second most abundant natural polysaccharide next to cellulose-. Generally, it found in the composition of crustacean shells. Chitin consists of ß (1→4)-2-acetamido-2-deoxy-d-glucopyranose (GlcNAc) as a repeating unit.

Deacetylation of chitin yields chitosan, which is actually a copolymer GlcNAc and ß-(1→4)-2-amino-2-deoxy-dglucopyranose with deacetylation greater than 60%.
Chitosan has abroad field applications ranging from cosmetics, artificial skin, photography, food and nutrition, ophthalmology and wastewater treatment. [**Knorr., 1984; Kurita., 1998; Muzzarelli., 1996; Razdan and Pettersson, 1994**].
Chitosan has three reactive groups, i.e., primary (C-6) and secondary (C-3) hydroxyl groups on each repeated unit, and the amino group (C-2) on each deacetylated unit
These reactive groups are readily subjected to chemical modifications to alter mechanical, physical and chemical properties of chitosan.
The presence of amine groups in the polymeric chain leads to the possibility of a several chemical modifications, including the preparation of Schiff bases (–RC=N–) by reaction with aldehydes and ketones [**Moore and Roberts, 1981; Muzzarelli, Jeunieux and Gooday, 1985**]. The reaction of chitosan with aromatic aldehydes in acetic acid to produce the corresponding Schiff bases has been described by **Tirkistani, 1998.** This author prepared, characterized and investigated the thermal properties of several chitosan derivatives.
Several chitosan schiff bases have been prepared and published in literature; reductive arylation of chitosan with salicylaldehyde has been reported along with its application in metal chelation [**Baba et al., 1997**]. **Crini et al. 1997** synthesized N- benzyl sulfonated derivatives of chitosan and reported their one-dimensional and two-dimensional NMR spectra. **Sashiwa and Shigemasa 1999** prepared a series of N-aryl chitosans. **Stevens and co-workers** synthesized a series of 24 N-alkyl and N-aryl chitosans with different degrees of substitution that were soluble in dilute aqueous acetic acid [**Rabea et al**., **2004, 2006, 2005**].
Chitosan Schiff bases have been pointed to as promising antibacterial agents. In last few years several chitosan was prepared by coupling various types of aldehyde with free amine groups of chitosan. **Pengcheng et al, 2005**, described preparation of five kinds of Schiff bases of chitosan and carboxymethyl chitosan through coupling it with 2-hydroxy-5-chlorobenzaldehyde, 2-hydroxy5-nitro benzaldeh-yde and 2-hydroxy benzaldehyde. The resultants Schiff bases were investigated for its antioxidant activity. Citral chitosan Schiff base was synthesized and tested as antimicrobial agent by **Xiaoxiao Jin et al, 2009**. Benzyl- and salicyl- chitosan were prepared and investigated for antifungal activity **[Pengcheng et al., 2007**]. **Cavalheiro** group prepared several derivatives of chitosan and salicylaldehyde derivatives Schiff bases **[Jose´E. dos Santosa et al., 2005]. Jiangtoa. and Hedong** prepared several derivatives of chitosan schiff base by coupling with p- aminobenzoyl derivatives **[Jiangtoa and Hedong, 2011].** It has been reported that chitosan can inhibit fungal growth and activate the defense response of plants **[Hadwiger and Beckman, 1980; Pearce and Ride, 1982; Kauss et al., 1989**]. This property makes chitosan potentially an antifungal agent.

In the current work, a new chitosan schiff base was prepared by coupling chitosan with Cinnamaldehyde that consist about 70% of cinnamon essential oil.

2. Materials and methods

2.1 Materials

Shrimp skeletons provided from commercial resource, Sodium hydroxide pellets (Purity 99-100 %, M.W.40). Cinnamaldehyde (Purity 98 %, M.W.132, Scharlau, Spain). Acetic acid (Purity 99.8%, M.W.60.05). Sulfuric acid (Purity 98%, M.W.96). Ethanol (Purity 99.9%, M.W.46.07). International co for Supp&Med. Industries, (Egypt).

Bacteria

Five bacterial strains were utilized for evaluating antibacterial activity of chitosan and its derivative. These include three Gram-negative strains (*Pseudomonas aeruginosa, Proteus vulgaris, Shigella*) and another two Gram positive (*Staphylococcus aureus, Streptococcus pyogenes*).The strains were incubated overnight at 37 °C in nutrient broth (peptone 1%, yeast extract 0.5%, NaCl 1%, pH 5.5).

Fungi

Three fungi strains were utilized for evaluating antifungal activity of chitosan and modified chitosan. These include *Fusserium, Aspergillus Niger and Penicillium*).The strains were incubated for three days at 28 °C in nutrient broth (sucrose 3%, Mg SO_4 0.05 %, KCl 0.05% $NaNO_3$ 0.3%, K_2HPO_4 0.1% and trace of $FeSO_4$).

2.2 Methods

2.2.1. Chitosan Modification

2.2.1.1. Preparation of chitin from shrimp Shells

According to **Islama et al., (2011),** the process mainly involved the following steps: Demineralizations of Shells. In this step, the shells were suspended in 5% HCl at room temperature in the ratio of 1:14(w/v) overnight. After 24

hours, the shells were quite squashy and were rinsed with water to remove acid and calcium chloride.**,** The de-mineralized shells were then treated with 5% NaOH at room temperature for 24 hours with a solution to solid ratio of 12:1(v/w). The residue was then collected and washed to neutrality in running tap water and then distilled water. The obtained product is chitin.

2.2.1.2. Preparation of chitosan from chitin

According to **Rigby** method **(1936),** preparation of chitosan is simply deacetylation of chitin. Removal of acetyl groups from the chitin was achieved by using 50% NaOH solution with a solid to solvent ratio of 1:50 (w/v) at 100-120 ^{o}C for 12 hours. The resulting chitosan was washed to neutrality in running tap water and rinsed with distilled water.

2.2.1.3. Chitosan purification

According to **Signini and Campana Filho (1999),** chitosan sample was dissolved in 2% acetic acid and left overnight. The solution was then filtrated through cheesecloth to remove contaminants and undissolved particles. Chitosan was then precipitated with 5 % sodium hydroxide, collected and washed with distilled water to remove the excess of alkali.

2.2.1.4. Preparation of chitosan – cinnamaldehyde Schiff base

Previously purified chitosan (1g) was dissolved in 50 ml of 2% acetic acid and stirred at room temperature for 6 hr. the resulting viscous solution was filtered through cheesecloth to remove undissolved particles and 10 ml of ethanol containing definite amount of cinnamaldehyde was added to solution under stirring to have homogenous solution. This mixture was stirred for 6 h at 50^{o}C. The formation of a deep yellow gel refers to formation of the chitosan Schiff base. The resulting product was added to excess of 5% sodium hydroxide solution. The precipitate was filtered and washed with distilled water and ethanol several times to remove un-reacted cinnamaldehyde, the product was filtered and dried in a vacuum oven at 60^{o}C overnight.

Five different molar ratios of chitosan - cinnamaldehyde were prepared and coded as samples CH-Cin1, CH-Cin 2, CH-Cin 3, CH-Cin 4, and CH-Cin 5 and the native chitosan (CH-Cin 0). The preparation conditions were listed in Table 2.1.

Table 2.1: Preparation conditions of Chitosan - Cinnamaldehyde Schiff base

Sample code	Chitosan : Cinnamaldehyde W:V	Time	Temperature ℃
CH-Cin 0	1:0	---	---
CH-Cin 1	1:0.025	6 hr	50
CH-Cin 2	1 :0.05	6 hr	50
CH-Cin 3	1 :0.125	6 hr	50
CH-Cin 4	1 :0.25	6 hr	50
CH-Cin 5	1 :0.5	6 hr	50

2.2.1.5. Qualitative determination of degree of substitution

0.05 g of polymer sample was dissolved well in 25 ml acetic acid (2%). The absorbance was measured at wave-length 310 nm referenced to acetic acid solution (2%) that characteristic of the phenyl nucleus of cinnamaldehyde.

2.2.2. Modified chitosan structure verification

2.2.2.1. Infrared spectroscopic analysis.

The structures of the chitosan and chitosan derivatives were investigated by carrying FT-IR spectroscopic analyses using Fourier Transform Infrared Spectrophotometer (Shimadzu FTIR - 8400 S, Japan).

2.2.2.2. Thermogravimetric Analysis (TGA).

TGA Analysis of chitosan and chitosan derivatives was carried out using Thermogravimetric Analyzer (Shimadzu TGA –50, Japan).

2.2.2.3. UV-Vis Spectroscopic analysis

0.05 g of polymer sample was dissolved in 25 ml of acetic acid 2%. The electronic absorbance of samples in quartz cell scanned from 190 -1000 nm was recorded.

2.2.3. Physico-chemical properties

2.2.3.1. Determination of ion exchange capacity of chitosan and its Schiff bases.

Chitosan is insoluble in sulfuric acid. A known weight of chitosan or chitosan derivatives were added to known volume of 0.1 M H_2SO_4 solution and the mixture was kept aside for 2 h. The mixture was filtered and an aliquot was titrated against standard solution of sodium hydroxide. Similarly control titration without the addition of chitosan was also run. From the difference in the volume of NaOH required for neutralization, ionic capacity of chitosan samples were calculated using following equation (2.1):

Ion exchange capacity = (V_2-V_1) a / w (meq/g) eq. (2.1)

Where V_2 and V_1 are the volumes of NaOH required for complete neutralization of H_2SO_4 in the absence and presence of chitosan, respectively, a is the normality of NaOH and w is the weight of sample taken for analysis [**Ramnani and Sabharwal., 2006**].

2.2.3.2. 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 [**Mohy Eldin et al., 2012**]. The solubility was determined by the following equation (2.2);

Solubility% =1- $[W_1/W_0]$ X 100 eq. (2.2)

Where W_1 and W_0 are weight of insoluble part and Total weight of sample respectively

2.2.3.3. Water uptake

Water uptake (%) estimation was performed by placing a weighed sample previously dried. After 6 hr the sample was then filtered off, carefully bolted with a filter paper and weighed. The water uptake was calculated by applying the following equation (2.3);

Water uptake % = [(M – Mo) / Mo] X 100 eq. (2.3)

Where M is the weight of the swelled sample at time t and M_0 is the weight of the dry sample. [**Mohy Eldin et al., 201**2]

2.2.4. Bio-evaluation of chitosan derivatives

2.2.4.1. Antibacterial Activity

Antimicrobial activity of the chitosan and chitosan derivatives was measured following the method of **Muhannad et al., (2002) and Jeon and Kim, (2000).** Briefly, the bacteria were inoculated in a Luria- Bertani medium (LB medium) (1% peptone, 0.5% yeast extract, and 1% NaCl). The inoculation was conducted at 37◦C for 24 h with shaking. The obtained bacterial suspension was diluted with the same peptone medium solution 100 times. 0.1 mL of diluted bacteria suspension was cultured in a 10 mL liquid peptone medium, which contains 0.1 mL of chitosan solution (1%) sterilized under 121◦C for at least 20 min. The inoculated medium was maintained at 37◦C for 18 h with shaking. The number of bacteria was counted by using the ultraviolet absorbance of culture medium at 620 nm.

2.2.4.2. Antifungal test

According to **Kuo, et al (1999)**, Antifungal assays were performed. In test plates, chitosan and modified chitosan were, respectively, mixed with sterilized potato dextrose agar (PDA), at different concentrations (100, 200, 400, 600 and 800 mg/L). Then the given mycelium of fungi was transferred to these plates and incubated at 27℃. Under the same condition, the same mycelium was incubated in control plates with non-samples-doped PDA. All incubations were terminated when the mycelium in control plates reached the edge. Finally the growth of the given mycelium in test plates was compared with that of the same mycelium in control plates, and the antifungal index was calculated from equation 3.4 ;

Antifungal index (%) = $(1-D_a/D_b)$ x 100 eq. (3.4)

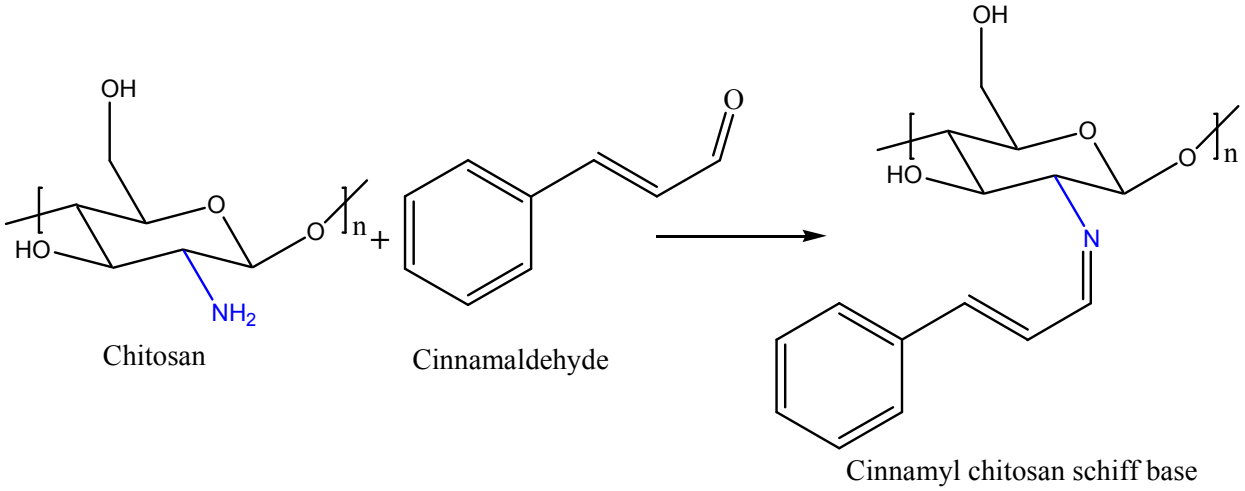
Where D_a is the diameter of the growth zone in test plates and D_b is the diameter of growth zone in control plates.

3. Results and discussion

In this work, cinnamaldehyde–chitosan Schiff bases with different degrees of substitution were prepared and characterized from physic-chemical points of view. The formation of Schiff bases was verified through monitoring changes of chemical structure using FT-IR, TGA, DSC, spectroscopic analysis and finally solubility. The impact of structure changes on properties like water uptake and morphology was monitored.

3.1. Immobilization of chitosan with cinnamaldehyde

The goal of the preparation part is coupling of cinnamaldehyde molecules with chitosan. Chitosan primary amino groups react with the active carbonyl groups of cinnamaldehyde to produce corresponding Schiff base as present in scheme 1. The optimal conditions of Schiff base preparation were studied. Three controllable factors were selected to study, namely: reaction time, temperature and molar ratio of chitosan to cinnamaldehyde.



Scheme 1: Reaction scheme of Chitosan – Cinnamaldehyde Schiff base preparation

3.1.1 Effect of molar ratio

Figure 3.1, shows the effect of various molar ratios between chitosan and cinnamaldehyde on the formation of Schiff base through monitoring the variation of absorbance at 310 nm referring to phenyl nucleus of cinnamaldehyde. From the figure, it's clear that increasing the cinnamaldehyde ratio to chitosan increases the formation of Schiff base between its aldehyde groups and amino groups of chitosan. This consumption of the amino groups leads consequently to decrease of its ion exchange capacity. The increase of absorbance at 310 nm was found inversely proportional to the decease of ion exchange capacity.

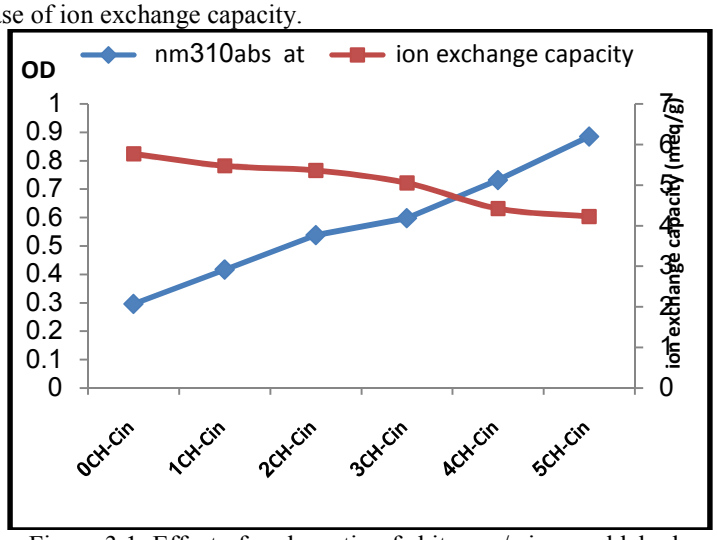


Figure 3.1: Effect of molar ratio of chitosan / cinnamaldehyde

3.1.2. Effect of reaction time

The effect of variation reaction time from 15 minutes up to 6 hours was studied and the results obtained are presented in Figure 3.2. It can be seen that after 15 min small noticeable change was observed of the absorbance reading at 310 nm up to 2 hours. After that, the readings were leveling off. This observation is proportional with ion exchange capacity measurements. This indicates the affinity of Schiff base formation reaction between cinnamaldehyde and chitosan

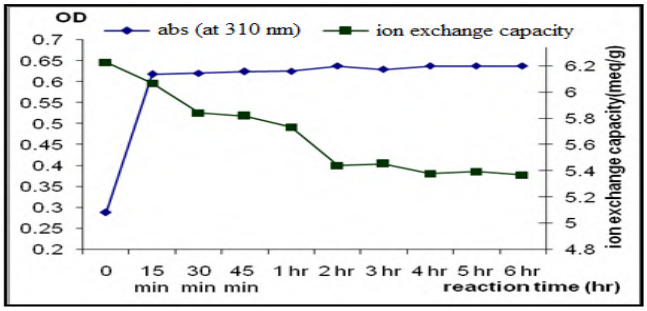


Figure 3.2: Effect of reaction time

3.1.3. Effect of reaction temperature

The temperature profile of the reaction was studied in the range from 20 °C to 80 °C at molar ration 1: 0.125 and reaction time 6 hr. (Figure 3.3).

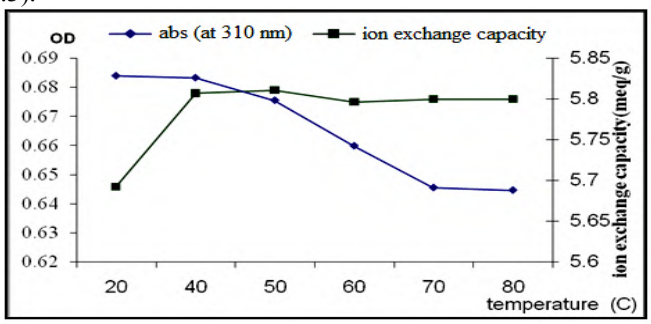


Figure 3.3: Effect of reaction temperature

By increasing the temperature from 20 to 40 °C, a small increase of the substitution degree was observed. After that there is no change by elevation of reaction temperature up to 80 °C. Generally, activation energy of the reaction is low. so, coupling of amine and aldehyde can be done in room temperature. This is due to stability of the produced Schiff base compared to aldehyde and amine. [**Dhar and Taploo., 1982**]

3.2 characterization of cinnamaldehyde- chitosan Schiff base

3.2.1. Physical properties

3.2.1.1. Water uptake

Water uptake of the chitosan and chitosan Schiff base samples was determined and presented in Figure 3.4. It was shown that there is a decrease of water uptake of the samples by increasing degree of substitutions. This was a direct result of changing the hydrophilic nature of chitosan by modification. Chitosan as polysaccharide polymer gain its hydrophilic nature from significant group's (hydroxyl and primary amine). The introduction of phenyl substituents clearly increases the hydrophobic character.

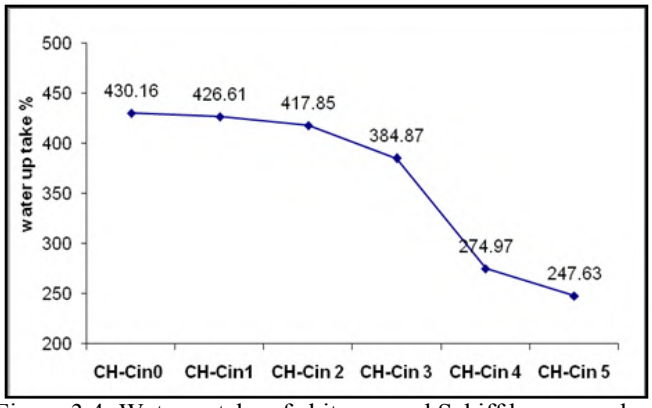


Figure 3.4: Water uptake of chitosan and Schiff base samples

3.2.1.2. Solubility

Solubility of chitosan and prepared chitosan Schiff bases were studied 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.

Chitosan was almost soluble at acidic pH till pH 6 after that it will be precipitate at neutral and alkaline pH. See Table 3.1. This was attributed to cationic nature of amino group at acidic pH. At low substituted Schiff bases CH-Cin1and CH-Cin 2, the solubility was not clearly affected at pH ranges from 3 to 5. Clear effect was observed of CH-Cin 3 to CH-Cin 5 where the solubility dramatically decreased with variation of pH.

Table 3.1: Solubility percent of chitosan and chitosan Schiff bases at different pHs

	CH-Cin 0	CH-Cin 1	CH-Cin 2	CH-Cin 3	CH-Cin 4	CH-Cin 5
pH3	100	100	100	97.65	69.56	58.17
pH4	100	99.89	97.11	74.19	49.25	30.64
pH5	96.97	98.56	96.57	60.11	36.48	20.75
pH6	88.33	85.66	82.86	56.28	16.28	8.49
pH7	0	0	0	0	0	0
pH8	0	0	0	0	0	0

Coupling of amino groups with aldehyde decreases the free amino groups. This was reflected on the solubility of modified polymer and decreases its solubility to almost half at pH3 in case of high substitution (CH-Cin 5).

3.2.2. Structure verification of polymer

3.2.2.1. Spectral characterization

The FT-IR spectra of chitosan and five Schiff bases samples are shown in Figure 3.5. The figures exhibit a broad band at 3200-3600 cm^{-1} corresponds to the stretching vibration of $–NH_2$ and OH groups of chitosan. A new band was generate at 1634.3 cm^{-1} attributed to –C=N vibration. Also, Bands of 1602, 1441, 757 and 647 cm^{-1} result to phenyl groups were observed and their intensities were increased with increasing the cinnamaldehyde molar ratio used in samples preparation which confirmed the formation of Schiff bases. 1171, 1072.4, 1357cm^{-1} bands resulting from C-O and C-O-C vibration were recognized.

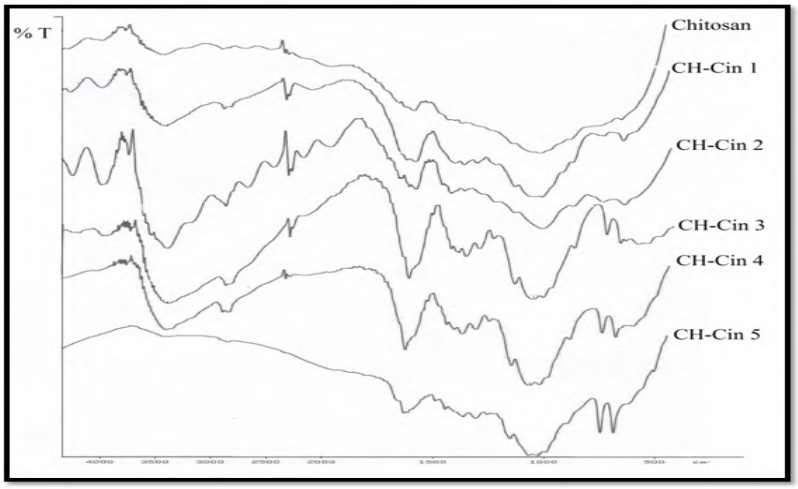


Figure 3.5: FT-IR spectrum of chitosan and five different Chitosan schiff bases

3.2.2.2. UV-Vis Spectra

Electronic spectra of chitosan and five samples Schiff bases were measured on range from 190 nm to 1000 nm (Figure 3.6). Chitosan and modified chitosan showed increase of peak intensity at wave length 310 nm with red shift by increasing of substitution degree. [**Perrin et al., 1980; Morrill et al., 1974**]

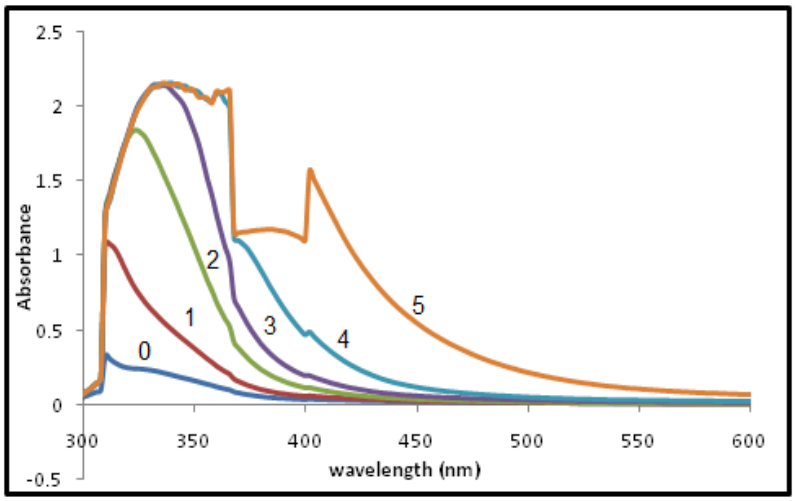


Figure 3.6: Electronic spectra of (0) Chitosan and five different chitosan schiff bases, (1) CH-Cin1, (2) CH-Cin 2, (3) CH-Cin 3, (4) CH-Cin 4, and (5) CH-Cin 5

In fact, chitosan peak at 310 nm was attributed to n - **σ** *transition. By substitution, the amine groups are coupled with conjugated phenyl groups which donate nitrogen with steam of electrons and degrease the energy level of **σ** *. After coupling a new transition will appear resulting from n- **π** * which generate a new peak at 402 nm as shown in Table 3.2. [**Perrin et al., 1980; Morrill et al., 1974**]

Table 3.2: Electronic absorbance peak of chitosan and modified chitosan

	First peak		Second peak	
	Wave length cm^{-1}	Intensity	Wave length cm^{-1}	Intensity
CH-Cin 0	310	0.333	-------	-------
CH-Cin 1	310	1.091	402	0.06
CH-Cin 2	324	1.842	402	0.115
CH-Cin 3	336	2.150	402	0.195
CH-Cin 4	340	2.156	402	0.486
CH-Cin 5	340	2.156	402	1.508

3.2.2.3. Scan electron Microscope (SEM)

The surface morphological analysis of chitosan and the five modified samples was studied using scanning electron microscope (SEM). The SEM graphs, Figure 3.7, show dramatic increase of surface roughness as increase the modification. This is explained by changing of internal structure of polymer. Immobilization of cinnamaldehyde molecules into chitosan backbone gives the polymer some hydrophobic nature. This will induce a change in the internal forces between polymer chains (i.e.; hydrogen bond, Van der Waals forces, etc) and may also induce changes of solid state crystallinity.

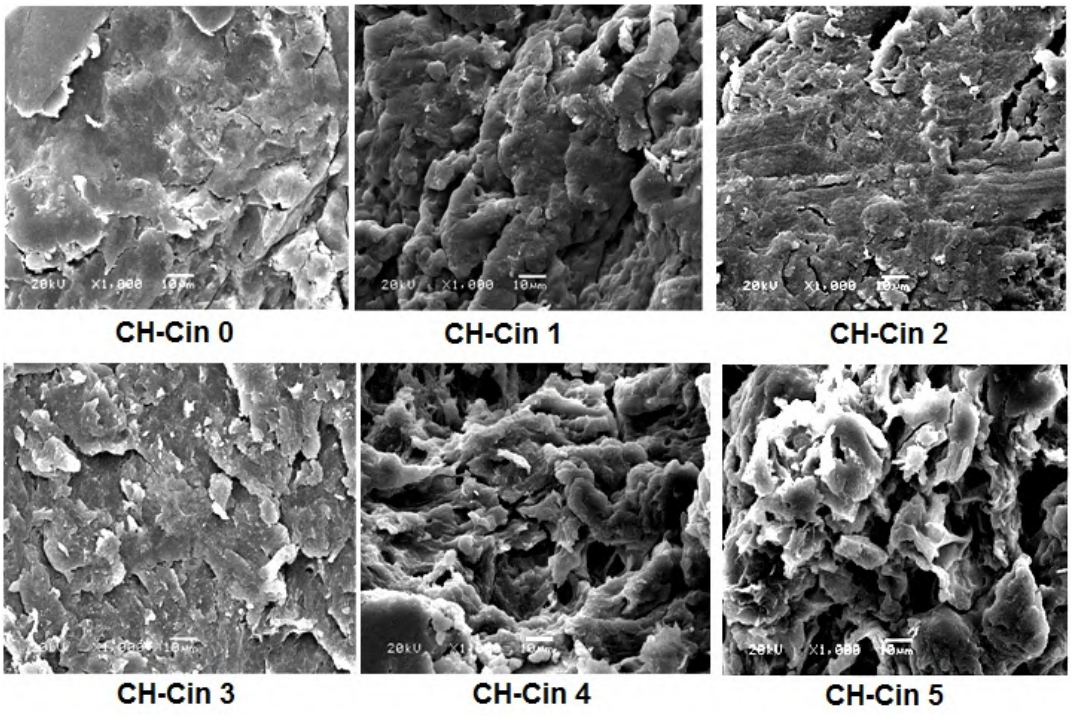


Figure 3.7: Scan electron microscope of chitosan and five different chitosan schiff bases, CH-Cin1, CH-Cin 2, CH-Cin 3, CH-Cin 4, and CH-Cin 5

3.2.3. Thermal analysis

3.2.3.1. Thermal gravimetric analysis (TGA)

Thermal gravimetric analyses of chitosan and the five different modified chitosans were carried out using TGA50 SHIMADZU JAPAN, (Figure 3.8).

The first depression recognized from zero to 200℃ was attributed to evaporation of moisture content of sample. According to Table 4, it was shown that increase of moisture content in slight substitution and that will be decrease in higher one. In fact, coupling of cinnamaldehyde will first deform the crystal structure of chitosan and increase the affinity of water molecules penetration that can be confirmed from the external structure seen on scan electron microscope plots. In high substitution, the increase of hydrophobicity of samples decreases its water content.

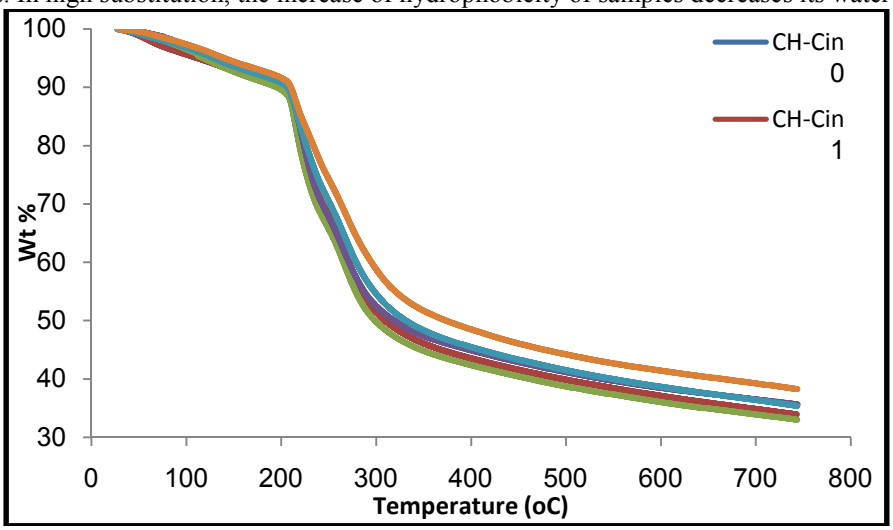


Figure 3.8: TGA analysis of Chitosan and five different chitosan schiff bases

According to **Pawlak and Mucha**, the main depression of chitosan TGA curve ranged from 220 to 350 °C was a result of oxidative decomposition of the chitosan backbone. In this stage first depression was resulted from destruction of amine groups to form crosslinked fragment and the second decomposition step, which appears at high temperature, may result from the thermal degradation of a new crosslinked material formed by thermal crosslinking reactions occurring in the first stage of degradation process. [**Pawlak and Mucha., 2003**]

Table 3.3: Peak temperature and weight loss of TGA analysis

	0-200 ℃	T50	W% $_{at\ 750}$
CH-Cin 0	9.17	318.99	33.95
CH-Cin 1	9.89	309.31	33.95
CH-Cin 2	10.42	298.67	33.03
CH-Cin 3	9.17	318.91	35.68
CH-Cin 4	9.20	331.55	35.43
CH-Cin 5	8.38	373.81	38.3

According to this explanation, the thermal stabilities of formed Schiff bases increased with increase of substitution degree of primary amino groups which was recognized in CH- Cin 4 and CH-Cin 5 where T_{50} is significantly increased from 319℃ (chitosan) to 331 and 374℃ respectively. The same behavior was observed at higher temperature; 750℃.

3.2.3.2. Differential Scanning Calorimetric analysis (DSC)

Differential scanning calorimetric analyses of chitosan and the five Schiff bases are illustrated by Figure 3.9.

The DSC curve of chitosan and its derivatives gives an endothermic peak at about 100°C, due to absorbed moisture and another step around 222°C, which is due to the glass transition though the baseline step is somewhat small. [**Takahashi et al., 2008**] DSC values of various derivatives are given in the Table 2.4. The exothermic peak, which appears in the temperature range at about 250 °C, corresponds to the decomposition of the polymer.

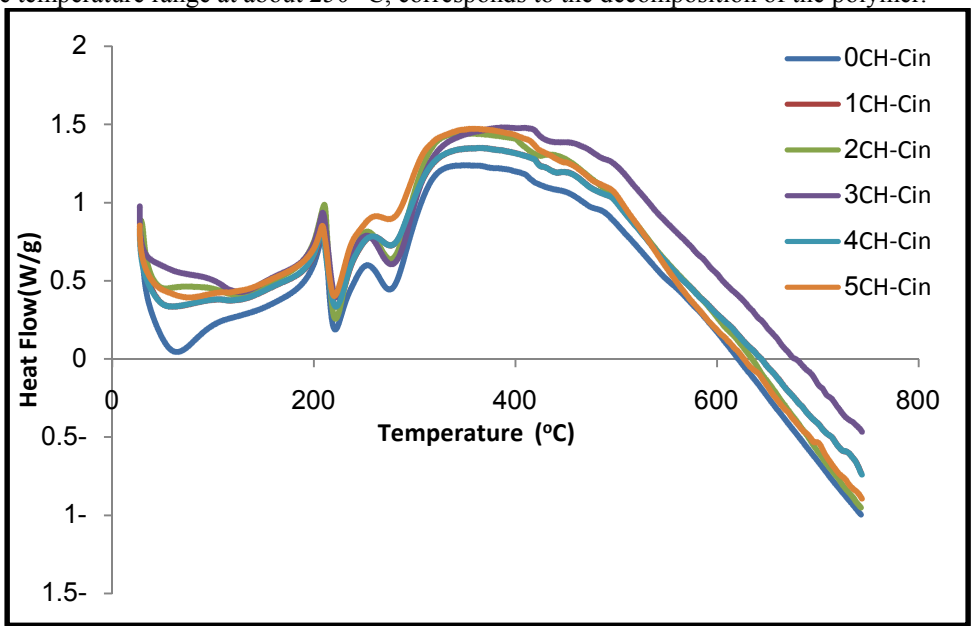


Figure 3.9: DSC analysis of Chitosan and five different chitosan schiff base.

The increase of exothermic Peaks temperature for higher substituted chitosan Schiff bases, CH-Cin4 and CH-Cin5, is in an agreement with observed thermal stability behavior observed in TGA measurements.

Table 3.4: Peak temperature of DSC analysis for chitosan and chitosan Schiff bases.

	First endothermic	Second	Exothermic

	peak ℃	endothermic (T_g)	peak ℃
CH-Cin 0	64.19	221.13	253.13
CH-Cin 1	53.1	221.36	253.08
CH-Cin 2	84.9	220.98	251.565
CH-Cin 3	126.94	222.55	252.49
CH-Cin 4	58.48	221.8	258.42
CH-Cin 5	75.20	220.1	261.45

3.3. Bio-evaluation of chitosan derivatives

3.3.1. Antibacterial evaluation of chitosan and chitosan Schiff bases

Different mechanisms for antimicrobial activity of chitosan have been proposed and recorded in the literature, but the exact mechanism is still unknown. The most accepted one is the interaction of the positively charged chitosan with the negatively charged residues at the cell surface of many fungi and bacteria. This causes extensive cell surface alterations and alters cell wall permeability **[Tsai and Su, 1999; Helander et al., 2001; Tokura et al., 1997],** that let to leakage of intracellular substance, such as electrolytes, UV-absorbing material, proteins, amino acids, glucose, and lactic dehydrogenase. Consequently, chitosan inhibits the normal metabolism of microorganisms and finally leads to death of the cell.

In this study, a new derivatives of chitosan was prepared by transformation of amino groups to Schiff bases, antibacterial activity was tested and compared to chitosan against five bacterial strains (Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Proteus vulgaris, Shigella).

3.3.1.1. Effect of degree of substitutions

Antibacterial activity of chitosan and chitosan Schiff base with different degree of substitution were studied (Figure 3.10).

It was shown that antibacterial activity of the Schiff bases are higher than that of chitosan and will increase by increasing the degree of substitution. This attributed to the introduction of cinnamaldehyde nucleus into chitosan backbone. This indicates that substitution of amino groups with cinnamon nucleus will promote chitosan antibacterial power.

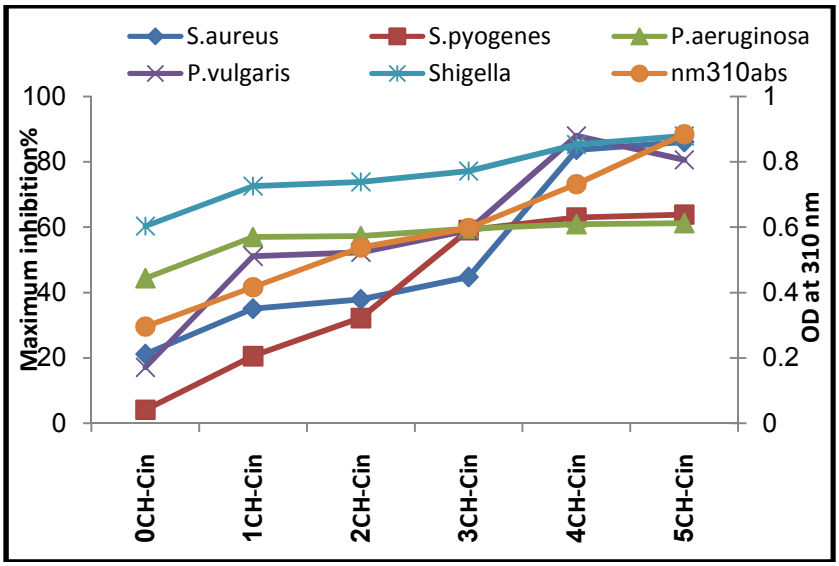


Figure 3.10: Antibacterial activity of chitosan and five different Schiff bases with different degree of substitutions against five different bacterial species

3.3.1.2. Effect of media pH

The surrounding pH of chitosan plays an essential role on its applications. Chitosan is soluble only on acidic pH due to the formation of polycationic nature of polymer. Effect of culture media pH on the antibacterial activity of chitosan and modified chitosans were studied and illustrated by Figures (3.11- 3.15). In general, it is clear that increase of media pH above pH 5 decreases its antibacterial activity. This can be explained by limitation of chitosan solubility at neutral and alkaline solutions. Chitosan was almost soluble at acidic pH till pH 6 after that it will be

precipitate at neutral and alkaline pH. (See Table 3.1). The only observed expectation was recognized with Shigella bacteria where the antibacterial activity was observed unchanged for almost all their formed Schiff bases , CH-Cin1-CH-Cin4, until pH 6 which are lighter than that of chitosan. CH-Cin1 shows higher antibacterial activity against the rest of tested bacterial strains compared with chitosan and higher substituted Schiff bases. This indicates the contribution of conformational charges of Schiff bases molecules in addition to the solubility in the antibacterial action. Moreover, the structure of the cell wall plays an essential role in the interaction process with the polymer.

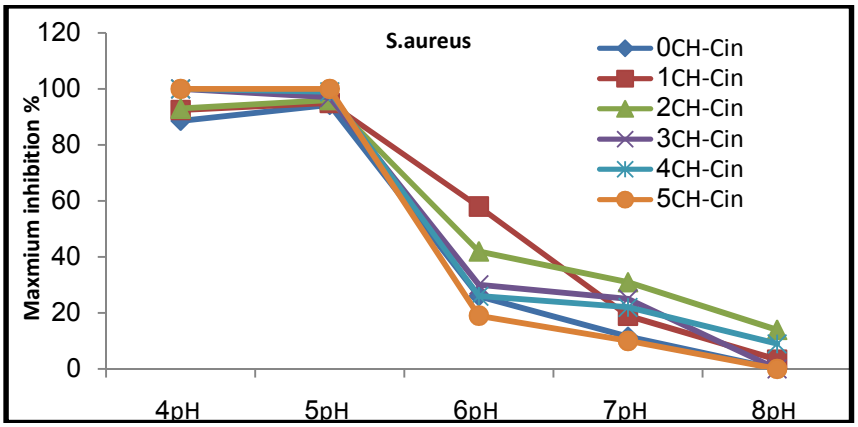


Figure 3.11: Effect of media pH on antibacterial activity of chitosan and Schiff bases with against Staphylococcus aureus.

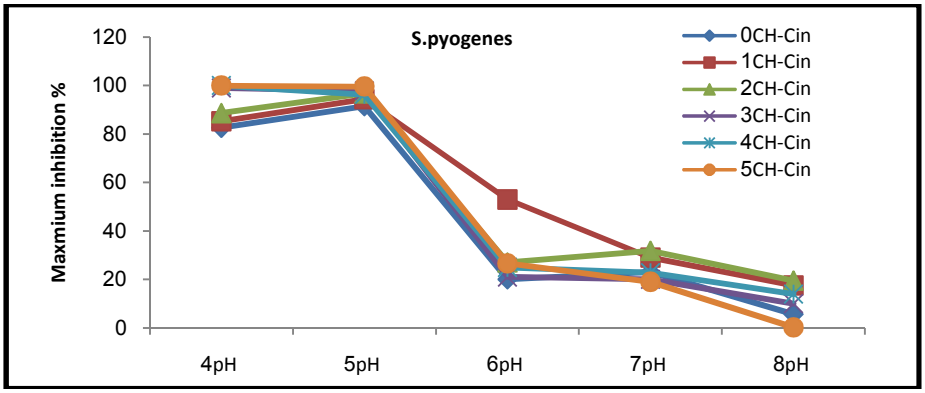


Figure 3.12: Effect of media pH on antibacterial activity of chitosan and Schiff bases with against Streptococcus pyogenes.

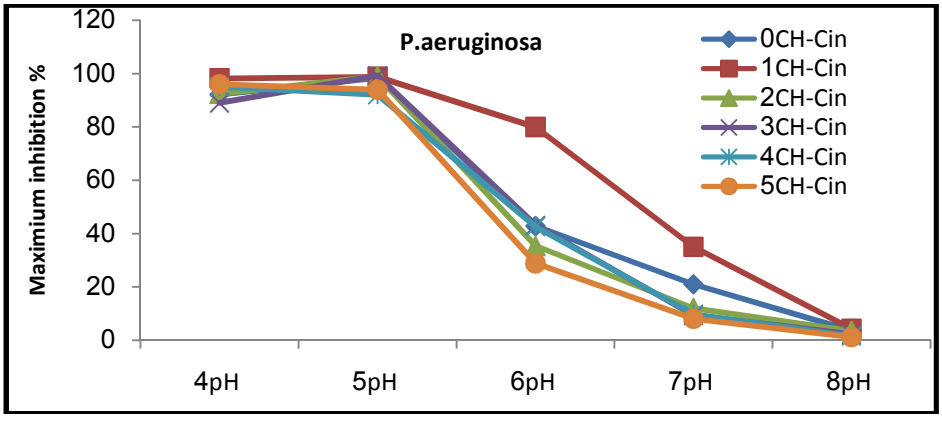


Figure 3.13: Effect of media pH on antibacterial activity of chitosan and Schiff bases with against Pseudomonas aeruginosa.

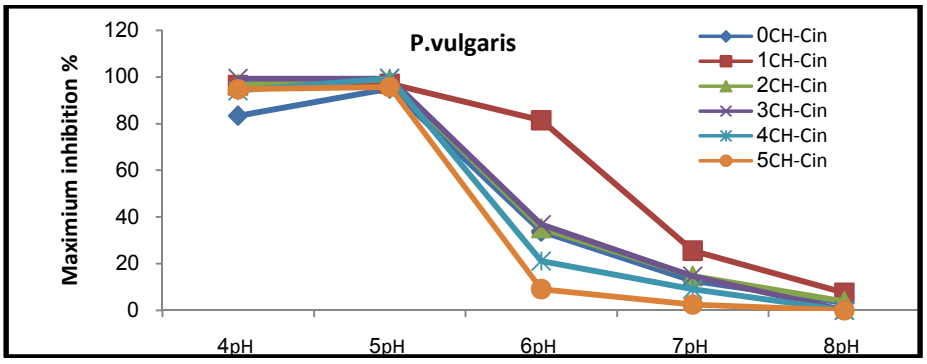


Figure 3.14: Effect of media pH on antibacterial activity of chitosan and Schiff bases with against Proteus vulgaris.

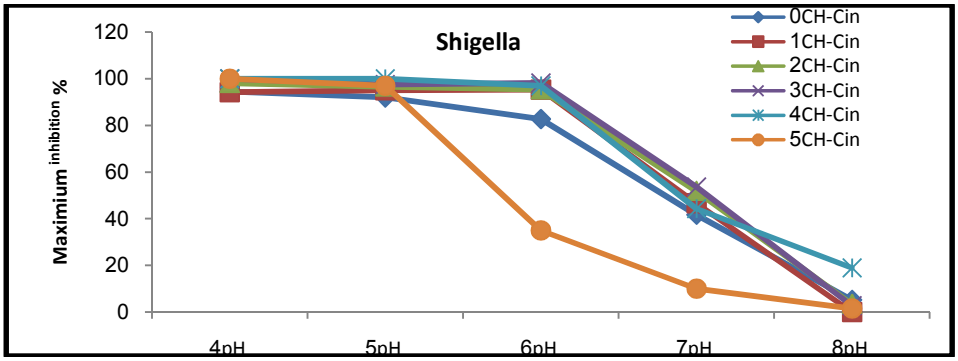


Figure 3.15: Effect of media pH on antibacterial activity of chitosan and Schiff bases with against Shigella

Coupling of the amino groups with aldehydes decreases the free amino groups. This reflected on the solubility of modified polymer and decreases its solubility to almost half at pH3 in case of high substitution (CH-Cin5).

3.3.2. Antifungal evaluation of chitosan and Schiff bases

Several studies were done to evaluate chitosan as antifungal agent. In this part, the antifungal activity of chitosan Schiff base against three different fungi strains (Fusserium, Aspergillus Niger and Penicillium) was evaluated and compared with chitosan itself. Factors such as polymer dose and degree of substitution were tested and presented to give a complete overview on antifungal activity.

3.3.2.1. Antifungal activity against Fusserium

Antifungal activity of chitosan and chitosan Schiff bases with different degree of substitution was tested. Table (3.5) show antifungal index of chitosan and chitosan cinnamaldehyde Schiff bases with different concentration compared with that of controlled one. It clear also that there is a depression in the fungi growth by increased substitution. No growth at all was observed with Schiff bases CH-Cin3, at the highest dose 800 mg/l, this dose decreases to 600 mg/l with CH-Cin4 and CH-Cin5.

Table 3.5: Antifungal index of chitosan and chitosan Schiff bases with different content of substitution against Fusserium.

Dose (mg/l)	CH-Cin 0	CH-Cin 1	CH-Cin 2	CH-Cin 3	CH-Cin 4	CH-Cin 5
100	11.77	11.77	12.94	14.11	16.47	17.65
200	17.65	17.65	18.2	20	21.18	22.35
400	23.53	25.88	35.29	38.83	55.29	100
600	41.18	36.47	44.7	60	100	100
800	44.71	45.88	71.77	100	100	100

3.3.2.2. Antifungal activity against Penicillium

Antifungal activity of chitosan and the five different substituted Schiff base derivatives was tested and presented Table (3.6). The table results represent a dramatic decrease in the fungi growth as increase of substitution and also by increase of loading dose of polymer. This behavior show the effect of substitution on the killer ability of chitosan against fungi the daily growth charts showed

Table 3.6: Antifungal index of chitosan and chitosan Schiff base with different content of substitution against Penicillium.

dose (mg/l)	CH-Cin 0	CH-Cin 1	CH-Cin 2	CH-Cin 3	CH-Cin 4	CH-Cin 5
100	33.33	34.85	36.36	46.97	50	51.515
200	37.88	39.39	37.88	50	51.52	53.03
400	40.91	42.42	46.97	54.45	56.06	100
600	45.45	46.97	54.55	62.12	100	100
800	53.03	54.55	62.12	69.697	100	100

3.3.2.3. Antifungal activity against Aspergillus Niger

Table 3.7 summarizes the antifungal index of chitosan and chitosan cinnamaldehyde Schiff base with different loading dose compared with that of controlled one. The dramatic decrease of daily growth of fungi is a result of antifungal activity of the polymer. This action was increased by increasing loading dose of the polymer solution. No growth at all was observed with Schiff bases CH-Cin3, at the highest dose 800 mg/l, this dose decreases to 600 mg/l with CH-Cin4 and CH-Cin5.

Table 3.7: Antifungal index of chitosan and chitosan Schiff base with different content of substitution against Aspergillus Niger.

Dose (mg/l)	CH-Cin 0	CH-Cin 1	CH-Cin 2	CH-Cin 3	CH-Cin 4	CH-Cin 5
100	15.07	16.44	16.44	17.81	26.03	30.14
200	17.81	19.18	19.18	20.55	27.34	35.62
400	24.66	26.03	20.55	23.29	31.51	38.36
600	31.51	28.77	23.29	38.36	39.73	100
800	34.25	36.99	34.25	41.1	100	100

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