ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Novel Aminated Chitosan-Aromatic Aldehydes Schiff Bases: Synthesis, Characterization and Bio-evaluation

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Manuscript Info

Manuscript History:

Received: 15 December
Final Accepted: 25 January 2015
Published Online: February 2015

Key words:

Aminated chitosan; aromatic aldehydes; Schiff bases; TEM; antimicrobial activity

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Abstract

Novel aminated chitosan-aromatic aldehyde Schiff bases have been synthesized, characterized and finally their antimicrobial activities were evaluated. P-hydroxy benzaldehyde and vanillin were reacted with aminated chitosan via the original and the introduced amino groups. The chemical structure of the obtained Schiff bases was verified through elemental analysis, IR spectroscopy, ^{1}HNMR spectra and TGA. The following microorganisms were chosen to test the antimicrobial activity of the synthesized polymers, the Gram-negative bacterium *(Escherichia coli* (NCIM 2065), *Salmonela typhi, and Pseudomonas aeruginosa*), and the Gram-positive bacterium (*Staphylococcus aureus*). Moreover, the fungi (*C. albicans (*SC5314), *Cryptococcus neoformans*, *Aspergillus flavus* and *Aspergillus niger*) were used. The antimicrobial activity of aminated chitosan on fungi, Gram-positive bacteria, and Gram-negative bacteria show small inhibitory effect against fungi species, an increase in the inhibition of Gram-negative more than Gram-positive bacteria is observed. Aminated chitosan modified with p-hydroxy benzaldehyde and aminated chitosan modified with vanillin showed small inhibit effect against fungi species, however they shows a higher inhibitory effect against a wide variety of Gram-positive bacteria and Gram-negative bacteria. Diameter of inhibition zones were ranged between 9-17 mm on Gram-negative bacteria, 18 mm on Gram-positive bacteria.

1. INTRODUCTION

Chitin is a natural polysaccharide that is usually obtained from shells of crustaceans such as crab, shrimp, and crawfish [Li et al., 2007a]. It is a copolymer of 2-acetamido-2-deoxy-D-glucose (N-acetyl-glucosamine, GluNAc) and 2-amino-2-deoxy-D-glucose (N-glucosamine, GluN) units randomly or block distributed throughout the biopolymer chain depending on the processing method used to derive the biopolymer [Khor and Lim., 2003]. Chitosan is a partially N-deacetylated derivative of chitin. The term chitosan is usually used when glucosamine units predominate or the polymers become soluble in a dilute acid solution. Conversely, the term chitin is used [Rúnarsson et al., 2007]. As a natural renewable resource, chitosan possesses unique properties such as biocompatibility, biodegradability, non-toxicity, and excellent film-forming ability, and has important applications in the biomedical, agriculture, functional food, wastewater purification, environmental protection, biotechnology, and cosmetics domains [Majeti and Kumar., 2000; Rinaudo., 2006; Kima et al., 2006]. Although chitosan should be useful for even more numerous applications, its use suffers severe limitations because it is insoluble in neutral or

alkaline media owing to its rigid and compact crystalline structure and strong intra- and intermolecular hydrogen bonds [Cravotto et al., 2005; Harish Prashanth and Tharanathan., 2007]. Chitosan has both reactive amino and hydroxyl groups that can be used to chemically alter its properties under mild reaction conditions [Li et al., 2007b].
The presence of amino groups leads to the possibility of several chemical modifications, including the preparation of Schiff bases by reaction with aldehydes and ketones. The reaction of chitosan with aromatic aldehydes to produce the corresponding Schiff bases has been described [Guo et al., 2007; Guinesi and Cavalheiro., 2006; Dos Santos et al., 2005].
Sashiwa and Shigemasa prepared a series of *N*-aryl chitosans, however, they only reported water solubility [Sashiwa, and Shigemasa., 1999]. Stevens and co-workers, who 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., 2005; Rabea et al., 2004; Rabea et al., 2006]. They reported that all *N*-aryl chitosans had higher insecticidal and fungicidal activities than chitosan. This implied that the aromatic moieties could attribute to these activities.
This study concerns the preparation and characterization of novel aminated chitosan -aldehydes (p-hydroxyl benzaldehyde and vanillin) Schiff bases and evaluation their antimicrobial activities against fungi, Gram-positive bacteria, and Gram-negative bacteria.

2. Materials and methods

2.1. Materials

Chitin, Ethylenediamine (EDA), p-hydroxybenzaldehyde, p-benzoquinone (pBQ) were purchased from Aldrich, Milwaukee, Wisconsin, USA and were used as received without further purification.

Vanillin and Glacial acetic acid were purchased from El-Gomhouria Chemical Company, Tanta, Egypt and were used as received.

Microorganisms

The following microorganisms were chosen to test the antimicrobial activity of the synthesized polymers, The Gram-negative bacterium *(Escherichia coli* (NCIM 2065), *Salmonela typhi, and Pseudomonas aeruginosa*), the Gram-positive bacterium (*Staphylococcus aureus*), were isolated, identified and certified by Bacteriology lab., Microbiology Departement, Faculty of Science, AL-Azhar University. The fungi (*C. albicans (*SC5314), *Cryptococcus neoformans, Aspergillus flavus* and *Aspergillus niger*) were used, these isolated were identified and certified by Fungi culture collection, Faculty of Science, Assuit University. The bacteria strains were maintained on nutrient agar and nutrient broth, while the fungi were maintained on sabouraud agar

2.2. Methods

2.2.1. Preparation of aminated chitosan

In 100 conical flask, 4.0 g of chitin was dispersed in 50 ml distilled water at (pH=10), p-benzoquinone (pBQ) was dissolved in it and stirred for 6 hr. the activated chitin (AC) was filtered off and washed well with distilled water. Then the product (AC) was dispersed in 50 ml solution of ethylenediamine in distilled water and stirred for 6 hr. the obtained aminated chitin (AMC) was filtered off and washed well with distilled water. According to Rigby and wolfarn method [Rigby., 1936], the chitin derivatives was treated with 50% aqueous solution of NaOH at 120-150 ^{o}C for 6 hr the obtained aminated chitosan was filtered off and washed well with distilled water till the pH decrease to 7.0 the product was characterized by IR [Mohy Eldin et al., 2012].

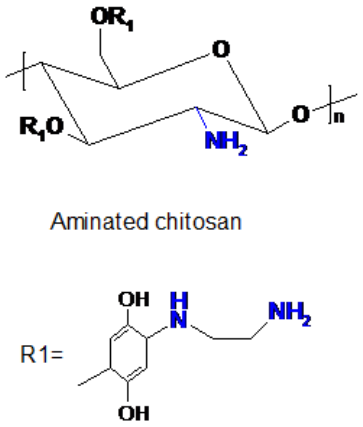


Figure 1: Chemical Structure of aminated chitosan (AMCH)

2.2.2. Modification of the Aminated Chitosan (AMCh) with Aromatic Aldehyde Derivatives

2.2.2.1. Reaction of Aminated Chitosan with P-hydroxy benzaldehyde *(I)*

To a solution of (1.18 g, 9.67 mmol) p-hydroxy benzaldehyde in 30 ml absolute ethanol was added (2 g, 4.84 mmol) of aminated chitosan portion wise with continuous stirring. Glacial acetic acid 1 ml was added to the mixture. The system was fitted to reflux at 80-90 ^{o}C for 2 days. The reaction mixture was then filtered off, washed with ethanol to remove the unreacted aldehyde. The product **(I)** was collected, dried under vacuum at 40^{o}C for 48 hrs. The yield was 1.9g (73%). The product was characterized by elemental analysis, IR spectroscopy, ^{1}HNMR spectra and TGA.

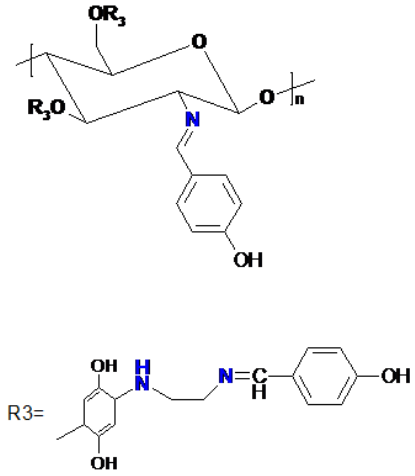


Figure 2: Chemical structure of (I)

2.2.2.2. Reaction of aminated chitosan with vanillin (II)

The title product was synthesized as described earlier for the synthesis of product (I) using the following quantities: aminated chitosan (3 g, 7.26 mmol), vanillin (2.2 g, 14.4 mmol) was added and 1 ml glacial acetic acid. The system was fitted to reflux at 80-90 ^{o}C for 2 days. The reaction mixture was then filtered off, washed with ethanol to remove the unreacted vanillin. The product was collected, dried under vacuum at 40^{o}C for 48 hrs. The yield was 2.7g (68%). The product **(II)** was characterized by elemental analysis, IR spectroscopy, ^{1}HNMR spectra and TGA.

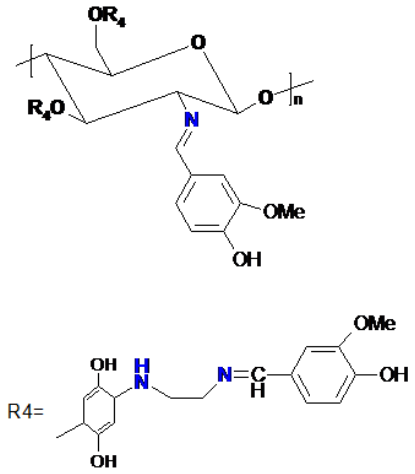


Figure 3: Chemical structure of (II)

2.2.3. Antimicrobial assessment

2.2.3.1. Antimicrobial activities

A loopful of each bacterium culture was spread to give the single colonies on the nutrient agar (3g peptone; 5g NaCl; 5g beef extract; 20 g agar per liter, pH=7.4) and incubated at 37ºC for 24 hours. However, fungi were placed onto sabouraud (10g peptone; 20 g glucose, pH=5.4) and incubated at 30 ºC for 24 hours [Mala et al., 2009].

2.2.3.2. Cut plug method for screening of antimicrobial activity for tested polymers:

Cut plug method recorded by Pridham et al., 1956 was employed to determine the antimicrobial activity of the chosen products and the procedure was as follows:

Freshly prepared spore suspension of different test microorganisms (0.5mL of about 10^6 cells/mL) was mixed with 9.5 mL of melting sterile Sabouraud's dextrose medium for *Candida albicans*, *Cryptpcoccus* neoformans, *Aspergillus niger, Asperegillus flavus,* or nutrient agar medium for *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi* at 45°C, poured on sterile Petri dishes, and left to solidify at room temperature. Regular wells were made in the inoculated agar plates by a sterile cork borer of 0.7 mm diameter. Each well was filled with 20 mg of each tested powder. Three replicas were made for each test, and all plates were incubated at 27 ^{o}C for 3 days for fungi, and at 32°C for 24 hours for bacteria. Then the average diameters of inhibition zones were recorded in millimeter, for all plates.

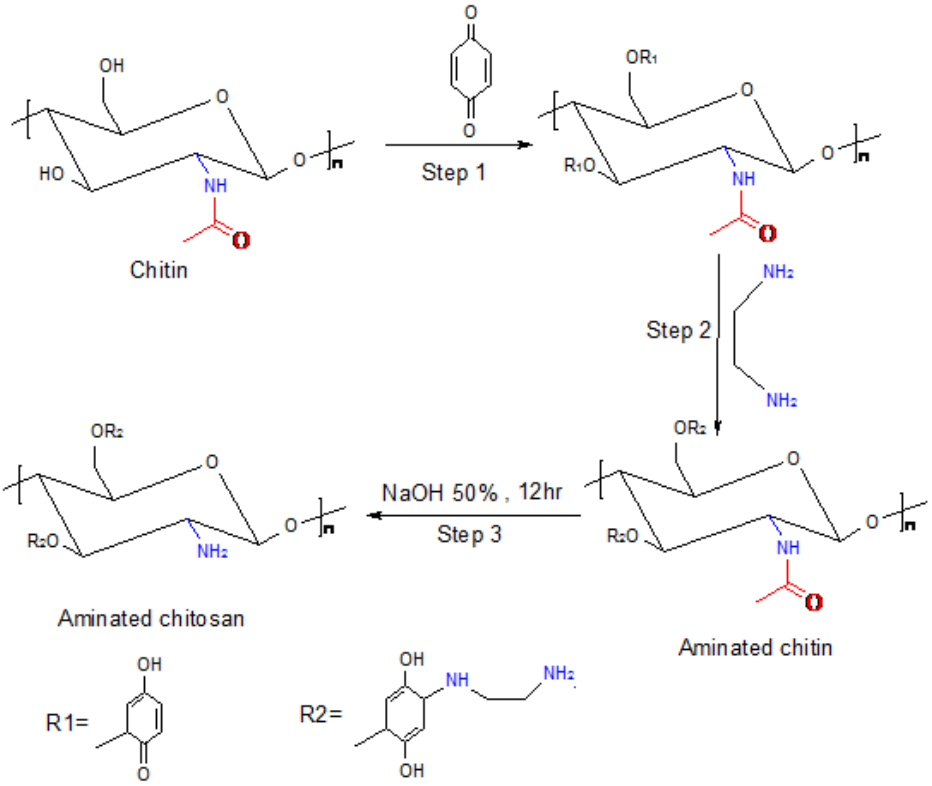
2.2.3.3. MIC determination for the most efficient antimicrobial polymers against test microorganisms (Measuring the Organisms Surviving Ratio):-

Half-fold serial dilutions were made for selected polymers in order to prepare concentrations of 6.25, 12.5, 25, 50 and 100 mg/mL in distilled water, zero concentration was considered as a negative control. A previously prepared pure spore suspension of each test microorganism (0.5mL of about 10^6 cells/mL) was mixed with 9.5 mL of each concentration in sterile test tubes, incubated at 27°C for 3 days for fungi, and at 32°C for 24 hours for bacteria, then optical density of growth was measured by spectrophotometer (Optima SP-300, Japan) at 620 nm for each incubated mixture. The results were represented graphically, and MIC was recorded for each tested material [Schindhelm et al., 1985].

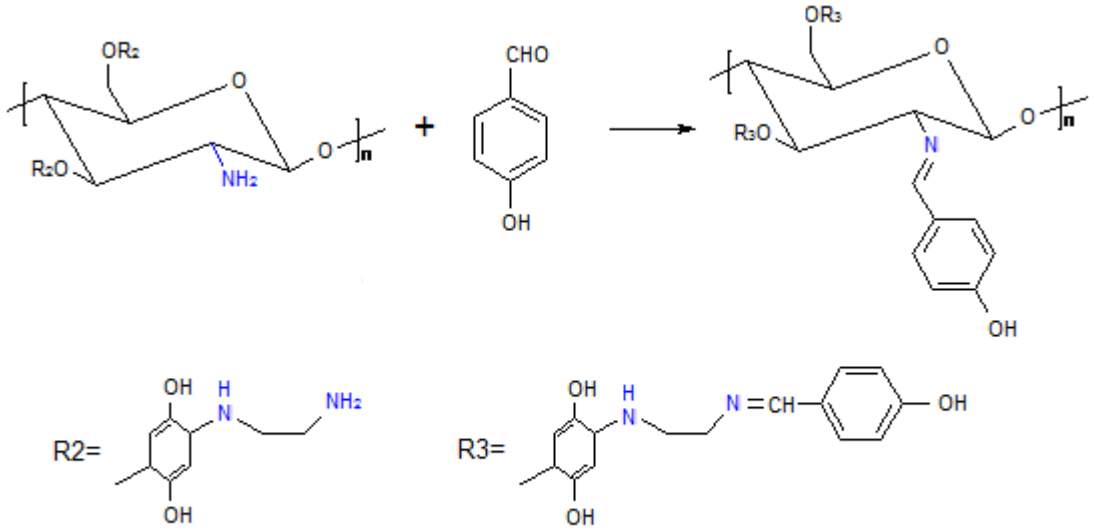
3. Results and discussion

The structure of chitosan is useful to the synthetic chemist interested in site selective modification. Such modifications have resulted into several derivatives of chitosan with distinct properties and applications. The presence of multiple nucleophilic groups within the chitosan backbone requires suitable synthetic protocol in order to obtain the desired selectivity. The synthetic transformation steps performed are often reactively simple, exploiting the difference in the nucleophilicities of primary amino group at(C-2) versus the two hydroxyl groups at (C-3 and C-6). The greater reactivity of amino groups rather than hydroxyl groups was established by previous investigation [Jukka et al., 2006]. However, the degree of selective substitutions varies greatly with the reaction condition. Several derivatives of chitosan were prepared to increase the amine content of chitosan via grafting it on the primary amino groups of the backbone of chitosan polymer chains itself, but the antibacterial activity of the product was decreased [Jukka et al., 2006]. In this investigation, external amino groups are introduced onto the hydroxyl groups of chitosan starting from chitin. P-Benzoquinone (PBQ) is used as activating agent and ethylene diamine (EDA) as a source of

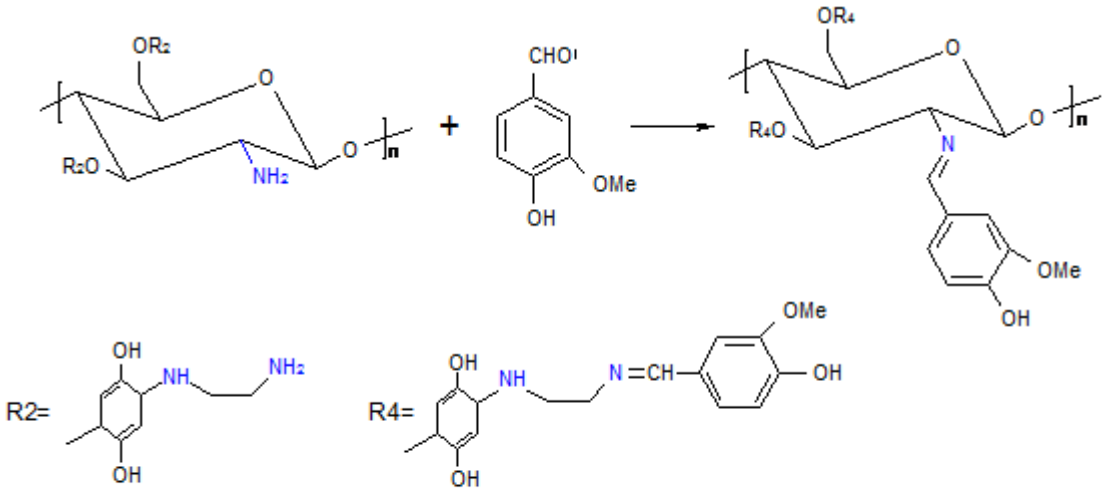
external amino groups **Scheme** 1 and the product was characterized by IR [Mohy Eldin et al., 2012]. Aminated chitosan was reacted with P-hydroxy benzaldehyde and vanillin to give the corresponding schiff base I and II (scheme 2 and 3).



Scheme 1**:** Synthesis of aminated chitosan



Scheme 2: Schiff base formation between aminated chitosan (AMCh) and p-hydroxy benzaldehyde **(I).**



Scheme 3: Schiff base formation between aminated chitosan (AMCh) and vanillin **(II)**

The IR spectra of aminated chitosan (AMCh) shows peaks at 3421 cm^{-1} for (OH), strong absorption band at 2922cm^{-1} for Aliphatic (C-H), at1639 cm^{-1} for (N-H) and showed peaks at 1153 cm^{-1} for O –bridge, at 1084 cm^{-1} for C-O and at 896 cm^{-1} (Benzene ring); Figure 1. The IR spectra of aminated chitosan (AMCh) modified p-hydroxy benzaldehyde **(I)** shows peaks at 3425 cm^{-1} for (OH), strong absorption band at 2920cm^{-1} for (C-H), at1599cm^{-1} for (N-H) and showed peaks at 1158 cm^{-1} for O –bridge, at 1518 cm^{-1} for C=N , at 1066 cm^{-1} C-O and at 896 (Benzene ring); Figure 4.

The IR spectra of aminated chitosan (AMCh) modified vanillin **(II)** shows peaks at 3422 cm^{-1} for (OH), strong absorption band at 2921cm^{-1} for (C-H), at1598 cm^{-1} for (N-H) and showed peaks at 1157 cm^{-1} for O –bridge, at 1525 cm^{-1} for C=N , at 1029 cm^{-1} C-O and at 817 cm^{-1} (Benzene ring); Figure 4.

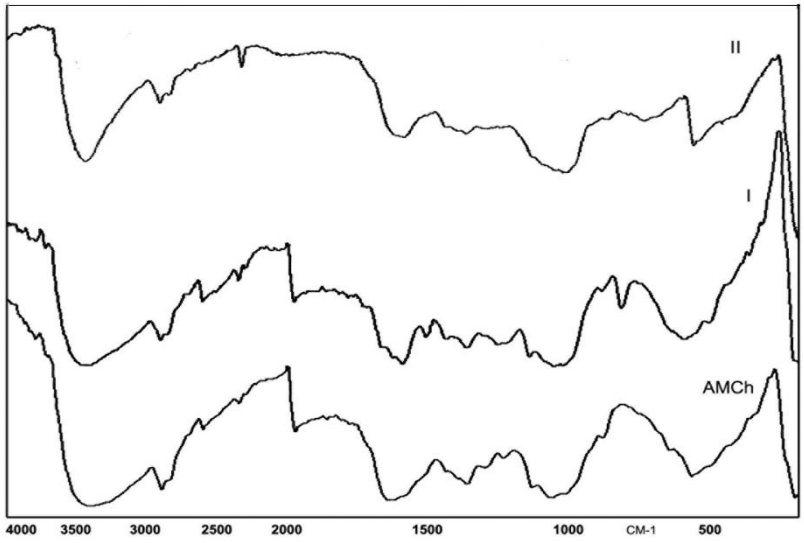


Figure 4**:** IR spectra of (AMCh), (I) and (II) derivatives in the region 400-4000 cm^{-1}

The elemental analysis was tabulated in **Table** 1 and it was a good agreement with the calculated value that prove its structure.

Table 1: Elemental microanalysis of aminated chitosan derivatives **(I-II)**

Polymer code	%C		%H		% N	
	Calc.	Found	Calc.	Found	Calc.	Found
I	60.1	52.16	7.25	6.69	7.1	5.82
II	59.1	42.74	5.8	4.83	8.0	7.39

The ^{1}H-NMR Spectrum (600 MHz, d^{6} DMSO, ppm, $Si(CH_3)_4$) of aminated chitosan (AMCh) modified vanillin **(II)** was characterized by the appearance of peaks at 0.96-1.3ppm (triplet, HC-CH-CH), at 1.2 ppm (doublet, HC-CH-O), at 1.9 ppm (singlet, O-CH_3), at 2.3-2.7 ppm (quartet, NH-CH_2-CH_2-N), at 3.3 ppm (triplet -CH_2-CH_2-N), at 6.8-7.4 ppm (multiplet, H_{arom}); Figure 5.

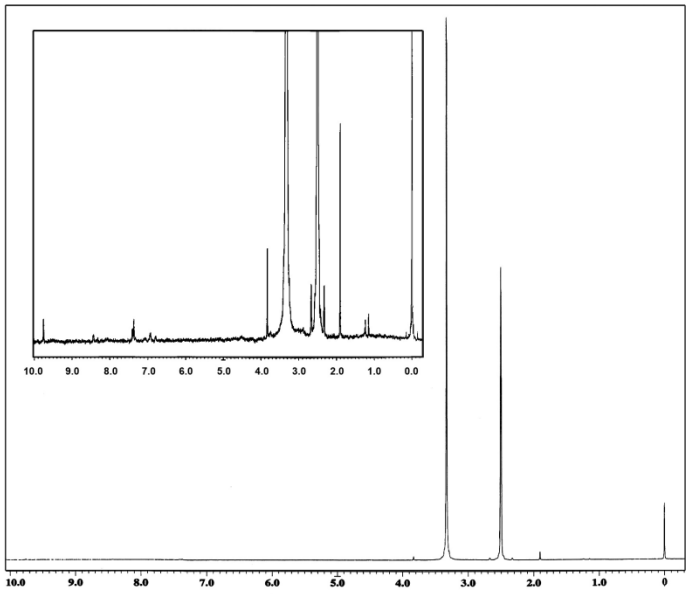


Figure 5: ^{1}H- NMR of polymer (II)

TGA curves of aminated chitosan (AMCh) is shown in Figure 6 is divided into three steps. The first depression may be attributed to the loss of reticular water around 100 ^{o}C that more than 10 % of sample. Second depression at 275 ^{o}C was mainly decomposition stage of the polysaccharide. The weight loss of aminated chitosan is 34% which is caused by the weight of functional group coupled chitosan. According to **Pawlak and Mucha**, the main depression of chitosan derivatives TGA curve ranged from 220 to 350 ^{o}C was a result of oxidative decomposition of the chitosan backbone. In this stage depression was resulted from destruction of amine groups to form crosslinked fragment and after that a decomposition of it, 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]**.**

TGA of modified chitosan with p-hydroxybenzaldehyde *(I)* shows first depression at the temperature around 100 ^{o}C equivalents to about 8 % of sample weight attributed to evaporation of moisture content. The results show degrease of derivative hydrophilicity that that of stating Aminated chitosan. Second depression at 250-325 ^{o}C with weigh loss about 29% corresponding to oxidative decomposition of the chitosan backbone. The TGA curve of modified chitosan with vanillin (II) as shown in Figure 6. The first one the polymer loss 7% of its weight assigned to the loss of water. The second stage starts at 260 ^{o}C and reaches at 320 ^{o}C corresponds to the decomposition (thermal and oxidative) of chitosan, vaporization and elimination of volatile products. The result as shown in Figure 6 indicates that there's increase in water content of the polymers by amination of chitosan modified by aldehyde this result confirm increase of compound hydrophilicity. Also modified chitosan schiff base exhibited higher thermal stability than that of aminated chitosan.

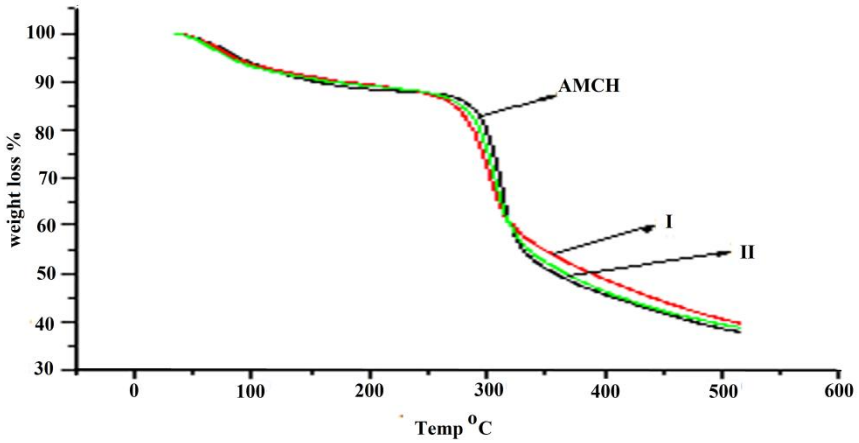


Figure 6: TGA of polymers (AMCh, I, II)

Antimicrobial activity of the modified chitosan and modified chitosan derivatives

The antimicrobial activities of modified chitosan and modified chitosan derivatives against *Candida albicans, Cryptococcus neoformans, Aspergillus niger, Asperegillus flavus, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella typhi* were examined using cut plug method and visible count methods as described before in the materials and methods.

Antimicrobial assessment of the aminated chitosan

The antimicrobial activity of aminated chitosan on fungi, Gram-positive bacteria, and Gram-negative bacteria show small-moderate inhibitory effect against all fungi species. An increase in the inhibition of Gram-negative more than Gram-positive bacteria was observed (Table 2). This is may be attributed to the physiological difference in the structure of the cell wall of the two strains [Mohy Eldin et al., 2012]. In fact, Gram negative bacteria have thick layer of phospholipids rather than the peptidoglycan comparing to the gram positive which has thin layer of peptidoglycan. The negative charges of the phospholipids enhance the adhesion power of poly cationic polymer on the cell wall [Mohy Eldin et al., 2012].

This result confirmed the rupture of the cell wall rather than the nuclear protein interaction mechanism. The interaction of the amine groups of modified chitosan with the cell wall decreases their selective permeability, which leads to leakage of the intracellular substances, such as electrolytes, UV absorbing material, protein, amino acids, glucose, and lactate dehydrogenase. As a result, chitosan and modified chitosan inhibit the normal metabolism of microorganisms and finally lead to the death of this cell [Sudarshan et al., 1992]**.**

Antimicrobial assessment of the aminated chitosan-Schiff base derivatives

As shown in **Table** 2, aminated chitosan modified with p-hydroxy benzaldehyde **(I)** shows moderate inhibit effect against *Aspergillus flavus* fungi only while no effect at all has been observed against other three fungi. On the other hand, aminated chitosan modified with vanillin **(II)** showed moderate inhibit effect against *Cryptpcoccus neoformans* and *Asperigllus niger* fungi species only. However, they show a higher inhibitory effect against a wide variety of Gram-positive bacteria and Gram-negative bacteria. Diameter of inhibition zones were ranged between 9-17 mm on Gram-negative bacteria, 18 mm on Gram-positive bacteria. The mechanism of antibacterial activity of chitosan and its derivatives is still not resolved. However, it is proposed that the positive charge density of aminated chitosan absorbed onto the negatively charged cell surface of bacteria leads to the leakage of proteinaceous and other intracellular constituents [Muzzarelli et al., 1990; Helander et al., 2001; Liu et al., 2004; Je and Kim., 2006]. The additional effect derived from the hydrophobic–hydrophobic interactions between the aryl substituent and the hydrophobic interior of the bacterial cell wall is proposed from our results. Similar results were reported by Kim et al [Je and Kim., 2006].

Table 2**:** Diameters of inhibition zone (mm) of tested polymers number (AMCh, I, and II) against different species of microorganisms.

Polymers Code	Inhibition zone (mm)		
	AMCh	I	II
Fungi			
Candida albicans	6	0.0	0.0
Cryptpcoccus neoformans	9.5	0.0	9
Aspergillus flavus	5	9	0.0
Asperigllus niger	6	0.0	11
Bacteria			
Escherchia coli	12	17	0.0
Staphylococcus aureus	9	18	8
Salmonela typhi	7	0.0	12
Pseudomonas aeruginosa	8	15	9

The minimal inhibitory concentrations (MICs)

The growth inhibiting effect was quantitatively determined by the colony forming units /mL (CFU/mL) of the surviving cell number as shown in Table 3. The inhibition percentage of (I) and (II) Schiff base was assayed with different concentrations (0, 6.25, 12.5, 25, 50 and 100 mg/mL). The 0 mg/mL concentration performed 100% surviving ratios on cells. The polymers (I) gave a higher MIC value at 25 mg/mL against *Staphylococcus aureus* while polymer (II) gave a higher MIC value at 50 mg/mL against *Asperigllus niger* respectively as shown in Table 3.

Table 3: The ratio of surviving cell numbers of some microbial in the presence of polymers (I and II).

Polymers	Percentage of surviving cells (% Optical density)						Microorganism
	Concentration (mg/mL)						
	0.0	6.25	12.5	25	50	100	
I	100	71	52	32	31	31	*Staphylococcus*
II	100	68	57	43	29	29	*A. niger*

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