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Extraction and Partial Characterization of Deacetylated Chitin from Cuttlefish *Sepia kobiensis* and their Free Radical Inhibition Efficacy

Aarthi Lakshmanan[1], Yagniyasree Manogaran[2,3], Pasiyappazham Ramasamy[2,3]*
[1]*Department of Physiology, Saveetha Dental College & Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, Tamil Nadu – 600077, India*
[2]*Polymer Research Laboratory (PR Lab), Centre for Marine and Aquatic Research (CMAR), Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, Tamil Nadu – 602105, India*
[3]*Department of Prosthodontics and Implantology, Saveetha Dental College & Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai, Tamil Nadu – 600077, India*

Abstract

This research aimed to extract and describe a functional polymeric compound from Sepia kobiensis cuttlebone and investigate its potential to impede the oxidation process. Cuttlebone waste is readily available and regularly discarded, making it an ideal resource for extracting useful bioactive compounds. The isolation technique created a pure polymeric substance by solvent extraction, precipitation, and purification. Several analytical methods, such as Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and field emission scanning electron microscopy (FESEM), were utilized to describe the isolated polymeric material. The study found that the separated material has a specific polymeric structure with functional groups linked to antioxidant action. Furthermore, the study evaluated the bioactivity of the isolated substance by measuring its ability to suppress oxidation processes in a model system. The material demonstrated high antioxidant activity by preventing reactive oxygen species (ROS) formation and scavenging free radicals. This action was attributed to the presence of antioxidant moieties within the structure as well as its polymeric nature. Finally, a bioactive polymeric substance with significant antioxidant properties was successfully isolated and characterized from the cuttlebone of Sepia kobiensis. The findings demonstrate how cuttlebone debris may be a valuable source of bioactive compounds and aid in the development of naturally occurring antioxidants for application in the culinary, cosmetic, and pharmaceutical industries. Further research is required to properly investigate its bioactivities and understand the underlying mechanisms of action.

Keywords: *Biopolymer, Deacetylated chitin, FESEM, Innovative, Superoxide radical, Sepia kobiensis.*

Introduction

Chitin is insoluble in most solvents due to its stiff structure. Thus, chitin undergoes chemical changes to improve its solubility. The most often utilized chitin derivative is deacetylated chitin, which is created via partial deacetylation [1]. Chitin is a popular functional material because it has many great qualities, such as being biocompatible, naturally breaking down, non-toxic, and able to adsorb things [2]. However, this bio-functional polymer has limitations in terms of processing capacity due to its lack of solubility in most natural solvents [3]. Deacetylated chitin is a naturally occurring polymer that is food-safe, non-antigenic, and

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****Corresponding Author: ramkanth281@gmail.com***

biodegradable. Manufactured from chitin, it offers a multitude of health benefits, including significant antibacterial and antioxidant properties [4, 5]. Deacetylated chitin has several biological uses. Multiple investigations have established its powerful antioxidant and antibacterial characteristics, potentially making it an antimicrobial agent alone or in combination with alternative polymers [6].

Beta1-4 linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), randomly distributed across the polymer, made deacetylated chitin. Deacetylated chitin is a naturally occurring positively charged polymer, unlike the bulk of complex sugars, which are neutral or charge anionically. Because of this property of deacetylated chitin, multilayer structures can be made, and it can interact electrostatically with both man-made polymers and naturally occurring negatively charged substances [7, 8]. Deacetylated chitin is the second most prevalent polysaccharide behind cellulose. Deacetylated chitin, a byproduct of deacetylated chitin, is a naturally occurring carbohydrate. Many industries, including food and medicine, use deacetylated chitin. Its low toxicity, excellent biocompatibility, and antimicrobial properties are among its advantages. Furthermore, deacetylated chitin dissolves efficiently in several organic and inorganic acids, including hydrochloric and acetic acids, whereas cellulose is only somewhat soluble in them [3].

Researchers have discovered several biological properties of deacetylated chitin, such as immunoactivity [7], anticancer activity, antioxidant capacity [9], and antimicrobial properties [10]. It has a wide range of uses, including the treatment of wastewater, the biological sciences, and the culinary, medical, and agricultural sectors. The molecular weight and degree of deacetylation of deacetylated chitin significantly influence its biological actions [11]. Deacetylated chitin has a wide range of biological and medicinal uses, including medication delivery [12], water filtration [13], and tissue engineering scaffolding [14]. Because of its distinct biological properties, deacetylated chitin has received a lot of interest in recent decades. The purpose of this study is to provide up-to-date information on the use of deacetylated chitin's natural characteristics and derivatives in medical and therapeutic applications.

Antioxidants are substances that either prevent or delay the oxidation of substrates that cells can oxidize. Natural antioxidants have recently received increased attention due to their ability to prevent a variety of chronic ailments [15]. The most common way to obtain chitin is from the exteriors of crustaceans, which is the inner shell (cuttlebone) of fish that lack spines. *Sepia kobiensis* is another potentially abundant source of chitin and deacetylated chitin. In India, people abandon cuttlefish inner shells in vast quantities as rubbish, thereby polluting the environment and endangering human health. The goal of this study was to isolate and discover a bioactive polymeric substance from *Sepia kobiensis* cuttlebone that might have antioxidant and oxidation-inhibitory effects.

Materials and Methods

Materials

Butylated Hydroxyanisole (BHA), EDTA, potassium ferricyanide, linoleic acid, Ascorbic acid, Butylated Hydroxyanisole (BHA), ferrozine, and $KmnO_4$ were purchased from Sigma Chemical Co. $FeCl_2$ and H_2O_2 were purchased from Merck Co. Analytical grade chemicals were also used.

Extraction of Chitin and Deacetylated Chitin

Rahul Varma's calcified and separated protein approach was used to recover chitin from the cuttlebone of *Sepia kobiensis* [16]. The mass of the dried chitin sample was then measured, which allowed the chitin content to be calculated. Maintaining a neutral pH is crucial for obtaining a pure sample. Chitin can

undergo a chemical reaction to produce deacetylated chitin. The chemical process is the most often used method for producing deacetylated chitin. The Islem Younes and Marguerite Rinaudo method of chemical deacetylation transformed the resultant chitin into deacetylated chitin [17].

Structural and Thermal Characterization

Fourier Transform-Infrared (FT-IR) Spectral Analysis of Deacetylated chitin

A BRUKERS ALPHA II FTIR A spectrum analyzer was used to evaluate deacetylated chitin that was isolated from *Sepia kobiensis*. A spectrum analyzer was used to test the deacetylated chitin extracted from *Sepia kobiensis*.

Field Emission Scanning Electron Microscopy (FESEM)

The surface properties and microstructure of deacetylated chitin were investigated using FESEM. Using the Hitachi Hus-4 vacuum vaporizer, a small amount of gold/palladium (40/60) was applied to the specimen, and the alloy evaporated swiftly at 20 V. The research was carried out at various magnification levels using an increased potential of 0.5 and 30 kV.

X-ray Diffraction (XRD)

The Shimadzu XRD-6000 device measured XRD intensity based on sample orientation and angle of diffraction (2θ). The diffraction patterns were used to identify the dimension and location of the crystallites, and the samples' crystal structures were identified following a thorough examination of their structural attributes.

In Vitro Antioxidant Assays

Scavenging Ability on 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH)

Using the Shimada et al. [18] and Ramasamy et al. [1] method, the scavenging activity of DPPH radicals was investigated. The preparation of the samples involved a range of concentrations, from 0.1 to 10 mg/ml. After dissolving in 4 mL of a 2g/l acetic acid solution, it is combined. One millilitre of DPPH radical-containing methanol solution was added to these samples once more. As a result, 10 mM/l of DPPH is the final concentration. The mixture was well shaken and then allowed to sit in the dim light for half an hour. A UV/vis spectrophotometer was used to measure the absorbance at 517 nm about a blank. BHA and ascorbic acid are used as controls.

The scavenging ability was estimated utilizing the formula below:

$$\text{Efficiency for scavenging (\%)} = \frac{(\Delta \text{A517 of control} - \Delta \text{A517 of sample})}{\Delta \text{A517 of control}} \times 100.$$

Superoxide Radical Scavenging Assay

The superoxide scavenging activity was measured by applying the Nishikimi et al. [19] and Ramasamy et al. [1] techniques. The sample was combined at concentrations ranging from 0.005-0.4 mg/ml with 0.1 M of phosphate buffer at pH 7.4, PMS (30 mM), NBT (72 mM), and NADH (338 mM) in the chemical reaction mixture. The mixture was allowed to incubate at room temperature for five minutes. The sample's absorbance was then measured at 560 nm using a UV/vis spectrophotometer against a blank. The blank symbolizes the negative control.

Using the following formula, the superoxide radical scavenging capacity was determined.

$$\text{Scavenging ability (\%)} = \frac{(\Delta A560\ of\ control - \Delta 560\ of\ sample)}{\Delta A560\ of\ control} \times 100.$$

Chelating Ability of Ferrous Ions

The ferrous ion binding capacity was calculated using Dinis et al.'s methodology [20] and Ramasamy et al [1]. When ferrocene and ferrous ions combine, a crimson hue is formed. The complex formation is hampered in the presence of chelating chemicals, which reduces the development of red colour. The colour reduction measurement is used to determine the chelator's binding capacity. One millilitre of

acetic acid, at levels that varied from 0.1 to 10 mg/ml, was applied to each sample. Subsequently, it was combined with 0.1 ml of 2 mM/l ferrous chloride and 3.7 ml of methanol. To the process, 0.2 ml of 5 mM/l ferrozine was added. A UV/vis spectrophotometer was used to determine the mixture's absorbance at 562 nm relative to a blank. Lower absorption is correlated with higher chelating power. Making use of EDTA as a measure of control.

Results

Yield of Chitin and deacetylated chitin

The findings of this investigation revealed that the yields of chitin and deacetylated chitin were 36.65% and 35.38%, respectively.

FT-IR Analysis

Figure 1 shows that the bands had an intensity of around 3365 cm^{-1}. Because of the polysaccharide's hydroxyl-stretched vibration, very broad bands were expected. When hydroxyl chains exist, a noticeable peak between 3500 and 1000 cm^{-1} indicates few hydrogen connections. The weak absorption bands were caused by stretched C-H vibrations in the CH_2 groups. The absorptions at 1627-1565 cm^{-1} were induced by stretching stresses of the CHO and C=O bonds. The strong absorption bands ranged from 3365 to 1411 cm^{-1}, indicating the C-H bond's deforming oscillations. The C-O and C-C link band sites are denoted by the polysaccharide absorbance range of 1082-656 cm^{-1}.

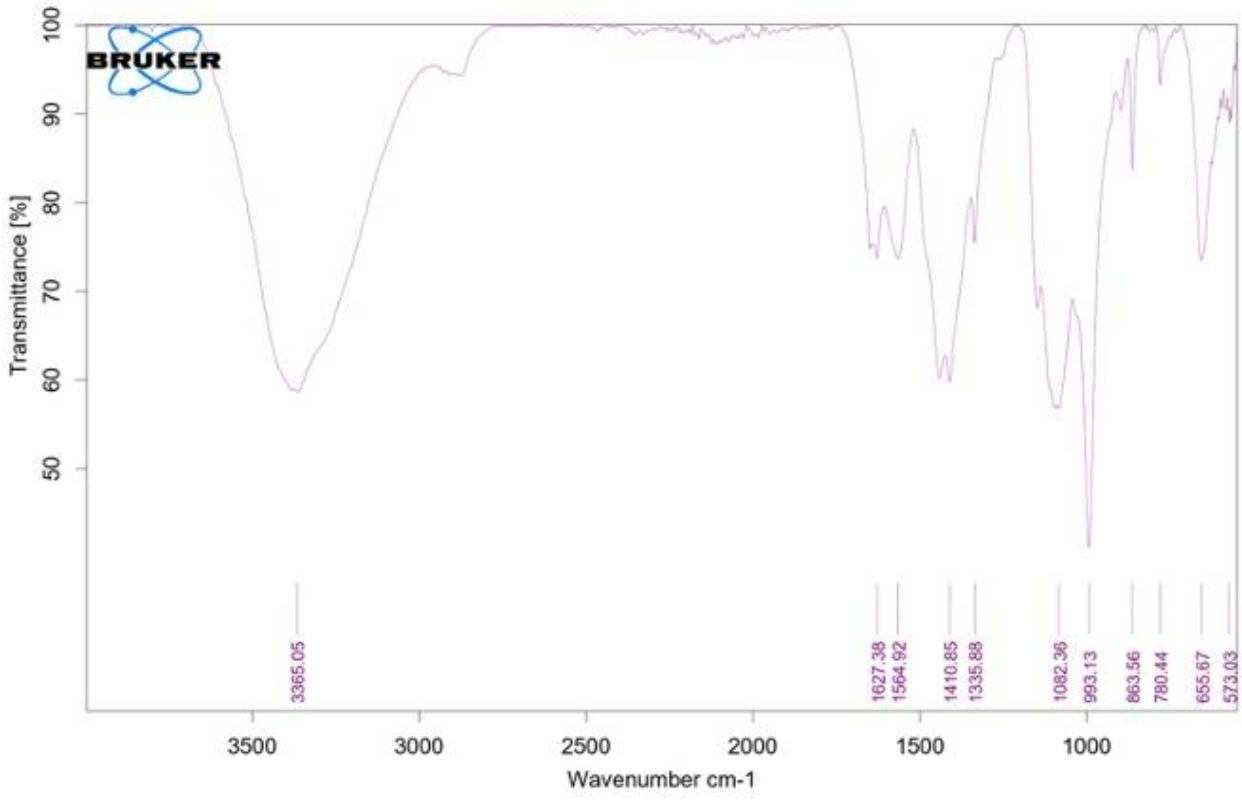

Fig. 1. FT-IR Spectral Analysis of *Sepia kobiensis* Deacetylated Chitin

FESEM Analysis

Field Emission Scanning electron microscope pictures (Figs. 2A, 2B) depict the deacetylated chitin's surface form. It also showed a smooth, non-porous membranous phase with crystallites, dome-shaped openings, and microfibrils, which are small, topologically added, dome-shaped hollows. Microfibers conform to the surface's structure and hardness.

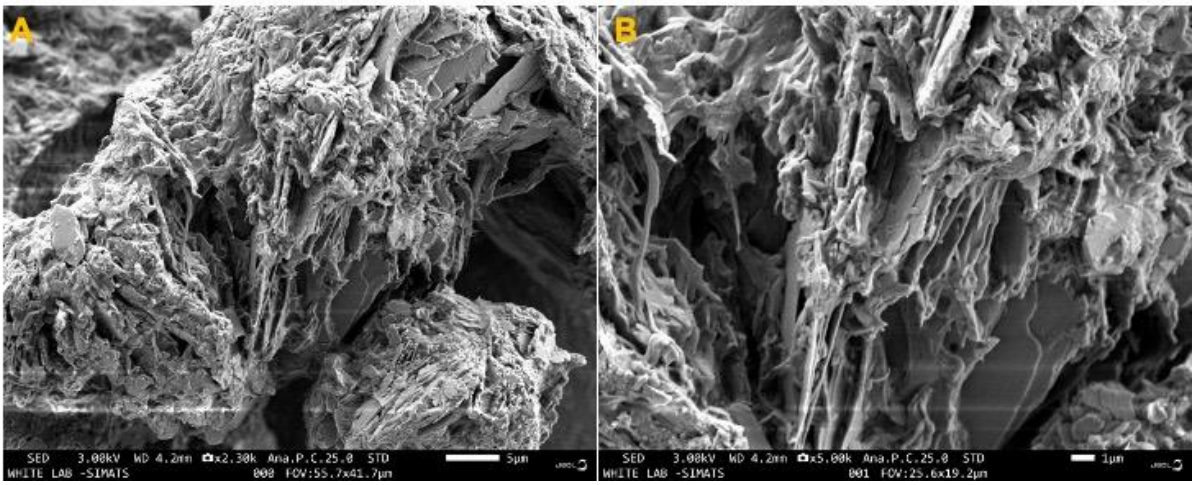

Fig. 2. FESEM images of *Sepia kobiensis* Deacetylated Chitin

XRD Analysis

Deacetylated chitin is crystal-rich in structure. Crystallized areas contribute to mechanical characteristics and stability. XRD analysis of deacetylated chitin revealed two broad and strong peaks at $2\theta = 10°$ and $2\theta = 40°$, along with weak peaks at 11°, 17°, 19°, 23°, 30°, 32°, 33°, and 37° (Fig. 3).

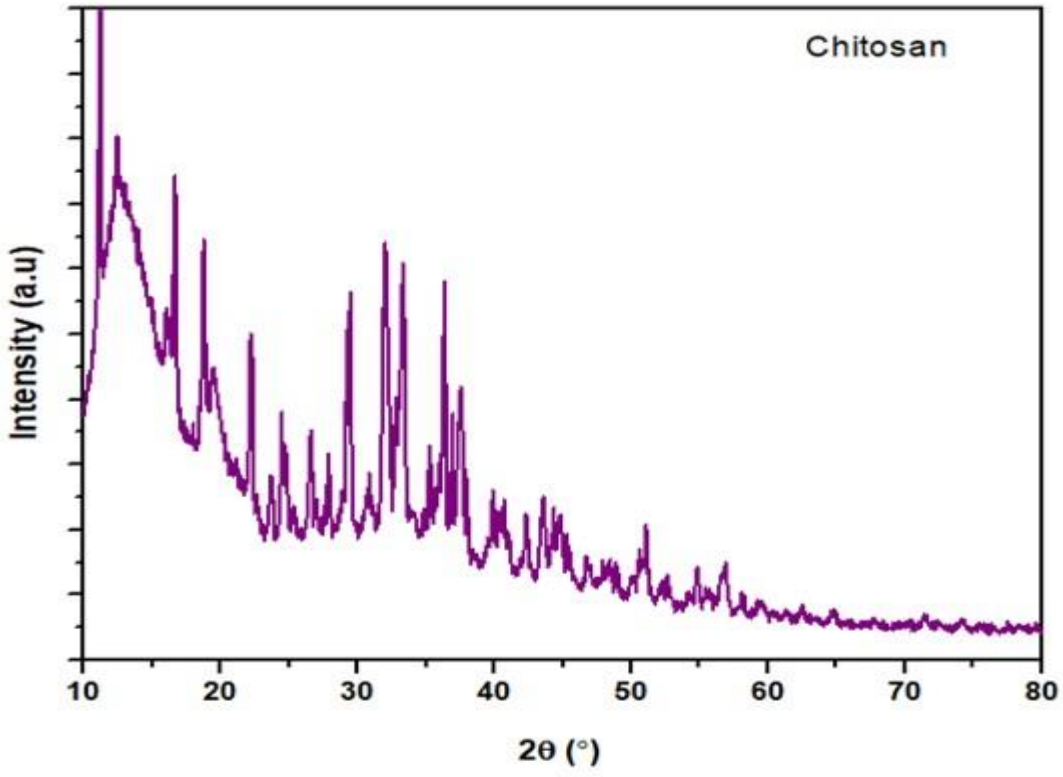

Fig. 3. XRD Spectrum of *Sepia kobiensis* Deacetylated Chitin.

In vitro Antioxidant Activity of Deacetylated Chitin

Scavenging Activity on DPPH

The DPPH assay is used to determine how well deacetylated chitin scavenges unstable radicals. The decrease in stable DPPH radicals is responsible for the yellow colour in this technique. Antioxidants were designed to donate hydrogen, which is how non-radical DPPH-H was generated. Hydrogen radical scavenging is the main process of antioxidation,

and DPPH uniquely absorbs hydrogen free radicals at 517 nm. Furthermore, studies have demonstrated that DPPH can identify the proton-scavenging activity of deacetylated chitin. In this study, deacetylated chitin made from *Sepia kobiensis* could remove toxins of 9.08 to 58.17% at concentrations ranging from 0.1 to 10 mg/ml. At concentrations of 0.1–10 mg/mL, ascorbic acid demonstrated scavenging activity ranging from 18.36% to 78.68% (Fig. 4A).

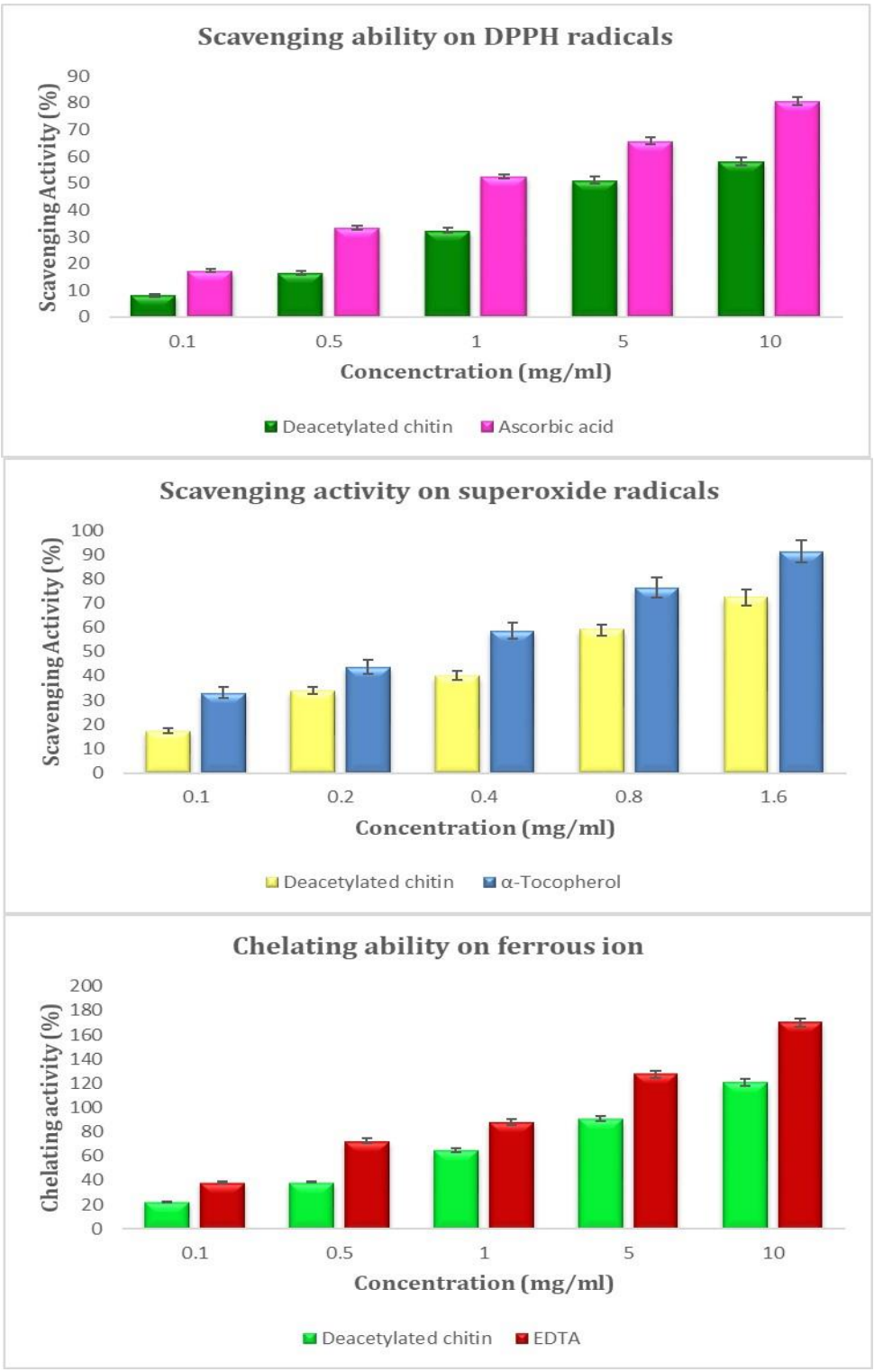

Fig. 4. A. Scavenging Ability of DPPH Radicals, B. Scavenging Ability of Superoxide Radical, C. Chelating Ability on Ferrous Ions

Superoxide Radical Scavenging Activity

Researchers have demonstrated the efficiency of deacetylated chitin levels between 0.1 and 1.6 mg/mL in scavenging superoxide radicals at concentrations ranging from 18.38 to 74.19%. At concentrations ranging from 0.1 to 1.6 mg/ml, alpha-tocopherol showed a scavenging ability of 32.11 to 89.46%. In comparison to the results, deacetylated chitin outperformed alpha-tocopherol. Furthermore, studies have shown that alpha-tocopherol from Sepia kobiensis outperforms deacetylated chitin in terms of scavenging ability (Fig. 4B).

Chelating Activity on Ferrous Ions

The research found that deacetylated chitin from Sepia kobiensis could bind to Fe^{2+}ions with a range of strengths, from 22.20 to 120.60% at concentrations of 0.1 to 10 mg/mL. At 0.1–10 mg/ml, EDTA demonstrated a chelating capability ranging from 40.82 to 170.32%. In comparison to deacetylated chitin and EDTA, EDTA has a stronger chelating impact. This demonstrated EDTA's excellent capacity to bind Fe^{2+} ions (Fig. 4C).

Discussion

The exterior bones of fungi, insects, and crustaceans are primarily home to chitin, the next naturally occurring biopolymer. Hydrolysis in alkaline solutions commonly produces deacetylated chitin. Chitin synthesis varies between species, as evidenced by recorded yields of 20% in *Sepia officinalis* cuttlebone [21] and 36.06%, 36.55%, and 22.18% in *Loligo lessoniana*, *Loligo formosana*, and *Penaeus monodon*, respectively [22]. In this investigation, we extracted 36.65% of the chitin from the *Sepia kobiensis* cuttlebone. It's worth noting that the substance known as chitin is hydrophobic, which limits its applicability. Deacetylated chitin is a well-known chitin derivative that researchers have created to boost chitin's solubility and widen its uses. *Nerita crepidularia* operculum and shell made 35.43% of deacetylated chitin, while *Doryteuthis sibogae* gladius's shell and operculum made 33.02% [23]. Deacetylated chitin, a deacetylated version of chitin, acts as the starting point for additional alterations. However, the production of deacetylated chitin was higher than that of *Donax scortum*. *S. pharaonis* [24]. This study determined that 35.38% of the deacetylated chitin recovered from the cuttlebone of *Sepia kobiensis*.

Deacetylated chitin's FTIR images frequently contain distinctive peaks related to its functional groups. For instance, a peak at 3400–3500 cm^{-1}, indicating the vibratory stretching of N-H bonds, identifies an amino group (-NH_2). In deacetyl, the amide group's (CONH) carbonyl stretching vibration (C=O) shows up as a thin band in deacetylated chitin, with a centre point between 1650 and 1655 cm-1 [25]. The deacetylated chitin polymer matrix regularly displays stretching vibrations of the C-O-C glycosidic links at distances ranging from 1050 to 1150 $cm^{-1.}$ Furthermore, the 890-1150 cm^{-1} range reflects the C-O and C-N bond bending vibrations as peaks, providing further structural insights into deacetylated chitin [26]. The measurement of deacetylated chitin oscillatory shifts between 656 and 3365 cm^{-1} indicates the presence of deacetylated chitin. We detected Raman peaks at 1411 cm^{-1} in deacetylated chitin.

One can use deacetylated chitin to create a variety of products, such as films, tiny spheres, and nanoparticles. FESEM was utilized to investigate the distribution, dimensions, and form of the particles within these frameworks. This data is required to improve production operations and adapt deacetylated chitin substances for the desired applications. FESEM imaging may be used to compare various deacetylated chitin compositions, methods of processing, and changes. By visually analyzing changes in surface structure and shape, scientists may enhance present compositions or determine which deacetylated chitin materials perform best for a particular use [27]. The

deacetylated chitin FESEM images show an intriguing substructure consisting of a network of intertwined fibres or particles. It appears to have a smooth outer layer with holes and faults. The deacetylated chitin fibres and particles had a wide range of diameters, indicating possible changes in the manufacturing procedure or the presence of aggregates. Past findings are difficult to compare because the samples were collected from diverse sources and regions and treated using a variety of SEM imaging methodologies [27].

Deacetylated chitin's XRD pattern frequently shows peak values corresponding to its crystallographic areas. The peaks' positions and intensities offered information regarding the deacetylated chitin framework's molecular makeup and organization. Scientists observed that our deacetylated chitin test outcomes resembled those of Rasti et al. Mollusc chitin, the source of deacetylated chitin, displayed a strong, clear reflection between 30 and 35° [28].

Many materials, including deacetylated chitin, can undergo the DPPH test to evaluate their antioxidant activity. Several studies have looked into how deacetylated chitin and its derivatives can remove DPPH, which suggests that they may be antioxidants. Deacetylated chitin may capture DPPH radicals because of its amino groups, which provide electrons for neutralizing unstable radicals. According to our investigation, *Sepia prashadi* has a scavenging activity of 62.17%. At 10 mg/ml, deacetylated chitin from *S. lessoniana* had a scavenging ability of 55.48% [29]. When diluted to 10 mg/ml, crab-deacetylated chitin C60 scavenged 28.4% of DPPH radicals [30]. The current study demonstrated a scavenging ability of 58.17% at 10 mg/ml.

Nature produces superoxide radicals and other ROS in metabolic processes in cells. Environmental stress has linked them to a variety of disorders, such as cancer, coronary artery disease, and cognitive disorders. Superoxide radical scavenging tests assess a chemical's antioxidant activity by determining its capacity to neutralize or scavenge damaging radicals. Every deacetylated chitin concentration test showed a significant capacity to remove superoxide radicals. Two different amounts of α-tocopherol and *Sepia prashadi* deacetylated chitin were tested and found to be 42.17%, 61.92%, and 76.19% effective at getting rid of superoxide radicals [31]. This study discovered that scavenging superoxide radicals at 1.6 mg/mL reduced them by 74.19%.

The chemistry of deacetylated chitin is noteworthy due to its propensity to bind substances. Various fields such as manufacturing, agriculture, medicine, and environmental studies utilize this skill. Deacetylated chitin polysaccharide, derived from chitin, has excellent chelating capabilities due to its amino groups' ability to bind to metal ions. This property makes deacetylated chitin valuable in a variety of applications. Deacetylated chitin from *Sepia prashadi* showed 27.59% ferrous ion chelation activity at a concentration of 10 mg/ml. At 10 mg/ml, EDTA showed a strong binding activity of 74.92%. Fungal deacetylated chitin exhibits its greatest chelating strength at 1 mg/mL [32]. At a dosage of 10 mg/ml, the findings revealed 170.32% activity. The experiment's results suggest that we can effectively utilize *Sepia,* a biomass outcome, to retrieve boron from affluence. Researchers are studying an adsorbent that is recyclable, inexpensive, and easy to use. The extraction and characterization of bioactive chemicals from organic materials are gaining popularity due to their potential use in a range of disciplines, including food preservation and healthcare. Recently, researchers recognized the cuttlebone of the marine invertebrate *Sepia kobiensis* as a potential source of bioactive compounds with antioxidant properties.

The data revealed that the polymeric material extracted from *Sepia kobiensis* cuttlebone has high antioxidant properties. Antioxidant tests, such as the superoxide

radical experiment and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging test, established this. The isolated polymeric material displayed concentration-dependent antioxidant activity, indicating that it can effectively halt oxidation processes. We also investigated how the separated polymeric component functions as an antioxidant. The polymeric substance was shown to be capable of chelating transitional metals and scavenging free radicals, both of which are known to trigger and increase oxidative stress. Furthermore, the polymeric substance has demonstrated a remarkable ability to diminish, which enhances its antioxidant properties.

Conclusion

Overall, taking out and studying the bioactive polymeric material from Sepia kobiensis' cuttlebone showed that it can control oxidation processes by being an antioxidant. These findings demonstrate the potential of this natural molecule for a wide range of uses, including the creation of nutraceuticals, functional foods, and antioxidant-based medicines. To optimize the output and biological activity of the resulting polymeric material, additional research into the link between structure and function is required, as well as improvements to the separation and purification procedures.

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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