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Preparation and Characterization of Chitosan Nanoparticles from Crab shell and Determination their Activity as Antibacterial and Antioxidant

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Abstract:- Back ground: Chitosan nanoparticles (CS-NPs) are potential materials for medical application like antibacterial, antioxidant and anticancer. **Objective:** This study was focused on preparation of CS-NPs from wasted crab shell, determination their antibacterial and antioxidant activity. **Methodology:** The CS-NPs were prepared by using the ionic gelation method, characterization of CS-NPs includes Scanning Electron Microscopy (SEM) and X-ray Diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR Analysis) and determination their antibacterial against (*E. coli* and *Streptococcus mutanse*) and antioxident activity by DPPH test. **Results:** crystalline nature of the prepared nanoparticles with average size around 32.2 nm scale with rod shape. The synthesized CS-NPs showed antibacterial efficacy against clinical bacterial isolates of MDR in both Gram positive, *Streptococcus mutanse*, and Gram negative, *E. coli* By using the disk diffusion method. This activity might be attributed to the unique biological and physicochemical properties of the CS-NPs, which facilitate the disruption of bacterial cell membranes, from results underscore the potential of crab shellas an eco-friendly and efficient biological agent for the synthesis of CS-NPs with potent antibacterial properties. The prepared CS-NPs revealed a significant high antioxidant activity compared to ascorbic acid in the same concentration. **Conclusion:** It can be concluded that CS-NPs can be prepared from carb sell in an eco-friendly way and this CS-NPs can used as a cost-effective antibacterial.

Keywords: CS-NPs, Crab shell, Anti-bacterial and antioxidant.

Supplementary information The online version of this article (https://doi.org/xx.xxx/xxx.xx) contains supplementary material,which is available to authorized users.

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Introduction:

In the realm of nanobiotechnology, there are great expectations from biomaterials-based nanoparticles since they are biodegradable, biocompatible, non/low immunogenic, and nontoxic. As a result, they are widely used in the tissue engineering [4], food industry [5], medication and gene delivery [1-3], etc. One kind of biopolymer is chitosan (CS), which is produced by deacetylating chitin with a strong base. Chitin is a linear biopolymer made up of N-acetyl-D-glucosamine units connected by β (1,4) glycosidic linkages. Biocompatible, biodegradable, and non-toxic, CS has been extensively researched and may be used in many facets of life. Applications for computer science (CS) are now being explored in a number of fields, including food processing [12], preservative supplement [7], antioxidant [8], antibacterial [9], anti-cancer [10], antidiabetic [11], and wastewater remediation in the environment [6]. The ionic gelation approach [13] uses sodium tripolyphosphate (TPP)[14] and barium chloride[15] to create chitosan nanoparticles. These cross-linking agents join two components with opposing charges to create nanoparticle[16].

Because of these characteristics, CS has been shown to be effective against both Gram-positive and Gram-negative bacteria. Electrostatic interactions between the functional group of CS and the bacterial cell wall, cell membrane, and cytoplasmic components are the primary mechanism of antibacterial activity [17]. According to Moon et al. [18], CS solution effectively inhibits microorganisms. Kusnadi et al. [19] also reported similar findings, showing that as DD rose, so did the activity of CS in suppressing S. aureus and E. coli bacteria. Still, these experiments used CS at a quite high dosage. Because of this, CS has been changed to CS-NPs, and as NPs are smaller than CS, their physical characteristics have changed significantly from those of CS. CS-NPs combine the best qualities of NPs and CS, including tiny size, higher surface area, and quantum size effects [20]. According to Kritchenkov et al., ionic gelation-derived CS-NPs had more antibacterial activity than CS against S. aureus and E. coli. In the same findings that Mubarakali et al. [21] reported.

Studies have been done on the antioxidant qualities of chitosan derivatives [22]. Furthermore, antioxidant effects of fungal chitosan from shiitake stipes have also been examined [23]. However, antioxidant activities of chitosan produced from crab shells are not available .[24]

In this study, prepared chitosan nanoparticals from crab shell, characterization of CS-NP prepared were investigated by XRD, FTIR and FE-SEM, then antimicrobial and anti-oxidants of CS-NPs activity are study.

Materials and Methodes:

Bacterial Isolates

The bacteria were isolated from burn and wound patients at the Hillah Teaching Hospital in Iraq. All samples were isolated and purified using conventional bacteriological methods such as culture on blood and MacConkey's agar plates for 24–48 hours at 37°C. The Vitek-2 compact system (Biomérieux) was used to confirm all of the isolates.

Solution and Media

We acquired Mueller-Hinton media and Mueller-Hinton agar from Hi-Media in Mumbai, India. Ceftriaxone (CRO-3Oμg), ampicillin (AM-10μg), cepaalothin (KF-30μg), chloramphenicol (C-30μg), and meropenem (MEM-10μg) were among the several antibiotic disks that were acquired from Bioanalyse, Turkey[25] .

Extraction of chitosan from carb shell

After several adjustments, chitosan isolation was done (Puvvada et al., 2012) [26]. The stages in the technique were as follows, along with figure (1):

Preparation of shell powder

Carp shells were purchased from the Iraqi local market. After a thorough water wash, all remaining protein residue and contaminants were removed from the shells. Before being powdered to create a homogenized product, the shells were dried for 48 hours at 60 ^{o}C in an oven.

Washing

Distilled water was used to wash 10 grams of powdered carb shell in order to remove any soluble contaminants. Until all contaminants are gone, this procedure is done many times. Subsequently, the residue was maintained at 80ºC in an oven until weight stability.

1- De-proteinization (DP)

Crustacean shell waste was handled at boiling point temperature with a diluted sodium hydroxide solution (3% NaOH) to dissolve the present proteins. The time of reaction was from (1-1.5 hr.) with Continuous mixing with a magnetic stirrer, Long-term alkaline therapy under serious circumstances triggers de-polymerization and de-acetylation. It was used comparatively elevated proportions of solid/alkali solution 1:10 (w/v)be used to achieve uniformity in response. Then, washing the residue with distilled water for half hour and dried in the oven .

2- De-mineralization (DM)

After the step of removing the proteins from the shells by (De-proteinization) method, it was disposed of the metals in the item by (De-mineralization) method from the product from earlier method. The shells subject to de-proteinize shells for demineralizing at room temperature with 1N HCl bath with continuous stirring for half an hour with a powerful solvent proportion of 1:15 (w/v) as demonstrated and then filtered. The filtrate was cleaned with distilled water and oven-dried for 30 min. It can get Chitin product after completing this process.

3- De-acetylation (DA)

In this process, its separated an acetyl group (CH3-C-) from chitin by treating chitin at 90˚C for 2hr with 50% NaOH solution, with a proportion of solid/solution of 1:10 (w/v). The produced chitosan was cleaning to neutrality in running water of tap, and then rinsed with distilling water, filtering and drying in the oven at 60 ° C to get the final chitosan product .

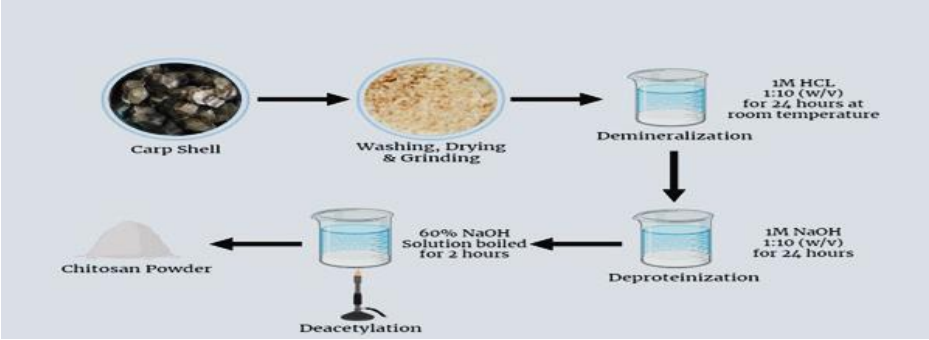


Figure (1): Steps of Carb shell chitosan extraction

Characterization of Chitosan Nanoparticles:

UV-Vis Spectroscopy examines chitosan nanoparticle form and stability in an aqueous combination. The X-ray diffractometer was utilized to evaluate chitosan nanoparticles synthesised from dried carb shell powder. The XRD shape reveals chitosan nanoparticles are crystalline. FTIR spectrometers evaluated the chemical composition of produced chitosan nanoparticles. FTIR identified the extract's active ingredients for chitosan nanoparticle synthesis. CS-NP dimensions and shapes were determined using scanning electron microscopy SEM of the samples. A thin layer was created by dropping specimens on foil. The excess solution was oven-baked at 80°C [27].

Antibiotic susceptibility and multiple drug resistance (MDR) determination

Every discovered bacterial isolate is being studied to assess how resistant they are to five different antibiotics. Ceftriaxone (CRO-3Oµg), ampicillin (AM-10µg), cepalothin (KF-30µg), chloramphenicol (C-30µg), and meropenem (MEM-10µg) are among the several antibiotic disks. All experiments were applied on plates containing Mueller-Hinton agar (Carl Roth, Germany) by employing technique of disk-diffusion of Kirby-Bauer. Following the guidelines of the Clinical Laboratory and Standards Institute (CLSI), each identified bacterial isolate was re-suspended and compared to a standard turbidity of 0.5 McFarland [equivalent to 1.5*10**-** colony forming unit per millilitre (cfu/ml)]. After that, the isolates were tested for antibiotic sensitivity. Each disc's surrounding zones of inhibition were assessed and categorized as sensitive, intermediate, and resistant [25].

Antibacterial Activity of CS-NPs

The antibacterial activity of CS-NPs was evaluated against the study's bacterial isolates, which were cultured on nutrient agar slants. The Clinical and Laboratory Standards Institute provided a description of the antibacterial activity. A dilution of five different concentrations of CS-NPs (500, 250, 125, 62.5, and 31.25 µg/ml) in sterile deionized water is used to investigate the effects of the NPs against the bacteria under investigation. The isolates were initially incubated at room temperature for 15 minutes, and then they were incubated at 37°C for the whole night. After a period of incubation, positive findings were noted when the inhibition zone was seen around the well. A digital Vernier caliper was then used to quantify the inhibition zone diameter in millimeters [25].

DPPH radical assay:

The activity of CS-NPs production from carb shell powder was measured by DPPH (1,1-diphenyl,2-picryl-hydrazil) free radical scavenging50. Chitosan nanoparticles are utilized at doses of 12.5, 25, 50, and 100µg/ml. ELISA reader spectrophotometrically calculated sample radical scavenging against stable DPPH radical. DPPH decrease at 517nm caused a deep-violet to light-yellow colorimetric change. Positive controls include ascorbic acid [28]. The inhibition percentage has been calculated by the following equation:

$$\text{Inhibition\%} = \frac{(\text{Absorbance of -ve control} - \text{Absorbance of sample})}{\text{Absorbance of -ve control}} \times 100$$

Results and Discussion:

The results of this study revealed that the obtained chitosan has off-white in colour, odourless and in a form of semi-crystalline powder as in figure (1). The product resulting from the biological methods used for the synthesis chitosan nanoparticles were characteristic by: XRD, FTIR and FE-SEM.

Description of the synthesized chitosan nanoparticles :

X-Ray Diffraction (XRD Examination):

Chitosan crystallinity influences a material's capacity to absorb water or metal ions [29]. The crystal structure of CS-NPs was measured using XRD method (6000 XRD) at a step, with a voltage (40 KV), electric current (30 mA), scanning rate (2º/min), and range (10º to 80º). Fig. 1. shows pattern of XRD of bio-synthesized CS-NPs observed the peaks at showing 4 sharp strong diffraction peaks at 28.11°, 32.21°, 40.11°, and 59.42° corresponding to lattice planes (111), (200), (220), and (311) respectively. planes of CS-NPs are metallic and have crystal structure [30-33], as in Figure (2).

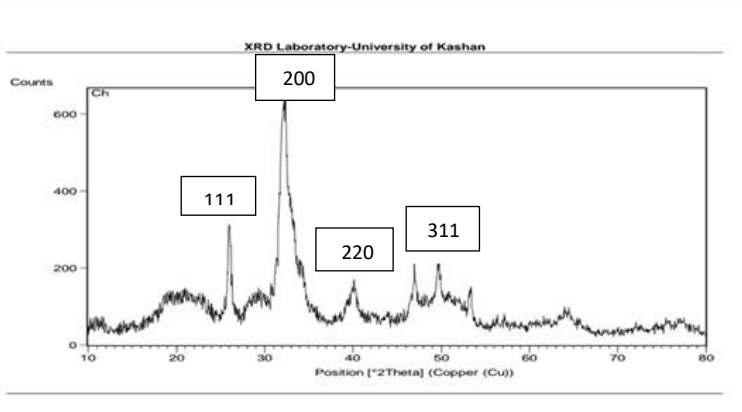


Figure (2): X-ray diffraction results for CS-NPs synthesized by using carb shell

Fourier transform infrared spectroscopy (FTIR Analysis):

The CS-NP FTIR spectrum is given in Figure (3). Table 1 lists the spectrum peaks: 3408.73, 1628.55, 1447.23, 1635.00, 1562.33, and 877.16 cm-1 represent N-H bending vibrations of primary and secondary amines, O-H stretching of phenolic and alcoholic groups, C-H bending in rings, C-O stretching of carboxylic acids and alcohols, and C-C stretching (in rings) and PO $^{-2}$ group. The PO $^{-2}$ group in the CS-NP spectrum was also found in [1]. The peak at 842.40, indicating the PO^{-2} group, is a significant divergence in the spectrum of CS-NP, supporting its development by other characterizations [34,35].

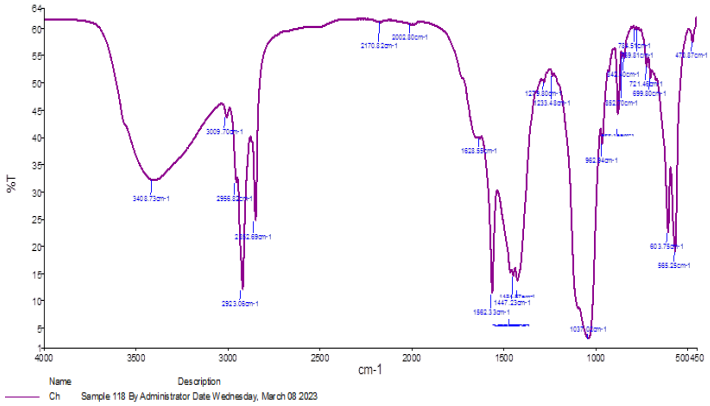


Figure (3): FTIR spectra pattern of CS-NPs synthesized by using carb shell

Table (1): FTIR of CS-NPs

Functional group	Wavelength (cm^{-}')
OH stretch	3408.73
C-H bend	1279.80
N-H bend	1628.55
C-C stretch (in ring)	1279.80
C-O stretch (alcohol)	1628.55
PO_4^{-2}	877.16

Field Emission-Scanning electron Microscopy (FE-SEM):

The surface morphology of CS-NPs was studied using FE-SEM. FE-SEM images revealed that the CS-NPs prepared by the crab shell tend to be spherical in shape with an average scale of 32.2 nm in size [31,32], Figure (4).

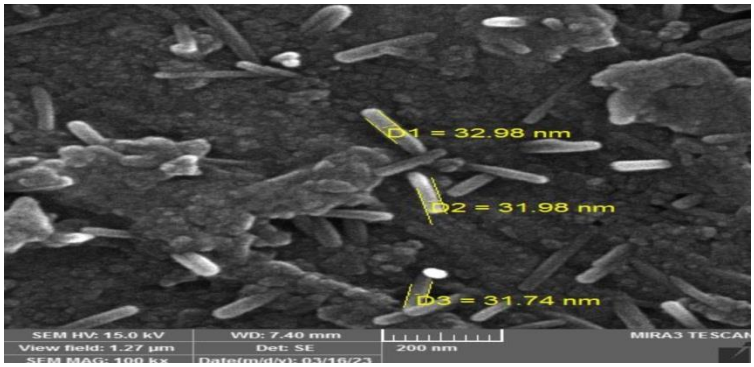


Figure (4) : FE-SEM of CS-NPs preparation from crab shell with 32.2 nm

Antibacterial activity of CS-NPs by disc diffusion method:

Each kind of bacteria's antibiotic sensitivity was determined using the modified Kirby-Bauer disc diffusion method. The test findings demonstrated that, in accordance with the CLIS, both species of bacteria were resistant to the medicines administered.

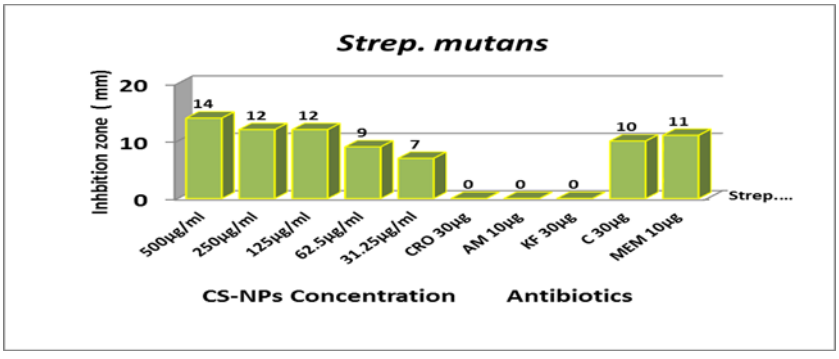
Because E. coli and Streptococcus mutanse bacteria are among the most common in fisheries products throughout the process of fish quality degradation, this research investigated CS-NPs against these bacteria. Meat and protein-containing foods like fish are susceptible to deterioration due to the Strep. mutanse bacteria [36]. In the meanwhile, new fish becomes infected with E. Coli because it spreads readily via polluted water [37]. If these bacteria's development is not inhibited, foodborne illnesses will result, endangering both fishermen and customers.

The antibacterial activity test used the disc diffusion technique to assess CS-NPs' capacity to stop the development of Strep. mutanse and E. coli bacteria. CS-NPs have strong, broad-spectrum antibacterial efficacy against microorganisms that are resistant to many drugs. The effects of several antibiotics on bacterial isolates were compared. The emergence of a clear or inhibitory zone encircling the paper disc indicates a satisfactory outcome when using disc diffusion to evaluate an extract or compound for antibacterial activity [38]

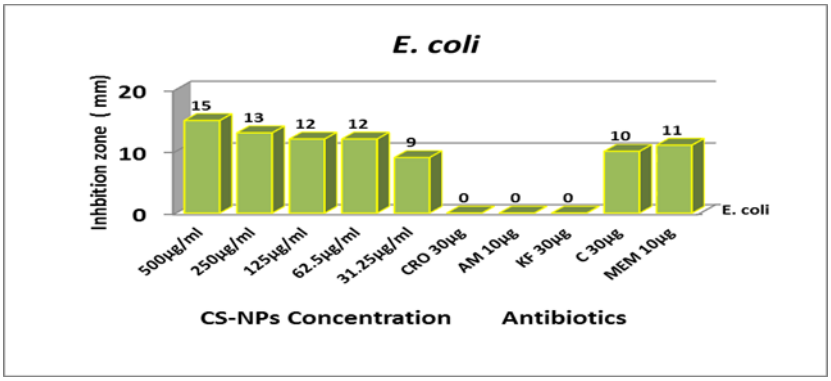
With an inhibitory zone surrounding the test point, a quantitative study of crab shell CS-NPs antibacterial properties may be possible. As shown in Table (2), the findings of the CS-NPs activity test were able to inhibit the growth of *Strep*. *mutanse* and *E. coli* with three different concentrations. CS-NPs antibacterial properties may be related to its extremely potent positive charge, which attracts negatively charged amino acid molecules that are used to make proteins in germs. Given the electrostatic interaction between these positive and negative charges, the membrane experiences a leaky pressure. Microbes are unable to proliferate because of this imbalance in the osmotic pressure within the cell. Furthermore, the microbial cell dies as a result of intracellular hydrolysis activities in the cell wall that liberate cell electrolytes [39], Figure (5-7).

Table 2: Antibacterial activity test disc diffusion method

Chitosan			
E. coli		***Streptococcus mutanse***	
Con. µg/ml	Inhibition zone mm	Con. µg/ml	Inhibition zone mm
500	15	500	14
250	13	250	12
125	12	125	12
62.5	12	62.5	9
31.125	9	31.125	7



Figure(5): Antibacterial action *Strep. mutans*



Figure(6): Antibacterial action *E. coli*

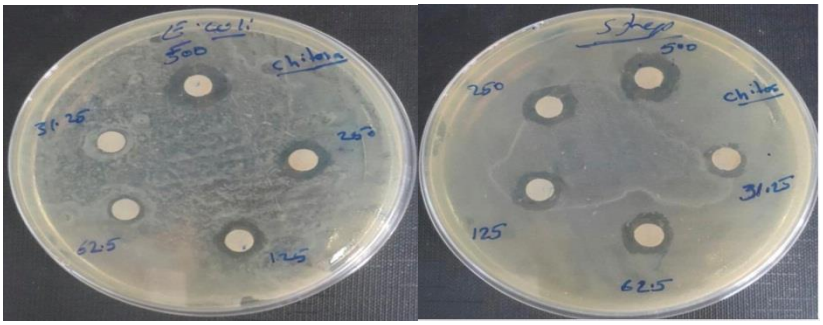


Figure (7). Antibacterial activity of CS-NPs against *E. coli* and *Strp. mutans*

Free radical scavenging activity assay [DPPH]:

Scavenging hydrogen radicals is an essential antioxidant process. DPPH (2,2-diphenyl-1-picrylhydrazyl) is detected because its hydrogen-free radical absorbs at 517 nm and its purple hue disappears quickly when reacting with proton radical scavengers [40].

concentrations of CS-NPs used (12.5, 25, 50, 100, and 200 µg/ml) demonstrated the highest antioxidant activity (78.88% at 200 µg/ml), whereas the concentrations of CS-NPs used (100, 50, 25, and 12.5 µg/ml) demonstrated antioxidant activity (59.76, 58.82, 43.36, and 29.36%), respectively. Ascorbic acid's antioxidant activity at the same concentration was (82.06, 74.81, 52.62, 40.36 and 37.96%) respectively, Figure (8). T-student test revealed significantly different between CS-NPs and ascorbic acid in all concentrations, however the previous results reveled a promising antioxidant activity of CS-NPs that biosynthesized by carb shell [41] .

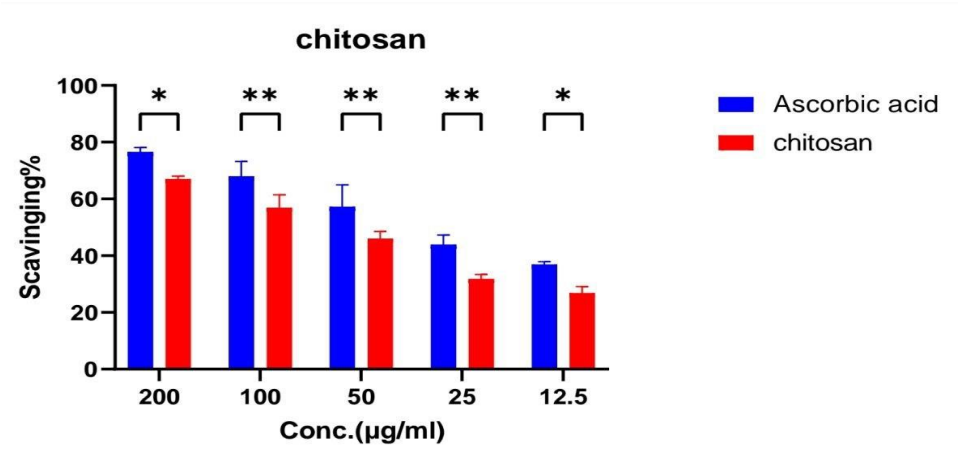


Figure (8). Free radical scavenging activity (%) of CS-NPs different concentrations compared to Ascorbic acid as a positive control

The quinoid substance generated by phenol oxidation may be adsorbed on nanoparticles to stabilize suspension [42]. It is widely known that phenolic compounds may directly counteract oxidative action with other substances due to their redox characteristics as reducing factors, hydrogen donors, and singlet oxygen quenchers [43].

Conclusion:

Carb shell can be used to bio- synthesis of CS-NPs in an environmentally friendly manner. These nanoparticles work well as anti-bacterial and anti-oxidants. It may also be synthesized on a large scale, used to target resistant infectious bacteria it is strongly advised that it be used as a more affordable alternative to conventional anti-bacterial agents, or used as a preservative agent in fishery products. Moreover, the ability of CS-NPs to exert anti-oxidant activity and add value to food products.

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