Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(2): 487 – 504 (2024)
www.ejabf.journals.ekb.eg

Effect of Crab Chitosan and Chitosan Nanoparticles on Experimental Animals: An Acute/ A Chronic Toxicological Study

Abdelrahman S. Talab[1*], Ahmed M. S. Hussein[2], Marwa E. El-Shamarka[3], Doha A. Mohamed[4], Mohie M. Kamil[2], Hala E. Ghannam[1]

[1]National Institute of Oceanography and Fisheries, Cairo, Egypt
[2]Food Technology Department, Food Industries and Nutrition Research Institute, National Research Center, 12622 Dokki, Cairo, Egypt
[3]Department of Narcotics, Ergogenic Aids and Poisons, National Research Center, Cairo, Egypt
[4]Nutrition and Food Sciences Dept., National Research Center, 12622 Dokki, Cairo, Egypt
[*]**Corresponding Author: Abdelrahman_saidh@yahoo.com**

ARTICLE INFO

Article History:
Received: March 4, 2024
Accepted: March 28, 2024
Online: April 4, 2024

Keywords:
Chitosan nanoparticles,
Chronic,
Oral toxicity,
Histopathology

ABSTRACT

Millions of tons of crab shells are annually discarded. Crab shells contain several valuable compounds such as chitosan that can be transformed into chitosan nanomaterials. The unique properties and applications of nanotechnology in targeting fish processing technology have received increased importance during the last twenty years. The application of nanotechnology in fishery products is rapidly increasing, and investigating the toxicity and safety of these nanoparticles is essential before their nutritional use. Therefore, this study aimed to determine the acute and chronic toxicity of crab chitosan and its nanoparticles (ChNPs) on albino mice and sprague Dawley rats, respectively. Acute toxicity revealed that crab chitosan and chitosan nanoparticles were completely safe up to 10g/ kg of mice body weight which is equivalent to 38.75g/ 70kg of man body weight, as there was no mortality among used mice. Chronic toxicity testing using 200mg/ kg crab chitosan and 100 & 200mg/ kg chitosan nanoparticles revealed no changes in all the assayed biochemical parameters when compared with those of control rats. No changes were detected in the lipid profile (T-Ch, TG, HDL-Ch& LDL-Ch) of rats given chitosan in normal and nano form in comparison with normal rats. Levels of plasma protein, albumin, globulin and A/G ratio showed no significant differences among different groups. Normal levels of kidney functions (creatinine & urea) and liver enzymes (ALT & AST) for all studied groups were obtained compared to normal rats. Histopathological examinations of liver sections showed nearly normal appearance in all studied groups. In conclusion, the prepared crab chitosan nanoparticles can be added to the non-toxic (safe) class of materials. It seemed to be an upcoming product that can be used safely as food additives.

INTRODUCTION

Recently, marine products waste is increasing continuously. Annually, about 6 to 8 million tons of shell waste from lobster, shrimp, and crab are produced worldwide. As people increasingly recongnize the importance of the huge amounts of marine waste products, the main source of commercial chitin has shifted to the shells of crustaceans (crab and shrimp) **(Yan & Chen, 2015)**. Significant economic and environmental

implications have been raised due to the discarded crustacean shells which occupy large spaces in landfills, therefore reducing waste disposal capacity has become an important issue. Landfilling as a waste disposal method contributes to climate change, and it is ten times greater than other methods. Therefore, sustainable waste management becomes critical to decrease these impacts **(Sieber *et al.*, 2018)**. Landfill waste accumulation, soil and water pollution, and evolving bad odors are adverse environmental consequences that rise from improper disposal of crustacean shells **(Topic *et al.*, 2023)**.

Nanoparticles (NPs) have great potential, especially in the production of nano-chitin and nanochitosan through nanotechnology. These materials retain great the characteristics of the normal chitin while offering a high surface area, aspect ratio, and abundant functional groups. This technology can be widely used in water treatment, food industry, green electronics, medical field, cosmetics, and more **(Kurita, 2006)**. Produced nanoparticles (NPs) are having at least one dimension less than 100nm **(De Berardis *et al.*, 2010)**. In the industrial sector, nanotechnology has produced a great revolution. The fields of electronics, aerospace engineering and biotechnology are attracted to nano-sized materials due to their physicochemical and electrical properties **(Bahadar *et al.*, 2016)**. The global nanotechnology market has expanded very fast, and its market value increased by tenfold, from 1.8 billion USD in 2020 to an expected level of more than 33 billion USD in 2030 **(Divyanshi, 2019)**. Due to their small size, large surface area, and polydispersity of nanomaterials, they exhibit new biological properties that differ from those of the original material. Unlike the original particles, where <1% of total atoms on the surface, nanoparticles (NPs) have >80% of total atoms are on their surface. These large number of surface atoms will affect the physicochemical properties of the nanoparticles **(Singh, 2016)**. Worldwide, chitosan has an increasing application in different fields, such as food processing and packaging, wound dressings and bandages. Hence, cytotoxicity assessment of chitosan nanoparticles is a must and of great importance **(Frigaard *et al.*, 2022)**.

Several studies discussed the antimicrobial effects of chitosan nanoparticles. However, investigation of their toxicity in experimental animals is limited. In the present study, acute oral toxicity for crab chitosan and nanochitosan was performed on male and female albino mice. Mice were monitored for 48h to examine the signs of toxicity that would appear. Moreover, a chronic toxicological study for 30 days on crab chitosan (200mg /kg, orally) and nanochitosan (100 & 200mg/ kg, orally) was done using Sprague Dawley male rats. This study would provide a comprehensive insight into the toxicological effects of chitosan nanoparticles.

MATERIALS AND METHODS

Materials

Crab exoskeletons were bought from the local fish market and then extracted using the method of **Toan (2009)**.

Chitosan preparation

Initially, during 36 hours, crab wastes were suspended in 4% HCl at room temperature in a 1:14 (w/v) ratio. The demineralized shells were treated with 5%

NaOH at 90°C for 24 hours, resulting in a 12:1 (v/w) solvent to solid ratio, in order to deproteinize them. Following the incubation period, the shells were sun dried and cleaned in running tap water until they were neutral. The resultant product was chitin, which was deacetylatied using 70% NaOH solution at a solid to solvent ratio of 1:14 (w/v) and incubated for 72 hours at room temperature. The residues were then rinsed with deionized water, filtered, dried in the sun, and finely grounded to produce chitosan **(Dutta *et al*., 2004)**.

Chitosan nanoparticle preparation

Chitosan nanoparticles (CS/NPs) were prepared using conventional particle size reduction procedures, such as the ball milling process (Ball milling Model: PQ-N2 Planetary Ball Mill, Gear Drive 4- station – planetary Ball mill, 220 v), as described by **Inkyo *et al.* (2006)** and **Joni *et al*. (2016)**. A 200ml agate vessel, containing 130 zirconia beads with diameters ranging from 0.5 to 1.5mm (75 beads 0.5mm, 30 beads 1.0mm, and 25 beads 1.5mm), was used to ball mill 25g of chitosan powder at 4000rpm in a high energy planetary ball mill. After adjusting the milling time for each sample, the dry chitosan powder was ground into fine particles for 90 minutes.

Method of milling

A ball mill was used to grind the dry chitosan into a fine powder. The resulting powder was chosen to have a more consistent micron size (around 37μm) through the use of 400 mesh sieves, and it was then put through a bead milling procedure in accordance with the approach of **Ibrahim *et al*. (2019)**. Following the milling procedure, chitosan was freeze-dried for two days at 50°C and 0.014mbar (LABCONCO, Kansas City, USA) to achieve a moisture content of 4%.

Examination

The produced chitosan and chitosan nanoparticles' moisture and ash contents were measured in accordance with the guidelines of **AOAC (2007)**. According to the method of **Mohanasrinivasan *et al.* (2014)**, the yield was estimated. The capacity to bind fat and water has been assessed in accordance with the method of **Wang and Kinsella (1976)**.

Fourier transform infrared (FTIR) spectroscopy: Using an infrared spectrophotometer model (4100 Jasco, Japan) in the 400– 4000cm–1 range, the samples of produced chitosan and chitosan nanoparticles were characterized in KBr pellets.

Degree of deacetylation (DD): The three forms of chitosan's DD were ascertained using an FTIR instrument. The percentage of the acetylated amine group was estimated by the following formula: DD (%) = 100 - [(A1629.85cm-1- A3450.65 cm-1)/1.33× 100] **(Struszczyk, 1987)**.

To characterize the produced chitosan and chitosan nanoparticles, scanning electron microscopy (SEM) with an accelerating voltage of 20kV, and a magnification range of 5,000 was applicable.

Using energy dispersive analysis of X-ray spectroscopy, the element contents of chitosan samples were assessed (JED-2300 analysis station, Joel). Joel's JED-2300 analysis station employed EDAX (Energy Dispersive analysis of X-Ray Spectroscopy) analysis to characterize the chitosan.

Methods

The experimental study on acute and chronic toxicity received the approval from the Ethics Committee of the National Research Center (NRC) in 2023. Then, the work was done during the same year at the Animal House of NRC, according to the guidelines of the Ethics Committee and following the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Animals and experimental design

Male Sprague Dawley rats weighing 100- 110g were used in the chronic toxicity study. All animals were housed in plastic cages (6 rats/ cage) in a well-ventilated environment (temperature about 25± 2ºC, relative humidity about 55%) with a daily illumination of 16h of light. They were fed on dry pellets with free access to water during the experiment. They were left for 2 weeks in this environment before the beginning of the experiment to ensure their good health. Adult albino mice (male and female, weighing 21- 25g) were used in the determination of the acute toxicity.

Oral acute toxicity test

Acute toxicity of crab chitosan and chitosan nanoparticles was carried out according to **Goodman *et al.* (2011)**. The animals were housed in suitable conditions to ensure their good health. Acute toxicity was carried out in accordance with the methods of Food Safety Toxicological Assessment. Seventy two mice (36 males & 36 females) underwent fasting for 4h, then they were divided into 12 groups that received a single oral dose of 0.5, 1, 2, 4, 5, 10g/ kg BW of either chitosan or chitosan nanoparticles. The animals were monitored well for the presence of any changes in behavior or signs of toxic symptoms and mortality. Observations of the fur, skin, mucous membranes, eyes, behavior, somatomotor activity, respiratory system, central and autonomic nervous system were made directly, within 30 min, and 4h after oral administration. Animals observation was continued for a total of 48h, with assessments made at 8-hour intervals. The body weights of the used mice were recorded at the end of 48h. All used mice were euthanized then subjected to autopsy and macroscopic observations. Mortality rate of mice was recorded in case there were any dead animals.

Chronic toxicity

Twenty-four male rats were divided randomly into four groups (n= 6). The first group served as control in which they received the vehicle, while group two, three and four received commercial chitosan (200mg/ kg), low and high dose of chitosan nanoparticles (100 and 200mg/ kg), respectively for 30 days. Doses of NPs were selected according to previous studies of **Liu and Huang (2013)**. At the end of the experiment, in tubes containing heparin, blood samples were collected from all rats of the different groups after overnight fasting. Centrifugation was performed at 3000rpm for 15min until clear plasma were obtained. These samples were then preserved at −20°C until the begining of biochemical tests. Rats were euthanized at the end of the experiment using cervical dislocation. Livers were collected from the 4 groups. They were preserved in 10% neutral buffered formalin for further histopathological examinations.

Clinical chemistry

Plasma was used for the determination of kidney functions (creatinine and urea), liver enzymes (aspartate aminotransferase (AST), and alanine aminotransferase (ALT)). Plasma albumin and total protein were assayed as indicator of the nutritional status. Plasma globulin and the ratio of albumin to globulin (A/G ratio) were calculated. Moreover, the lipid profile, including total cholesterol high density lipoprotein-cholesterol (HDL-Ch), low-density lipoprotein-cholesterol (LDL-Ch), and triglycerides levels, were estimated. The biochemical parameters were analyzed according to the instructions of the commercial kits (Erba Mannheim diagnostic). Liver samples were removed from all rats for histopathological studies.

Histopathological examinations

Liver specimens from all groups were collected then fixed immediately in 10% formalin saline (pH 7.0) and processed by the conventional methods to obtain paraffin sections for histopathological examinations. Afterward, the tissues were treated with grades of alcohol and xylol, embedded in paraffin and sectioned at 5µm thickness. Hematoxylin and Eosin (H and E) stain was used for studying the histopathological changes **(Bancroft & Gamble, 2013)**. Microscopic grading of the liver sections were carried out to determine the degree of severity of the observed histopathological lesions, following the method described by **Baliga *et al* (1999)**, with some modifications.

Statistical analysis

The results were expressed as Mean ± SE, and statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan's test to analyze the obtained results.

RESULTS

Functional and physicochemical properties of the crab chitosan and its nanoparticles recorded (16.33%- nd) yield; (6.13- 6.20%) moisture; (8.16- 8.16) nitrogen; (2.60- 2.90) ash; (745- 750) water binding capacity; (525- 530) fat binding capacity; (86- 88) degree of deacetylation, respectively. The origins of the polymer can attribute to the differences in chitosan extraction yield **(Luo *et al*., 2019)**. Similar results were reported in the studies of **Ghannam *et al*. (2016)**, **Olaosebikan *et al*. (2021)**, **Yarnpakdee *et al*. (2022)** and **El-araby *et al*. (2022a)**.

Characterization of chitosan samples

Functional groups of crab shells chitosan and its nanoparticles samples were identified using the Fourier transform infrared (FTIR) spectroscopy. The FTIR spectra of chitosan and chitosan nanoparticles samples are shown in Fig. (1). On the other hand, Table (1) summarizes the characteristic bands of crab chitosan and its nanoparticles samples.

Table 1. The FTIR bands (cm^{-1}) of crab chitosan and its nanoparticle samples

Functional groups and vibration modes	Crab chitosan	Crab chitosan nanoparticles
Stretching vibrations of hydroxyl groups (OH) Asymmetric/sym metric stretching of the amine bonds (NH_2)	3859.41 3642.43 3263.14	3861.13 3283.31
C-H aliphatic stretching vibration (CH_2)	2921.73 2852.21	2872.99 2326.58
Amide frequencies of C=O bond stretching of amide I	1642.64 1551.73	1810.28 1550.93
N-H bending vibrations of NH_2 groups of the amide II	1410.03 1326.68	1424.99
CH_2 deformation vibrations in the CH_2OH groups	1204.70	1315.57
Symmetrical angular deformation of CH_3 in $NHCOCH_3$ groups	1154.07	1199.02 1150.54
C-N stretching vibrations of amide III	1067.91 1028.20	1060.90 1024.37
Symmetric/asymmetric stretching signals of the C-O-C bridge (glycolsidic linkage)	952.62 711.35	898.42 798.05
C-O stretching vibration in secondary and primary OH groups	637.89 418.89	591.85 418.58

The FTIR peaks for crab chitosan and crab chitosan nanoparticles were observed at 3859.41- 3263.14 and 3861.13- 3283.31cm^{-1} (N-H and O-H stretching vibrations), 2921.73- 2852.21 and 2872.99- 2326.58cm^{-1} (axial stretching of C-H in the polymer chain), 1642.64- 1551.73 and 1810.28- 1550.93cm^{-1} (amide I vibration modes), 1410.03- 1326.68 and 1424.99cm^{-1} (N-H straining vibrations of NH_2 groups), 1204.70 and 1315.57cm^{-1} (CH_2 deformation vibrations), 1154.07 and 1150.54cm^{-1} (symmetrical angular deformation of CH_3 in $NHCOCH_3$ groups), 1067.91- 10.28.20 and 1060.90- 1024.37cm^{-1} (amide III vibration modes), 952.62- 711.35 and 898.42- 798.05cm^{-1} (C-O-C bridge), 637.89- 418.89 and 591.85- 418.58cm^{-1} (C-O stretching vibration of alcohol groups), respectively. The obtained results showed that, crab chitosan and its nanoparticles had high concentration and did not detect any contaminant. Similar results were recorded in the works of **Rakkhumkaew *et al.* (2018)**, **Varma and Vasudevan (2020)** and **Panda *et al.* (2022)**.

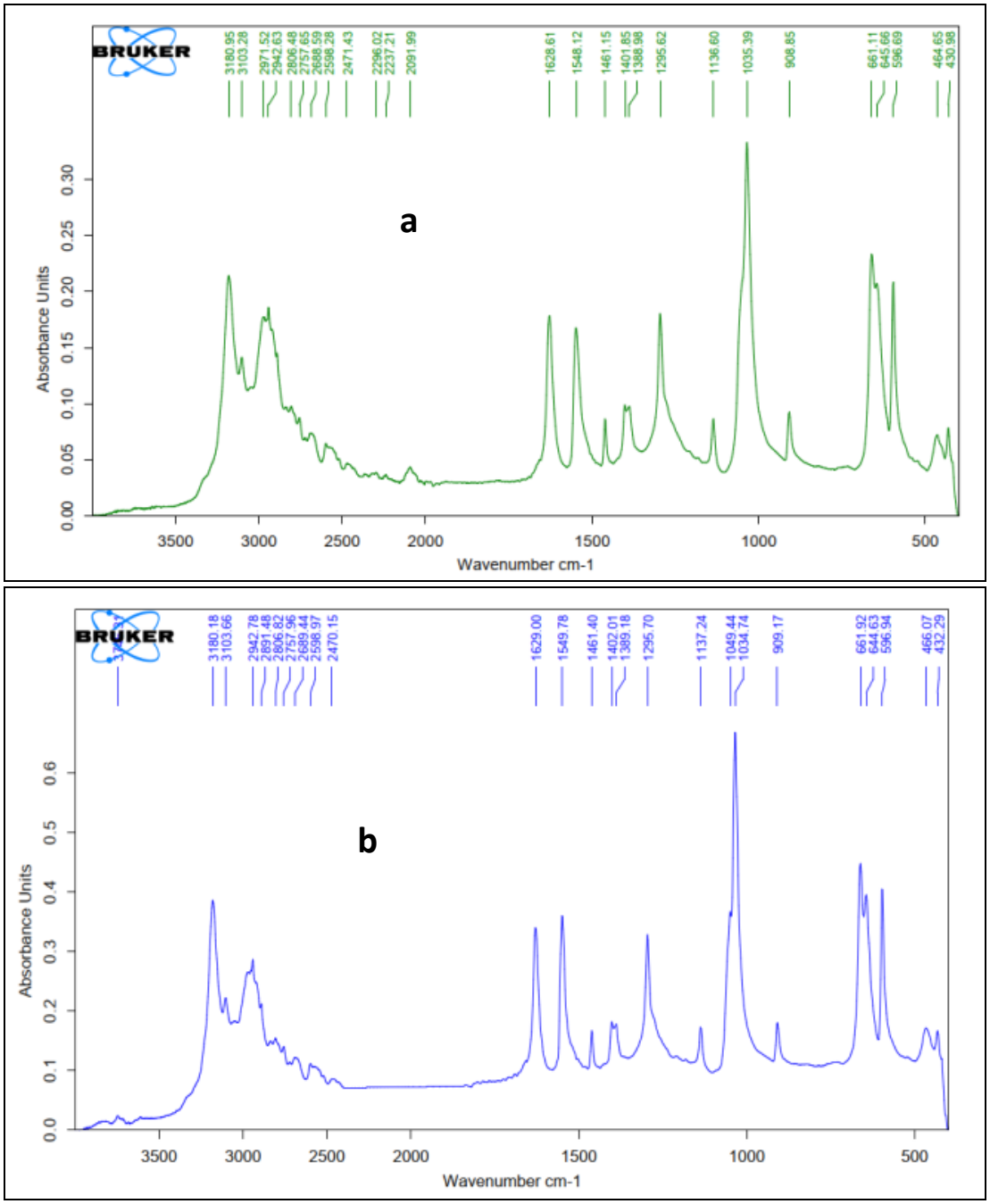

Fig. 1. FTIR of : **(a)** Crab chitosan and **(b)** Crab chitosan nanoparticles

X-ray diffraction (XRD)

Fig. (2a, b) exhibits the characterization of chitosan and chitosan nanoparticles samples by X-ray diffraction (XRD) technique. Fig. (2a, b) displays the characterization of chitosan and chitosan nanoparticles samples via the X-ray diffraction (XRD) technique. The XRD studies of crab chitosan showed a sharper peak at 25.421° (4756.36 counts/ s) and a maximum peak at 14.835° (8384.45 counts/ s), while, crab chitosan nanoparticles revealed stronger peaks at 17.578° (687.760 counts/ s) and a maximum reflection at 33.605° (1012.17 counts/ s). The XRD results of the chitosan samples showed the crystalline nature of obtained polymers. Similar

results have been obtained in previous studies **(Rasti *et al.*, 2017; Ibitoye *et al.*, 2018; Kuyyogsuy 2020; El-araby *et al.*, 2022b)**.

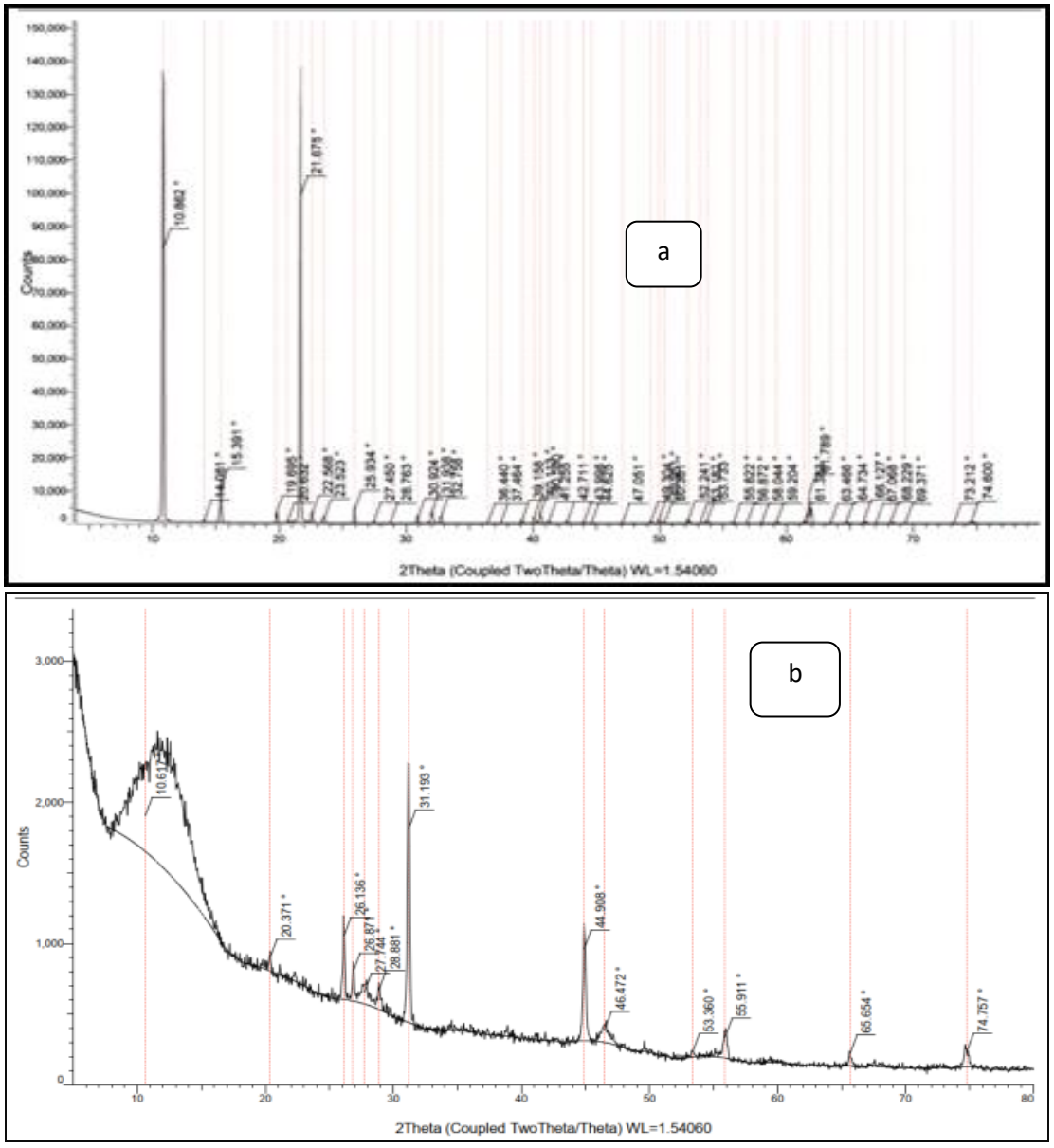

Fig. 2. X-ray diffraction patterns of: **(a)** Crab chitosan and **(b)** Crab chitosan nanoparticles

Energy dispersive X-ray spectroscopy (EDAX)

EDS or EDAX is an X-ray non-destructive analysis used to identify the elemental composition of materials. Its principle is based on applying an external X-ray stimulation on electrons of K bands within atoms, in order for one or more of this electron to leave its orbit, and be replaced by an electron from L band, with the emission of radiation energy kα; this last electron will be also replaced by an electron from M band, with the emission of Lα radiation energy. Furthermore, the electron of K band can be replaced directly by an electron from M band with the emission of Kβ radiation energy. All of these energies radiation will be captured by the device's

collector and processed in order to display a spectrum with different peaks. Each peak corresponds to a unique chemical element with its atomic and weight concentration **(Lovejoy *et al*., 2012)**.

Table 2. Elementary EDAX analysis for crab chitosan and its nanoparticles

Element	Weight %	Atomic %	Net Int.	Error %
Crab chitosan				
C K	35.86	46.84	47.17	9.93
N K	3.45	3.86	1.28	78.18
O K	41.76	40.95	54.31	12.38
NaK	4.53	3.09	14.28	16.95
MgK	0.34	0.22	1.93	70.06
P K	1.63	0.82	13.24	16.04
K K	0.53	0.21	3.84	60.4
Ca K	7.36	2.88	43.76	7.75
Cu K	4.54	1.12	7.39	23.77
Crab chitosan nanoparticles				
C K	35.61	44.05	104.09	8.76
N K	6.84	7.26	6.31	26.94
O K	44.44	41.28	134.36	10.89
Na K	7.31	4.73	55.96	11.03
Mg K	1.33	0.81	16.94	17.03
Si K	1.41	0.74	27.45	12.13
Ca K	3.07	1.14	39.34	13.24

All EDX spectrum chromatograms of crab chitosan and its nanoparticles are presented in Fig. (3a, b). The obtained results recorded in Table (2) and plotted in Fig. (3) in order to compare easily the elemental weight and atomic concentration percentages in each chitosan samples. The obtained results indicated that all samples contained carbon (C), nitrogen (N) and oxygen (O), with different intensities related to their concentration in each chitosan and chitosan nanoparticles samples. While, phosphorus (P), calcium (Ca), potassium (K), sodium (Na), magnesium (Mg), silicate (Si), and copper (Cu) were found in shrimp and crab chitosan and their nanoparticles. This result proves the incapacity of diluted acid to remove all minerals from crab shells, contrary to concentrated acids which may leave just a few minerals traces **(Eddya *et al*., 2020)**.

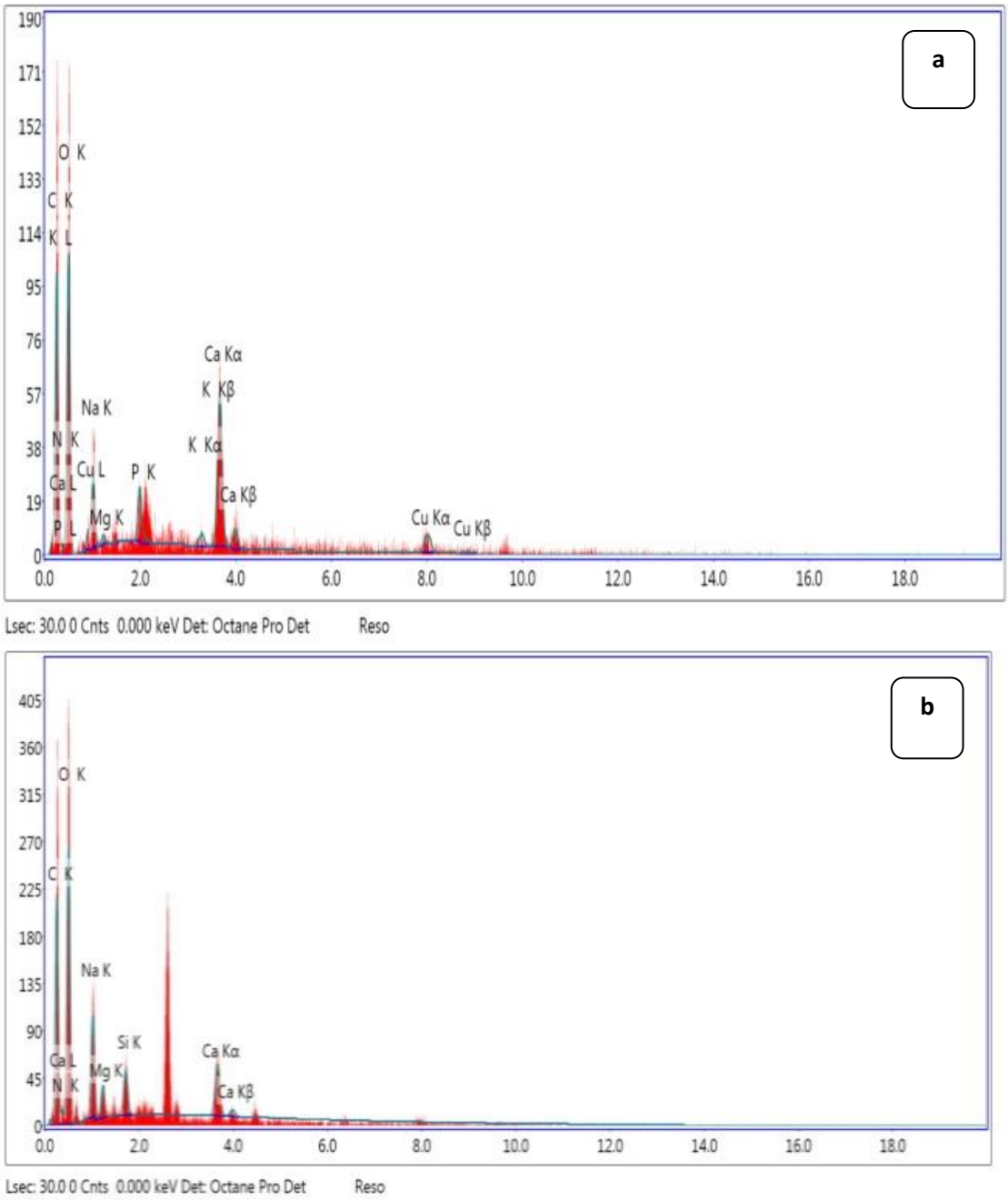

Fig. 3. EDAX of: **(a)** Crab chitosan and **(b)** Crab chitosan nanoparticles

Scanning electron microscopy (SEM)

The morphological characteristics of crab chitosan and its nanoparticles were evaluated by a scanning electron microscopy (Fig. 4). The SEM micrographs showed that, there are obvious differences in the roughness and surface morphology among the different types of chitosan and its nanoparticles types in addition to a relatively smooth top surface and fibrous structures. Crab chitosan presented a hard and rough surface morphology at 40µm, while crab nanoparticles had fibrous structures at 40µm. These results are in agreement with those of **Ghannam *et al.* (2016)**, **Ibitoye *et al.* (2018)**, **Tolesa *et al.* (2019)** and **El-araby *et al.* (2022b)**.

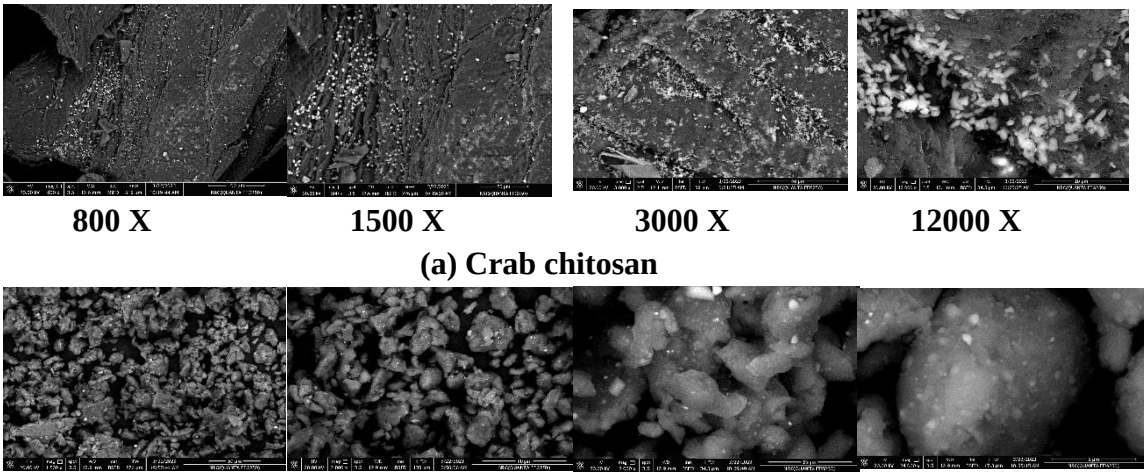

Fig. 4. SEM of **(a)** crab chitosan and **(b)** crab chitosan nanoparticles

Acute toxicity

Acute oral toxicity test aims to evaluate the toxicity of a substance *in vivo*, species sensitivity, and the target organ. Hazard preliminary information after acute exposure can be easily obtained from this test so we can determine LD50 value and undergo subsequent toxicity tests to establish the ideal doses to be given from tested materials and preparations. Finally, an acute toxicity determines the category of materials, as well as the labeling information **(Estuningtyas *et al*., 2018)**. Our oral acute toxicity testing results indicate that there were no side effects (weakness, salivation, tremors, seizures, diarrhea, lethargy, coma or mortality) observed in the used mice during the first 4h after treatment, either with crab chitosan or chitosan nanoparticles. Monitoring the mice during the next 48h didn't show any abnormalities in behavior or signs of toxicity in all treated groups after oral administration. Furthermore, food consumption and body weight of mice in all groups showed no differences.

Acute lethal toxicity test revealed that crab chitosan and chitosan nanoparticles were completely safe up to the highest used dose (10g/ kg bw), which is equivillant to 38.75g/ 70kg bw in humans **(Paget & Barnes, 1974)**.

Acute toxicity testing showed that chitosan nanoparticles did not have side effects or mortality at the dose >10000mg/ kg BW. Therefore, humans can safely consume crab chitosan or chitosan nanoparticles **(OECD, 2001)**. Similar results were reported in the study of **Estuningtyas *et al*. (2018)**, who evaluated the acute toxicities (lethal dose [LD50]) of the mahkotadewa extract given alone or in combination with chitosan nanoparticles in mouse model acute toxicity.

Biochemical analysis

Biochemical results for all treated groups are presented in Table (3). As noticed from Table (3), rats that received 200mg/ kg crab chitosan and 100 & 200mg/ kg chitosan nanoparticles showed normal levels for all the studied parameters.

Plasma lipid profile (T-Ch, HDL-Ch, LDL-Ch & TG) of rats given chitosan and nano form of it appeared a normal style (no significant differences when compared with those of control group).

Plasma levels of albumin, globulin, protein, and A/G ratio appeared normal in all treated groups. Liver enzymes (AST & ALT) and kidney functions (creatinine & urea) of all groups were normal when compared with those of control rats. The normal levels in kidneys and liver biochemical parameters were confirmed by normal histopathological pictures of livers from different groups, showing no pathological changes in liver sections from different groups.

Table 3. Nutritional and biochemical parameters of different groups

Parameter	Normal control group	Chitosan nanoparticles low dose (100mg/ kg) group	Chitosan nanoparticles high dose (200mg/ kg) group	Crab chitosan (200mg/ kg) group
T-Ch (mg/dl)	$73.17^{a}\pm0.946$	$74.67^{a}\pm1.519$	$74.50^{a}\pm2.171$	$74.00^{a}\pm2.190$
TG (mg/dl)	$59.67^{a}\pm1.401$	$60.67^{a}\pm0.843$	$61.67^{a}\pm1.022$	$60.50^{a}\pm1.765$
HDL-ch (mg/dl)	$44.50^{a}\pm0.428$	$43.50^{a}\pm0.428$	$43.67^{a}\pm0.494$	$43.80^{a}\pm0.307$
LDL-Ch	$21.00^{a}\pm0.516$	$21.33^{a}\pm0.803$	$21.67^{a}\pm0.759$	$21.50^{a}\pm0.563$
Total protein (g/dl)	$7.50^{a}\pm0.063$	$7.40^{a}\pm0.163$	$7.37^{a}\pm0.161$	$7.45^{a}\pm0.068$
Albumin (g/dl)	$3.85^{a}\pm0.099$	$3.77^{a}\pm0.105$	$3.68^{a}\pm0.079$	$3.75^{a}\pm0.056$
Globulin (g/dl)	$3.66^{a}\pm0.139$	$3.63^{a}\pm0.191$	$3.68^{a}\pm0.125$	$3.70^{a}\pm0.085$
A/G ratio	$1.07^{a}\pm0.065$	$1.06^{a}\pm0.076$	$1.01^{a}\pm0.040$	$1.02^{a}\pm0.034$
AST (U/l)	$39.50^{0a}\pm0.612$	$40.12^{a}\pm0.654$	$40.67^{a}\pm0.615$	$40.30^{a}\pm0.667$
ALT (U/l)	$19.83^{a}\pm0.654$	$20.67^{a}\pm0.161$	$20.50^{a}\pm0.177$	$20.25^{a}\pm0.574$
Creatinine (mg/dl)	$0.630^{a}\pm0.011$	$0.640^{a}\pm0.012$	$0.645^{a}\pm0.014$	$0.642^{a}\pm0.013$
Urea (mg/dl)	$26.35^{a}\pm0.721$	$26.83^{a}\pm0.703$	$27.17^{a}\pm0.833$	$26.98^{a}\pm0.539$

In each column, the same letters mean non-significant difference, while different letters mean significant difference at 0.05 probability.

The data are expressed as mean values ± standard error.

Histopathological pictures

Microscopic examination of the liver sections in the control group showed a normal histological picture, with a central vein surrounded by the hepatocytes. Between the strands of hepatocytes, hepatic sinusoids were observed, and distinct nuclei were exhibited, as shown in Fig. (5A).

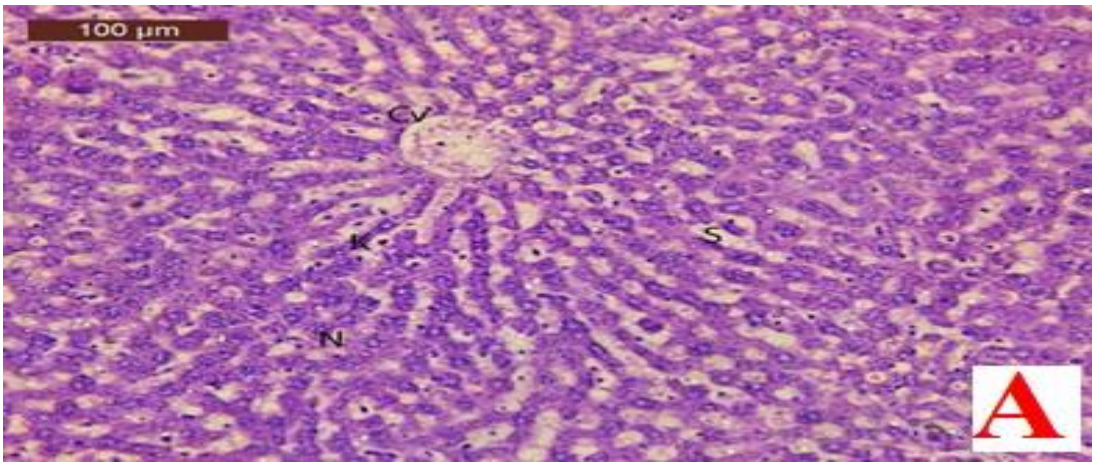

Fig. 5A. A photomicrograph of rat liver of control group showing normal hepatic architecture, central vein (CV), blood sinusoids (S), and nucleus (S)

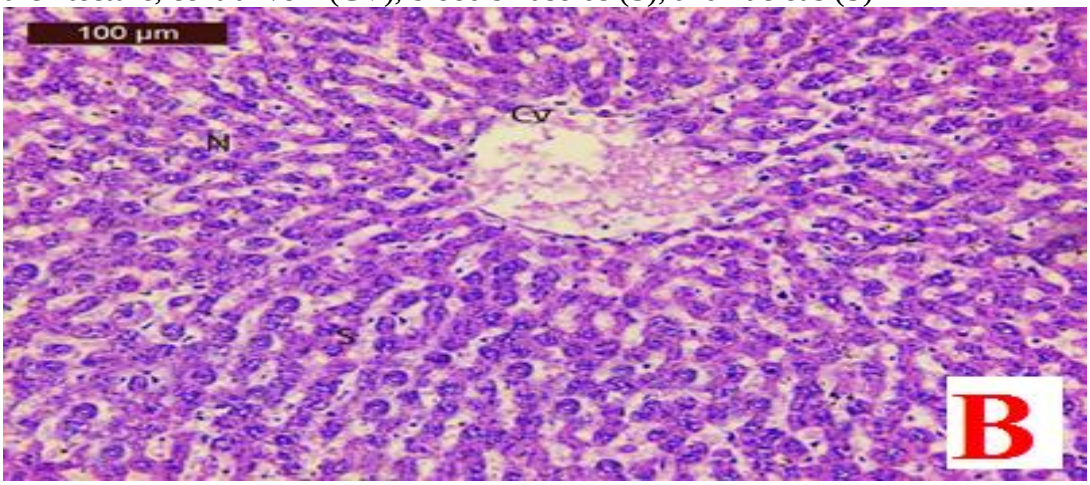

Fig. 5B. A photomicrograph of liver of NCH nano-chitosan high dose group showing nearly normal appearance of histological structure, normally blood sinusoids (S), and nucleus (S) with mild dilated of central vein (CV)

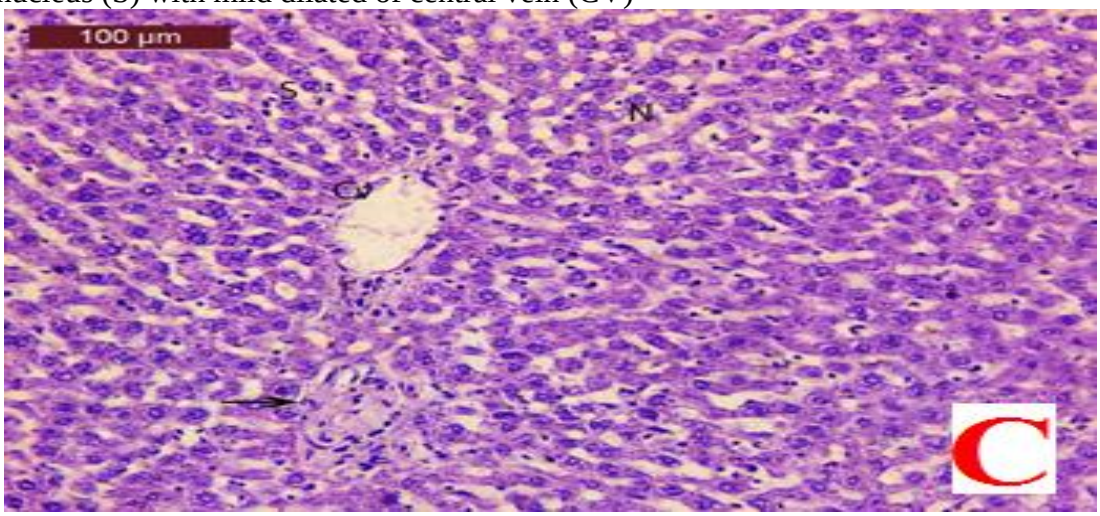

Fig. 5C. A photomicrograph of liver of NCLK nano-chitosan low dose group showing nearly normal appearance of histological structure, normal central vein (CV) and mild inflammatory cells aggregation in some areas (arrow)

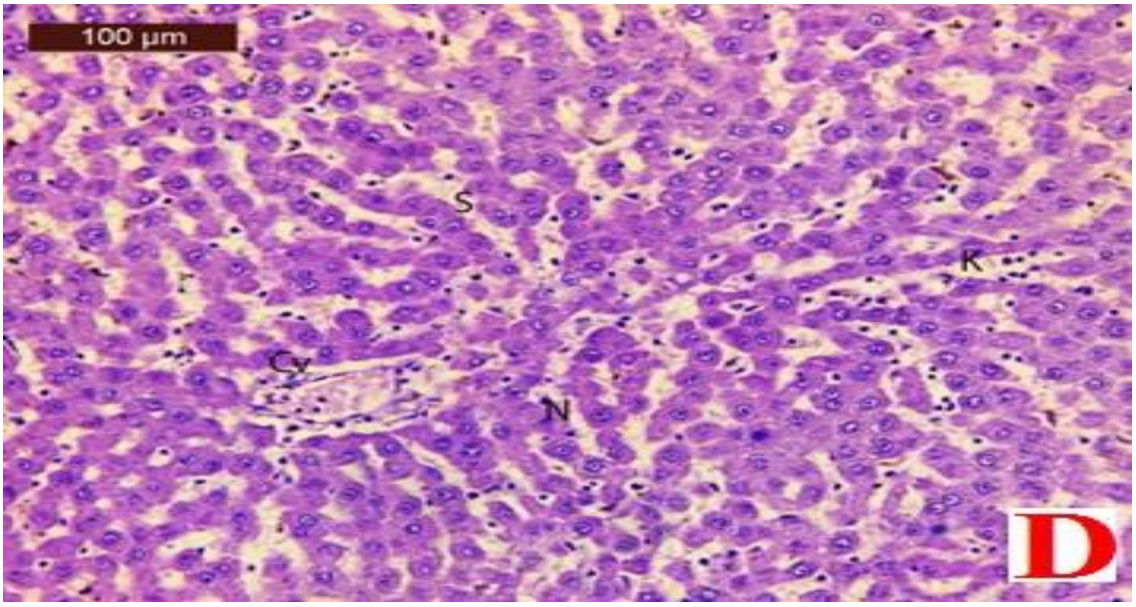

Fig. 5D. A photomicrograph of liver of normal chitosan CHYK group showing nearly normal appearance of histological structure, with mild blood sinusoids (S) and increased in Kupffer cells (K)

Animals treated with chitosan nanoparticles in the high dose group showed a nearly normal appearance of histological structure, normally blood sinusoids and nucleus with mild dilated of central vein (Fig. 5B). Sections of the liver tissue treated with chitosan nanoparticles in the low dose group showed a nearly normal appearance of histological structure, normal central vein, and mild inflammatory cells aggregation in some areas (Fig. 5C). Whereas, sections of the liver tissue treated with crab chitosan group showed a nearly normal appearance of histological structure, with mild blood sinusoids, and increased Kupffer cells (Fig. 5D).

Our results agree with some *in vivo* studies reported by **Liu *et al.* (2015)**, **Jin *et al.* (2017)**, **Zhao *et al.* (2017)** and **Yan *et al.* (2020)**, who detected no pathological changes and no difference in biochemical parameters, and no obvious toxicity when compared with results of the control group, indicating a high level of safety of chitosan nanoparticles when used orally or intramuscularly in mice, rats and chickens.

Additionally, the current results coinside with the opinion of **Frigaard *et al.* (2022)** who concluded in their review that, the majority of chitosan nanoparticles showed an extremely low cytotoxicity regardless of the method of cytotoxicity assay, cell lines used, particle composition, formed derivatives, and animal types used during *in vitro* and *in vivo* studies. Furthermore, chitosan nanoparticles showed less cytotoxicity compared to free chitosan, which increases the hypothesis on the safety of chitosan nanoparticles. Chitosan nanoparticles seem to be a safe upcoming product worldwide since free chitosan is already available in the market with an increasing demand on it.

CONCLUSION

This study aimed to evaluate the acute and chronic toxicity of different concentrations of crab chitosan and its nanoparticles (ChNPs) in experimental animals

(Albino mice & Sprague Dawley rats). Acute lethal toxicity test revealed that commercial crab chitosan and chitosan nanoparticles were completely safe since no adverse effects appeared up to the highest used dose (10g/ kg bw) that is equivalent to 38.75g/ 70kg bw in humans. Chronic toxicity test revealed that, there were no changes in all the biochemical parameters that were measured when compared with those of normal rats. Plasma lipid profile (T-Ch, HDL-Ch, LDL-Ch & TG) of rats given chitosan in normal and nano form appeared to be normal when compared with normal rats. Plasma levels of albumin, globulin, protein, and A/G ratio appeared the same in different treated groups. Liver enzymes (AST & ALT) and kidney functions (urea & creatinine) showed no alterations among the studied groups and the normal control group. According to histpathological studies for livers of animals treated with nanochitosan, the high dose group showed a nearly normal appearance of histological structure, normally blood sinusoids and nucleus, with mild dilated of central vein. In conclusion, chitosan and chitosan nanoparticles didn't cause any alterations in liver enzymes (AST & ALT) or kidney functions (urea & creatinine) of all studied groups since they appeared in normal levels when compared with control rats. This study showed that crab chitosan and chitosan nanoparticles were completely safe for use as food additives, however more studies are still needed to explain the detailed mechanism.

REFERENCES

AOAC (2007). Official Methods of Analysis of the Association of Official Analytical Chemistry (AOAC) International, 18th ed., Gaithersburg, Maryland, USA.

Bahadar, H.; Maqbool, F.; Niaz, K. and Abdollahi M. (2016). Toxicity of nanoparticles and an overview of current experimental models. Iran Biomed J., 20(1):1-11

Baliga R.; Ueda N.; Walker P.D. and Shah S.V. (1999). Oxidant mechanisms in toxic acute renal failure. Drug Metab Rev J., 31:971-997.

Bancroft, J.D. and Gamble, M. (2013). Theories and Practice of Histological Techniques. 6thed. New York, London and Madrid: Churchil Livingstone.

De Berardis, B.; Civitelli G.; Condello M.; Lista P.; Pozzi R.; Arancia G. and Meschini S. (2010). Exposure to ZnO nanoparticles induces oxidative stress and cytotoxicity in human colon carcinoma cells. Toxicology and applied pharmacology, 246(3): 116-127.

Divyanshi, T.S.B. (2019). Nanotechnology Market by Type (Nanodevices and Nanosensors) and Application (Electronics, Energy, Chemical Manufacturing, Aerospace & Defense, Healthcare, and Others): Global Opportunity Analysis and Industry Forecast, 2018–2025: Allied Market Research. Portland, OR: Allied Market Research, 2019; Available at: https://www.alliedmarketresearch.com/ nanotechno-logy-market.

Dutta, P.K.; Dutta J. and Tripathi, V.S. (2004). Chitin and chitosan: chemistry, property and application. J. Sci. Ind. Res., (63):20-31.

Eddya, M., Tbib, B. and EL-HamiK, H. (2020). A comparison of chitosan properties after extraction from shrimp shells by diluted and concentrated acids. Heliyon, 6, e03486.

El-araby, A.; El Ghadraoui, L. and Errachidi, F. (2022a). Physicochemical properties and functional characteristics of ecologically extracted shrimp chitosans with different organic acids during demineralization step. Molecules, 27: 8285.

El-araby, A.; El Ghadraoui, L. and Errachidi, F. (2022b). Usage of biological chitosan against the contamination of post-harvest treatment of strawberries by *Aspergillusniger*. Front. Sustain. Food Syst., (6): 881434.

Estuningtyas, A.; Widiasari, S. and Kusmardi, K. (2018). Acute toxicity of chitosan nanoparticles containing mahkotadewa (*Phaleria Macrocarpa*) leaf extract and anti-inflammatory effects in a dextran sodium sulfate-induced mouse model of ulcerative colitis. International Journal of Applied Pharmaceutics, (10): 6-10.

Frigaard, J.; Jensen, J.L.; Galtung, H.K. and Hiorth, M. (2022). The potential of chitosan in nanomedicine: an overview of the cytotoxicity of chitosan based nanoparticles. Front Pharmacol., 4(13): 880377.

Ghannam, H.E.; Talab, A.S.; Dolganova, N.V.; Hussein, A.M.S. and Abdelmaguid, N.M. (2016). Characterization of chitosan extracted from different crustacean shell wastes. Journal of Applied Science, 16: 454-461.

Goodman, G.; Goodman, S. and Gilman, A. (2011). Principles of Toxicology. In: The Pharmacological Basis of Therapeutics. Goodman, A.G.; Gilman, A. (Eds.). 2011; 12th Edn Macmillan, New York.

Ibitoye, E.B.; Lokman, I.H.; Hezmee, M.N.M.; Goh, Y.M.; Zuki, A.B.Z. and Jimoh, A.A. (2018). Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. Biomed. Mater., 13: 025009.

Ibrahim, A.I.; Ebeid, H.M.; Kishk, Y.F.M.; Abdel Fattah, A.A. and Mahmoud, K.F. (2019). Effect of grinding and particle size on some physical and rheological properties of chitosan. Arab Universities Journal of Agricultural Sciences, Ain Shams Univ., Cairo, Egypt, 27(2): 1513-1527.

Inkyo, M.; Tahara, T.; Iwaki, T.; Iskandar, F.C.; Hogen, J.; Okuyama, K. and Colloid, J. (2006). Experimental investigation of nanoparticles dispersion by beads milling with centrifugal bead separation, J. Colloid Interf. Sci., (304): 535-540.

Jin, Z.; Li, D.; Dai, C.; Cheng, G.; Wang, X. and Zhao K. (2017). Response of live newcastle disease virus encapsulated in N-2-hydroxypropyl dimethylethyl ammonium chloride chitosan nanoparticles. Carbohydr. Polym., 171, 267-280.

Joni, I.M.; Panatarani, C. and Maulana, D.W. (2016). Dispersion of fine phosphor particles by newly developed beads mill, in The 2nd Padjadjaran International Physics Symposium 2015 (PIPS-2015): Materials Functionalization and Energy Conservations, AIP Conf. Proc. 1712, edited by I M. Joni et al. (American Institute of Physics, Melville NY, 2016; 050019-1–050019-6.

Kurita, K. (2006). Chitin and Chitosan: Functional Biopolymers from Marine Crustaceans. Mar. Biotechnol., 8 (3) 203-226.

Kuyyogsuy, A. (2020). Preparation and characterization of chitosan obtained from pacific white shrimp shells and its in vitro antifungal activity. Asian J. Chem., (32): 2515-2519.

Liu, Q.; Zheng, X.; Zhang, C.; Shao, X.; Zhang, X.; Zhang, Q.; *et al*. (2015). Antigen- Conjugated N-Trimethylaminoethylmethacrylate Chitosan Nanoparticles Induce strong Immune Responses after Nasal Administration. Pharm. Res., 32 (1): 22-36.

Liu, Z. and Huang, G.N. (2013). Exposure to silver nanoparticles does not affect cognitive outcome or hippocampal neurogenesis in adult mice. Ecotoxicol Environ Saf., (87):124-130.

Lovejoy, T.C.; Ramasse, Q.M.; Falke, M.; et al. (2012). Single atom identification by energy dispersive x-ray spectroscopy, Appl. Phys. Lett., 100 (15): 154101.

Luo, Q.; Wang, Y.; Han, Q.; Ji, L.; Zhang, H.; Fei, Z. and Wang, Y. (2019). Comparison of the physicochemical, rheological, and morphologic properties of chitosan from four insects. Carbohydr. Polym., (209): 266-275.

Mohanasrinivasan, V.; Mishra, M.; Paliwal, J.; Singh, S.; Selvarajan, E.; Suganthi, V., et al. (2014). Studies on heavy metal removal efficiency and antibacterial activity of chitosan prepared from shrimp shell waste. Biotech., (4): 167-75.

OECD, (2001). Organization for Economic Cooperation and Development. OECD Guidelines for Testing of Chemical, 407-8.

Olaosebikan, A.O.; Kehinde, O.A.; Tolulase, O.A. and Victor, E.B. (2021). Extraction and Characterization of Chitin and chitosan from *Callinectes amnicola* and *Penaeus notialis* shell wastes. J. Chem. Eng. Mater. Sci., (12): 1-30.

Paget, P. and Barnes, T. (1974). Evaluation of Drug Activities Pharmacometrics. eds. Laurrence, D.R.; Bacharach, A.R. Academic Press, London and New York 1974; pp. 135-140.

Panda, P.K.; Dash, P.; Chang, Y.H. and Yang, J.M. (2022). Improvement of chitosan water solubility by fumaric acid modification. Mater. Lett., (316): 132046.

Rakkhumkaew, N. and Pengsuk, C. (2018). Chitosan and chitooligosaccharides from shrimp shell waste: characterization, antimicrobial and shelf life extension in bread. Food Sci. Biotechnol., 27: 1201-1208.

Rasti, H.; Parivar, K.; Baharara, J.; Iranshahi, M. and Namvar, F. (2017). Chitin from the molluscchiton: extraction, characterization and chitosan preparation. Iran. J. Pharm. Res., 16: 366.

Sieber, V.; Hofer, M.; Brück, W.M.; Garbe, D.; Brück, T. and Lynch, C.A. (2018). An integrated bio-refinery for processing chitin-rich bio-waste to specialty chemicals. In P. Rampelotto, & A. Trincone (Eds.), Grand challenges in marine biotechnology 2018; 555-578 Cham: Springer.

Singh, A. (2016). Engineered Nanoparticles: Structure, Properties and Mechanisms of Toxicity. Boston, MA: Elsevier, 2016.

Struszczyk, H. (1987). Microcrystalline chitosan. Preparation and properties of microcrystalline chitosan. J. Appl. Polym. Sci., (33): 177-189.

Toan, N.V. (2009). Production of chitin and chitosan from partially autolyzed shrimp shell materials. The Open Biomaterials Journal (1): 21-24.

Tolesa, L.D.; Gupta B.S. and Lee M.J. (2019). Chitin and chitosan production from shrimp shells using ammonium-based ionic liquids. Int. J. Biol. Macromol. (130): 818-826.

Topic Popovic, N.; Lorencin, V.; Strunjak-Perovic, I. and Coz-Rakovac, R. (2023). Shell waste management and utilization: Mitigating organic pollution and enhancing sustainability. Applied Sciences, 13(1): 623.

Varma, R. and Vasudevan, S. (2020). Extraction, characterization, and antimicrobial activity of chitosan from horse mussel *Modiolus Modiolus*. ACS Omega (5): 20224-20230.

Wang, J.C. and Kinsella, J.E. (1976). Functional properties of novel proteins: Alfalfa leaf protein. J. Food Sci., (41): 286-292.

Yan, N. and Chen, X. (2015). Sustainability: Don't waste seafood waste. Nature, 524 (7564), 155-157.

Yan, Y.; Sun, Y.; Wang, P.; Zhang, R.; Huo, C.; Gao, T.; et al. (2020). Mucoadhesive Nanoparticles-Based Oral Drug Delivery Systems Enhance Ameliorative Effects of Low Molecular Weight Heparin on Experimental Colitis. Carbohydr Polym., (246):116660.

Yarnpakdee, S.; Kaewprachu, P.; Jaisan, C.; Senphan, T.; Nagarajan, M. and Wangtueai, S. (2022). Extraction and physic-chemical characterization of chitosan from mantis shrimp (*Oratosquillanepa*) shell and the development of bio-composite film with agarose. Polymers, 14: 3983.

Zhao, K.; Li, S.; Li, W.; Yu, L.; Duan, X.; Han, J.; et al. (2017). Quaternized chitosan nanoparticles loaded with the combined attenuated live vaccine against Newcastle disease and infectious bronchitis elicit immune response in chicken after intranasal administration. Drug Deliv., 24 (1):1574-1586.