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Extraction, Characterization and Antioxidative Potential of a Bioactive Polymeric Material from the Cuttlebone of *Sepia brevimana*

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Abstract

This study aimed to extract and characterize a functional polymeric chemical from Sepia brevimana cuttlebone and explore its potential to inhibit oxidative processes. Cuttlebone waste is widely available and frequently discarded, making it an excellent source for extracting beneficial bioactive chemicals. The isolation process involved solvent extraction, precipitation, and purification to produce a pure polymeric material. Several analytical techniques, including Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and scanning electron microscopy (SEM), were employed to characterize the isolated polymeric material. The study revealed that the isolated material possesses a unique polymeric structure with functional groups associated with antioxidant activity. The bioactivity of the substance was assessed by evaluating its efficiency in inhibiting oxidative processes in a model system. The material demonstrated excellent antioxidant activity by inhibiting the generation of reactive oxygen species (ROS) and scavenging free radicals. The presence of antioxidant moieties and the polymeric nature of the structure contributed to this activity. In conclusion, a bioactive polymeric material with strong antioxidant capabilities was successfully extracted and characterized from Sepia brevimana cuttlebone. The findings highlight the potential of cuttlebone waste as a rich source of bioactive chemicals, offering a sustainable pathway for producing natural antioxidants for applications in the culinary, cosmetic, and pharmaceutical industries. Further research is needed to comprehensively analyze its bioactivities and understand the underlying mechanisms of action.

Keywords: *Anti-oxidant, Biomedical, Cuttlebone, Cuttlefish, Pharmaceuticals, Sepia brevimana.*

Introduction

Chitosan is famous for its ability to fight harmful substances in the body. Many studies have shown that chitosan helps stop harmful molecules called reactive oxygen species (ROS) and prevents fats from becoming hazardous in food and living things. There are several proposed ways that chitosan may have antioxidant effects [1, 2]. Chitosan can pick up free radicals and attach to metal ions by giving away hydrogen or pairs of unused electrons [3, 4]. Chitosan can interact with metal ions in different ways, such as sticking to them, swapping ions, and binding tightly to them [5]. The hydroxyl groups (OH) and amino groups (NH) in chitosan are key parts that help it act as

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an antioxidant. However, it can be challenging to separate them because chitosan has a rough structure and tight hydrogen bonds [3].

Chitosan is a cationic polymer with a large molecular mass. 1,4-linked glucosamine and various amounts of N-acetylated glucosamine residues make up this linear polymer. It is usually made by breaking down chitin in an alkaline environment. Chitin can come from shellfish shells or the cell membranes of many different types of bacteria and fungi [6, 7]. It has generated great interest as a healthcare material due to its peculiar biological features, which include anticancer, immune stimulatory [8], and antimicrobial [9, 10]. Oxidative stress, induced by radicals produced by oxygen, is assumed to have a significant role in a range of degenerative diseases, as well as the natural process of ageing. Normal metabolic processes or external substances and agents create reactive oxygen species (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide (H_2O_2), which can readily trigger lipid peroxidation in the membrane, leading to the formation of lipid peroxide. These ROS can damage a wide range of essential biomolecules.

In the last few decades, several studies have discovered the antioxidant properties of chitosan derivatives. There is an increasing fascination with discovering natural antioxidants that may safeguard the human body from free radical damage and slow the progression of many chronic illnesses [11]. Natural antioxidants consist of a diverse spectrum of chemicals, including phenolic substances, nitrogen-containing substances, and carotenoids [12, 13]. However, recent discoveries have revealed the antioxidant activities of several biological polysaccharides. The antioxidant properties of chitosan and its related compounds have attracted the most attention [14]. Despite extensive research on the antioxidant properties of chitosan and its derivatives, a deeper understanding of its precise mechanisms of action remains limited. The challenges associated with separating functional groups like hydroxyl and amino groups due to chitosan’s rigid structure have yet to be fully addressed. Additionally, while studies focus on chitosan's interactions with free radicals and metal ions, variations in its activity across different derivatives and environmental conditions are underexplored. There is also insufficient data linking chitosan’s antioxidant potential to specific biological outcomes in vivo. Lastly, the development of scalable methods for enhancing and utilizing chitosan’s antioxidant properties in practical applications remains a significant gap. In this study, we looked at chitosan's antioxidant properties using a variety of tests, such as DPPH/superoxide radical scavenging and ferrous ion chelation, to learn more about how antioxidants work and how they can damage cells.

Materials and Methods

Extraction of Chitin

For deproteinization from marine cuttlefish, 40% NaOH (w/v) was applied, and the deproteination procedure was carried out for 72 h. After deproteinizing the cuttlebone with NaOH, we washed them with water. The outer layers were then treated with 4% concentrated HCl to remove calcium, with a demineralization period of 20–24 h, followed by a water wash and drying. The chitin was then obtained by deproteinizing and decalcifying the cuttlebone.

Conversion of Chitosan from Chitin

For the production of chitosan, dehydrated, deproteinated, and decalcified chitin was soaked in water. To deacetylate, wet chitin was placed in a 20 M sodium hydroxide solution and stirred. The deacetylation method lasted 48 h. After deactivation, chitosan granules were cleaned, crushed, and dried out in a ventilated oven at 60-70°C. Chitosan was extracted from marine cuttlefish excrement and used in further antioxidant investigations.

Structural and Thermal Characterization

Fourier Transform-Infrared (FT-IR) Spectral Analysis of Bioactive Polymers

A BRUKERS ALPHA II FTIR A spectrum analyzer was used to assess the deacetylated chitin extracted from *Sepia brevimana*. A spectrum analyzer was utilized to evaluate the bioactive polymers isolated from *Sepia brevimana*.

Scanning Electron Microscopy (SEM)

The surface characteristics and architecture of bioactive polymers were studied using SEM. Using the Hitachi Hus-4 vacuum vaporizer, a small quantity of gold/palladium (40/60) was added to the specimen, which evaporated quickly at 20 V. The study was conducted at various magnification levels, with an enhanced potential of 0.5 and 30 kV.

X-ray Diffraction (XRD)

The Shimadzu XRD-6000 gadget assessed XRD intensity using specimen orientation and angle of diffraction (2θ). The diffraction patterns were utilized to determine the size and placement of the crystallites, and the specimen's crystal frameworks were determined after a detailed investigation of their structural characteristics.

In vitro Antioxidant Property

Scavenging Ability on 1, 1-Diphenyl-2-Picrylhydrazyl Radicals (DPPH)

The DPPH radical-scavenging capacity of chitosan at a concentration of 1 mg mL^{-1} was determined as described by Dong et al. [15]. Specimens dissolved in 500 lL of distilled water (1 mg/mL) were mixed with 375 lL of 99.5% ethanol and 125 lL of 0.02% DPPH in 99.5% ethanol. The blends were incubated for 60 min in the dark at ambient temperature, and the reduction in DPPH radical was measured at 517 nm using an ultraviolet-visible spectrophotometer. In its radical form, DPPH has an absorption band at 517 nm that disappears when reduced using an antiradical. Lower absorbance of the reaction mixture indicated more DPPH-free radical elimination capability. The DPPH radical-scavenging ability was determined in the manner that follows:

Radical-scavenging activity = $\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}$ X 100 Absorbance of control.

Superoxide Radical Scavenging Assay

The technique of Bersuder et al. [16] was performed to determine chitosan's superoxide scavenging ability. Chitosan (0.05–0.5 mg/mL), PMS (30 µM), NADH (338 µM), and NBT (72 µM) were added to a reaction mixture along with phosphate buffer (0.1 M pH 7.4). The mixture was left to sit at room temperature for five minutes. The absorbance was measured at 560 nm against a blank. The ability to scavenge superoxide radicals was calculated using the formula as follows: Scavenging effect

$$(\%) = \frac{(1 - \text{Asample 560 nm})}{\text{Acontrol 560 nm}}.$$

Chelating Ability on Ferrous Ions

The approach of Xing et al. [17] investigated the strong antioxidant properties of ferrous ion-chelating compounds. Chitosan had a significant inhibitory effect on superoxide radical elimination activity, which was concentration-dependent. A strong scavenging impact (20.4–88.6%) of super radicals was visible at all tested levels of chitosan and was explored using the method of detecting the ferrous iron-ferrozine complex at 562 nm to monitor chitosan's Fe2+ chelation capacity. Briefly, the reaction mixture, which comprised chitosan at different levels, FeCl2 (2 mM), and ferrozine (5 mM), was reduced to an overall capacity of 0.8 mL with water, well agitated, and incubated for 10 minutes at ambient temperature. The combination's absorbance was determined at 562 nm compared to a blank. EDTA acted as a positive control. The ability of

chitosan to chelate ferrous ions was found using the formula as follows:

$$\text{Chelating effect (\%)} = \frac{(1 - \text{A sample 562 nm})}{\text{A control 562 nm}}.$$

Results

Yield of Chitin and Bioactive Polymers

The results of this study showed that the yields of chitin and deacetylated chitin were 34.46% and 36.58%, respectively.

FT-IR Analysis

Fig. 1 indicates that the bands exhibited an intensity of approximately 3413.26 cm^{-1}. The polysaccharide's hydroxyl extended vibration was predicted to produce very wide bands. When hydroxyl chains exist, a visible peak between 3500 and 1000 cm^{-1} suggests few hydrogen linkages. Stretched C-H vibrations of the CH_2 groups led to weak absorption bands. Absorptions at 1636.18–1545.22 cm^{-1} were caused by stretching tensions in the CHO and C=O bonds. The strong absorption bands from 3413.26 to 1405.07 cm^{-1} indicate the C-H bond's changing oscillations. The C-O and C-C link band sites are represented by the carbohydrate absorbance, which ranges from 1018.10 to 638.58 cm^{-1}.

Scanning Electron Micrographs (SEM)

The surface structure of deacetylated chitin is visible in the scanning electron microscopy images (Figs. 2A, 2B). It also had a smooth, non-porous membranous phase with crystallites, dome-shaped holes, and microfibrils, which are tiny, topographically added dome-shaped holes. Microfibers adhere to the surface's architecture and toughness.

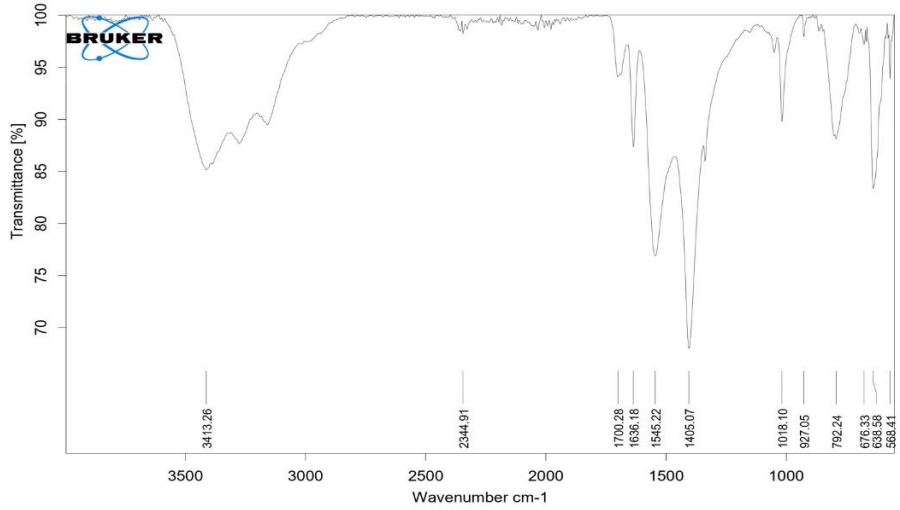

Figure 1. FT-IR Spectrum of S. *brevimana* Bioactive Polymers

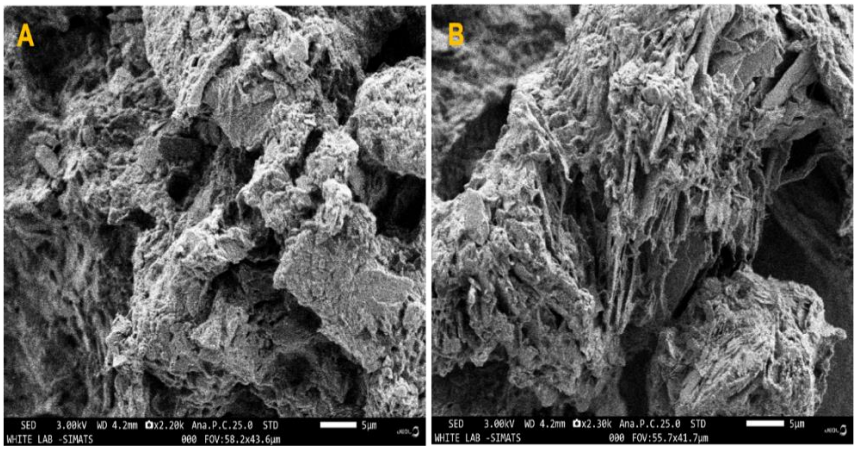

Figure 2A, 2B. SEM Images of *Sepia brevimana* Bioactive Polymers

X-ray Diffraction (XRD)

Bioactive polymers have a crystal structure. Crystallized zones contribute to mechanical properties and stability. XRD examination of deacetylated chitin showed two wide and prominent peaks at 2θ = 10° and 2θ = 30°, with weak peaks at 12°, 17°, 19°, 23°, 30°, 34°, and 37° (Fig.3).

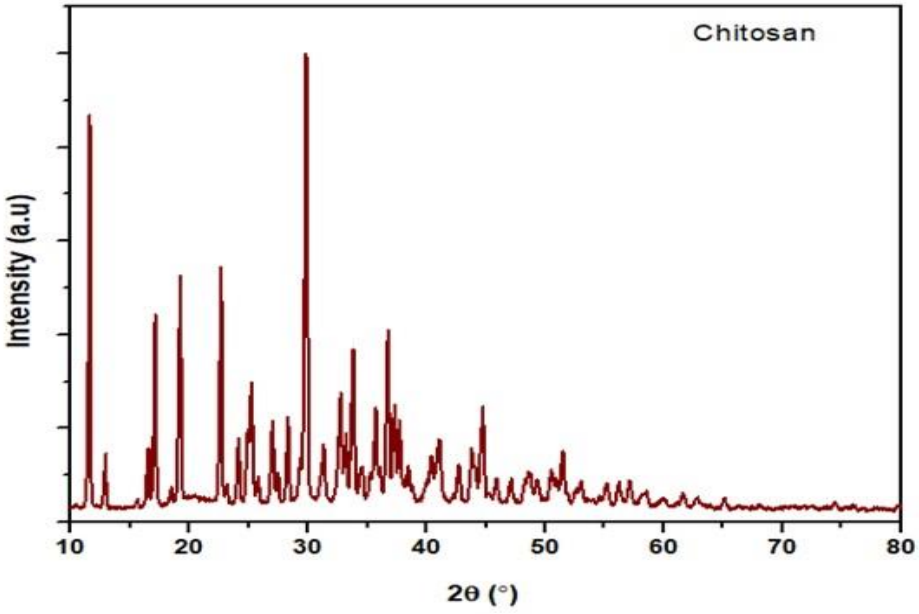

Figure 3. XRD Spectrum of *Sepia brevimana* Bioactive Polymers

In Vitro Antioxidant Activity of Bioactive Polymer

Scavenging Activity on DPPH

The DPPH test measures how successfully deacetylated chitin scavenges unstable radicals. The yellow colour in this approach is due to the reduction in stable DPPH radicals caused by bioactive polymers. The purpose of antioxidants is to donate hydrogen, thereby producing non-radical DPPH. The basic method of antioxidation is hydrogen radical scavenging, and DPPH has a unique absorption of the hydrogen-free radical at 517 nm. Furthermore, studies have demonstrated that DPPH can detect the proton-scavenging action of deacetylated chitin. In this study, deacetylated chitin from *Sepia brevimana* demonstrated a scavenging ability of 12.08-48.57% at doses ranging from 0.1 to 10 mg/mL. The scavenging activity of ascorbic acid ranged from 25.36 to 71.68% at doses of 0.1–10 mg/mL (Fig. 4A).

Superoxide Radical Scavenging Activity

Studies have shown that bioactive polymer levels between 0.1 and 1.6 mg/mL are effective in scavenging superoxide radicals at levels ranging from 23.38 to 60.19%. Alpha-tocopherol demonstrated 30.11 to 81.46% scavenging efficacy at doses that ranged from 0.1 to 1.6 mg/mL. According to the findings, deacetylated chitin beat alpha-tocopherol. Furthermore, studies have shown that alpha-tocopherol outperforms bioactive polymers derived from *Sepia brevimana* in terms of scavenging ability (Fig. 4B).

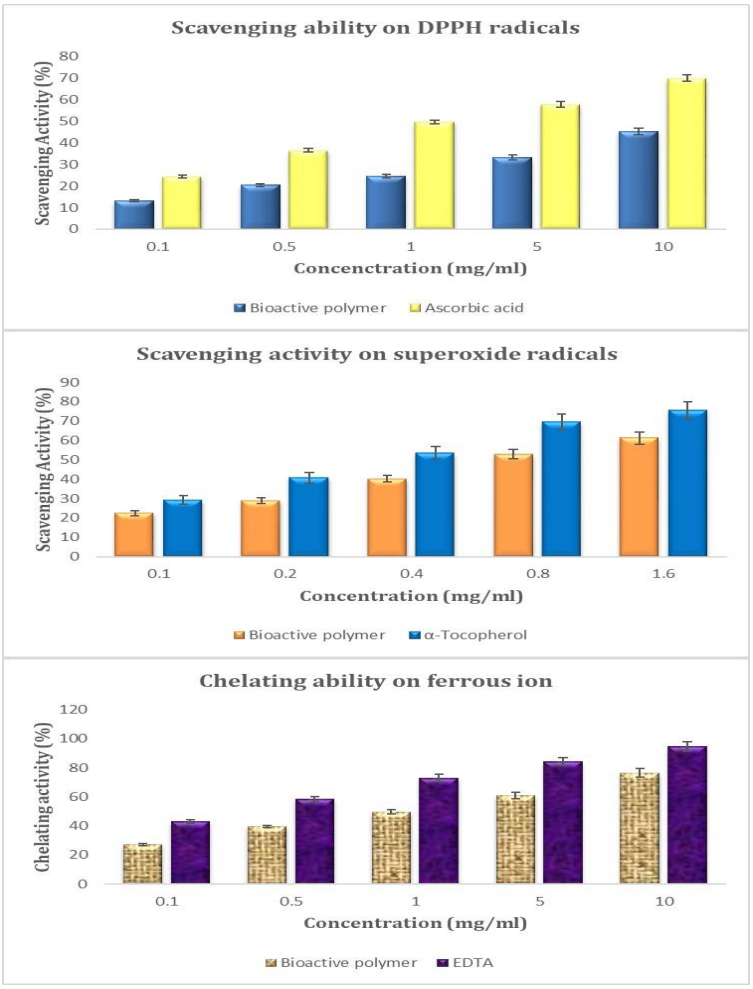

Figure 4. A. Scavenging Ability of DPPH Radicals, B. Scavenging Ability of Superoxide Radical, C. Chelating Ability on Ferrous Ions

Chelating Activity on Ferrous Ions

The results of this study showed that deacetylated chitin from *Sepia brevimana* could bind to Fe^{2+}ions with a success rate ranging from 27.20 to 79.60% at concentrations of 0.1 to 10 mg/mL. At concentrations ranging from 0.1 to 10 mg/ml, EDTA chelated 41.82 to 98.32%. Compared to deacetylated chitin and EDTA, EDTA exhibits a greater chelating action. This proved EDTA's excellent ability to bind Fe^{2+} ions (Fig. 4C).

Discussion

Chitin, the next naturally occurring biopolymer, primarily resides in the outer bones of fungi, insects, and crustaceans. Hydrolysis in alkaline solutions typically creates deacetylated chitin. Different species make chitin in different ways, as shown by the 20% yield in Sepia officinalis cuttlebone [18] and the 36.06%, 36.55%, and 22.18% yields in *Loligo lessoniana*, *Loligo formosana*, and *Penaeus monodon*, respectively [19]. In this study, we recovered 34.46% of the chitin from the *Sepia brevimana* cuttlebone. It's worth mentioning that chitin is hydrophobic, which limits its use. Deacetylated chitin is a renowned chitin derivative that researchers developed to increase chitin's solubility and expand its applications. *Nerita crepidularia* operculum and shell made 35.43% of deacetylated chitin, while *Doryteuthis sibogae* gladius's shell and operculum made 33.02% [20]. Deacetylated chitin a deacetylated variant of chitin serves as the starting point for further modifications. However, the fraction of deacetylated chitin synthesis was higher than that of *Donax scortum* and *S. pharaonis* [21]. In this work, the

quantity of deacetylated chitin extracted from *Sepia brevimana* cuttlebone was 36.58%.

Deacetylated chitin FTIR images usually show different peaks associated with its functional groups. For example, a peak at 3400–3500 cm^{-1} distinguishes an amino group ($-NH_2$) and indicates the vibratory stretching of N-H bonds. When you stretch (C=O) deacetylated chitin, the amide group's (CONH) carbonyl vibration shows up as a narrow band with a centre between 1650 and 1655 [22]. The deacetylated chitin polymer matrix often shows stretching vibrations of the C-O-C glycosidic links at frequencies between 1050 and 1150 cm^{-1}. Also, the 890-1150 cm^{-1} range shows the bending vibrations of the C-O and C-N bonds as peaks, which gives us more information about the structure of deacetylated chitin [23]. You can detect deacetylated chitin by measuring periodic variations between 638.58 and 3413.26 cm^{-1}. Deacetylated chitin exhibited Raman peaks at 1411 cm^{-1}.

Deacetylated chitin may be used to generate a variety of products, including films, microspheres, and nanoparticles. SEM was used to study the distribution, size, and shape of the particles inside these frameworks. This information is essential to optimize manufacturing processes and tailor deacetylated chitin compounds to the intended uses. SEM imaging may be used to compare different deacetylated chitin compositions, methods of processing, and variations. By visually analyzing changes in surface structure and shape, scientists can improve current compositions or discover which deacetylated chitin materials perform best for a certain application [24]. The SEM image of deacetylated chitin reveals an unusual substructure made up of a system of interconnected fibres or particles. It looks to have a smooth exterior layer that contains holes and flaws. The sizes of the deacetylated chitin fibres and particles varied, indicating that the production technique had changed or that aggregates were present. Comparing previous findings is challenging due to the acquisition of samples from various sources and locations and their processing using a variety of SEM imaging procedures [24].

The XRD pattern of deacetylated chitin typically reveals peak values that correlate to crystallographic regions. The positions and intensities of the peaks revealed information about the molecular composition and organization of the deacetylated chitin framework. Scientists found that our deacetylated chitin test results matched those of Rasti et al. [25]. Mollusc chitin, the source of deacetylated chitin, demonstrated a robust, clear reflection within the 30 to 35° range [25]. You can use the DPPH test to determine the antioxidant activity of several materials, including deacetylated chitin. Many studies have examined how well-deacetylated chitin and its derivatives remove DPPH, suggesting they may be antioxidants. Deacetylated chitin can catch DPPH radicals because its amino groups can offer electrons to neutralize unstable radicals. According to our findings, *Sepia prashadi* has a scavenging activity of 62.17%. At 10 mg/ml, deacetylated chitin from *S. lessoniana* demonstrated a scavenging activity of 55.48% [26]. Researchers found that when diluted to 10 mg/mL, crab-deacetylated chitin C60 could neutralize 28.4% of DPPH radicals [27]. The current investigation found that deacetylated chitin had a scavenging ability of 48.57% at 10 mg/mL.

Cells naturally produce superoxide radicals and other ROS during metabolic activities. They have been linked to a wide range of environmental stress-related illnesses, including carcinoma, cardiovascular disease, and cognitive impairments. Superoxide radical scavenging assays determine a chemical's antioxidant activity by measuring its ability to neutralize or scavenge harmful radicals. Each deacetylated chitin concentration examined has shown a substantial ability to eliminate superoxide radicals. When α-tocopherol and *Sepia prashadi* deacetylated chitin were diluted

to 0.5 mg/ml, they were able to remove 42.17%, 61.92%, and 76.19% of superoxide radicals [28]. The study found that scavenging superoxide radicals at 1.6 mg/ml decreased them by 60.19%.

Deacetylated chitin chemistry is notable for its ability to bind substances. Various fields such as industry, agriculture, medical, and environmental research apply this talent. Deacetylated chitin polysaccharide generated from chitin has good chelating properties due to its amino groups' propensity to attach to metal ions. This characteristic makes deacetylated chitin useful for a broad variety of purposes. At 10 mg/ml, deacetylated chitin from *Sepia prashadi* was able to bind and remove 27.59% of the ferrous ions. At 10 mg/ml, EDTA had a high binding activity of 74.92%. The highest chelating effectiveness of fungal deacetylated chitin was observed at 1 mg/mL [29]. At 1 mg/mL, chitosan isolated from shrimp shells chelated ferrous ions by 62.6%. However, EDTA had 68% chelating activity [30]. The results showed 79.60% activity at a dose of 10 mg/mL.

The experiment's results demonstrate the efficient use of sepia, a biomass product, to recover boron from wastewater. The adsorbent under investigation is recyclable, inexpensive, and simple to use. The extraction and characterization of bioactive substances from organic materials is gaining popularity due to their potential applications in a wide range of sectors, including culinary preservation and medicine. A possible source of bioactive chemicals with antioxidant qualities has recently been identified: the cuttlebone of the marine invertebrate *Sepia brevimana*. The results showed that the polymeric substance that was taken from the cuttlebone of *Sepia brevimana* is a powerful antioxidant. Antioxidant assays, such as the superoxide radical experiment and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging test, proved this. The isolated polymeric substance demonstrated concentration-dependent antioxidant activity, showing that it may effectively inhibit oxidation processes. We also looked at how the divided polymeric component acts as an antioxidant. The polymeric material was demonstrated to be capable of chelating transitional metals and scavenging free radicals, both of which have been shown to induce and exacerbate oxidative stress. Furthermore, the polymeric material has shown a remarkable ability to degrade, which boosts its antioxidant properties.

Conclusion

The findings of this study underscore the significant potential of chitosan as a natural antioxidant, which holds great importance for public health. Reactive oxygen species (ROS) and oxidative stress are major contributors to various chronic and degenerative diseases, including cardiovascular conditions, diabetes, neurodegenerative disorders, and cancer. By demonstrating chitosan's ability to scavenge harmful free radicals like DPPH, hydrogen peroxide, and superoxide anion, as well as its ferrous ion chelation properties, this research highlights its potential to mitigate oxidative damage in biological systems. The application of chitosan as a natural antioxidant in food systems can enhance food safety and extend shelf life by preventing lipid peroxidation and oxidative spoilage, which directly reduces health risks associated with consuming rancid or oxidized foods. Moreover, exploring the broader *in vivo* mechanisms and applications of chitosan could pave the way for novel preventive and therapeutic strategies to combat oxidative stress-related diseases, significantly improving public health outcomes. This study contributes to advancing sustainable and health-promoting solutions in both the food and healthcare industries.

Declaration of Interest

The authors declare no competing financial interests or personal relationships that could influence the work reported in this study.

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