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Antimicrobial and Toxicological Evaluation of Food Grade Chitosan From Crab (*Callinectes sapidus*)

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ABSTRACT: Food grade chitosan was produced from crab (*Callinectes sapidus*) shell using unconventional methods of demineralization, deproteinization and deacetylation. The antimicrobial susceptibility and toxicological evaluation of the chitosan was invesyigated using standard procedures. Chitosan produced from *Callinectes sapidus* was found to contain moisture (0.75±0.00 %), nitrogen (6.94±0.13 %), and ash (0.75±0.00 %). Functional characterization revealed a degree of deacetylation (89.0±2.50 %), viscosity (91.0±5.00 cp), solubility (96.4±0.65 %), water binding capacity (270±6.20 %), fat binding capacity (505.3±18.20 %) and molecular weight (152±1.30 KDA) respectively. Susceptibility profile of some food-borne pathogens to crab chitosan revealed a minimum inhibitory concentration (MIC) which ranged from 100 - 1200µg/ml. Chitosan was active against *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* 0157: H7, *Salmonella typhimurium, Saccharomyces cerevisceae and Penicillium expansum.* Among the bacteria isolates, *Staphylococcus aureus* had the least MIC of 200µg/ml with the highest zone of inhibition of 40mm at a concentration of 1600µg/ml. *Escherichia coli* 0157: H7 had the highest MIC of 1200µg/ml as the most resistant of the pathogens examined. *Saccharomyces cerevisceae and Penicillium expansum* were highly susceptible to crab chitosan with MIC of 100 and 200µg/ml respectively. Gram-positive organisms were more susceptible to chitosan and this was concentration dependent. Acute toxicity of chitosan at a single dose of 5000mg/kg on male and female albino rats (*Rattus norvegicus*) of Wistar strain did not produce any sign of toxicity. The reult of the sub-chronic toxicity of chitosan revealed no changes in animal behaviour. Body weights, organ weights, haematological indices remained at normal levels. The non-toxicity of this food grade chitosan will be useful for shelf-life extension of foods.

Keywords: Chitosan, *Callinectes sapidus*, Toxicity, Bioactivity, Food

Introduction

Chitosan is a natural polysaccharide comprising copolymer of glucosamine and N-acetylglucosamine. It can be obtained by the deacetylation of chitin from crustacean shells (No and Meyers, 1989). Chitin and chitosan have very similar chemical structures. Chitin exhibits structural similarity to cellulose and differs from it with the replacement of C-2 hydroxyl residues by acetamide groups (Kurita, 1998).

Chitosan is a biocompatible, biodegradable, non-antigenic material which can be shaped in fibre particles, films and gels. Chavasit and Torres (1990) reported that one of the most relevant applications for chitin and chitosan is as a functional ingredient in foods. Chitin and chitosan can be used as food additives. Most importantly, the food processing industry extensively uses polysaccharides in food product development and processing for the purpose of imparting desirable functional properties such as thickening, gelling, emulsifying and whipping. They are also required as extenders, adhesives and crystal controlling agents for the production of sauces, baking goods, ice cream, confectionery, cold cuts, snacks, fruit juice, jams, jellies, cheese and several others. Given that both chitin and chitosan are forms of polysaccharide, they have great potential in the food industry (Knorr, 1984). Chitin and chitosan can be used as functional ingredients for texture control in foods, through increasing interactions among functional groups. They can also be used as emulsifiers because of their remarkable ability to bind water (230 – 440% w/w) and fat (170 – 315% w/w) with crystalline chitin being the best emulsifier. Good water uptake of chitosan has been found to be significantly higher than that of crystalline cellulose (Knorr, 1982, 1984).

The chelating properties of chitosan is advantageous in the removal of heavy metals, dyes, pigments, acids and organic solids such as proteins. This has resulted in utilizing about 50% of the currently produced chitosan in clarification, coagulation and flocculation processes in food processing waste and wastewater treatment. It is also employed in downstream processing for the removal or recovery of microorganisms. Several studies have demonstrated the effectiveness of chitosan for coagulation and recovery of suspended solids in processing wastes from poultry, seafood and vegetable operations (Bough, 1975, 1976). These studies indicate that chitosan can reduce the suspended solids of various food processing wastes by 70-98%. Chitosan is also effective for dewatering activated sludge suspensions resulting from biological treatment of brewing and vegetable canning wastes (Bough, 1976).

Fruit juice production requires clarifying agents to reduce turbidity. Chitosan has proved to be very effective in the removal of colloids and dispersed particles from fruit concentrates because of its net positive charge, without affecting colour (Chavasit and Torres, 1990). Addition of water-soluble and acid soluble chitosan to fruit juice processing has been as effective as treatment with silica sol/ gelatine/ bentonite, obtaining 2.9 FTU (formazin turbidity units). Acid soluble chitosan is more effetive at low concentrations with maximum clarification being obtained at 0.7g/l. However, water-soluble chitosan is preferred because it is easier to manipulate and does not affect aroma (Soto-Peralta *et al.*, 1989).

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Most chitosan production studies have focused on shrimps, mussels, lobsters, and antartic krill using the traditional method of HCl demineralization (No and Meyers, 1995; Tsai and Su, 1999; Zheng and Zhu, 2003). The objective of this study was to produce a food grade chitosan from the blue crab (*Callinectes sapidus*) that can be used in shelf-life extension of foods. Attention is therefore given to unconventional production, antimicrobial susceptibility and toxicological profile of chitosan from the blue crab (*Callinectes sapidus*)

Materials and Methods

Sources of Microorganisms

The microorganisms used in the study were bacteria (gram-positive and gram-negative) and fungi. *Salmonella typhimurium, Escherichia coli* 0157:H7 and *Listeria monocytogenes* were stool samples obtained from Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. *Staphylococcus aureus (SAUBT$_1$),* a clinical wound isolate was obtained from the Department of Medical Microbiology, University of Benin Teaching Hospital (UBTH), Benin City, Nigeria. The yeast and mould *Saccharomyces cerevisiae and Penicillium expansum* were isolated from deteriorating pineapple fruit waste samples obtained from Uselu market, Benin City, Nigeria.

Identification of Isolates

The microorganisms employed in the experiment were characterized based on shape, size and colour of colony and inspected by light microscopy. The bacteria were Gram-stained (Roberts *et al*., 1995). Phenotypic profiling of both gram-positive and gram-negative bacteria was undertaken using API 50CHB and API 20E strips (BioMerieux, Marsielle, France) respectively. The yeast isolated from the fruit sample was identified (Barnett *et al*., 2000) on the basis of colony morphology, microscopic appearance and carbon source utilization patterns using API 20C AUX 20210 Kit, (BioMerieux, France). The mould was characterized according to the methods of Barnett and Hunter (1972).

Source of Crab

Fresh blue crabs (*Callinectes sapidus*) were obtained from Yanga market, Benin City, Nigeria. They were transported in clean black polyethene bags to the laboratory and stored at -8±1^{0}C until utilized.

Preparation of Crab Shells

Crab shells were prepared from the crabs as described by Omogbai (2012). Preceding the preparation of crab shell, the frozen crabs were thawed at room temperature (28±2^{0}C) and the fleshy parts were mechanically removed leaving the shell. The shells were washed under running tap water to remove soluble organics, adherent proteins and other impurities. The shells were then dried in the oven (Model DHG-9023A, GPU Shanghaisuipu, China) at 70^{o}C for 36h when completely dried products were obtained. Dried crab shells were ground with a laboratory blender, sifted with 20- (0.841mm) and 40 (0.425mm) mesh sieves. The dried ground shell was stored in opaque plastic bottles at room temperature (28±2 ^{0}C) until used for chitosan production or analysed for chemical composition.

Unconventional Chitosan Production Studies

In the unconventional method of chitosan production, the deproteinization and demineralization steps, as well as the sequential treatment process were modified to produce food grade chitosan according to Omogbai (2012) Chitosan was produced by altering the normal sequence of deproteinization, demineralization, decoloration and deacetylation (DPMCA) to demineralization, deproteinization, deacetylation and decoloration (DMPAC). Demineralization of crab shells was achieved using 1N Lactic acid with a shell to acid ratio of 1:20 for 1.5 h. In deproteinization experiment finely ground crab shells was mixed with 3% NaOH in a quick-fit flask (Pyrex,England) fitted with a condenser at a solid to liquid ratio of 1:10(w/v). The shells were then deproteinised using standard autoclaving conditions at 121 ^{0}C for 20 min. The treated shells were filtered with a 100 mesh sieve (1.063 mm), washed to neutrality under a running tap water and then rinsed with deionised water. This was followed by deacetyiation with 40 % NaOH in a microwave oven for 20 min. The product obtained was decolorised first with acetone for 10 min and then bleached with sodium hypochloride for 5 min. The sample was finally washed with deionised distilled water and dried for 2.5 h to obtain a crispy chitosan powder (Omogbai, 2012).

Susceptibility of Microbial Isolates to Chitosan

Agar Well Diffusion Assay: The antibacterial activity of chitosans was studied against different strains of bacteria and fungi by agar diffusion method described by Hugo and Russel (1998). The media- Muller-Hinton agar (for bacteria) and malt extract agar (for fungi) were distributed in aliquots of 20ml and autoclaved at 121 ^{0}C for 15 min, cooled to 45 ^{0}C before the addition of each test strain of bacteria or fungi. Target cells (0.2 ml of inoculum) were seeded onto the molten medium and then poured into petri dishes. Wells (5 mm in diameter were then made on the solidified agar using a sterilized stainless steel punch/cork borer. The wells were filled with 0.2 ml volume of the different concentrations of chitosan solution. Acetate buffer (pH 6.0) was used as a control. A pre-incubation diffusion time of 30min was allowed. The agar plates were incubated at 37 ^{0}C for 24 h for bacteria and 30 ^{0}C for 72 h for fungi respectively. Inhibitory activity was indicated by a clear zone surrounding the well. The diameters of clear inhibition zones delimited were measured in millimeters using a compass and metre rule.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) test was carried out by two-fold serial broth dilution of chitosan solution following the modified method of Hacek *et al.* (1999).

Toxicological Studies of Chitosan

The oral acute toxicity of chitosan was carried out as outlined below

Experimental Animals

The male and female albino rats (*Rattus norvegicus*) of Wistar strain, weighing between 135 and 152g were obtained from the animals holding unit/farm in Ikpoba Hill, Benin City, Nigeria. The animals were housed individually in cages in the animal house under

hygienic well ventilated and standard environmental conditions (temperature: 28 ± 2^{O}C; humidity: 50-55%; photoperiod: 12 h natural light/ dark cycle) with free access to commercial rat feed (Bendel Feeds and Flour Mills Limited, Ewu, Nigeria) and tap water before the commencement of the experiment.

Experimental Protocol

Acute Toxicity: Acute toxicity of chitosan (DMPAC) was carried out according to the Organisation of Economic Co-operation and Development (OECD) guideline for testing of chemicals, TG420 (OECD, 2001). A total of ten (10) rats were randomly divided into two groups of five (5) animals per sex. Chitosan at a single dose of 5,000 mg/kg body weight was administerd orally to the treated group, while the control group received water only. Body weight, signs of toxicity and mortality were observed after the administration at the first to sixth hour and once daily for 14 days. All rats were fasted on the 15th day for 16-18 h, and then sacrificed for necropsy examination (Ibeh, 2005; Carol, 1995). The internal organs were excised and weighed.

Subchronic Toxicity: Seven days after acclimatization, the animals were divided into five (5) groups of 20 animals. At the commencement of the experiment they were starved overnight with free access to water. The control group (group I) were fed with the commercial rat feed and received water but animals in group II, III and IV were given fruit juice (orange) containing chitosan intra-gastrically at concentration of 400 mg/kg, 800 mg/kg and 1600 mg/kg for 90 days.In order to assess reversibility effect, the extract at the dose of 1600 mg/kg was administered once daily to the fifth (satellite) group of rats for 90 days, and kept for another 28 days post-treatment. Toxic manifestations such as signs of toxicity, mortality and body weight changes were monitored daily. The animals were sacrificed after fasting on 91st and 118th (satellite group) day for necropsy examination (Ibeh, 1998; Carol, 1995; OECD, 1981).The body weight evolution and weight of the organs from the control and the test group were compared using the t-test run on the software SPSS for windows.

Haematological Examination

For haematological analysis, 3 ml of blood were collected by cardiac puncture into heparinized vials and stored at 10 ^{0}C for analysis the same day. The packed cell volume (PCV), haemoglobin (Hb) concentration, red blood cell (RBC) and white blood cell (WBC) count were determined using the standard techniques described by Dacie and Lewis (1991) and Jain (1986). The differential WBC counts, mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated (Jain, 1986).

Statistical Analyses and Design

Reported values are averages from three independent trials. Experimental data were subjected to statistical analyses of mean, standard deviation and analysis of variance (ANOVA). The significant value was evaluated using the t-distribution test ($\alpha = 0.05$) using appropriate computer software (Ogbeibu, 2005).

Results and Discussion

Characteristics of Unconventionally Produced Crab Chitosan

The physicochemical characteristics of unconventionally prepared crab chitosan were determined as shown in Table 1. Crab chitosan was found to contain moisture 0.75% and Nitrogen 6.94%. The ash content was reduced considerably from 45.6 % to 0.75 % with the application of lactic acid for demineralization. This chitosan exhibited a molecular weight of 152×10^3 daltons, degree of deacetylation of 89.0 %, viscosity of 91.0 centipoise, solubility of 96.4 %, water binding capacity and fat binding capacity of 270.0 % and 505.3 % respectively.

Table 1: **Physico-chemical Analysis of Unconventionally Produced Chitosan (DMPAC) using Lactic Acid as Demineralization Agent**

Parameter	Amount (%)
Moisture	0.75±0.00
Nitrogen	6.94±0.13
Ash	0.75±0.00
Degree of deacetylation	89.0±2.50
Solubility	96.4±0.65
Water binding capacity	270±6.20
Fat binding capacity	505.3±18.20
Molecular weight (Daltons)	$152x10^3$±1.30
Viscosity (cP)	91.0±5.00

NOTE: Molecular weight and Viscosity are measured in Kilodaltons (KDa) and Centipoise (cP) respectively.

The effect of chitosan (DMPAC) concentration and its antimicrobial properties were studied in the range of 50µg/ml to 1600µg/ml Table 2. At a concentration of 50µg/ml none of the bacteria isolate tested was susceptible to chitosan. At a concentration of 1600 µg/ml chitosan (DMPAC) produced large zones of inhibition with *Staphylococcus aureus* which recorded the largest zone of inhibition of 40 mm.The least zone of inhibition at this concentration was 23 mm recorded with *Escherichia coli* 0157: H7. Among the isolates *Saccharomyces cerevisieae* was most sensitive with a zone of inhibition of 8mm at 100 µg/ml. The minimum inhibitory concentration (MIC) for the isolates tested was in the range 100 µg/ml to 1200 µg/ml. *Saccharomyces cerevisieae* had the least MIC while *Escherichia coli* 0157: H7 had the highest followed by *Listeria monocytogenes* with an MIC of 600µg/ml Table 3.

Table 2: Susceptibility Testing of Microbial Isolates to Chitosan (DMPAC)

Concentration (µg/ml)	Zone of inhibition (mm)					
	Microbial isolates					
	SA	**LM**	**EC_H**	**ST**	**SC**	**PE**
1600	40	27	23	26	29	27
1400	37	24	21	25	26	27
1200	36	21	15	23	23	23
1000	34	18	0	20	22	20
800	34	14	0	16	20	18
600	26	10	0	12	16	14
400	20	0	0	10	13	09
200	18	0	0	07	10	07
100	0	0	0	0	08	0
50	0	0	0	0	0	0

SA= *Staphylococcus aureus*
LM= *Listeria monocytogenes*
EC_H= *Escherichia coli* 0157:H7
ST= *Salmonella typhimurium*
SC= *Saccharomyces cerevisiae*
PE= *Penicillium expansum*

Table 3: Minimum Inhibitory Concentrations of the Microbial Isolates

Microorganism	Minimum inhibitory concentration (µg/ml)
Staphylococcus aureus	200
Listeria monocytogenes	600
Escherichia coli 0157:H7	1200
Salmonella typhimurium	400
Saccharomyces cerevisiae	100
Penicillium expansum	200

Toxicological Studies of Chitosan

Acute Toxicity Study of Chitosan

In acute toxicity study, when chitosan at a single dose of 5,000 mg/kg was orally administered to the rats, there was no noticeable difference in the feeding habit throughout the 14days. There was no obvious hair loss in any of the rats. The fur appearance was full lustre and eye colour remained sparkling and normal throughout the period of study. The body temperature remained at 38.3 ± 0.13^0C and the faecal pellets were also normal. No diarrhoea was seen in any of the rats. The experimental animals were not sluggish but smart as in the control group and showed no sign of toxicity. None of the rat observed died during the experimental period. Toxicity evaluation was further carried out by observing both body weight gain and internal organ weight as shown in Tables 4 and 5 respectively. There was no significant change in these parameters relative to the control group.

Table 4: Body Weights of Rats in the Acute toxicity of Chitosan

Body weight (g)	Day 0	Day 7	Day 14	Weight gain on Day 14
	Female			
Control	138.40±11.15	169.00±6.35	185.80±6.26	47.40±6.30
Chitosan5000mg/kg	137.83±1.30	167.40±3.50	186.00±3.32	48.17±4.52
	Male			
Control	137.26±2.49	184.30±5.24	219.60±7.43	82.34±5.12
Chitosan5000mg/kg	139.60±3.45	191.40±2.46	202.20±2.63	62.40±2.72

Values are expressed as mean±standard deviation., ***n = 5****.*
There were no significant differences at $p < 0.05$

Table 5: Organ Weights of Rats in the Acute Toxicity Testing of Chitosan

C	Organ weight (g)	
	CONTROL	**CHITOSAN 5,000mg/kg**
Female		
Lung	1.07±0.04	1.16±0.03
Heart	0.90±0.01	0.88±0.02
Liver	7.04±0.29	7.12±0.55
Spleen	0.58±0.01	0.61±0.01
Adrenal	0.04±0.00	0.58±0.00
Kidney	0.89±0.05	0.92±0.02
Ovary	0.07±0.00	0.07±0.00
Male		
Lung	1.20±0.03	1.19±0.04
Heart	0.94±0.02	1.06±0.06
Liver	7.78±0.03	8.89±0.46
Spleen	0.81±0.01	0.64±0.01
Adrenal	0.04±0.00	0.04±0.00
Kidney	1.05±0.03	1.03±0.02
Ovary	1.33±0.04	1.32±0.01

Values are expressed as mean ±standard deviation of triplicate determinations, ***n = 5.***
There were no significant differences at p<0.05.

Subchronic Toxicity Study of Chitosan

In subchronic toxicity of chitosan, there were no changes in animal behavior and no toxic signs were detected in the experimental rats. Both salivation and respiratory activity were normal. The body weight gains in treated groups of male and female rats showed a slight increase but were not significantly different from those of the control group. The exception was the male and female rats which received chitosn at the dose of 1600 mg/kg/day (Table 6). The organ weight of rats in the subchronic toxicity of chitosan is shown in Table 7. The female group treated with the chitosan at the dose of 400 mg/kg /day, had the liver weight significantly higher ($p < 0.05$) than the control. In contrary, the weight of heart and kidney were significantly lower than those of their control values in female rats treated with 800 and 1600mg/kg/day of chitosan. The satellite female group showed a significant ($p<0.05$) decline in the kidney weight when compared with the control.

Table 6: Body Weights of Rats in the Subchronic Toxicity Testing of Chitosan

	Body weight (g)			
	Day 0	**Day 90**	**Day 118**	**Weight gain on day 90**
Female				
Control	150.65±2.28	273.15±3.50	-	122.50±5.06
Chitosan 400 mg/kg	148.50±2.84	265.40±6.34	-	116.90±4.25
Chitosan 800 mg/kg	145.60±3.11	262.18±5.55	-	116.58±2.43
Chitosan 1,600 mg/kg [a]	142.00±2.65	256.56±3.40*	-	114.40±2.33
Chitosan 1,600 mg/kg [b]	142.00±3.74	260.40±3.32	289.00±7.76	118.40±3.55
Male				
Control	178.70±5.70	408.00±13.21	-	229.30±10.45
Chitosan 400 mg/kg	171.45±3.95	401.20±6.24		229.75±12.73
Chitosan 800 mg/kg	180.50±2.86	398.70±10.06	-	218.20±6.07
Chitosan 1,600 mg/kg [a]	178.70±2.89	367.50±12.23	-	188.80±10.08*
Chitosan 1,600 mg/kg [b]	185.40±7.19	377.34±9.46	415.20±5.65	191.94.20±12.05

Values are expressed as mean ±standard deviation., ***n = 10.***
a: Group treated with Chitosan at 1,600 mg/kg/day for 90days
b: Satellite group treated with Chitosan at 1,600 mg/kg/day for 90days followed by no treatment for 28 days.
**Significantly different from control, p<0.05*

In the male group (Table 7), there was a significant ($p<0.05$) decrease in the weights of lung and heart of rats, treated with 400 mg/kg/day of chitosan**,** while those of heart and kidney decreased in the animals treated with 800 mg/kg/day. At the dose of 1600 mg/kg/day, a significant weight decrease was found not only in liver and heart, but also kidney and spleen as compared with those of the controls.

Table 7: Organ weights of rats in the sub-chronic toxicity testing of Chitosan

		Chitosan Treatment			
Organ	**Control**	**400mg/kg**	**800mg/kg**	**1,600mg/kg[a]**	**1,600mg/kg[b]**
Female					
Lung	1.32±0.01	1.36±0.03	1.20±0.03	1.28±0.01	1.35±0.02
Heart	1.03±0.03	0.98±0.02	0.95±0.01*	0.89±0.02	1.04±0.02
Liver	5.44±0.16	6.79±0.21*	5.73±0.15	5.94±0.24	5.81±0.14
Spleen	0.69±0.02	0.71±0.04	0.66±0.10	0.64±0.01	0.72±0.03
Adrenal	0.07±0.00	0.06±0.00	0.05±0.00	0.35±0.00	0.05±0.00
Kidney	0.97±0.04	0.99±0.03	0.87±0.02*	0.88±0.01*	0.84±0.02
Ovary	0.15±0.00	0.11±0.00	0.13±0.00	0.14±0.04	0.06±0.00
Male					
Lung	1.87±0.10	1.42±0.03*	1.51±0.01	1.36±0.01	1.64±0.05
Heart	1.49±0.06	1.30±0.04*	1.29±0.03*	1.18±0.03*	1.38±0.02
Liver	10.65±0.32	10.34±0.28	10.08±0.40	9.18±0.42*	9.86±0.23
Spleen	0.92±0.04	0.83±0.03	0.85±0.03	0.75±0.01*	0.90±0.02
Adrenal	0.04±0.00	0.04±0.00	0.06±0.00	0.04±0.00	0.04±0.00
Kidney	1.28±0.03	1.23±0.02	1.18±0.18*	1.12±0.03*	1.25±0.01
Testis	1.93±0.02	1.91±0.03	1.90±0.01	1.88±0.03	1.88±0.02

*Values are expressed as mean ±standard deviation***., n = 10.**
a: Group treated with Chitosan at 1,600 mg/kg/day for 90days
b: Satellite group treated with Chitosan at 1,600 mg/kg/day for 90days followed by no treatment for 28 days.
**Significantly different from control, p<0.05*

The haematological parameters of female and male rats subjected to subchronic toxicity test are shown in Tables 8 and 9, respectively. The concentrations of red blood cells (RBC) and haemoglobin (Hb) in females treated with 400 mg/kg/day of chitosan were slightly lower (Table 8), than those of the control values ($p < 0.05$). In the male groups treated with 400 and 800 mg/kg/day chitosan**,** haemoglobin concentration was significantly ($p < 0.05$) lower than in the control values (Table 9). In the satellite male group, a slight but significant decrease in the concentration of red blood cells was observed. Thus significant increase of mean corpuscular volume (MCV) and mean corpuscular heamoglobin (MCH) was shown. However the differences in these values were minor and remained within normal ranges for rats.

Table 8: Haematological values of female rats in the sub-chronic toxicity testing of Chitosan

	Chitosan Treatment				
	Control	**400mg/kg**	**800mg/kg**	**1,600mg/kg[a]**	**1,600mg/kg[b]**
Red blood cells (10^6/µl)	6.81±0.04	6.42±0.10*	6.72±0.05	6.78±0.10	6.82±0.05
Haemoglobin (g/dl)	14.63±0.15	13.76±0.28*	14.30±0.12	14.45±0.21	14.58±0.15
Hematocrit (%)	40.68±0.34	39.34±0.45	40.35±0.28	40.00±0.57	41.37±0.23
Meancorpuscularvolume volume (fl)	59.75±0.25	61.10±0.26	60.25±0.54	59.13±0.50	54.67±5.41
Mean corpuscular haemoglobin (pg)	21.43±0.12	21.90±0.15	21.40±0.22	21.38±0.18	21.37±0.11
Mean corpuscular haemoglobin Concentration (g/dl)	35.95±0.26	35.81±0.30	35.62±0.24	36.20±0.22	35.29±0.13
Platelet (10^5/µl)	7.52±0.20	7.49±0.83	7.83±0.22	8.33±0.23	8.45±0.00

*Values are expressed as mean ±standard deviation***, n = 10.**
a: Group treated with Chitosan at 1,600 mg/kg/day for 90days
b: Satellite group treated with Chitosan at 1,600 mg/kg/day for 90days followed by no treatment for 28days.
**Significantly different from control, p<0.05*

Table 9: Haematological values of male rats in the sub-chronic toxicity testing of Chitosan

Parameter			Chitosan Treatment		
	Control	**400mg/kg**	**800mg/kg**	**1,600mg/kg[a]**	**1,600mg/kg[b]**
Red blood cells (10^6/µl)	7.90±0.12	7.66±0.10	7.61±0.13	7.79±0.16	7.20±0.05*
Hemoglobin (g/dl)	15.55±.23	14.75±0.11*	14.75±0.18*	15.48±0.25	15.10±0.10
Hematocrit (%)	46.27±0.67	43.86±0.05	45.60±0.72	44.50±0.50	43.65±0.65
Mean corpuscular vol.	57.00±0.33	58.37±0.00	58.65±0.21	60.53±3.45*	57.65±0.26
Mean corpuscular hemoglobin (pg)	19.43±0.35	19.39±0.12	19.41±0.02	19.66±.30	20.82±0.17*
Mean corpuscular hemoglobin Concentration (g/dl)	33.67±0.12	33.58±0.00	33.37±0.10	33.92±0.35	34.28±0.13
Platelet (10^5/µl)	8.40±0.20	8.29±0.21	8.37±0.03	8.73±0.45*	8.86±0.10*

*Values are expressed as mean ±standard deviation***., n = 10.**
a: Group treated with Chitosan at 1,600 mg/kg/day for 90days
b: Satellite group treated with Chitosan at 1,600 mg/kg/day for 90days followed by no treatment for 28 days.
**Significantly different from control, p<0.05*

The alteration of the regular processing sequence of chitosan production had profound effect on its antimicrobial activities as revealed in the results. Chitosan (DMPAC) possess strong antimicrobial activity on the isolates tested. It is plausible that producing chitosan by the DMPAC method resulted in highly protonated polymer with high degree of deacetylation (DD) which must have accounted for its strong antimicrobial activity. Majeti and Kumar (2000) reported that the functionality of chitosan to a large extent is dependent on the charged amino groups (protonation) in the molecule which is a function of degree of deacetylation.

The antimicrobial activity of chitosan (DMPAC) showed marked inhibition of various pathogenic and food spoilage organisms. However, the inhibitory effects differed with concentration and the type of organism whether bacteria or fungi. However, chitosan (DMPAC) demonstrated relatively stronger bactericidal effects on Gram-positive bacteria than on Gram-negative bacteria in the presence of 1600 µg/ml chitosan as evidenced from the larger zones of inhibition obtained. The antimicrobial activity of chitosan (DMPAC) observed in this study is in agreement with the report of Yang *et al*. (2007). Tsai and Su (1999) investigated the antibacterial activity of shrimp chitosan against *Escherichia coli* while Chung *et al*. (2003) reported the effect of abiotic factors on the antibacterial activity of chitosan against *Staphylococcus aureus* and *Escherichia coli*. The inhibitory effect of chitosan on fungi; yeasts and moulds differed with the type of fungi. Susceptibility increased with increase in concentration of chitosan. This is in agreement with previous reports of El-Ghaouth *et al.* (1992). The susceptibility of yeasts and moulds to chitosan (DMPAC) in the present study is not only informative but would be helpful, using chitosan as a natural preservative in foods prone to spoilage by such organisms.

In toxicological studies, alteration or changes in general behaviours, body weight and internal organ weight are critical for the objective evaluation of the effect a compound on experimental animals. This is so, since such changes are often the first signs of toxicity (Auletta, 2002). In acute toxicity study, chitosan at a single dose of 5,000 mg/kg did not shown any toxicity signs (body weight, internal organ weight, and general behaviours). The results suggest that chitosan is practically non-toxic after an acute level exposure in rats. From the evaluation of its subchronic toxicity at doses of 400, 800 and 1600 mg/kg/day for 90 days, both female and male rats treated doses presented no signs of behavioral changes and toxic signs as evidenced by no hair loss, the normal appearance of respiration pattern, colour of eyes and body surfaces, frequency and nature of movement, both involuntary and voluntary (Chan *et al*, 1982; Auletta, 2002). The differences in body weight and body weight gain could be attributed to the physiological variation in rats such as food intake and metabolism. Furthermore all of the increase and decrease in internal organ weights were minor changes which difference may have been due to the variation in size of internal organs and/or body weight of the animals (Bailey *et al*., 2004).

Bone marrow is one of the target sites for the adverse effects of test substances. Since blood cells are mainly produced in the bone marrow, any test substance that affects the bone marrow could inhibit certain enzyme activities involved in the production of haemoglobin in red blood cells and thus reduce the ability of the blood to distribute oxygen through-out the body. Thus in haematological examinations significant changes found in this study are within normal ranges as reported by Feldman *et al*. (2000). Furtherance to the above, the physical examination during the experimental period indicated that all animals were healthy. Therefore, these results suggest that chitosan did not cause any adverse haematological effects.

Conclusion

The non-toxic and antimicrobial effect of uncoventionally prepared chitosan (DMPAC) on food-borne pathogens shows it has a potential to be applied for the non-thermal shelf-life extension of acidic foods such fruit juices.

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