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Synthesis and Antimicrobial Activities of Cationated Chitosan

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Abstract

This study investigates the synthesis and antimicrobial study of cationated chitosan. The study was first carried out by the modification of chitin using 30% NaOH to obtain chitosan which was then use to synthesize cationated chitosan by treating the chitosan with 2, 3-epoxypropyltrimethylammonium chloride. The results obtained reveal that the chitosan have a degree of deacetylation of 67% which was soluble in 1% acetic acid and then characterized for moisture content, viscosity, UV-visible spectroscopy, the FT-IR spectra showed absorption peaks 3432 cm^{-1} and 3254 cm^{-1} due to OH and NH_2 functional groups respectively, 2923 cm^{-1} due to CH_3, 1617 cm^{-1} due to C=O, 1069 cm^{-1} due to C-O-C stretching frequency vibration, DSC spectra showed that the chitosan was more thermally stable than the cationated chitosan, the antimicrobial studies reveal that the sample have a zone of inhibition of 28 mm, MIC value of 6.25 mg/ml and MBC value of 12.5 mg/ml. The cationated chitosan can be used in the treatment of ailment caused by some of these microorganisms.

Keywords: Chitosan; Cationated chitosan; Antimicrobial activity

Introduction

Contamination by microorganisms is of great concern in a variety of areas, such as medical devices, healthcare products, water purification systems, hospitals, dental office equipment, food packaging, food storage, household sanitation, etc. [1,2]. Antimicrobial polymer, also known as polymeric biocides is a class of polymers with antimicrobial activity, or the ability to inhibit the growth of microorganisms such as bacteria, fungi or protozoans. Antimicrobial polymer may enhance the efficiency and selectivity of currently used antimicrobial agents, while decreasing associated environmental hazards because antimicrobial polymers are generally non-volatile and chemically stable and do not permeate through skin. Therefore, they

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can reduce losses associated with volatilization, photolytic decomposition and transportation. Chitin, poly [β-(1-4)-N-acetyl-D-glucosamine) is a natural polysaccharide of major importance, This kind of linear muco-polysaccharide is abundant in nature mainly present in animal support or tissue of lower evolutionary organisms such as crustaceans, insects and mushrooms, only less abundant in nature than cellulose[3]. Chitin, the second-most abundant biopolymer, and its deacetylated product, chitosan are high molecular-weight biopolymers and are recognized as versatile environmentally friendly raw materials in many applications [4]. Chitin maybe regarded as cellulose with hydroxyl at position C2 replaced by an acetamide group.

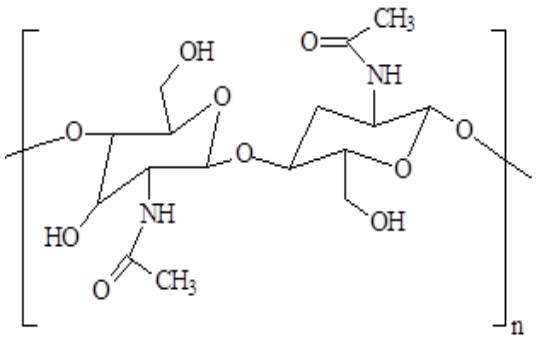


FIG. 1. **Chemical structure of chitin (Lertsuttiwong et al.,2002).**

Chitosan, poly [(1,4)-β-linked 2-amino-2-deoxy-D-glucose] [polyglucosamine], is the N-deacetylated form of chitin with amino groups along with the chitosan structure (polyglucosamine) also known as 2-amino-2-deoxy-(1,4)-β-D-glucopyranan[5,6]. This N-deacetylation is almost never complete. Chitosan is considered to be the most widely distributed biopolymer, it is a cationic, non-toxic, biodegradable and biocompatible polyelectrolyte with a pKa of approximately 6.5 [6,7]. The solubility of chitosan in acidic solutions is the generally accepted criterion for identifying chitin and chitosan. Chitosan polymer is hydrophobic in nature although consisting of hydrophilic functional groups such amine groups and hydroxyl groups. Chitosan can be easily modified by chemical methods due to the presence of the amino groups and the hydroxyl groups, to prepare modified chitosan. Recently there has been a growing interest in the chemical modification of chitosan in order to improve its solubility and widen its application [8].

Materials and Methods

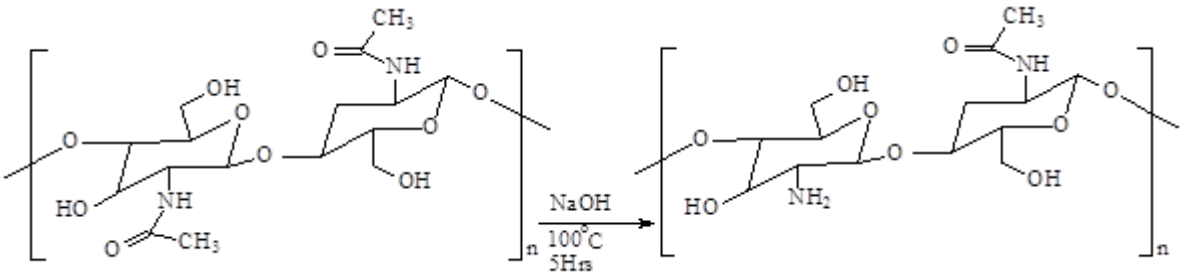
Materials

Chitin, hydrochloric acid, sodium hydroxide, acetic acid, 2,3-epoxylpropyltrimethylammonium chloride, acetone, melamine, dimethylsulfuroxide (DMSO), Mueller Hinton agar, Mueller Hinton broth, potato dextrose agar and nutrient agar.

Modification of chitin to chitosan

Chitin was deacetylated in 30% (w/v) NaOH for 5 h at 100°C using a heating mantle. After deacetylation, the chitosan was washed thoroughly with water followed by distilled water. The resulting chitosan was dried to constant weight at 65°C, as shown in the reaction scheme below.

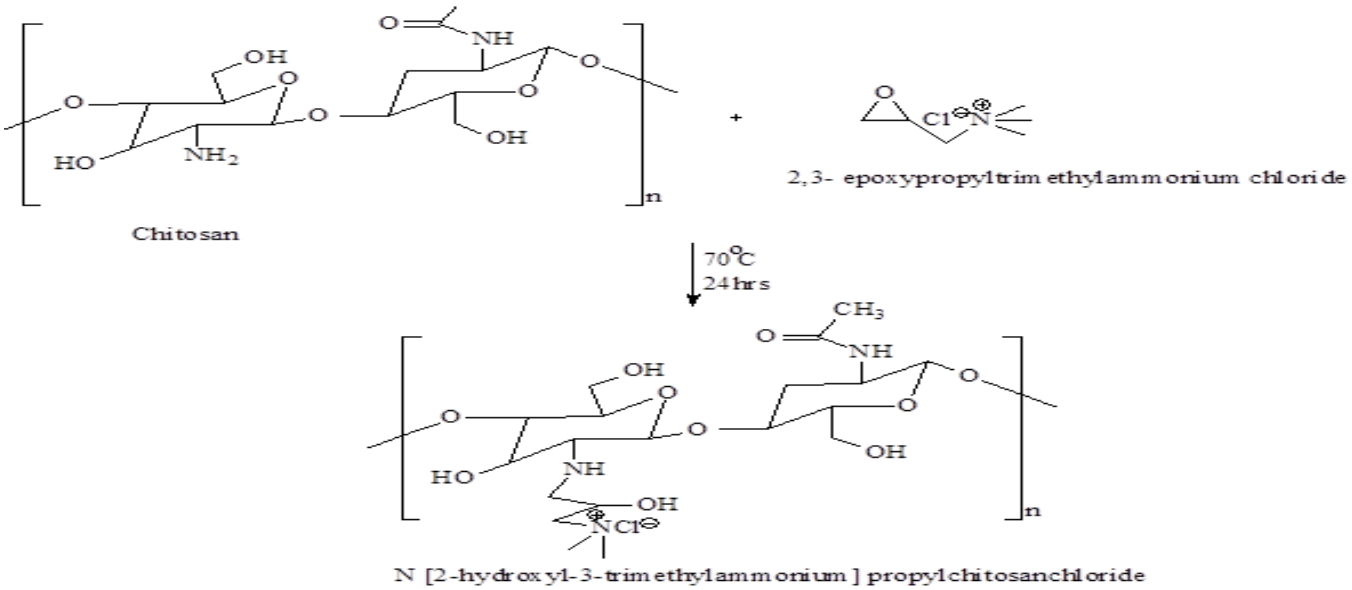
SCHEME 1**. Extraction of chitosan from chitin.**



Synthesis of cationated chitosan

Chitosan (1 g, 5.78 mmol) was dissolved in 1% acetic acid (50 ml), and then 2, 3-epoxypropyltriammonium chloride (2.52 g, 16.6 mmol) was added. The reaction was carried out at 70°C for 24 h. The reaction mixture was then cooled and poured into cold acetone. The product was collected by filtration, washed with acetone several times, and vacuum dried at room temperature as shown in the reaction scheme below.

SCHEME 2. **Synthesis of cationated chitosan.**



Characterization of Antimicrobial Polymer

Solubility analysis

Solubility analysis of the sample was performed in different selected solvents. For this purpose, the sample was added to the solvent at the concentration of 5 mg/mL at 25°C and their solubility was evaluated.

Viscosity

Viscosity of chitosan was determined using a Brookfield. Chitosan solution was prepared by dissolving 1 g of Chitosan in 1% acetic acid to obtain 1% concentration on a dry basis at 25°C.

Determination of percentage moisture content

The crucible was dried in an oven at 80°C for 20 min, cooled in a desiccator and weighed (W_1) g, 2 g of the sample was then placed into the crucible and reweighed (W_2) g, the crucible with the sample was dried in the oven at 105°C until a constant weight was obtained after successive cooling in desiccator and weighing. It was finally transferred from the oven to the dessicator to cool and then quickly weigh (W_3) g. The percentage moisture content was calculated using formula:

$$100\% \text{ moisture content} = \frac{(w_2 - w_3) \times 100}{w_2 - w_1} \qquad (1)$$

Where;

$W_{1=}$ initial weight of empty crucible.

W_2= weight of crucible with sample before drying.

$W_{3=}$ weight of crucible with sample after drying.

Determination of ash content

The crucible was dried by holding in the Bunsen flame for about two min, then transferred into a dessicator to cool before weighing (W_1) g. 2 g of the sample was weighed inside the crucible (W_2) g, the crucible with the sample was heated gently in a Bunsen burner in a fume cupboard till the smoke ceased, which was then transferred to the muffle furnace, preheated at 550°C. The heating was continued until all the carbon had been burnt away; the crucible was taken away with a pair of tong and immediately covered and placed in a desiccator to cool before weighing (W_3) g. The ash content was calculated using the formula:

$$\% \text{ ash content} = \frac{(w_3 - w_1) \times 100}{w_2 - w_1} \qquad (2)$$

Where;

W_1=initial weight of empty crucible.

W_2=weight of crucible with sample before ashing.

W_3=weight of crucible with ash.

Determination of degree of deacetylation

Dried chitosan (0.2 g) was dissolved in 20 cm^3 0.1 M hydrochloric acid and 25 cm^3 deionized water. After 30 min of continuous stirring, another portion of deionized water (25 cm^3) was added and stirring continued for 30 min. When the Chitosan had completely dissolved, the solution was titrated with a 0.1 M sodium hydroxide solution using a pH meter to monitor the pH. Degree of deacetylation (DA) of chitosan was calculated using the formula:

$$DA(\%) = 2.03x\frac{V_2 - V_1}{M + 0.0042\left(\frac{V_2}{V_1}\right)} \qquad (3)$$

Where;

V_1=volume of NaOH used to neutralize the HCl

V_2=volume of NaOH used to neutralize the amine

M=mass of chitosan used

Fourier-transform infrared spectroscopy analysis (FT-IR)

The sample was thoroughly mixed with KBr, the dried mixture was pressed which resulted into a homogeneous sample disk. The measurement was carried out over the frequency range of 400-4,000 cm^{-1} using the Agilent FTIR spectrophotometer [9].

UV-Visible spectroscopy (UV-VIS)

The UV-Visible absorption spectra were measured using Agilent UV-visible spectrophotometer. 0.1 M acetic acid solution of the sample was used while for the cationated sample distilled water was used and the spectra recorded from 200-650 nm wavelength frequency range [10].

Differential scanning calorimetry (DSC)

The differential scanning calorimeter (TA Instrument, Q200, USA) was employed to study the thermal property of the sample. 2.5 mg of the sample was placed in an aluminum pan and sealed. The lid was perforated before sealing. Empty

closed aluminum pan was used as the reference. Samples was scanned from a temperature range of 50°C to 450°C at a heating rate of 10°C/min under nitrogen atmosphere.

Antimicrobial Studies

Antimicrobial profile (sensitivity test) of the polymer using agar well diffusion method

The standardized inocula of both the bacterial and fungus isolates were streaked on sterilized Mueller Hinton and potato dextrose agar plates respectively with the aid of a sterile swab sticks. Wells were pounced on each inoculated plates with a sterile cork borer with a diameter of 6 mm. The wells were properly labeled according to different concentrations of the polymer which were 50, 25, 12.5 and 6.25 mg/ml. each well was filled up with approximately 0.2 ml of the polymer solution.

The inoculated plates with the polymers were allowed to stay on the bench for about one hour; this was to enable the polymer solution to diffuse into the agar. The plates were then incubated at 37°C for 24 h (plates Mueller Hinton agar) while the plates of potato dextrose agar were incubated at room temperature for about 3-5 days.

At the end of the incubation period, the plates were observed for any evidence of inhibition which appeared as a clear zone that was completely devoid of growth around the wells (zone of inhibition) the diameter of the zones were measured using a transparent ruler calibrated in millimeter.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the sample was determined using the broth dilution method [11,12]. Mueller Hinton broth was used as the diluent. The lowest concentration of the polymer solution showing inhibition for each organism when the polymer solution was tested during sensitivity test was serially diluted in the test tubes containing Mueller Hinton broth. The organisms were inoculated into each tube containing the broth and the polymer solution. The inoculated tubes were then incubated at 37°C for 24 h. At the end of the incubation period the tubes were examined for the presence or absence of growth using turbidity as a criterion, the lowest concentration in the series without visible sign of growth (turbidity) was considered to be the minimum inhibitory concentration (MIC).

Determination of minimum bactericidal concentration (MBC)

The result from the minimum inhibitory concentration (MIC) was used to determine the minimum bactericidal concentration (MBC) of the polymers.

A sterilized wire loop was dipped into the test tubes that did not show turbidity (clear) in the MIC test and a loopful was taken and streaked on a sterile nutrient agar plates. The plates were incubated at 37°C for 18 h-24 h.

At the end of incubation period, the plates were examined for the presence or absence of growth. This is to determine whether the effects of the polymers are bacteriostatic or bacteriocidal [11,12].

Results and Discussion

The degree of deacetylation of the chitosan extracted was determined using potentiometric titration with a degree of deacetylation of 67% which is higher than that reported by [13] of 50.64% for shrimp shell lower than the reported degree of deacetylation of 98.38%-98.79% achieved by [14]. This is an indication that chitosan has been extracted in the work, since the necessary condition as stated by some literature is that the degree of deacetylation should be above 50% and it should be soluble in acidic media [15].

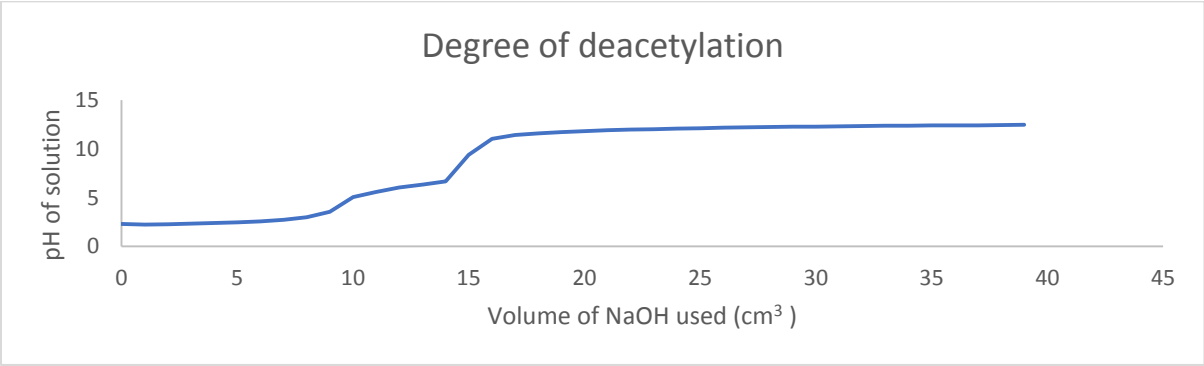


FIG. 2. **Degree of deacetylation of chitosan.**

TABLE 1. **Physicochemical characterization of the uncationated and cationated chitosan.**

Parameters	**Uncationated**	**Cationated**
Solubility	1% Acetic acid	Water
Moisture content (%)	8.07	8.26
Ash content (%)	5.88	-
Degree of deacetylation (%)	67	-
Viscosity (cp)	144.7	-

The deacetylated chitin (chitosan) obtained was tested with acetic acid to see its dissolution in organic acid, it was soluble in 1% acetic acid. [16]. stated that the solubility of chitosan is controlled by the distribution of the acetyl groups remaining along the chain. This is a necessary condition to ascertain that chitosan have been obtained from chitin, the cationated chitosan was tested in water which was soluble in it [17].

The moisture content of the uncationated chitosan as shown in TABLES 1 and 2 is 8.07% while that of the cationated chitosan have a moisture content of 8.26% this increase in moisture content is attributed to the hydrophilic nature of the cationated chitosan. The ash content is an indication of the effective removal of inorganic mineral from the source of chitin, the chitosan extracted has ash content of 5.88%. The ash content is due to the presence of calcium carbonate which is found in large amount in shrimp shells [18]. Viscosity is an important parameter in the conventional determination of molecular weight of chitosan [19]. Higher molecular weight chitosan often provides highly viscose solutions, which may not be desirable for industrial application. The chitosan extracted have a viscosity of 144.9 cp. From literature the viscosity of chitosan ranges from 60 to 780 cp [20].

The UV spectra of the chitosan noticeably shown in the UV spectrum at 273 nm the value is in agreement with the report of [21]. However, the absorption bands of the cationated chitosan exhibited a shift in intensity with an absorption band of 231 nm the decrease in wavelength could be ascribed to the solvent effect used in the dissolution, since chitosan was soluble in acetic acid while the cationated chitosan was soluble in water.

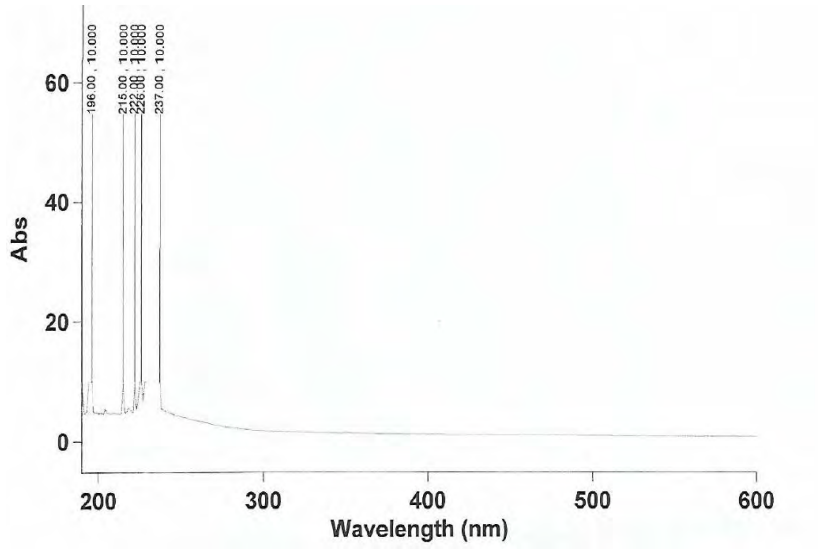


FIG.3. **UV-Visible spectra of uncationated chitosan.**

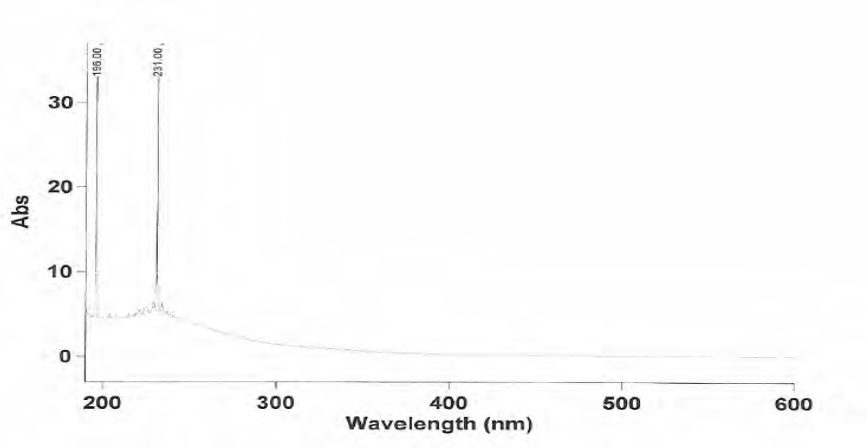


FIG.4. **UV-Visible spectra of cationated chitosan.**

The FT-IR spectra of the chitosan which showed a broad peak at 3500-3300 cm^{-1} for OH and NH_2 stretching vibration [22-24], the amide I band or C=O and amide band or NH deformation are appearing at 1651 cm^{-1} and 1580 cm^{-1} respectively [23-26]. Peaks at 1155 cm^{-1} to 1148cm^{-1} and 1077 cm^{-1} to 1069 cm^{-1} are due to C-O and C-O-C stretching frequencies respectively [27,28]. The chitosan saccharide ring stretching was observed at 895 cm^{-1} [29-31]. The cationated chitosan FT-IR spectra showed that there was bonds breakage and formation since the amino group in the chitosan is more reactive and where responsible for the reaction with the cationating agent. The FT-IR spectra of the cationated chitosan revealed absorption at 1640-1636 and 1550 cm^{-1} coressponding to C=O groups. The shift is attributed to the equilibrium between the cyclic form of chitosan, it also showed absorption at 1420-1416 cm^{-1}, which indicates the presences of CH_2 groups, in addition it showed an absorption peak at 3339-3272 cm^{-1} corresponding to OH and NH_2 groups of the chitosan, as well as absorption at 1460 cm^{-1} indicating the presence of the tertiary amine connecting the alkyl groups.

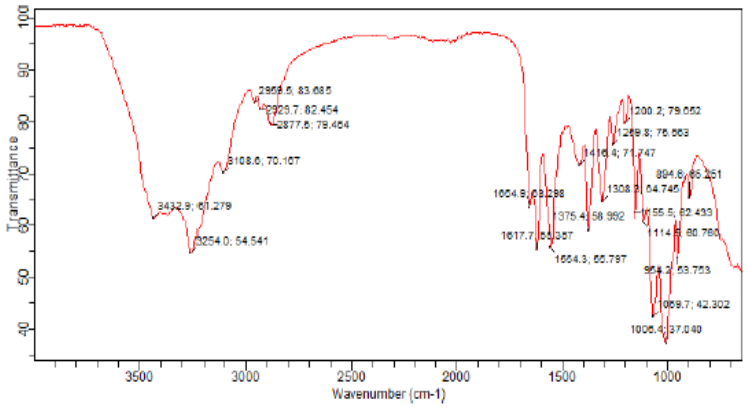


FIG. 5. **FT-IR spectra of uncationated chitosan.**

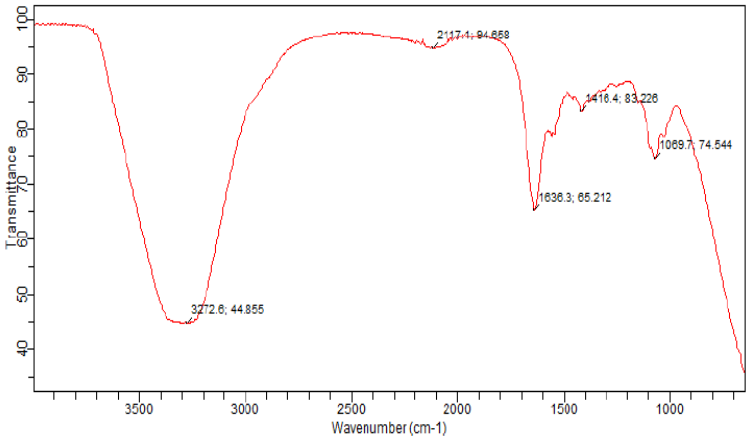


FIG. 6. **FT-IR spectra of cationated chitosan.**

The DSC curves of the chitosan showed endothermic peak at 79.33°C. The endothermic peak, often called dehydration temperature is due to the evaporation of water molecules [32,33]. This value is in close agreement with the report of of 79°C. The presence of the dehydration temperature suggest that some bound water was still not removed from the sample after drying, while it gives an exothermic transition at 345°C which likely corresponds to chitosan decomposition since polysaccharide do not melt.

The cationated chitosan had a lower value of the dehydration temperature at 76.11°C, while an exothermic peak at 110°C, this decrease in the temperature may be attributed to the hydrophilic nature of the cationated chitosan.

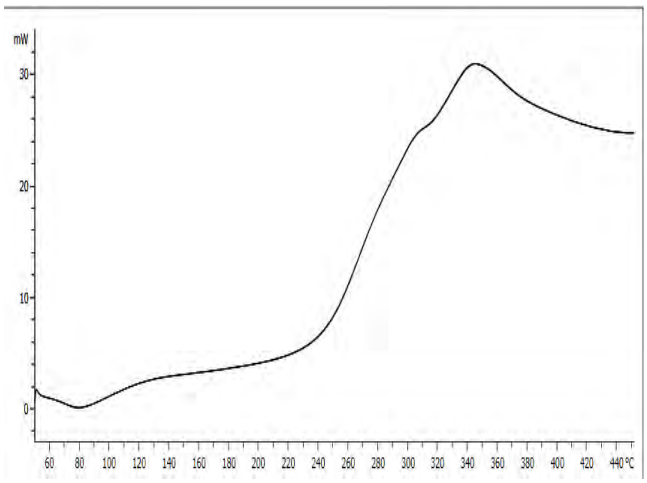


FIG. 7. **DSC spectra of uncationated chitosan.**

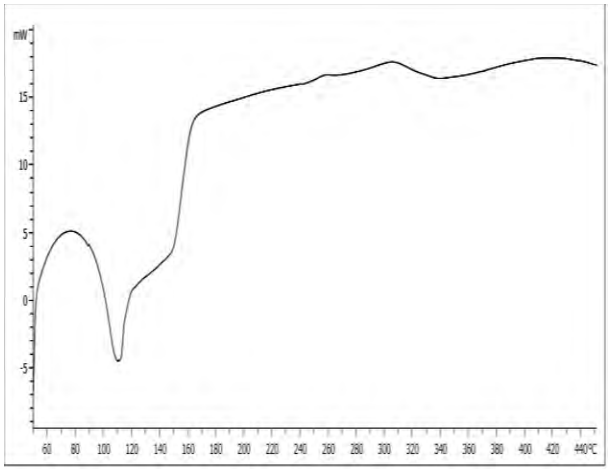


FIG. 8. **DSC spectra of cationated chitosan.**

TABLE 2. **Zone of inhibition of the uncationated and cationated chitosan.**

Test organisms	**Uncationatedchitosan**	**Cationatedchitosan**
Staphylocous aureus	50	25
Bacillus subtiles	50	25
Escherichia coli	50	25
Salmoenella typhi	50	12.5
Klebsiella pneumoniae	50	25

TABLE 3. **Minimum inhibitory concentration uncationatedand cationated chitosan (mg/ml).**

Test organisms	Uncationated chitosan	Cationated chitosan
Staphylocous aureus	25	12.5
Bacillus subtiles	25	12.5
Escherichia coli	25	12.5
Salmoenella typhi	25	6.25
Klebsiella pneumoniae	25	12.5

TABLE 4. **Minimum bacteriocidal concentration of uncationated and cationated chitosan.**

Test organisms	Uncationated chitosan	Cationated chitosan	Cipro	Fluco
Staphylocous aureus	20	28	35	0
Bacillus subtiles	19	22	32	0
Escherichia coli	17	26	37	0
Salmoenella typhi	16	20	38	0
Klebsiella pneumoniae	18	22	39	0

The antimicrobial studies, showed that the polymers have a strong activity against *Staphylococus aureus*, *Bacillus subtiles, Escherichia coli*, *Salmoenella typhi* and *Klebsiella pneumonia*. The zone of inhibition of the organisms that showed sensitivity ranges from 16-20 mm for uncationated chitosan, while the cationated chitosan have a zone in the 20-28 mm, the increase in the zone could be sign to the hydrophilic nature and positive charge deposit of the cationated chitosan since cell wall are negatively charged. The result of the minimum inhibitory concentration (MIC) of the uncationated and cationated chitosan as shown in (TABLE 3) the uncationated having an MIC value of 25 mg/ml while the cationated having an MIC value of 12.5 mg/ml for *Staphylococus aureus*, *Bacillus subtiles*, *Escherichia coli* and *Klebsiella pneumonia* and *Samoenella typhi* 6.25 mg/ml.

The MBC value of the uncationated chitosan is 50 mg/ml for all the tested organisms while the cationated chitosan have 25 mg/ml for all except 12.5 mg/ml for *Samoenella typhi* (TABLE 4).

Staphylococus aureus is known to play an important role in skin diseases including superficial and deep lesion. It is also implicated in causing sexually transmitted infections (STIs). The strong activity of the uncationated and cationated chitosan indicates that they can be effective against skin and sexually transmitted infections (FIG. 1-8).

Conclusion

Chitin was modified using 30%W/V of sodium hydroxide to obtain chitosan the chitosan was cationated using 2,3-epoxytrimethylammonium chloride, The degree of deacetylation was determined by potentiometric titration, the result obtained was 67%DD the physiochemical characterization such as solubility, moisture content and ash content were carried out chemical analysis such as FT-IR spectroscopy which reveal the presence of some functional groups such as OH,NH_2, C=O, C-O-C groups, the UV-Visible spectroscopy reveal degree of conjugation at the visible region and DSC which shows the thermal stability of the polymers were also carried out. The antimicrobial studies reveal that the polymers were sensitive and have a zone of inhibition in the range of 16 mm-28 mm, thus with an MIC value as low as 6.25 mg/ml and high as 25 mg/ml. From the work carried out it can be inferred that as the degree of deacetylation increases the antimicrobial activity also increases, however when chitosan was cationated it also increases the antimicrobial activity when compared with the uncationated chitosan.

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