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Sub-chronic Toxicity Study of a Characterized Food Grade Chitosan from Crab (*Callinectes sapidus*)

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Abstract
Food grade chitosan was produced by modification of the deproteinization and demineralization steps, as well as the sequential treatment process in traditional chitosan production process. Chitosan was functionally characterized using standard procedures. The sub-chronic toxicity of chitosan was assessed using clinical blood chemistry and histopathological parameters of male and female albino rats (Rattusnorvegicus) of Wistar strain. The physiochemical characteristics of food grade chitosan produced in this study show that ash, nitrogen and moisture content were 0.60±0.00 %, 6.94±0.06 % and 0.75±0.02 % respectively. The mean functional characteristic of molecular weight, degree of deacetylation, solubility, water binding capacity, fat binding capacity and viscosity were 152.00±1.30 kda, 89.00±0.50%, 95.40±0.65 %, 270.00±6.2 %, 487.00±7.40 % and 91.00±5.00 cp respectively. With the satellite group and in the female rats treated with chitosan, aspartate aminotransferase (ALT) and alanine aminotransferase (AST) were in the range 27.45±1.00 to 29.87±2.17 U/l and 97.00±3.23 to 100.40±1.25 U/l in comparison to their control values of 32.02±1.06 and 103.25±2.15 U/l respectively. However alkaline phosphatase (ALP) (51.78±5.40 U/l) was significantly ($p < 0.05$) different from the control (40.25±2.45 U/l). In the male category both ALT (50.60±5.04 U/l) and ALP (80.45±3.14 U/l) were significantly ($p < 0.05$) different from control values of 37.25±2.18 and 64.70±2.51 U/l respectively. Microscopic examination showed no treatment related histopathological alterations in the vital organs of liver and kidney of albino rats irrespective of chitosan levels used. Conclusively chitosan administered orally did not cause sub-chronic toxicities in female and male rats.

Keywords: Chitosan, characteristics, toxicology, fruit, juice

Introduction
Crabs are found all over the world. Most are marine occurring in all oceans even at its greatest depth. Others inhabit brackish water and fresh water habitats. Some are semi-terrestrial. Like all other invertebrates, crabs are habitat specific. Thus specific crabs are found in marine, brackish and fresh water habitats or on land [1,2] In aquaculture, the crab species of interest include: the mud or mangrove crab (*Scylla serrata*), swimming crab (*Partunustrituberculatus*), blue crab (*Callinectessapidus*) and barbean king crab (*Mithraxspinosissiumus*) [3]
The crab shell (also called carapace) is the outer covering or exoskeleton of a crab. The entire surface of the body of a crab, including most of the lining of the gut is covered by a tough cuticle which collectively constitutes the exoskeleton. This cuticle is heavily calcified over most of the outside of the body giving protection from predators and aiding the freedom which crabs enjoy on the sea bed [4] Typically, the cuticle is composed of four layers: the epicuticle, exocuticle, endocuticle and membranous layer. It is complete in every detail including the pigments that give distinctive colour and pattern to the shell. The crab shell contains chitin which when processed have diverse biotechnological applications [3] When reacted with concentrated sodium hydroxide or subjected to the enzyme chitin deacetylase, chitin undergoes deacetylation forming a derivative called chitosan [5]
Chitosan is a natural polysaccharide comprising co-polymer of glucosamine and N-acetylglucosamine. It can be obtained by the deacetylation of chitin from crustacean shells [6]. 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 [7] Chitin can be transformed into chitosan that has free amino groups by removing acetyl groups (CH_3-CO) from chitin molecules [4]
Some workers [8] reported that chitosan is insoluble in water, alkali and organic solvents, but soluble in most diluted acids with pH less than 6. When chitosan is dissolved in an acid solution, it becomes a cationic polymer due to the protonation of free amino groups on the C-2 position of the pyranose ring [9]. The cationic properties of chitosan in acidic solutions give it the ability to interact readily with negatively charged molecules such as lipids and cholesterol. In this respect, chitin and chitosan have attained increasing commercial interest as suitable resource materials due to their excellent properties including biocompatibility, biodegradability and absorption ability to form films and to chelate metal ions [10]
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

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(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 [11]

About 50% of the currently produced chitosan is employed in clarification, coagulation and flocculation processes in food processing waste and wastewater treatment owing to its chelating ability [12] Several studies have demonstrated the effectiveness of chitosan for coagulation and recovery of suspended solids in processing wastes from poultry, seafood and vegetable operations [13] 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 [13]

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 [14] 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 effective 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 [15]

Chitosan, a versatile hydrophilic polysaccharide has a broad antimicrobial spectrum to which gram-negative, gram-positive bacteria and fungi are highly susceptible [16]. Chitosan is extracted from the shells of shrimp, lobster, and crabs. It is a fibrous substance that might block absorption of dietary fat and cholesterol. The main sources are the marine crustaceans, shrimp and crabs. Chitosan exhibits variety of physicochemical and biological properties resulting in numerous applications in fields such as waste and water treatment, agriculture, fabric and textiles, cosmetics, nutritional enhancement, and food processing. The biocompatibility, biodegradability and bioactivity of chitosan make it a very attractive substance for diverse applications as a biomaterial in pharmaceutical and medical fields as well as the food and beverage industry [16, 17]

Toxicological studies on chitosan was reported by some authors [18,19]. The safety and haemostatic potential of chitosan was reported by [20] and noted that the sterilized films were devoid of pyrogen which will make it suitable for pharmaceutical usage. Current work on chitosan by some researchers has focused on isolation, characterization and applications [9, 12, 21, 22]. Today, consumers are clamouring for natural antimicrobials or bioactive substances for food preservation in the food and beverage industry. A biomaterial to be employed in the fruit juice preservation industry should be non-toxic, non-irritant or carcinogenic. Thus, in order to reduce or eliminate the possible bad effects of using chitosan, toxicity testing is required. Previous researchers did not report on the histopatholgy and clinical blood chemistry of animals used in their experimentation. Consequently there is limited information on the sub-chronic toxicity testing of food grade chitosan prepared from crab to be employed in fruit juice preservation.

The objective of this study is to produce and characterize a food grade chitosan from crab (*Callinectes sapidus*) that will be used for fruit juice preservation. In this study emphasis is given to unconventional chitosan preparation by modification of the traditional method, physiochemical and functional characterization and sub-chronic toxicity assessment of chitosan from *Callinectes sapidus.*

Materials and Methods

Source of Crab

Fresh blue crabs (*Callinectessapidus*) 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 obtained from the crabs as described by [17]. Prior to the preparation of crab shell, the frozen crabs were de-iced at room temperature (28±2 ^{0}C) and the fleshy parts were mechanically removed leaving the shell. Soluble organics, adherent proteins and other impurities were further removed by washing the shells under running tap water. The shells were then dried in the oven (Model DHG-9023A, GPU Shanghaisuipu, China) at 70 ^{0}C for 36h when completely dried products were obtained. Dried crab shells were ground with a laboratory blender, sifted with 20 (0.841mm) mesh 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.

Chitosan Production.

In chitosan production, the deproteinization and demineralization steps, as well as the sequential treatment process were modified to produce food grade chitosan [17]. Chitosan was produced by altering the traditional 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 20min. The treated shells were filtered with a 100mesh sieve (1.063mm), washed to neutrality under a running tap water and then rinsed with de-ionised water. This was followed by deacetylation 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 de-ionised distilled water and dried for 2.5 h to obtain a crispy chitosan powder [17]

Physiochemical Analyses of Chitosan

The protein content of samples was determined using Kjeldahl method with Nessler's reagent [23. The ash content of each sample was determined by dry-ashing in a muffle furnace at 600 ^{0}C [23] Moisture content of each sample was determined by the gravimetric method [23] by drying the sample to constant weight at 105 ^{0}C. The sample was weighed before and after drying.

Determination of Degree of Deacetylation.

The degree of deacetylation (DD) of chitosan samples was determined by a colloid titration method [24]. Chitosan (0.5 g) was dissolved in aqueous 5 % (v/v) formic acid solution (99.5g). One gram of chitosan/formic acid solution was diluted to 30ml distilled/deionised water in an Erlenmeyer flask. After adding 2 to 3 drops of 0.1% toluidine blue indicator (w/v), the solution was titrated with n/400 potassium polyvinyl sulphate solution (PVSK; factor = 1.01; Wako chemical, Japan). A single molecule of PVSK reacts with each deacetylated amino group in the chitosan molecule. The degree of deacetylation was calculated from the molar ratio of deacetylated amino groups in the chitosan molecule, which was estimated from the volume of PVSK solution consumed. Measurements were made in duplicates on each sample, and degree of deacetylation was calculated using the following formula:

DD (%) = $$\frac{x/161}{x/161 + y/203} \times 100$$

where:

x = (Amount of glucosamine in molecule) = $\frac{1}{400}$ x $\frac{1}{1000}$ x f x 161 x V.

$= 2.5 \times 10^{-6}$ f.161V

y = Amount of N-acetylglucosamine in molecule = 0.5 x $\frac{1}{100} - x$

$= 0.005 - x$

V = Titrated volume (ml) of n/400 pvsk

f = factor of PVSK Solution = 1.01 [24].

Determination of Molecular Weight (Mw)

For the determination of viscosity- average molecular weight of chitosan five different concentration (0.015625 ~1.0%) solutions of chitosan in 0.1M acetic acid/0.2M NaCl (1:1, v/v) were prepared. The solution was passed through a filter (Whatman #4) to remove insoluble materials. The ubbeloohde- type capillary viscometer (Canon-Fenske,No. OB) was used to measure the passage time of the solutions flowing through the capillary in a constant-temperature water bath at 25^{0}C. Three measurements were made on each sample. The running times of the solution and solvent were recorded as seconds (sec) and used to calculate intrinsic viscosity (ŋ). The viscosity-average molecular weight of chitosan solutions was calculated using the Mark Houwink equation which provides the relationship between intrinsic viscosity and molecular weight [25]

ŋ= K(Mw)^a…….. Mark Houwink equation

Where K and a are constants for given solute-solvent system and temperature. Values of K and a were 1.81×10^{-3} cm^3/g and 0.93 respectively.

ŋ = intrinsic viscosity

Mw = molecular weight

Determination of Viscosity

Viscosity was determined with a Brookfield viscometer, model LVDV-II + (Brookfield Engineering Laboratories, Stoughton, MA, USA). Chitosan solution was prepared, in 1% (v/v) acetic acid at 1% concentration on a moisture-free basis. The chitosan solution was then filtered using Miracloth (rayon polyester; EMD Biosciences, CA, USA) to remove insoluble materials. After that, the solution was allowed to stand for 2h to remove air bubbles. Measurements were made in triplicates on each sample using a RV No 2 spindle at 50rpm on solutions at 28±2^{0}C.Values were reported in centipoises units (cp) [23]

Determination of Solubility

The modified method of Brine and Austin [26] was used in solubility determination. Samples of crab chitosan powder (0.1g in triplicates) were placed in centrifuge tubes using known weights and then dissolved in 10ml of 1% acetic acid for 30min using an incubator shaker operating at 240rpm and at 28±2^{0}C. The solution was then immersed in a boiling water bath for 10min, cooled to room temperature, 28±2^{0}C and centrifuged at 10,000rpm for 10min. the supernatant was decanted. The undissolved particles were washed with 25ml distilled water and centrifuged at 10,000rpm. The supernatant was removed and undissolved pellets dried at 60^{0}C for 24h. Finally the dried particles were weighed and the percentage solubility determined by calculation:

% Solubility = $$\frac{(W_i + C_w) - W_f + C_w}{(W_i + C_w) - W_i} \; x\,100$$

Where:

Wi= initial weight of tube
Cw= weight of chitosan.
Wf= final weight of tube.

Determination of Water Binding Capacity (WBC)

Water binding capacity of chitosan was measured using a modified method of [27]. Water binding capacity was initially carried out by weighing a centrifuge tube containing 0.5 g of sample, 10ml of water was added with mixing on a vortex mixer for 1min to disperse the sample. The tube contents were left at ambient temperature (28±2 ^{0}C) for 30min with intermittent shaking for 5s every 10min and centrifuged (Model #Z383K, HERMLE-National Labnet Company, Woodbridge, NJ, USA) at 3,500rpm (6000 x g) for 25 min. The supernatant was then decanted and the tube was weighed again. Water binding capacity was calculated as follows:

$$WBC(\%) = \frac{\text{Water Bound(g)}}{\text{Initial Sample weight(g)}} \times 100$$

Determination of Fat Binding Capacity (FBC)

A modified method of [27] was used in measuring fat binding capacity of chitosan. A centrifuge tube containing 0.5 g of the sample was weighed and 10ml of oil was added. Soybean oil and olive oil were used in this experiment. The contents of the centrifuge tube were subjected to mixing on a vortex mixer for 1min to disperse the sample. The contents were left at 28±2 ^{0}C for 30 min with shaking for 5 s at every 10min and centrifuged (Model #z383K, HERMLE-National Labnet Company, Woodbridge, NJ, USA) at 3,500rpm (6,000 x g) for 25 min. The supernatant was decanted and the tube weighed again. Fat binding capacity (FBC) was calculated as follows:

$$FBC(\%) = \frac{\text{Fat bound(g)}}{\text{Initial sample weight (g)}} \times 100$$

Experimental Animals

Male and female albino rats (*Rattusnorvegicus*) 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 under hygienic well ventilated and standard environmental conditions (temperature: 28 ± 2^{0}C; humidity: 50-55%; photoperiod: 12h 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 [28, 29]

Sub-chronic 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 90days, and kept for another 28 days post-treatment. Toxic manifestations such as signs of toxicity, mortality and body weight changes were monitored daily [30]. The animals were sacrificed after fasting on 91st and 118th (satellite group) day for necropsy examination [31, 32]. 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.

Preparation of Serum

The method described by [33] was employed. Under ether anaesthesia, the neck area of the animal was quickly cleared of fur and skin to expose the jungular veins. The veins after being slightly displaced (to avoid contamination with interstitial fluid) were then sharply cut with a sterile scalpel blade. Blood was collected into centrifuge tubes and allowed to clot for 10 min. This was then centrifuged at 33.5 g for 15 min using Uniscope Laboratory centrifuge (model SM 800B, Surgifriend Medicals, Essex, England). The sera were aspirated with Pasteur pipettes into clean, dry, sample bottles and used within 12 h of preparation for the biochemical analysis.

Determination of Biochemical Parameters

The serum separated was used for the determination of diagnostic marker enzymes alanine aminotransferase (EC2.6.1.2) (ALT), aspartate aminotransferase (EC 2.6.1.1) AST [34] and alkaline phosphatase (ALP) activity was assayed by the procedure described by [35]. Clinical blood chemistry was assayed as described by [36].

Organ/ Tissue Pathology

The abdominal walls of the animals were quickly dissected through the linear alba and peritoneum using a scalpel blade. The liver, heart, kidney, spleen and lung of each rat were examined for gross lesions. A 0.5 cm^3 sample of each organ was fixed in 10% phosphate – buffered formalin and prepared for histological examination, following the method of [37] Different sections of each organ were examined for lesions using an Ortholux light microscope (Leitz – Weiltzer, Germany Gm Bh).

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) counts were determined using the standard techniques described by

[38, 39] The differential WBC counts, mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated [39]

Statistical Analysis and Design.

Reported values are averages from three independent trials. All experimental data were subjected to statistical analysis 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 [40]

Results and Discussion

Characteristics of Chitosan

The physiochemical characteristics of food grade chitosan (DMPAC) produced in this study is shown in Table 1. The ash, nitrogen and moisture content were 0.60±0.00 %, 6.94±0.06 % and 0.75±0.02 % respectively. Crustacean exoskeletons contain large amounts of calcium carbonate, depending on the source. Ash measurement is an indicator of the effectiveness of the demineralization (DM) step for removal of calcium carbonate. A high quality grade chitosan should have less than 1% of ash content. The ash content of 0.60±0.00 % indicates the effectiveness of the demineralization with acetic acid solution for the chitosan produced (Table 1). Its reduced level is important for ensuring good quality chitosan. Demineralization is conventionally accomplished by extraction with dilute hydrochloric acid to convert calcium carbonate to calcium chloride. Among such methods are those of [41, 42, 43]. However reaction time by these authors varied with preparation methods from 30 min [43] to over 2 days [41] Some researchers [43] using crayfish reduced ash content with 1N HCL from 63% to 0.3% while others [41] used 2M HCL to bring the ash content in the crab to nil. Demineralization was accompanished with 22% HCL and 6N HCL, respectively at room temperature (28±2 ^{0}C) by [44 and 45]. In the current study in which novel demineralization process was employed using organic acid (lactic acid), the total ash content of the crab shell was reduced from an initial value of 43.2% to less than one percent. Lactic acid was very efficient in the demineralization process.

Table 1 Physiochemical Characteristics of DMPAC Chitosan

Parameter	**Composition**
Ash	0.60±0.00 %
Nitrogen	6.94±0.06 %
Moisture	0.75±0.02 %
Degree of Deacetylation	89.00±0.50 %
Solubility	95.40±0.65 %
Water binding capacity	270.00±6.20 %
Fat binding capacity	480.70±7.40 %
Viscosity	91.00±5.00 cp
Molecular weight	152.0±1.30 kda

A moisture content of 0.75±0.02 % recorded for chitosan DMPAC compared well with the moisture content of the traditionally produced chitosan with a moisture content of 0.50 % [46] The high nitrogen content observed in this study may be due to the presence of protein residues reported by [47]. Protein is bound by covalent bonds forming stable complexes with chitin and chitosan. Thus it is very difficult to achieve 100% deproteinization. Even with complete deproteinization, residual nitrogen will still remain in the chitosans as amino ($-NH_2$) group [47]

The crab chitosan sample had a moisture content of 0.75%. This is considerably lower compared to the value (10 %) reported for commercial chitosan products by [10] The higher moisture content is probably due to the hygroscopic nature of chitosan as reported by [48,49] Thus it is very possible that the sample used was affected by moisture absorption during storage. Moisture absorption may be important by affecting water holding capacity of chitosans, when it comes to its processing and applications [49].

DMPAC chitosan prepared in this study had a low viscosity (91.0 ± 5.0cp) which is not significantly different from the value for some traditionally prepared chitosans by earlier authors [21, 49, 50]. The low viscosity will be particularly suitable for application in highly fluid foods such as fruit juices and wines. Shelf-life extension of fruit juices and wines requires natural preservatives which will not alter significantly the fluidity of these materials for sensory acceptability [51]. However different workers have reported on the variation of the viscosity of chitosan solutions, which ranges from 60 to 780 cp [49, 50]. This range of viscosity have also been observed by [52] with five commercially available chitosans. Viscosity of chitosans varied considerably from 60 to 5,110 cp depending on species [42].

The chitosan obtained had a molecular weight of 152±1.30 kda and degree of deacetylation of 89±0.50%. The molecular weights of chitosan have been reported to vary with the source and methods of preparation. Commercial chitosan products have molecular weight ranging from 100,000 to 1, 200,000 Daltons (Da) [10] The molecular weights of the crab chitosan (DMPAC) prepared in this study was lower when compared with

previous report [10, 22] It is possible that the chitosan underwent more depolymerization which resulted in lower molecular weights. The results from the present work showed that crab chitosan prepared unconventionally had high DD value. A high DD value increases the amount of positively charged groups available for flocculating a negatively charged material [53].
DMPAC had a high solubility of 96.4±0.65 %. The solubility of chitosan is to a great extent controlled by the degree of deacetylation and the deacetylation must be at least 85% complete in order to have the desired solubility [46].

Table 2: Differential white blood cell counts of rats in the sub-chronic toxicity testing of Chitosan

	Chitosan Treatment				
	Control	400mg/kg	800mg/kg	1,600mg/kg[a]	1,600mg/kg[b]
Female					
WBC ($x10^3$/µl)	3.06±0.20	2.83±0.25	2.84±0.30	2.65±0.19	2.97±0.00
Neutrophil (%)	19.45±0.10	17.65±0.36	17.41±0.45	18.55±0.78	11.15±0.25*
Lymphocyte (%)	73.30±0.13	74.50±1.85	74.00±0.07	75.68±1.02	84.56±0.45*
Monocyte (%)	5.78±0.47	5.28±0.25	6.35±0.22	5.30±0.32	5.98±0.65
Eosinophil (%)	0.64±0.25	1.12±0.25	1.41±0.08	1.22±0.10	2.21±0.15*
Basophil (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Male					
WBC ($x10^3$/µl)	4.01±0.20	1.12±0.03	3.70±0.16	3.76±0.23	3.15±0.26
Neutrophil (%)	14.51±0.03	1.15±3.00*	16.45±1.00	15.00±1.00	16.00±0.50
Lymphocyte (%)	77.50±1.05	70.18±0.60*	75.70±1.08	79.00±0.04	76.51±0.15
Monocyte (%)	5.55±0.36	5.67±0.05	5.24±0.53	5.36±1.20	5.60±0.20
Eosinophil (%)	2.45±0.36	1.54±0.22	2.35±0.25	2.51±1.00	1.20±0.00
Basophil (%)	0.00±0.00	0.81±0.00	0.00±0.00	0.00±0.00	0.00±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 28 days.
**Significantly different from control, p<0.05*
WBC = White blood cells

DMPAC chitosan had a water binding capacity of 270± 6.2%. This is low when compared to the water binding capacity (WBC) of conventionally processed crab chitosan. The difference in WBC between chitinous polymers have been adduced to dissimilarities in crystallinity, differences in the amount of salt forming groups and the residual protein content of the products [54]
The unconventional chitosan DMPAC showed low fat binding capacity (FBC) at 480.70± 22.60 % for soybean oil and 505.30±18.20 % for olive oil. This result is slightly similar to that reported at 314-535 % by [52] but higher than that (217-403%) reported by [50]. Thus reversing the sequence of steps in chitosan production had profound effect on the FBC. On the whole, production of chitosan with low molecular weight and high degree of deacetylation characteristics will be advantageous for application in shelf-life extension of tropical fruit juices to help cut-off microbial contaminants due to their potential antimicrobial effects [16]
The differential white blood cell count values of rats in the sub-chronic toxicity test are shown in Table 2. No significant ($p < 0.05$) difference was detected at any of the three doses administered compared to the control values in the female treated groups. However a significant decrease in neutrophil (from 19.45±0.25 % to 11.15±0.25 %) and significant increase in lymphocyte (from 73.30±0.13 % to 84.56±0.45 %) and eosinophil (from 0.64±0.25 % to 2.21±0.15 %) were observed in the female satellite group when compared to the control values. In the male rats treated with 400 mg/kg/day of chitosan, neutrophils (1.15±3.00 %) was significantly ($p < 0.05$) different from the control value of 14.51±0.03 %.The result of the clinical blood chemistry of rats in the sub-chronic toxicity testing with chitosan is shown in Tables 3 and 4. There was no marked difference in any of the specific activities of enzymes in chitosan-fed rats. In the female rats treated with chitosan, aspartate aminotransferase (ALT) and alanine aminotransferase (AST) were in the range 27.45±1.00 to 29.87±2.17 U/l and 97.00±3.23 to 100.40±1.25 U/l in comparison to their control values of 32.02±1.06 and 103.25±2.15 U/l respectively. However alkaline phosphatase (ALP) (51.78±5.40 U/l) was significantly ($p < 0.05$) different from

control (40.25±2.45 U/l). In the male category both ALT (50.60±5.04 U/l) and ALP (80.45±3.14 U/l) were significantly ($p < 0.05$) different from control values of 37.25±2.18 and 64.70±2.51 U/l respectively. The clinical blood parameters of glucose, total protein, albumin and total bilirubin had similar values as the control in both the male and female rats fed with chitosan. However the level of blood urea nitrogen (BUN) and creatinine were different. In particular in the female rats BUN (19.76±0.53 mg/dl) and creatinine (0.35±0.05mg/dl) were significantly ($p < 0.05$) different from control values of 23.67±1.04 and 0.46±0.02 mg/dl respectively. The male rats in the satellite group followed a similar trend with the female rats fed with chitosan at 1600 mg/kg/day.

Table 3: Clinical blood chemistry values of female rats in the sub-chronic toxicity testing of Chitosan

PARAMETER	CONTROL	CHITOSAN TREATMENT			
		400mg/kg	800mg/kg	1,600mg/kg[a]	1,600mg/kg[b]
Glucose (mg/dl)	104.56±0.45	100.20±1.05	101.08±2.0	95.90±1.51	106.38±2.00
BUN (mg/dl)	23.67±1.04	21.20±0.55	20.57±0.42*	21.65±1.13	19.76±0.53*
Creatinine (mg/dl)	0.46±0.02	0.45±0.00	0.42±0.01	0.41±0.01	0.35±0.05*
Total protein (g/dl)	5.15±0.03	5.13±0.00	4.98±0.04	5.28±0.15	5.30±0.17
Albumin (g/dl)	3.60±0.04	3.51±0.10	3.56±0.10	3.58±0.00	3.58±0.00
Total bilirubin (mg/dl)	0.17±0.01	0.13±0.02	0.13±0.2	0.15±0.00	0.16±0.00
Direct bilirubin (mg/dl)	0.00±0.00	0.00±0.00	0.00±0.00	0.23±0.01*	0.00±0.00
AST(U/I)	103.25±2.15	100.40±1.25	99.60±0.23	97.00±3.23	98.21±2.51
ALT (‘U/l)	32.02±1.06	27.45±1.00	27.78±1.36	29.50±2.06	29.87±2.17
ALP (U/l)	40.25±2.45	44.50±4.20	42.17±2.05	51.78±5.40*	30.01±1.06*

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*
BUN = Blood Urea Nitrogen, AST = Aspartate aminotransferase
ALT = Alanine aminotransferase, ALP = Alkaline Phosphatase

TABLE 4: Clinical blood chemistry values of male rats in the sub-chronic toxicity testing of chitosan

PARAMETER	CONTROL	CHITOSAN TREATMENT			
		400mg/kg	800mg/kg	1,600mg/kg[a]	1,600mg/kg[b]
Glucose (mg/dl)	122.27±1.45	125.35±3.06	120.00±0.00	121.50±2.07	128.30±1.15
BUN (mg/dl)	21.00±0.52	20.10±0.41	19.62±0.50	24.55±0.28*	21.71±0.11*
Creatinine (mg/dl)	0.29±0.02	0.27±0.00	0.25±0.03	0.31±0.00	0.37±0.01*
Total protein (g/dl)	5.57±0.06	5.73±0.04	5.68±0.12	5.55±0.02	5.41±0.05
Albumin (g/dl)	3.42±0.02	3.45±0.04	3.36±0.02	3.59±0.06	3.49±0.00
Total bilirubin (mg/dl)	0.11±0.01	0.14±0.01	0.13±0.01	0.13±0.01	0.11±0.00
Direct bilirubin (mg/dl)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
AST(U/I)	110.20±2.08	106.10±3.05	104.50±1.30	105.90±2.30	107.30±3.15
ALT (‘U/l)	37.25±2.18	39.10±1.06	35.09±1.08	50.60±5.04*	36.80±1.12
ALP (U/l)	64.70±2.51	69.07±3.01	67.82±4.15	80.45±3.14*	65.45±1.52

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 90 days followed by no treatment for 28 days.
**Significantly different from control, p<0.05.*
BUN = Blood Urea Nitrogen, AST = Aspartate aminotransferase
ALT = Alanine aminotransferase, ALP = Alkaline Phosphatase

The results of the histopathological assessment are shown in Plates 1-4**.** Gross examination of vital organs such as liver and kidney during autopsy did not reveal any abnormalities that could be attributed to chitosan feeding in both sexes of rats. Furthermore from microscopic examination, no treatment related histopathological alterations were observed in the vital organs irrespective of chitosan levels used. Detailed histological examination of the liver showed that it was characterized by normal hepatic cells with distinct nuclei and normal eosinophilic cytoplasm with normal sinusoids. The kidneys displayed normal renal architecture with normal glomeruli, proximal tubules and collecting ducts.

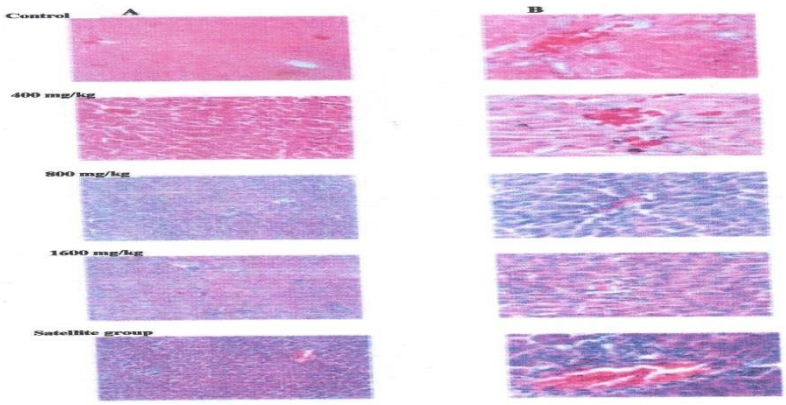

Plate 1: The histopathology of male liver from the control and treated groups in the sub-chronic toxicity of chitosan showing no macroscopic and microscopic damage
A = 10x Magnification, B = 40x Magnification

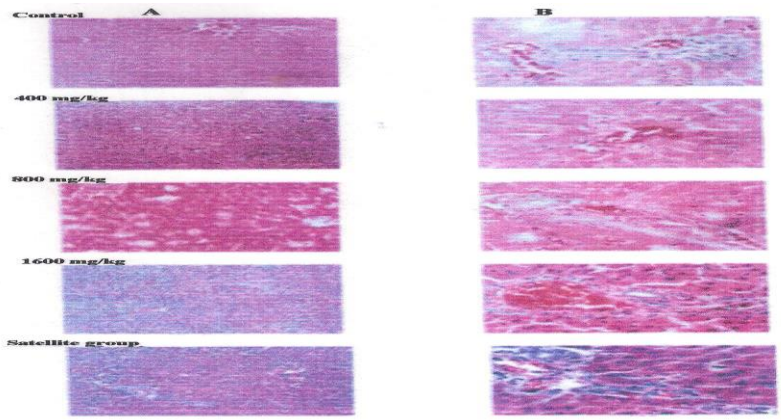

Plate 2: The histopathology of female liver from the control and treated groups in the sub-chronic toxicity of chitosan showing no macroscopic and microscopic damage
A = 10x Magnification, B = 40x Magnification

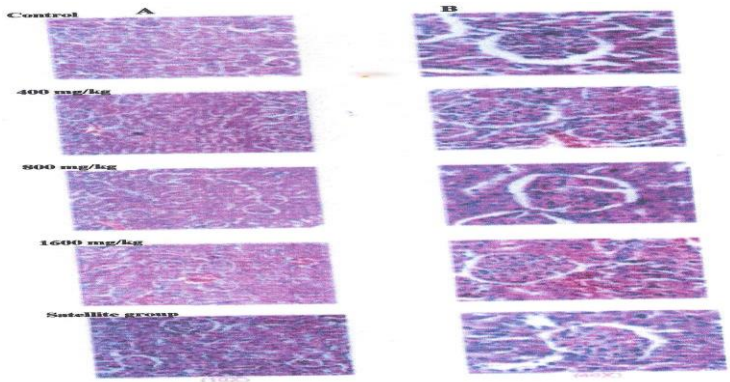

Plate 3: The histopathology of male kidney from the control and treated groups in the sub-chronic toxicity of chitosan showing no macroscopic and microscopic damage
A = 10x Magnification, B = 40x Magnification

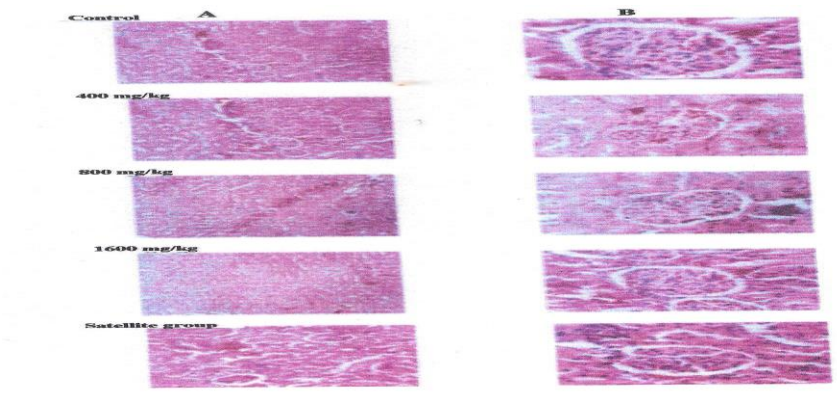

Plate 4: The histopathology of female kidney from the control and treated groups in the sub-chronic toxicity of chitosan showing no macroscopic and microscopic damage
A = 10x Magnification, B = 40x Magnification

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 [55]. 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 negative haematological or immunological effects.

The liver and kidney constitute one of the important internal organs in the body with several vital functions. Symptoms of disorder in those organs appear only in serious diseases which can be detected in chemical blood chemistry of the animal. In the experiment under study, although there were some alterations in the blood chemical values, these were found to be within the normal reference intervals for Wistar rats [56, 57]. Thus the repeated exposure of rats to chitosan over a long period had no toxic effects on them from the result of the clinical blood chemistry. A close look at the clinical blood chemistry revealed significant decrease in the total cholesterol in both male and female experimental rats. This result re-establishes the anticholesterolemic property of chitosan [58] The hypolipidemic property of chitosan has already been reported in high fat diet fed experimental animals [59] The cardioprotective effect of chitosan is probably related to its ability to inhibit the increased accumulation of lipids both in the systemic circulation and in the myocardium by its antilipidemic property.

The necropsy and histopathological examination were performed to further confirm whether or not the internal organs or tissues had been damaged. The results shown in Plates 1-4 did not reveal macroscopic or microscopic changes in investigated internal organs or tissues in any experimental rats. These results indicated healthy status of liver and kidney in the experimental rats. Conclusively chitosan administered orally did not cause acute and sub-chronic toxicities in female and male rats. Its usage in the preservation of tropical fruit juices should be carefully considered after chronic toxicity assessment has been carried out on the long-term safety of the polymer preservative.

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