Synthesis Chitosan nanoparticles from Animal Byproducts (Shrimp Shells) Characterization, Physical Properties and Toxicity of Polymeric Nanoparticles in vivo

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Abstract

The study included the extraction of Chitin (Ch) from shrimp shells and preparation of Chitin nanoparticles (NCh) using acid hydrolysis and ultrasound method. In addition, the preparation of chitosan (Chs) and Chitosan nanoparticles (NChs) by Deacetylation method, then we study some of the characteristics, including yield, diameter by Size Analyzer, Field Emission Scanning Electron Microscopy (FESEM), Fourier transform infrared (FTIR) spectroscopy, X-Ray Diffractometer (X-RD), Viscosity, Water Binding Capacity (WBC) and Fat Binding Capacity (FBC). The results showed, (a) decreased the percentage of yield of NChs to 8.20%, (b) the effective diameter of the natural chitosan using a Size Analyzer and was 28121.5 nm, (c) FESEM analysis revealed that the NChs displayed a nanoscale structure with diameters ranged from 20.93 - 30.20 nm (d) the degree of deacetylation (DD%) using FT-IR spectroscopy device, of NChs was (80.21%) compared to Chs 71.45%, and in addition, from the X-ray spectrum, two peaks in spectra of (Ch, NCh) and (Chs, NChs) were observed at the diffraction angles (2θ) which are (10.70-10.99) and (19.99-20.37). Results show increasing the crystallization coefficient of NCh and NChs, while viscosity, WBC and FBC of NChs decreased compared to Chs. The NChs did not show any toxic effect on human blood cells at concentrations of 100-1000 µg. ml^{-1}.

Keywords: polymer, nanopolymers, nanoparticles, chitin, chitosan, viscosity, binding, capacity. toxicity.

Introduction

Biopolymers are biodegradable polymeric materials and include polymers of plant origin (starch, cellulose, and protein) and polymers of animal origin (proteins and polysaccharide) or microbial (polyhydroxybutyrate). In addition to that, polymers are chemically manufactured from natural units such as polylactic acid, the decomposition of which leads to the production of non-toxic materials and does not cause any environmental pollution (5). Many researchers have studied Nano biopolymers due to its unique physical and chemical characteristics, including their size and surface area. A chitosan nanoparticles (NCh) is among the most important Nano biopolymers, because they have mechanical and functional characteristics compared to their natural sizes (17 and 2). Chitosan $(C_6H_{11}O_4N)n$ is one of the most abundant polymers in nature after cellulose, its differs from other polysaccharides due to the presence of an amine group that makes it characterized by multiple physical, chemical, and biological properties. In addition, Chitosan has the ability to form edible film, bind ions, and has antioxidant and antimicrobial properties (35 and 2). Chitosan is prepared from the deacetylation of Chitin using alkaline solutions, especially sodium hydroxide (NaOH), where Chitin is found in the exoskeleton of crustaceans and the secondary waste of marine fishing and shrimp is one of its most important sources. Both Chitin and Chitosan consist of units of N-acetyl D-glucosamine (2-acetamido-2-deoxy-D-glucose) and units of D-glucosamine (2-amino-2-deoxy-D-glucose), linked together by glycosidic linkages from type (β,1- 4). Chitosan has a positive charge as a result of containing an amine group. In addition, Chitosan is soluble in acidic solutions, which enables it to possess properties that can be used in food manufacturing and packaging (32, 35 and 39).

The method of acid hydrolysis has been widely used in preparing chitosan nanoparticles by using two steps, the first is acid hydrolysis using strong acids, especially hydrochloric acid (HCl) at a concentration of 3 M, thus producing chitin nanoparticles, while the second step is the process of deacetylation of chitin nanoparticles and production of chitosan nanoparticles using alkaline solutions such as 50% sodium hydroxide (NaOH) solution (1 and 40).

Chitosan nanoparticles have good physical, chemical, and biological properties compared to chitosan in natural size and are considered one of the most important biologically active and environmentally friendly compounds (3). Also, they are widely used in packaging various foods such as meat and fish, and many other applications (28 and 3). Chitosan nanoparticles have a diameter less than 100 nm, and the degree of deacetylation increases with increasing NaOH concentration and reaction temperature when preparing chitosan nanoparticles from chitin nanoparticles under alkaline conditions (11 and 19).

This study was aimed to produce chitosan nanoparticles from marine food wastes. As well as studying the properties of these nanopolymers in terms of effective groups using FTIR,

their diameters, morphological shape using SEM and their degree of crystallinity using XRD.

Material and Methods

Materials: Shrimp shells were obtained from the fish market in Basrah Governorate, Hydrochloric acid 37%, sulfuric acid 98%, NaOH, NaOCI2, acetone, ethanol, acetic acid, toluene, monochloroacetic and laboratory materials were obtained from Sigma Company.

Methods

Extraction of chitin from Shrimp Shells

Deproteinization was done by treating 1 g of shrimp shells with 10 ml of (NaOH,1M) for 20 h, and removing salts (demineralization) was done by treating the product with (HCl, 2N) for 16 h at a ratio of (1:5) (w/v). The product was then dried at 60 °C for 5 hours (14). Then decoloration was done by treating the product with acetone for 10 minutes, after which it was added to a solution of sodium hypochlorite (NaOCl, 0.315%) (30). Finally, the product was washed with distilled water until it reached neutralization and then dried at 60 °C for 5 hours.

Preparation of Chitin nanoparticles

Chitin nanoparticles was prepared by acid hydrolysis according to the method of (34) with some modifications, where a weight of 5 g of chitin was taken and 150 ml of HCI at a concentration of 3M was added to it at a temperature of 90 °C for 90 minutes, and 300 ml of distilled water was added to stop the reaction and transfer the product to a centrifuge at 8944 g for 10 minutes and the sediment is removed. As the centrifugation process was repeated three times and then transferred to dialysis membranes (12-14 kDa) in distilled water until the pH was reached to 7. Then the suspension produced from the acid decomposition was transferred to the Vibra-cell ultrasonic (75043) device for 10 minutes, at a frequency of 20 kHz, a power of 50 kW, at a rate of one minute run and one minute turn off and placed the suspended in a container containing ice to avoid high temperatures (28). Finally, the suspension was transferred to the freeze dryer (Hetosick Danemark) to obtain the chitin nanoparticles powder, and the yield was 51% of the weight of the chitin.

Preparation of chitosan and chitosan nanoparticles

Both chitosan and chitosan nanoparticles were prepared according to the method of (13 and 19) with some modifications. A weight of 1 g of chitin or chitin nanoparticles was taken and 25 ml of 40% NaOH was added to it with stirring for 8 hours at a temperature of 90 °C, and 50 ml of distilled water was added to it to stop the reaction. After that, it was transferred to the centrifuge at 5744g for 10 minutes, the centrifugation process was repeated three times, and after that, the precipitate was placed in dialysis membranes (12-14 kDa), it was placed in bath distilled water, and the distilled water of bath was changed every 12 hours until a neutral pH was reached. Finally, the product was transferred to

the lyophilizer for 48 hours and then chitosan and chitosan nanoparticles were obtained.

Characterization of chitosan and chitosan nanoparticles

Yield

The percentage of the Polymer yield was estimated according to the method of (24) by using the following equation:

$$\text{yield (\%)} = \frac{\text{polymer weight}}{\text{sample weight}} \times 100 \quad \dots \quad 1$$

Size Analyzer

Dimensions of chitosan extracted from shrimp shells were measured according to the method used by (8) using Size Analyzer type (90 Plus) of the Center for Nanotechnology and Advanced Materials of the University of Technology/Baghdad.

FESEM

This measurement was carried out in the laboratories of the University of Tehran according to the method of (33), several drops of suspended (chitin nanoparticles, chitosan nanoparticles,) at concentration of 0.01% were placed on a glass slide and left to dry at room temperature. Then the morphology of the polymers was studied using a scanning electron microscope (FESEM Device TESCAN Mira3, Company Model: Mira3, Country Czech Republic).

FT-IR

The active groups of polymers (chitin, chitosan) and nanopolymers (chitin nanoparticles, chitosan nanoparticles) were determined using the FT-IR (FT/IR-4100, Jasco, Japan) of the Polymer Research Center / University of Basra, and according to what was mentioned by (11), and the wavelength range was from 400 - 4000 cm^{-1}. The degree of deacetylation (DD%) of chitosan was calculated according to the following equation (2):

$$DD\% = 100 - [(\frac{A1655}{A3450}) \times \frac{100}{1.33}] \dots \quad 2$$

Where, A_{1655} and A_{3450} are The absorbance at 1655 and 3450 cm^{-1} respectively.

X-ray diffraction

The crystallinity index of polymers and nanopolymers was measured using an X-Ray Diffraction device (X-Pert pro) in the Department of Physics at the College of Science, University of Basra, and the Cu Kα radiation source was used in this device with a voltage of 40 kV and an angle of 2θ from 10 - 40 degrees (9). The degree of crystallinity of natural chitin and chitosan and chitin nanoparticles and chitosan nanoparticles was calculated according to the equation (3) mentioned by (22):

$$CrI = (\frac{I_{(110)} - I_{(am)}}{I_{(110)}}) \times 100 \quad \dots\dots \quad 3$$

Wher: CrI = Crystallinity index, $I_{(110)}$ = was the maximum intensity of the diffraction peak at 2θ = 20, $I_{(am)}$ = was the minimum intensity corresponding to the amorphous structure.

Viscosity

1 g of chitosan or chitosan nanoparticles were dissolved in 100 ml of 1% acetic acid solution, the viscosity of chitosan and chitosan nanoparticles solutions, were estimated using an Ostwald

Viscometer Type D, according to the method used by (29).

Water Binding Capacity (WBC) and Fat Binding Capacity (FBC)

0.5 g was mixed with 10 mL of distilled water or Corn oil for 30 minutes and then centrifuge at 1431g for 25 minutes. The water binding capacity of chitosan and chitosan nanoparticles were estimated according to the method used by (29), and the amount of WBC and FBC were calculated according to the following equation 4 and 6:

WBC (%) = [water bound (g)/ initial sample weight (g)] x100 …….. 4

Water bound (g) = [weight of the tube aftertreatment (g) - (weight of the empty tube(g) + weight of the sample (g))]… 5

FBC (%) = [Fat bound (g)/ Initial sample weight (g)] x 100 6

Fat bound (g) = [weight of the tube after treatment (g) - (weight of the empty tube (g) + weight of the sample (g))] …7

Detection of chitosan nanoparticles toxicity

The toxicity of chitosan nanoparticles was detected according to the method used by (27).1 ml of fresh human blood obtained from the medical unit of Basra University was taken and 20 ml of normal saline was added to it. Then, concentrations of 100-1000 µg. ml^{-1} of chitosan nanoparticles were prepared and 100 µL of each concentration was mixed with 2 ml of human blood suspension. The control sample was prepared by adding 100 µl of distilled water and the samples were incubated at 37 °C and determination of degeneration or sedimentation blood cells after 10, 30 and 60 minutes.

Statistical Analysis

The data were statistically analyzed according to a Complete Randomized Design (CRD) using statistical program SPSS (2018). The results were compared using the t-teat test to compare some of the characteristics and the differences were statistically significant at the level of (0.05), and each experiment was repeated three times.

Results and Discussion

Yield of Chitosan and Chitosan nanoparticle:

Table No.1 shows the lower percentages of the yield of chitosan nanoparticles compared to chitosan. It was 15.50 and 8.20%, respectively, the results were close to what was found by (16), who showed that the yield of chitosan from shrimp residues ranged between 13.12-17.36%, the decrease in the yield of chitosan nanoparticles is attributed to the treatment of chitin with 3 M hydrochloric acid, depolymerization of the chitosan, and loss of chitosan nanoparticles during washing.

Table 1. Yield of chitosan and chitosan nanoparticles

Properties	Chitosan	Chitosan nanoparticles
Yield%	15.50 ±1.25 a	8.20 ± 0.50 b

*Different letters in one rows were significantly different (P≤0.05).

Characterization of Chitosan and Chitosan nanoparticles

Size Analyzer

Figure 1 shows the effective diameter of chitosan extracted from shrimp shells, as the figure shows that the effective diameter of natural chitosan was 28121.5 nm, it may be due to the high degree of polymerization of chitosan (25).

FESEM

Figures 2 and 3 show the morphology and average diameter of chitin nanoparticles and chitosan nanoparticles particles using Field Emission Scanning Electron Microscopy (FESEM) to examine the morphology samples using different magnifications (200,100, 50, 5) kx. Figure2

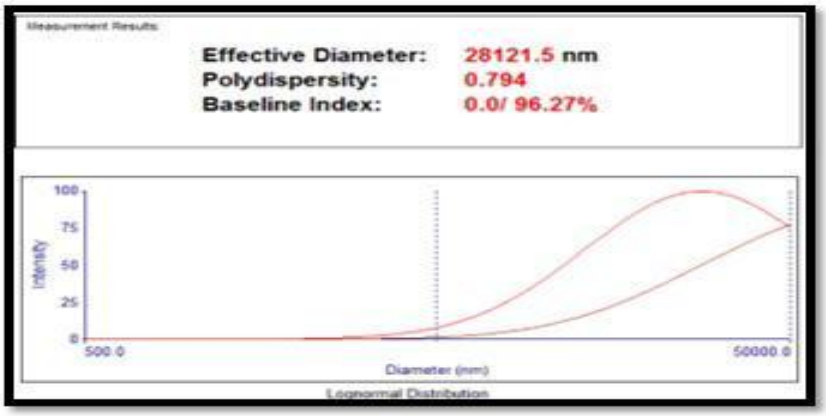

Figure 1. Size ratio for chitosan extracted from shrimp shells using Size Analyzer

showed that the average diameter of NCh resulting from the acid hydrolysis of Ch was (20.81-40.31) nm while Figure 3 shows that the diameters of Chs resulting from the deacetylation of NCh ranged between (20.93-30.20) nm, which is the nanoscale required to give the material the nanoscale character, and the nanoparticles were found in a spherical shape with an unregulated nanoparticle size distribution.

This result converged with that of (18), who found that the average diameters of NChs were 33.64 to 74.87 nm and have a spherical shape.

FT-IR spectroscopy analysis

Infrared spectroscopy is widely used to identify chemical groups in polymers.

Figure 4 represents the infrared spectrum of samples of chitin extracted from shrimp shells, chitin nanoparticles, chitosan, and chitosan nanoparticles prepared. It is noted that there is a wide band of spectral absorption at the wavelength (3394.1- 3451.96 cm^{-1}), which is due to the stretching vibration of O-H hydroxyl groups and N-H amino groups, which expresses the hydrogen bond (12), a less intense band at the wavelength (2872.45-2880.17 cm^{-1}) belongs to the aliphatic -CH_2 bond and two bands appeared at the wavelength (1656.55 and 1661.37 cm^{-1}) belonging to the carbonyl group of acetyl groups C=O, which are clear for chitin and chitin nanoparticles, respectively (15)

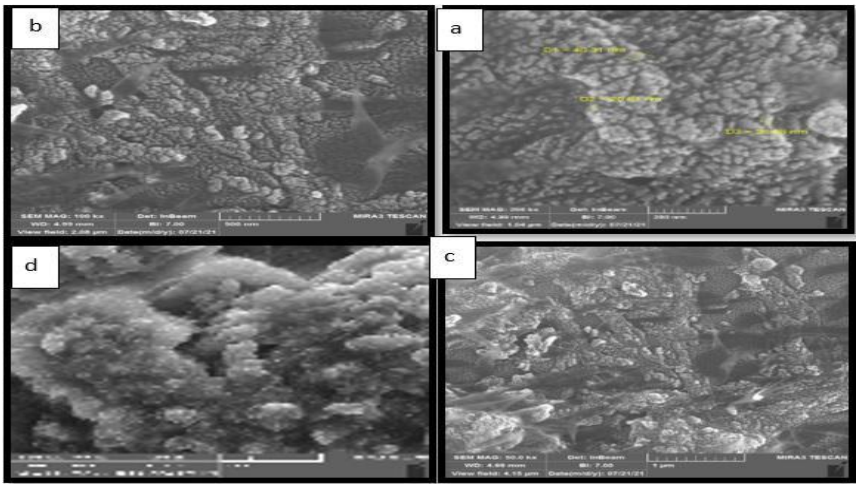

Figure 2. FESEM images of chitin nanoparticles with different magnification (a) 200kx, (b): 100kx, (c): 50kx, and (d): 5k

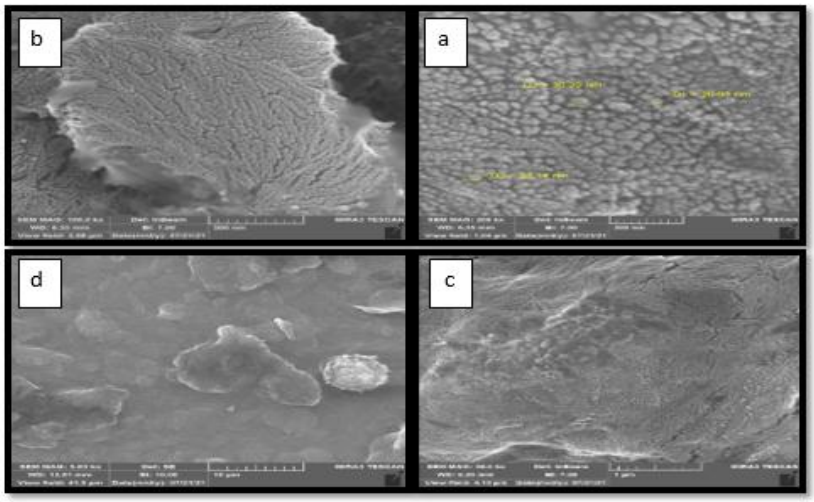

Figure 3. FESEM images of chitosan nanoparticles with different magnification (a) 200kx, (b): 100kx, (c): 50kx, and (d): 5kx.

In addition, the Figures show that there are no changes in the infrared spectra between chitin and chitin nanoparticles resulting from acid hydrolysis, which indicates that there is no change in the chemical composition of chitin nanoparticles (15). As for the amide group (amide II), it appeared clearly at the wavelength (1598.70 and 1599.99 cm^{-1}) of chitosan and chitosan nanoparticles, respectively, which indicates the conversion of chitin to chitosan (38), and the presence of the band at the frequency (1421.28-1425.14 cm^{-1}), it indicated the stretching vibration of the CH-group (14). The bands that ranged between wavelengths (1255.43- 893.844 cm^{-1}) indicated the stretching vibration of the C-O and C-O-C bonds, respectively (10).

The degree deacetylation (DD %) of chitosan and chitosan nanoparticles,

which was estimated by FTIR technique and shown in Table 2, was 71.45% and 82.20%, respectively. This is consistent with what was found by (23), when he showed that the DD % ranged between 75-82%, and it is one of the chemical properties of chitosan that affect the performance of chitosan in many applications. Also, the table shows an increase in DDA of chitosan nanoparticles compared to natural chitosan due to increased surface area and decreased particle size (20).

Table 2. degree of Deacetylation (DDA) of Chitin, Chitin nanoparticles, chitosan and chitosan nanoparticles from shrimp shells

Properties	Chitin	Chitin nanoparticles	Chitosan	Chitosan nanoparticles
DD%	30.60± 2.00a	32.87± 0.60a	71.45± 1.00b	80.21± 0.30 c

*Different letters in one row were significantly different (P≤0.05).

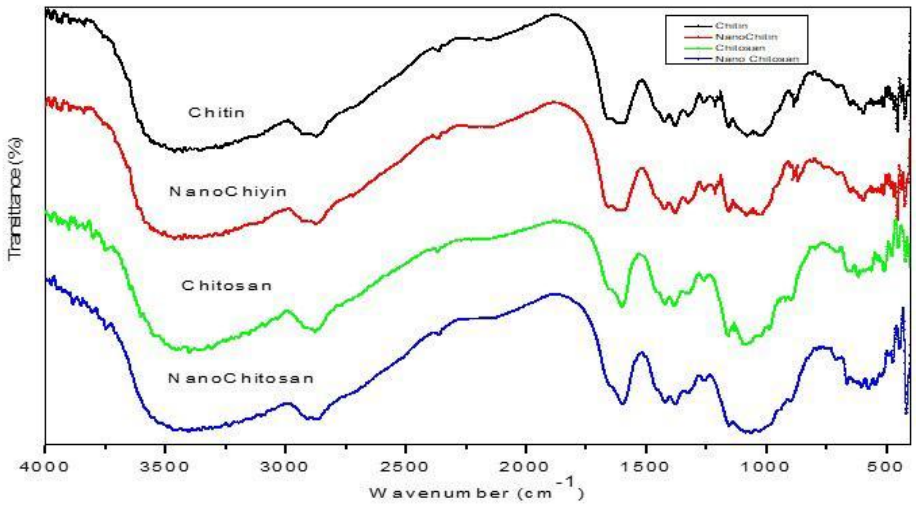

Figure 4. FTIR spectrum of chitin, chitin nanoparticles, chitosan and chitosan nanoparticles

X-Ray Diffraction (XRD)

The X-ray diffraction technique provides important information about the crystallinity and particle size of the synthesized compounds and polymers. Figure 5 shows the X-ray pattern of chitin (Ch), chitin nanoparticles (NCh), chitosan (Chs) and chitosan nanoparticles (NChs). It is observed that there is a low-intensity peak at the diffraction angle (2θ) 10.70º, 10.87º, 10.73º, and 10.99º, respectively, which represents the first order, and the presence of a high-intensity peak that represents the second order at the diffraction angle (2θ) of 19.99º, 19.99º, 20.16º, and 20.37º respectively, which corresponds to chitin, chitin nanoparticles, chitosan, and chitosan nanoparticles, respectively (31). The results are close to what was found by (36), which showed the presence of two clear and prominent peaks at angles (2θ) 10.40º and 20.40º in XRD which are the most important peaks of chitosan

extracted from shrimp shells. It is noted from the results of Table No. 3 that the crystallinity indexes of chitin nanoparticles increased, which amounted to 79.82% compared to chitin, which is 64.40%, and for chitosan and chitosan nanoparticles, the crystallinity indexes were 62.50 and 69.17%, respectively. It is noted that the crystallinity indexes of chitosan nanoparticles decreased compared to chitin nanoparticles due to the alkaline treatment and high temperatures during the deacetylation of chitin nanoparticles (40). The result is consistent with (33), which found a decrease in the crystallinity indexes from 86% to 47% when treated with 50% NaOH for 6 hours, also indicating a higher crystallinity index of chitosan nanoparticles compared to natural chitosan, which is attributed to acid hydrolysis when preparing chitin nanoparticles. The preparation of chitin nanowiskers from chitin by acid hydrolysis (HCI, 3N) led to a high crystallinity index from 20.0 to 45.6% (4). The presence of a clear peak at 2θ = 20º indicates the regular crystal structure of chitosan and chitosan nanoparticles (21).

Table 3. crystallinity index of chitin (ch), chitin nanoparticles (NCh), chitosan (Chs) and chitosan nanoparticles (NChs) from shrimp shells.

Properties	Ch	NCh	Chs	NChs
crystallinity index	64.40± 1.00c	79.82± 0.30a	62.50± 0.60c	69.17± 0.60b

*Different letters in one rows were significantly different (P≤0.05).

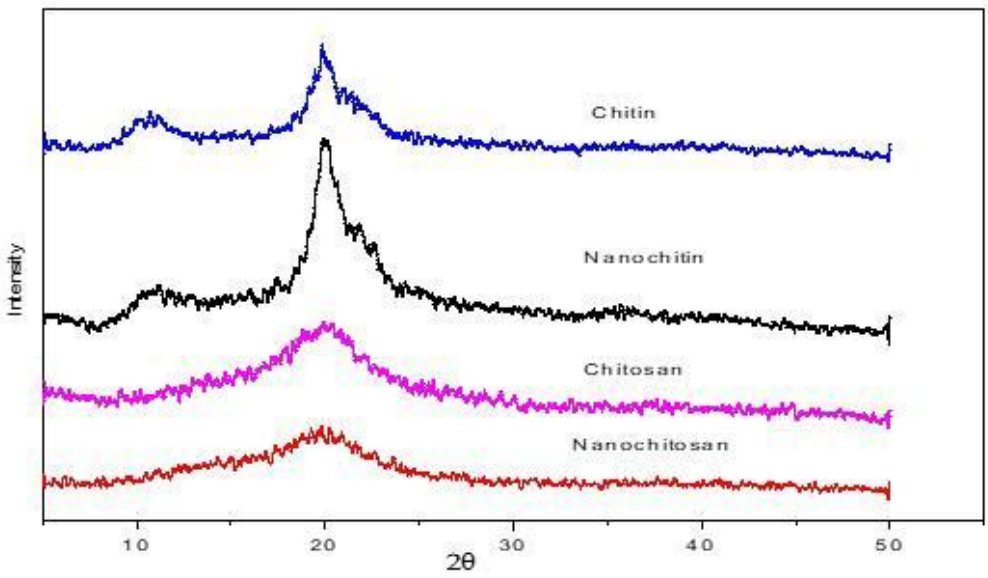

Figure 5. XRD of chitin, chitin nanoparticles, chitosan and chitosan nanoparticles

Physical properties of polymers and nanopolymers

Viscosity

Viscosity is an important characteristic that affects the physical properties of polymers and is highly dependent on molecular weight, solution concentration, ionic strength, pH, temperature, and methods of preparing solutions (25). Viscosity is greatly affected by physical processes such as grinding, heat, sterilization, and ultrasound, and the deacetylation of chitosan by acetone or sodium hypochlorite at any stage of its preparation process which leads to a significant decrease in its viscosity (29). Table No. 4 shows the values of viscosity and water-oil binding capacity for chitosan and chitosan nanoparticles.

It was noticed that the viscosity of chitosan nanoparticles was decreased significantly to 45.05 cP compared to the viscosity of chitosan, which was 72.42 cP. The decrease in the viscosity is due to acid hydrolysis and high heat treatment during the preparation, which leads to depolymerization, removal of acetyl groups in chitosan and lower molecular weight (25). viscosity is used as a parameter to determine the average molecular weight of a polymer (6). There is a direct relationship between the molecular weight and the viscosity of chitosan, and the results are in agreement with (7) who showed a decrease in the viscosity of chitosan nanoparticles compared to chitosan Because of the small size of the chitosan nanoparticles and less resistance for the mobility or flow of molecules and hence impart viscosity (6).

Water Binding Capacity (WBC)

It is noticed from Table No.4 a significant decrease in the water binding capacity of the nanopolymers compared to the normal-sized polymers, which have water-binding capacity 602.92% and 335.63%, for chitosan and chitosan nanoparticles respectively, the reason may be due to the low molecular weight of the nanopolymers. The results agreed with (25) which showed a decrease in the capacity of chitosan binding to water from 611% to 372% when the molecular weight decreased from 1.67×10^6 to 0.22×10^6 Da.

Fat Binding Capacity (FBC)

Table No.4 shows the fat binding capacity of chitosan and chitosan nanoparticles. It is noted that the fat-binding capability of nanopolymers decreased significantly compared to polymers of normal size. The fat binding capacity of chitosan and chitosan nanoparticles were 474.36% and 320.50%, respectively. The results were similar to those found by (37), which showed that the ability to bind fat to chitosan prepared from shrimp shells ranged between 420-481%.

Table 4. Physical properties of chitosan and chitosan nanoparticles

Properties	Chitosan	Chitosan nanoparticles
Viscosity (cp)	72.42 ± 3.31 [a]	45.05± 4.07 [b]
Water Binding Capacity (%)	602.92± 7.60 [a]	335.63 ± 8.50 [b]
Fat Binding Capacity (%)	474.36± 3.44 [a]	320.50 ± 8.70 [b]

*Different letters in one rows were significantly different (P≤0.05).

Detection of Toxicity of Nano polymer

Figures 6 show the results of the detection of toxicity of chitosan nanoparticles prepared from shrimp shells. The results showed that nanopolymers with concentrations of 100-1000 µg/ml and when incubating for 10, 30, and 60 minutes did not cause any changes in human blood cells such as degeneration or sedimentation, which indicates that there is no toxic effect of nanopolymers on human blood and at all concentrations. So, nanocomposites are safe for human consumption and can be used in various food industries. These results are in agreement with the findings of (26) who showed that there was no toxic effect of bacterial nanocellulose on male mice.

Figure 6. Detection of the toxicity of chitosan nanoparticles after 60 minutes.

Conclusion

Chitin (Ch), a by-product of marine animals, was successfully extracted from shrimp shells, and Chitin nanoparticles (NCh), chitosan (Chs), and Chitosan nanoparticles (NChs) were prepared. The yield of Chitosan nanoparticles decreased due to treated with chemical composites. The Chitosan nanoparticles were less than 100 nm, no apparent differences in chemical composition between Chitosan and Chitosan nanoparticles. An increase in the crystallinity index of Chitin nanoparticles and Chitosan nanoparticles due to acid hydrolysis. The physical properties such as viscosity, water-binding capacity and fat-binding capacity for Chitosan nanoparticles were decreased compared to natural Chitosan. As well as the toxicity of the prepared Chitosan nanoparticles was not detected.

Conflict of interest

The authors have no conflict of interest.

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