PHYSICOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF CHITOSAN PREPARED FROM Schistocerca gregaria (DESERT GRASSHOPPER) AND THE INVESTIGATION OF ITS ANTIMICROBIAL ACTIVITY

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ABSTRACT
Chitosan a versatile biopolymer was prepared from Schistocerca gregaria (desert grasshopper). The physicochemical parameters associated with the prepared chitosan were analyzed. Percentage yields (25.23%), ash content (3.25%), moisture content (1.72%), fat binding capacity (399.91%), water binding capacity (638.10%), pH (6.7-7.0), solubility (2% acetic acid) and degree of deacetylation (97.68%) were all established. FTIR and surface morphology (via SEM) analyses were carried out on the prepared chitosan. The activity of the prepared chitosan was tested against two gram positive bacterial species (Escherichia coli, Salmonella Typhi), one gram negative bacterial species (Staphylococcus aureus) and three fungi (Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus) by agar well diffusion methods. The results revealed that the chitosan inhibited the growth of the microbes in vitro.
Keys: Chitosan; desert grasshopper; physicochemical parameters; degree of deacetylation

INTRODUCTION

Chitosan a non-toxic biopolymer generally obtained by alkaline deacetylation of chitin, is a biocompatible (Park and Kim, 2010). It is biodegradable nature and exhibits promising use in a wide range of biomedical applications (Rinaudo, 2006). These include wound dressing, tissue engineering, implant coatings and in therapeutic agent delivery systems (Park and Kim, 2010). Chemically, chitosan is a linear heteropolysaccharide material that is composed of β-1, 4-linked –D-glucose amine (GlcN and N-acetyl-D-glucosamine (GlcNac) at varying ratios (Figure 1) (Acharyulu *et al*, 2013). The proportion of the GlcNac in relation to the GlcN is defined as the degree of acetylation (DA). In this, chitin can be differentiated from chitosan by DA being ≤0.5. This in aqueous acidic medium such as organic acids like acetic, formic, citric as well as inorganic acids such as diluted hydrochloric acid (Rinaudo, 2006). Chitosan is found naturally in certain fungi (*Mucoraceae*), it is prepared mainly from shrimp. It has also from a few species of insects (Liu *et al.*, 2012). However, it is mostly obtained from the thermochemical deacetylation of chitin in the presence of an alkali. Several methods have been proposed, most of them involving hydrolysis of the acetylated residue, using sodium or potassium hydroxide solutions. Also a mixture of anhydrous hydrazine and hydrazine sulfate has been reportedly used (Shilratan *et al.*, 2014).
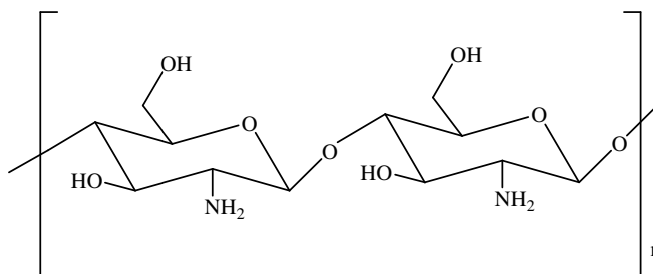
Figure 1: Structure of chitosan

The degree of deacetylation (DD) is the key property that affects the physical and chemical properties of chitosan, such as solubility, chemical reactivity and biodegradability and consequently their applications (Acharyulu *et al* 2013; Wanule *et al.*, 2014).

Chitosan hydrogels are used in drugs delivery systems both as epidermal and implants, because they are capable of maintaining constant drug concentration for a longtime (Sarmento, *et al.*, 2007).As a results of biocompatibility of chitosan hydrogels are not only used for drugs delivery systems, but for generation of hemodialysis membranes, biodegradable surgical seams, artificial skin burns healing agents, immobilizing enzymes and cells, among others (Gustavo, *et al.*, 2017).The modifications of chitosan structure through its amino group generated chitosan derivatives with great biomedical and industrial applications. However, there are no or fewer studies on the Physicochemical and functional characterization of pristine chitosan prepared from Desert grasshopper (*Schistocerca gregaria).*

MATERIALS AND METHODS
Materials
Desert grasshopper (*Schistocerca gregaria*) was collected from Maigatari local Market, Maigatari Local Government Area, Jigawa State, Nigeria. Hydrochloride acid (HCl), acetic acid, sodium hydroxide (NaOH), all from Sigma Aldrich and distilled water was obtained from the chemistry laboratory, at Bayero University, Kano.Dimethyl Sulfoxide (DMSO) obtained from Sigma Aldrich. Bacterial and Fungal isolates were obtained and identified at the Department of Microbiology, Faculty of Sciences, Bayero University, Kano. All solvents were of analytical grade and were used as received. Nutrient Broth (NB) and potato dextrose agar (PDA) were used as bacterial and fungal media respectively.

Methods
The grasshoppers wings were removed separately and the exoskeletons were sun-dried for three days. These were then ground into powder. The exoskeletons were used for the preparation of the chitosan using the following methods; which include demineralization, deproteination and deacetylation processes.

Initially, the grasshopper exoskeleton powder was dimineralized with 5% HCl, with solid to solvent ratio of 1:15(w/v). Constant stirring was then undertaken for 30mins at ambient temperature and was followed by vacuum filtration. The residue was washed in a running tap water, rinsed with distilled water and oven

dried at 60°C for 24hours. Furthermore, dimineralized powder was deproteinized with 3.5% NaOH solution in the ratio 1:15(w/v), for 30-40mins, with constant stirring. The mixture was vacuum filtered and the residue was washed in a running tap water. This was then rinsed with distilled water and oven dried at 60°C, for 24hours. The removal of the acetyl group from the chitin was achieved by autoclaving for 2hrs at 121°C, using 50% NaOH with a solid to liquid ratio of 1:15(w/v). The resulting chitosan was then washed and neutralized with tap water followed by rinsing with distilled water and oven dried at 60°C, for 24hours (Shilratan *et al.*, 2014).

Chitosan purification
According to the procedure reported by Signini and Campana Filho(1999),the chitosan sample was dissolved in 2% acetic acid and left to stand for 18 hours. The solution was then filtrated through a cheesecloth in order to remove contaminants and undissolved particles. The chitosan was then precipitated with 5 % sodium hydroxide, collected and washed in a running tap water before being rinsed, with distilled water in order to remove the excess of alkali. This process was repeated until the material reached neutrality and was then dried in a vacuum oven for 24hrs, at 60°C.

Determination of degree of deacetylation
0.1g of the chitosan sample was weighed and placed in a desiccator, for 12hrs. The sample was then removed from the desiccator and covered immediately to avoid air entry. The spectra of the chitosan sample were obtained using an infrared spectrophotometry (Shimadzu FTIR 8300 Spectrometer), with measurement conducted in the frequency range of 650 - 4000cm^{-1}. The DD of the chitosan sample was determined by comparing the absorbance of the measured peak to that of the reference peak. The DD was calculated from the absorbance (A) ratios using the baseline. The computation equation for the baseline is given by equation I:

$$DD = 100 - \left[\frac{A1655}{A3450} \times \frac{100}{1.33} \right] \dots\dots\dots\dots\dots$$
$$\dots\dots\dots\dots\dots\dots\dots\dots \text{(I)}$$

In equation I, A1655 represents the absorbance of the amide-I band at 1655 cm^{-1}as a measure of the N-acetyl group content of the material. A3450 represents absorbance of the hydroxyl band at 3450cm^{-1}. The factor '1.33' denotes the value of the ratio of A1655 / A3450 for the fully N-acetylated chitosan (Domszy and Roberts, 1985).

Ash content
The ash value of the chitosan was determined gravimetrically by weighing 1g of the prepared sample into previously ignited, cooled and tarred crucibles. The samples were heated in a muffle furnace at 650^0C for 6hrs.The crucibles were allowed to cool in the furnace for less than 30minsbefore they were placed into a desiccator which had a vented top. The ash content was calculated using equation II (Shilratan *et al.,* 2014).

$$\%Ash = \frac{Weight\,of\,residue\,(g)}{Sample\,weight(g)} \times 100 \quad \ldots\ldots(II)$$

Moisture content
The moisture content of the prepared chitosan was determined by the gravimetric method that was reported by Shilratan *et al.,* (2014). 1g of the sample was weighed and dried to constant weight in an oven at $I05^{\circ}$C for 24hrs. The moisture content was then calculated using equation III.

$$\text{Moisture content } (\%) = \frac{w_1 - w_2}{w_1} \times 100 \ldots\ldots(III)$$

Where W_1 =weight (g) of sample before drying and W_2 after drying.

Fat binding capacity of chitosan
The chitosan sample was measured using a modified method that reported by Wang and Kinsella (1976). Fat absorption determination was initially carried out by weighing a centrifuge tube that had contained 0.05g of the sample. To this 10ml of olive oil was added by mixing on a vortex mixture for 1min in order to disperse the sample. The content was left at ambient temperature for 30mins with intermittent shaking at every 10mins before being centrifuged at 2000 rpm, for 30mins. After this, the supernatant was decanted and the tube was weighed again. FBC was calculated using equation IV:

$$FBC\,(\%) = \frac{Fat\,bound\,(g)}{Sample\,weight} \times 100 \ldots(IV)$$

Solubility Test
0.01g of the chitosan samples were placed in three (3) different dry beakers and 20ml of 2% of acetic acid were then added to each beaker. Each mixture was then stirred using a magnetic stirrer for 30mins. The solubility of the material was determined by the method reported by Mohy *et al.* (2015).

Water uptake/water binding capacity
Water uptake (%) estimation was determined by placing a weighed sample of 0.02g of previously dried chitosanin 25mls of distilled water using an incubator shaker operating at 240rpm, for 6hrs at 25°C.The sample was then filtered off, carefully bolted with a filter paper and weighed. The water uptake was calculated on the basis of equation V.

$$\text{Water uptake \%} = \left[\frac{(M - M_o)}{M_o}\right] \times 100 \quad \ldots\ldots(V)$$

In equation V, M is the weight of the swelled sample at time t and$M_o$is the weight of the pre-swelled sample.

Antibacterial test
Antibacterial analysis was carried out using the standard agar well diffusion method. The suspension of each microorganism was rubbed with a swab on a solidified nutrient agar in petri dishes. Four different concentrations per petriplate of the test compound in DMSO were prepared and placed on the culture media before incubation was carried out at 37^0C, for 24hours. Activities were determined by measuring (in mm) the diameter of the zone showing complete inhibition. The results obtained were compared with the activity of cifrolaxacin (500mg/ml) as a standard antibacterial drug.

Antifungal Test
The invitro antifungal test of the chitosan was assayed using three fungal isolates(*A. niger, A. flavus, A. fumigatuses*)by agar well diffusion method. Potato dextrose agar(PDA) was used to prepare the culture media and the incubation was carried out at room temperature for 48hours.The results obtained were compared with the activity of ketoconazole(200mg/ml), as a standard antifungal drug.

Scanning Electron Microscopy (SEM)
The surface morphology of the chitosan was observed using SEM, model: UG 200 SEM, UG-microtech, UCK field, UK. To analyze the chitosan sample, the test sample was cut into pieces of various sizes and wiped with a thin gold-palladium layer using a sputter coater unit (Shashikala and Shafi, 2015).

RESULTS AND DISCUSSION
Structural confirmation by Infrared Spectroscopy
The FTIR spectrum of the chitosan (Figure 2) exhibits strong peak at 3257cm^{-1} which can be assigned due to the stretching vibration of O-H, superimposed to the N-H stretching band and inter hydrogen band of the polysaccharide.
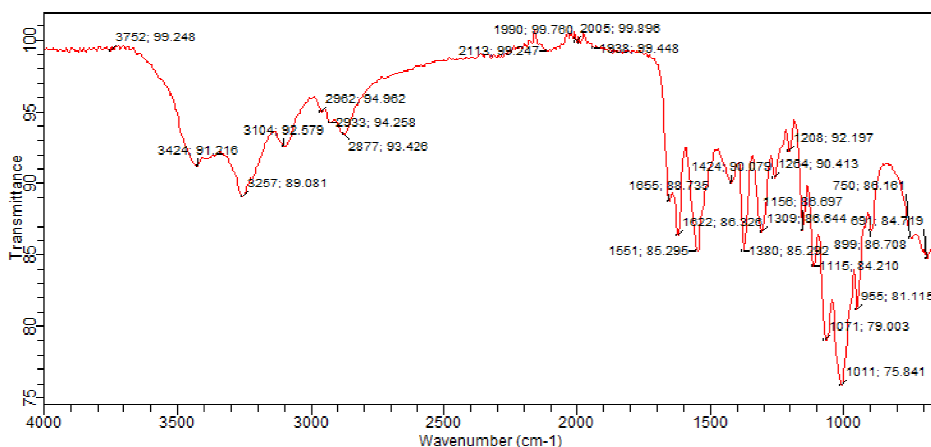
Figure 2:FTIR spectrum of the chitosan material

The C-H axial stretching band is shown at 2873 cm^{-1}.Primary amines have two weak absorption peaks at 1655 cm^{-1} and 1551cm^{-1},corresponded to amide (1) and amide (11) respectively. These indicate that the prepared chitosan has a high degree of deacetylation. Besides these, the bands at 1115 cm^{-1} and 1071cm^{-1} arise due to C-O stretching (1-4)-linked-β-D-glucosamine unit (Vadivel *et al.*, 2015).

Physicochemical and functional parameters

The physico-chemical and functional properties of the prepared chitosan, obtained from *schistocerca gregaria* (grasshoppers) were evaluated. The results were given in Table 1

Table 1: Physico-chemical and functional parameters of the prepared chitosan

Physico-chemical parameters	Values
Yield	25.23%
Fat binding capacity	399.91%
Moisture content	1.72%
Ash content	3.25%
pH	6.7-7.0
Solubility	Soluble in Acetic acid
Degree of deacetylation	97.68%
Water binding capacity	638.10%

Moisture content

The moisture content of the prepared chitosan (Table 1) was found to be 1.72%. This is within the range interactions of 1.6% - 2.1% for water absorption of the chitosan. The moisture ranges could be attributed to the extent of the drying process and the exposure of the sample to the atmosphere. Chitosan is hydroscopic in nature. It is likely that the prepared chitosan can be affected by small moisture adsorption during storage. The lower the moisture content of chitosan the better its shelf stability and hence its quality (Sneha *et al.*, 2014).

Ash content

Ash content is an important parameter that affects chitosan stability and viscosity. The ash content determined for the prepared chitosan (Table 1) was 3.25%, indicating the effectiveness of the demineralization step that was used in removing any minerals that the material might have contained (Shilratan *et al.*, 2014).

Solubility and pH

The prepared chitosan (Table 1) was found to be soluble in 2% acetic acid solution and insoluble in water, methanol, ethanol, tetrahydrofuran and dimethylsulfoxide. The pH of the prepared chitosan was in the range of 6.7-7.0, which indicates the pH neutrality of the prepared chitosan (Pillai *et al.*, 2009).

Degree of deacetylation

The degree of deacetylation (DD) is an important parameter affecting solubility, chemical reaction and biodegradability. DD may range from 30-90% depending on the available source and the preparation procedure of a material. This was calculated by using the FTIR data obtained from the prepared chitosan. The relationship proposed by Domszy and Roberts (1985) was used in the calculations.

The DD of the prepared chitosan was found to be 97.68% (Table 1). 100% DD is very rare. Commercial chitosan material with various DD in the range of 75-85% is generally found (Suneetha *et al.*, 2017).

Fat binding capacity

Fat binding capacity signifies how the chitosan easily bind to or adsorb to fat. Average fat binding capacity reported by Roul,(2001) for crab-based chitosan and crayfish-based chitosan for use in soya bean oil were 706% and 587% respectively, regardless of the type of the vegetable oil. The prepared chitosan sample showed desirable FBC value of 399.9% (Table 1). This is in agreement with 314% to 535% with an average of 417% being obtained and reported by Roul, (2001).

Water binding capacity

The binding capacity for the prepared chitosan (Table 1) was found to be 638.10%. The result was supported by similar observations made by Roul, (2001) for chitosan samples, wherein, WBC was in the ranged 581%-1150%, with an average of 702%. Also, Cho *et al.*, (2014) reported the WBC ranging from 458% to 805% for five commercial chitosan materials that were obtained from shrimp and crab shells(Cho *et al.*,2014).

Table 2: Sensitivity test of chitosan against bacterial and fungal isolates

Isolates	Concentrations (ug/ml)				Anti-bacterial Cifrolaxacin (500mg/ml)	Anti-fungal Ketoconazole(200ug/ml)
	5000	3000	2000	1000		
Staphylococcus aureus	13	10	8	-	22mm	
Escherichia coli	16	14	9	13	28mm	
Salmonella Typhi	17	15	12	10	31mm	
Aspergillus niger	15	13	11	9		27mm
Aspergillus flavors	14	11	9	-		30mm
Aspergillus fumigates	10	9	-	-		21mm

Antimicrobial activity study

The *invitro* antimicrobial activity of the prepared chitosan was investigated using standard agar well diffusion technique. Diameter of zones of inhibition by the chitosan at concentrations of 5000μg/mg, 3000μg/ml, 2000μg/ml and 1000μg/ml against the three bacterial and three fungal isolates used are presented in Table 2. The antibacterial activity results revealed that the chitosan was active against *Staphylococcus aureus*, *Escheria coli* and *Salmonella* Typhi at high concentrations. The results obtained were compared with the activity of the standard antibacterial drug cifrolaxacin. These show that the chitosan possessed potential antibacterial activity. The antifungal activity test revealed that the prepared chitosan was active on the tested fungal isolates namely *Aspergillus flavor*, *Aspergillus fumigatus*, *Aspergillus niger*. The results obtained were compared with standard antifungal drug ketoconazole(Table 2).

Scanning electron microscopy analysis

The chitosan produced fromthe Grasshoppers was examined using SEM analysis, model: UG 200 SEM, UG-microtech, UCK field, UK. The morphology data from the chitosan sample shows that the test material has arough surface, organized microfibrillar crystalline structure which was truant in chitosan, and that it possess layers of flakes that are of porous nature seen in some areas (Figure 3).Similar observations were reported by Yen *et al.*,(2009), Arabia *et al.*,(2013) and Muzzarelli *et al.*,(2014).
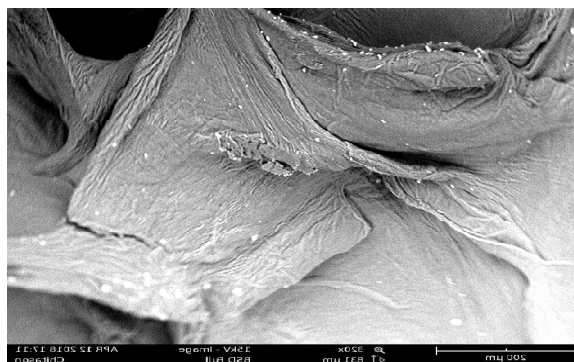

Figure 3: SEM microgram obtained from the chitosan material

CONCLUSION
Chitosan was prepared from *schistocerca gregaria* (desert grasshopper) and was characterized from the physicochemical points of view. The data from the FTIR analysis confirmed the formation of the chitosan. The SEM analysis was carried out in the same manner and had showed the surface morphological features of the prepared chitosan. Antimicrobial activity results indicated that the prepared chitosan had a promising antibacterial and antifungal activity. These suggested that the prepared material could be carefully used as food preservative.

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