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Potential Application of Chitosan Nanoparticles as Preservative Agent for Fishery Products

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ABSTRACT

Given its superior bioactivities and biocompatibility, chitosan (CS), a natural polymer that is biodegradable and nontoxic, is widely used in food and biomedical industries. The presence of anionic elements is required to achieve the superior gelling characteristic of CS, which is attributable to its polycationic nature. Additionally, compared to the free form, the chitosan nanoparticles (CSNPs) enhanced bioactivities, such as antioxidant and antibacterial activities and improved stability during storage and continuous release. This study attempted to explore the main uses of CSNPs as a fish preservation agent for *Sardinella aurita*. Antimicrobial activities of CS and CSNPs were tested against *S. aureus* and *E. coli.* Moreover, CS and CSNPs were sprayed over two fish groups, with a third group left untreated (control group). Results delineated the significant antibacterial potential of CSNP compared to CS and control (Gentamycin). Additionally, compared to the CS and control groups, the fish exposed to CSNPs had higher organoleptic indices in their eyes, gills, mucus, flesh, texture and smell. Conclusively, the spray made from shrimp shell waste as CSNPs has good antibacterial and preservation properties. When processing or transporting fisheries products, CSNPs might work as an antibacterial agent and a natural preservative.

INTRODUCTION

Most fishermen have long used synthetic preservatives such as formaldehyde to preserve unsold catches. Given its technical advantages of being simple to get, affordable and useful, formaldehyde is justified for use in preserving fresh fish **(Utama *et al.*, 2021)**. According to the Regulation of the Minister of Health No. 33 of 2012 concerning food additives, formaldehyde is a preservative with dangerous adverse effects. Formaldehyde is a carcinogenic and mutagenic chemical that can cause cell and tissue damage **(Desvita *et al.*, 2020)**. People also frequently use borax in addition to formaldehyde. Borax is a white, odorless, crystalline chemical that is soluble in water **(Xie *et al.*, 2017)**. It is frequently misapplied as a food additive to enhance the flavor and longevity of food products **(See *et al.*, 2010)**. It is typically used as a preservative, antiseptic and cockroach repellant. Therefore, a substitute for synthetic preservatives in the form of natural

preservatives is required. As a result, a natural preservative solution is required to reduce the use of synthetic preservatives including formaldehyde and borax. The chance of developing health issues is lower when using natural preservatives because they are derived from nature. Before distribution, fishermen's catches can be preserved using natural materials' antioxidant and antibacterial properties **(Lourenco *et al*., 2019)**. Examples of natural preservatives are salt, sugar and chitosan. Salt can inhibit the microorganisms' development and reduce water content so that food is more durable **(Dwivedi *et al*., 2017)**. Sugar can bind to water so that it can prevent food spoilage. According to **Goy *et al*. (2009)**, chitosan serves as an inhibitor of microbial progress. Shrimp is one of the crustaceans whose shells are used to make chitosan **(Li *et al*., 2021)**.

The shrimp (*Portunus pelagicus*) has a high commercial value, resulting in a significant enough demand for shrimp catching. Solid waste in the form of shell trash is produced in large quantities by the shrimp industry. The processing of shrimp is directly proportional to the waste produced, so handling efforts are needed to reduce the negative impact on the environment **(Bhattacharjee *et al*., 2019)**. One tiny shrimp produces trash that is made up of 57% shell, 3% body rejects and 20% boiled water. The problem of environmental pollution and garbage that keeps growing can be solved by using shrimp shell waste **(Nguyen *et al*., 2020)**.

Improved hazardous detection, shelf life and packaging techniques are now possible thanks to advanced nanotechnology, which has also sped up the process of addressing food safety issues involving microbial contaminants **(Bajpai *et al*., 2018)**. Food nanotechnology has the effect of enhancing the nutritional content and solubility of food. Nanotechnology can improve food safety, extend shelf life, improve flavor and detect pathogens/toxins/pesticides, as well as creating/making functional food **(Seabra *et al*., 2013)**. Chitosan nanoparticles, or nano-chitosan, are one of the by-products of the nanotechnology process. All prior research; however, concentrated on the production of chitosan and its use as an antibiotic. As far as we are aware, not many studies have been done on the production of nano-chitosan as a natural preservative for fisheries goods. Nano-chitosan is ideal for use as a natural preservative since it has a stronger antibacterial and antifungal absorption capacity than chitosan **(Abdeltwab *et al*., 2019)**.

The study's goals were to assess and ascertain the efficacy of nanochitosan spray as a natural preservative in captured fish. To increase shelf life and assure the food safety and security of fisheries products, research results were required to identify the best antibacterial, preservative and additive options. Fishermen would be able to easily apply this study to their catches, preventing large and ongoing losses.

MATERIALS AND METHODS

1. Sample preparation

Shrimp shells obtained from fishermen in Alexandria, Egypt, served as the study's primary source of data (Fig. 1a). They were cleaned from remaining meat and dirt using water, and then the shells were dried in the sun for approximately two days (Fig. 1b). After that, with

the aid of a blender and a sieve shaker, shrimp shells were ground into a powder measuring 60 meshes or 250 microns **(Supriyantini *et al*., 2018)**.

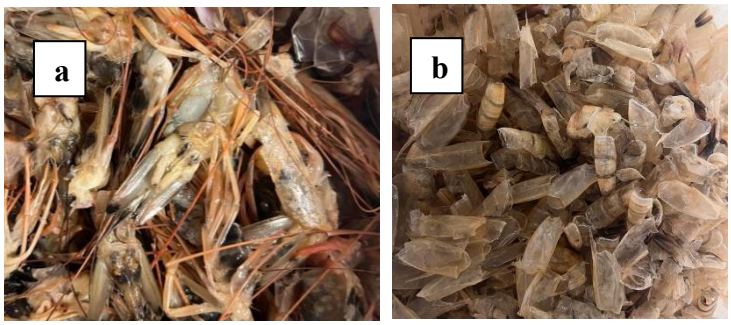

Fig. 1. Shrimp shell waste showing: (a) Raw shell waste, and (b) Cleaned shell waste after dryness

2. Chitosan production from shrimp shell waste

Demineralization, deproteination and deacetylation are three steps in the production of chitosan from shrimp shells **(Supriyantini *et al*., 2018)**. To demineralize the shrimp shell powder, a 1:7 (w/v) solution of 1.0 N HCl was added, and the mixture was agitated using a magnetic stirrer at 200 rpm for 30 minutes until becoming frothy. The mixture was baked at a temperature of 80-90°C for 1.0 hour, then cooled and filtered to obtain a solid, and finally cleaned by using distilled water to a neutral pH. The solid was heated to a constant weight and baked at a temperature of 100°C. After cooling to room temperature, the yield was measured and weighed. By adding 3.5% NaOH solution to the demineralized product at a ratio of 1:10 (w/v), stirring with a magnetic stirrer while heated for an hour at 70-80°C, and then cooling the mixture, deproteinization was achieved. The precipitate was filtered, rinsed with distilled water until the pH was neutral, and then heated at 100˚C to maintain a constant weight. It was left to cool down to room temperature, and the final weight was adjusted. The deproteinized product then moved on to the deacetylation stage, where it was dissolved in 50% NaOH at a 1:15 (w/v) ratio and stirred for 2 hours at 80–90°C, using a magnetic stirrer at 200 rpm. Afterwards, it was cooled and filtered. After washing with distilled water to achieve pH neutrality, the residue was baked to a consistent weight at 100°C. The yield was left to cool to room temperature before weighing. Chitosan is the end- product.

3. Preparation of nano-chitosan by ionic gelation

Using ionic gelation, 0.2% chitosan solution in acetic acid solution was made, and then 1% NaTPP solution was added with stirring speeds of 600 rpm for an hour. Dispersed solids, a kind of nano-chitosan, were produced **(Arsyi *et al*., 2018)**.

4. **Particle size and morphology determination using TEM**
   CNPs size and morphology were observed by transmission electron microscope (TEM), Faculty of Science, Alexandria University. All samples for TEM analysis were prepared by allowing a single drop of nanoparticle suspension to dry overnight at room temperature on a Formvar® coated 200-mesh copper grid (TAAB Laboratories Equipment Ltd., Aldermaston, England).
5. **Fish groups**

Nine *Sardinella aurita* fish samples were freshly collected from Abu-Qir Bay, Alexandria, Egypt. Each group had three fish after being split into three (Fig. 2). The first group (control group) (1-3) was sprayed with water; the second group (4-6) was sprayed with chitosan solution, and the third group (7-9) was sprayed with chitosan nanoparticles solution. Length (L) and weight (W) of each fish individual were determined in order to unify their starting W and L parameters (data not shown).

Fig. 2. Fish samples (*Sardinella aurita*) showing: (A) Control group; (B) Chitosan-sprayed group, and (C) Nano-chitosan sprayed group

6. Manufacturing and application of nano-chitosan spray

Nano-chitosan spray was obtained by mixing nano-chitosan powder and acetic acid solvent at a ratio of 3:1 (w/v) in a 100ml spray container, and the mixture was homogenized. The application of nano-chitosan spray was made by spraying it evenly on the surface of the fish. This is the same as what was done in the research of **Hamdayanti *et al.* (2012)**, who used a sprayer to apply a chitosan solution to the sample's complete surface in order to administer natural preservatives as a spray. Controls were sprayed with sterile water.

7. Antibacterial activity test with disc diffusion method

The disc diffusion method was executed by wetting the disc paper with a solution of CS and CSNPs, and then placing it on Mueller Hinton Agar covered with *E. coli* (ATCC 8739) and *S. aureus* (ATCC 6538). *S. aureus* is a Gram-positive spore-forming bacterium **(Lestari *et al.*, 2019)**, and *E. coli* is a short-rod Gram-negative bacterium **(Bambang *et al.*, 2014)**; these bacteria are often found in water and caught fish. After that, 2% of amoxilin was drip-applied, while it was incubated for 24 hours at 37°C. Disc paper with a clear zone surrounding it indicates a favorable disc diffusion **(Prasetiowati *et al.*, 2018)**.

8. Organoleptic test

The organoleptic test was performed on 9 individuals of *Sardinella aurita* that had been sprayed with chitosan and nanochitosan as well as control treatment for each fish species. An organoleptic evaluation was performed on the eyes, gills, mucus, meat, texture and smell based on the study of **Syafitri *et al.* (2016)**, and a thorough evaluation was given. The data obtained from the outcomes of the organoleptic assessment were descriptively analyzed to determine the level of freshness of the fish.

9. Preservative-effectiveness test

A preservative-effectiveness test was conducted by measuring the resistance time of fish against bacteria. The measurement started right after spraying the fish until it decayed. The long resistance of fish to bacteria shows the preservative effectiveness. This is similar to the research of **Cahyaningsih *et al.* (2021)**, who observed the physical changes of the sample until its optimal storage period (days) to determine the sample's level of resistance to test the preservative activity.

RESULTS AND DISCUSSION

Fish is a highly perishable item that needs to be handled, processed and distributed properly in order to be productively and economically used. The demand for fish is increasing, and reducing postharvest losses can significantly help meet this demand, while also enhancing the quality and quantity of fish for consumers and raising producer profitability. One alternative to overcome the lack of fish freshness during transportation can be done by adding natural preservatives **(Thulasiraman *et al.*, 2021)**. The antibacterial activity of chitosan has been highlighted to extend the product's shelf life. Natural nano-chitosan is a high-quality product with unique physicochemical characteristics. The physical cross-linking of nano-chitosan by ionic gelation with particularly negatively charged macromolecules such as sodium tripolyphosphate has been studied using a variety of methods. Additionally, coating chitosan nanoparticles with chemicals or natural antibacterial agents, antioxidants, enzymes, or active ingredients is a possibility **(Abdeltwab *et al.*, 2019)**. Since nano-chitosan has a bigger surface

area and a stronger attraction for bacterial cells than chitosan, it exhibited higher antibacterial activity during product storage than chitosan **(Ramezani *et al*., 2015)**.

1. Chitosan from shrimp shell waste

The chitosan yield was achieved by dividing the resulting chitosan by the initial weight of the sample multiplied by 100%. Chitosan yield declined from 100% at the beginning to 30.2% at the end, and this happened as the temperature and the concentration of the NaOH solution increased. The yield of shrimp shell chitosan was higher than that reported in the study of **Supriyantini *et al*. (2018)** who concluded that, the yield of chitosan from shrimp shells obtained reached 36.84%. The total chitosan produced from 250 grams of shrimp shells was 75.5g (30.2%) (Table 1).

Table 1. Results of the production process of chitosan

Stage	Product mass (g)	Yield %
Demineralization	107	42.8
Deproteination	82.3	32.9
Deacetylation	75.5	30.2

2. Nano-chitosan with ionic gelation

Nano-chitosan is the application of nanoparticle technology to chitosan. Chitosan from the deacetylation stage is then made into nano-chitosan using the ionic gelation method. Ionic gelation is a method that is widely used since the process is effective and simple and easily controlled. Chitosan is diffused in acetic acid; polyanions are then added, and with constant stirring, nanoparticles are spontaneously formed **(Qonitannisa *et al.*, 2020)**. This is the ionic gelation mechanism. Since natrium tripolyphosphate (NaTPP), a nontoxic polyanion chemical, interacts with chitosan through electrostatic forces to create ionic crosslinks, this substance is employed as a polyanion.

3. Particle size and morphology determination using TEM

TEM scans revealed that CSNPs had a diameter between 31 and 82nm, making them appear to be tiny and distinct; larger particles are due to the aggregation of single small particles that tend to fuse generating a larger entity. This aspect is clearly shown by subsequent TEM images. Air-dried samples are not completely desiccated, especially not nanogels as chitosan-TPP particles. During TEM analysis, when magnifying a CSNPs aggregate as much as possible, a very fast fusion (seconds/minutes) of single particles into one entity was observed: the heat of the electron beam promoted intermolecular links thanks to the still present aqueous environment inside the gel-network. In order to confirm that this behavior was caused by a linking agent, TEM images (Fig. 3) of chitosan solution were collected showing small particles of 40 nm in

diameter but with a completely different behavior. Upon magnifying on several close entities, their structure did not change, and no fusion occurred even if the ray was left on them for a considerable amount of time (data not shown).

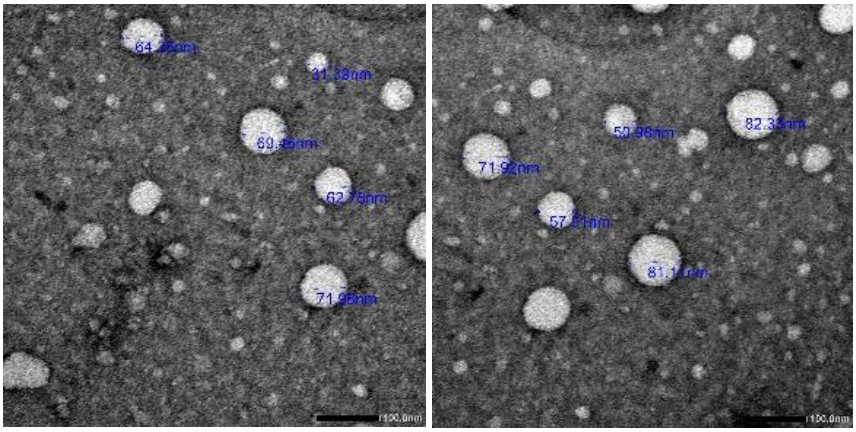

Fig. 3. TEM scans of chitosan nanoparticles. Scale bar: 100nm. TEM: transmission electron microscopy

4. Antibacterial activity test with disc diffusion method

This study tested nano-chitosan against *E. coli* and *B. subtilis* bacteria because these bacteria are amongst the most prevalent in fisheries' products during the process of fish quality deterioration. *B. subtilis* bacterium plays a role in the decay of meat and protein ingredients like fish **(Sari *et al.,* 2019)**. Meanwhile, *E. coli* spreads easily through contaminated water, thus contaminating fresh fish **(Sumampouw, 2018)**. If the growth of these bacteria is not stopped, it will cause foodborne diseases that will harm consumers and fishermen.

The disc diffusion method in the antibacterial activity test aimed to determine the potential of nano-chitosan in inhibiting the growth of *E. coli* and *S. aureus* bacteria. When utilizing disc diffusion to test an extract or compound for antibacterial activity, a favorable result is exposed by the appearance of a clear or inhibitory zone surrounding the paper disc **(S. Magaldi *et al.,* 2003)**.

With an inhibitory zone surrounding the test point, a quantitative study of shrimp nano-chitosan's antibacterial properties may be possible. As shown in Table (2), the findings of the nano-chitosan activity test were able to inhibit the growth of *S. aureus* and *E. coli* with three different concentrations. Nano-chitosan's 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. This imbalance in the osmotic pressure inside the cell hinders the growth of microbes. In addition, intracellular hydrolysis events occur in the cell wall, leading to the release of cell electrolytes, and thus causing the death of a

microbial cell **(Sarwono, 2010)**. On the other hand, chitosan was effective against *S. aureus* but not effective against *E. coli* in the tested concentrations (Fig. 4).

Table 2. Antibacterial activity test disc diffusion method*

Bacterial strain	Control (Gentamycin)	Chitosan	Chitosan nanoparticle
Staphylococcus aureus (ATCC 6538)	15.33 ± 0.19	16.50 ± 0.16	21.33 ± 0.19
Escherichia coli (ATCC 8739)	20.00 ± 0.00	NA	22.17 ± 0.09

*Antimicrobial activity was determined by using agar diffusion or disc diameter: 6.0mm.
*Results are means of three readings ± S.E.
* NA: No activity.
*Positive control: Gentamycin.

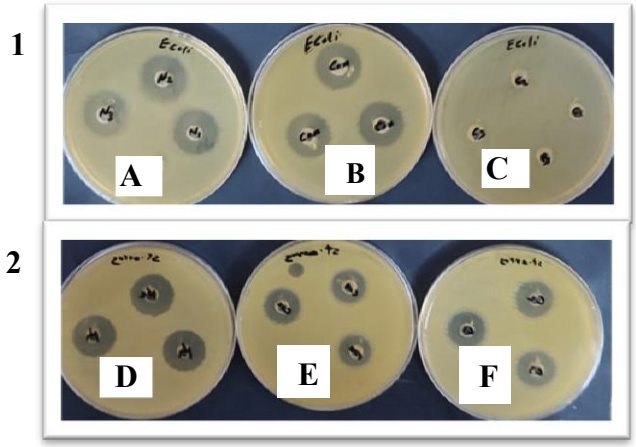

Fig. 4. Agar disk diffusion method of the following: (A and D) Chitosan nanoparticles; (B and E) Control (Gentamycin); (C and F) Chitosan against *E. coli* (1) and *S. aureus* (2) to evaluate the antimicrobial activities

5. Organoleptic test

The organoleptic test utilizes the human senses as the main tool in measuring the quality of a product. The organoleptic test parameters for tilapia, mullet, milkfish, mackerel, kipper and catfish comprised the eyes, gills, mucus, flesh and texture, whereas those for squid, cuttlefish, and shrimps included appearance, smell and texture. Aspects of fish product quality that can be assessed by these senses are depicted in Table (3). As we describe quality, it is important to note that it goes beyond just how delicious the fish is to eat: the eating quality is perhaps the most important aspect of overall quality and is greatly affected by how well the fish is kept, whether in ice or frozen storage. But quality also includes factors such as the fish's value, its processing suitability and its size, whether it is damaged or blemished. A judgment may occasionally be

made using just one sense. Therefore, identifying a whole fish as a whiting or a haddock is based solely on appearance. Some situations call for the utilization of two or more senses. For instance, just by looking at a fish, a suspicion that its freshness is questionable can be sparked. This suspicion can then be verified by the fish's aroma or flavor. On the fresh fish organoleptic assessment sheet, the test results are recorded **(based on FAO in partnership with Support unit for International Fisheries and Aquatic Research, SIFAR, 2001)**. The sample in the organoleptic test is suitable for consumption if it has an organoleptic score of at least 7 (Table 4). The organoleptic analysis data using chitosan and nano-chitosan spray are presented in the Table (3).

Table 3. Some quality aspects of fish products and the senses used to assess them **(**based on **(FAO, 2001)**

Sense	**Aspect of quality**
Sight	General appearance and condition, size, shape, physical blemishes, color, gloss and identity.
Smell	Brightness, off-odors and smells, stains, oiliness, rancidity and shadiness.
Taste	Glow, off-tastes and scents, blemish, greasiness, staleness, blackness, sourness, the primary tastes of acerbity, tartness, aroma and sugariness.
Tongue and finger contact	General texture, hardness, softness, elasticity, brittleness, roughness, smoothness, grimness, fusion, facility, moisture, drought, concision and existence of bones.
Hearing	Brittleness and crispness.

The organoleptic results on the first day showed that the fish sprayed nano-chitosan recorded an organoleptic value of more than 7, thus it was suitable for consumption. In contrast, the fish with the control treatment had an organoleptic value below 7; therefore, it was unsuitable for consumption. As for the chitosan-sprayed group, it demonstrated a freshness value lower than 7 but greater than 5. After 5 days, findings confirmed that the nano-chitosan spray had an organoleptic value of more than 5. Conversely, the fish with the control treatment and chitosan had an organoleptic value below 3 and 4, respectively (Table 5).

Biochemical spoilage of fish occurs with a marked change in pH due to the autolysis process, so it will accelerate the decline in fish quality. Fish's flavor and scent can be affected by the quality of fisheries' goods, which would lower their selling price because it is unfit for human consumption. **Nurhayati *et al*. (2019)** explained that, the decline in fish quality is influenced by the type, size of fish, bacteria or enzymes, oxidation, the fishing process, the environment and how the catch is handled; all contribute to the loss in fish quality, which affects the consumers' interest and purchasing power directed to the catches of fishermen that will be caught for sale.

Table 4. Sensory score sheet for fishery products (adopted from **(FAO, 2001)**).

Score	Odor	Flavor	Texture, taste, and appearance	Score
10	Originally mild scents of sweet, boiled milk, and flour that gradually became stronger	Initial flavors are watery, metallic, and starchy; they may eventually develop subtle sweetness as well as meaty flavors	Short stiff fibers that are brittle and dried	10
9	Seafood, seaweed, cooked meat, and uncooked green plants	Sweet, savory, whipped, vegetal, and distinctive		9
8	Loss of scent and a neutral odor	Characteristically sweet flavors yet less intense	Initially stiff but becoming softer with storage; the succulent, fibrous material starts off white and opaque but turns yellowish and waxy over time	8
7	Wood shavings, woodsap, and vanillin	Neutral	Initially stiff but becoming softer with storage, the succulent, fibrous material starts off white and opaque but turns yellowish and waxy over time.	7
6	Condensed milk, caramel, and toffee-like smells	Tasteless		6
5	Milk jug fragrance, cooked potato, and boiling garments	Tad sourness and faint “off” flavors		5
4	Lactic acid, acidic milk, and “byre-like”	Faint bitterness, sourness, and “off” tastes		4
3	Lower fatty acids (e.g., acetic or butyric acids), composted grass, soapy, turnip-like, and tallow-like flavors	Strongly bitter, rubbery, and barely sulfuric scents		3

Table 5. Organoleptic results of the tested fish groups

Group	**First-day score**	**Fifth-day score**
Group 1 (control)	6	3
Group 2 (chitosan-sprayed)	7	4
Group 3 (nanochitosan sprayed)	9	6

6. Preservative-effectiveness test

A preservative-effectiveness test is used to determine the ability of preservatives when applied to a fishery product based on the appearance of fish body upon spraying. Based on the effectiveness test results, data revealed that the nano-chitosan spray had the ability to preserve shrimp for up to 5 days (Fig. 5). Fish spraying with nano-chitosan was effective for shrimp preservation because it has a smaller particle size to be well absorbed on the surface of the shrimp. This finding concurs with that of **Abdeltwab *et al*. (2020)** who stated that, the antimicrobial activity and permeability of chitosan nanoparticles increase when their size gets smaller.

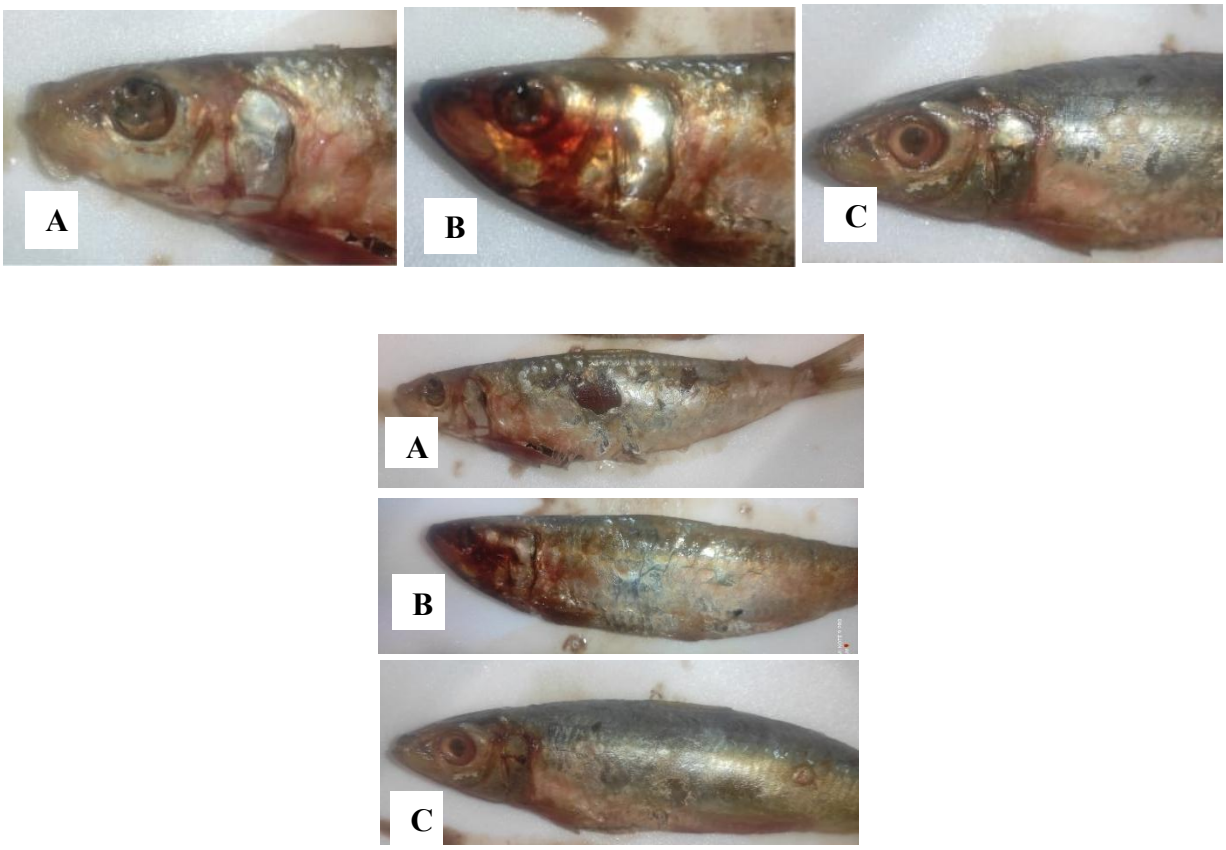

Fig. 5. The appearance of fish groups (preservation-effectiveness test) showing: (A) Control group; (B) Chitosan-sprayed group, and (C) Chitosan nanoparticles sprayed group

CONCLUSION

Chitosan nanoparticles can be used as a preservative agent in fishery products. The study found that the use of chitosan nanoparticles as a preservative agent in fishery products was more effective in inhibiting microbial growth than normal chitosan (CS), and they allow retaining high

sensory scores of the tested fish groups. Additionally, chitosan nanoparticles (CSNPs) have been prepared using several methods. Ionic gelation is the most exploited method for the preparation of CSNPs, showing high potential to enhance the absorption and availability; this could explain the effectiveness of CSNPs compared to CS. The augmented antimicrobial activity of CSNPs can enhance the shelf life of various foods. Moreover, the ability of CSNPs to exert antioxidant activity and add value to food products, such as tea polyphenols, vitamins, etc., can help reduce the lipid or protein oxidation of foods. Hence, nanostructured forms of CS can be considered as profitable value-added products, which are used in a wide array of foods, packaging, encapsulation, biomedical remedy and health promotion. Conclusively, spray nano-chitosan from shrimp shells was effective in the preservation process of *Sardinia aurita* given that it was able to inhibit the deterioration of fish quality for 5 days.

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AUTHORS' CONTRIBUTIONS

Under the direction of Dr. Haiam M. Aboul-Ela, College of Fisheries and Aquaculture Technology, Arab Institute of Science, Technology, and Maritime Transport, the first five authors completed this study as part of their graduation project in the previous year. From the beginning through the completion of the final publication, all writers contributed and discussed the findings. The contribution of each author is as follows: Haiam M. Aboul-Ela was responsible for the main conceptual ideas, supervising the work and writing the manuscript; Abdelaziz H. Elmotyam, Mayar A. Belal, Mina H. Fouad, Nada A. Mohamed, and Neveen E. Elkasas were equally responsible for collecting the data and drafting the manuscript.

CONFLICTS OF INTEREST

All authors of this research declare that they have no conflicts of interest.

DECLARATION OF GENERATIVE AI IN SCIENTIFIC WRITING

The authors declare that they did not use AI-assisted tools in this paper.

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DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available on request from the corresponding author.

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