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Research Article

CHARACTERIZATION OF BIOPOLYMER "CHITOSAN" FROM THE SHELL OF DONACID CLAM *DONAX SCORTUM* (LINNAEUS, 1758) AND ITS ANTIOXIDANT ACTIVITY

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ABSTRACT

To find out a novel source for natural antioxidant 'chitosan', from the shell of donacid clam *Donax scortum* which was characterised by UV-Vis spectroscopy, FT-IR spectroscopy, Potentiometric titration, Viscometry and DSC. Chitosan was evaluated for antioxidant efficacy through various antioxidant assays such as conjugated-diene method, DPPH radical scavenging assay, reducing power and metal ion chelating assay. This study has not only brought out the importance of shell for its utilization into a potential source for obtaining another natural antioxidant that may again form the basis for the source of active principle of a drug or the drug itself in future.

Keywords: *Donax scortum*, Chitin, Chitosan, FT-IR, DSC, Antioxidants, DPPH.

INTRODUCTION

Chitin is the universal component of adult mollusk shells as shown for a variety of species[1,2]. Chitin is a polysaccharide, composed of mainly repeating unit of β-(1 4) linked N-acetyl-D glucosamine obtained from the mollusc, crustaceans (crab, shrimp and crayfish), exoskeletons of insects, cell walls of fungi and some alga and microorganisms. It is found to be the second largest polymer in the world after cellulose[3-5]. The deacetylated derivative or chitosan, a copolymer of (1-4)-2-acetamido-2-deoxy- β- D-glucan and (1-4)-2-amino-2-deoxy- β- D-glucan, has been receiving great attention as novel functional material for its excellent biological properties such as biodegradation, immunological, antioxidant and antibacterial activities. *Donax scortum* is a bivalve mollusc, commonly called 'leather donax', which is known to be fished and marketed in India. Reactive Oxygen Species (ROS), including superoxide anion radicals, hydroxyl radicals and hydrogen peroxide etc., are often generated by oxidation product of biological reactions or exogenous factors[6]. ROS can readily react with most biomolecules including lipids, proteins, amines, lipoproteins, carbohydrates and DNA[7]. Excessive generation of ROS, induced by various stimulating factors such as certain pollutants and tobacco smoke, which exceed the antioxidant capacity of the organism, will lead to aging, cancer, and many other diseases[8]. Various exogenous antioxidants play an important role in the elimination of ROS and protect the cells against toxic effects of ROS[9]. However, the use of synthetic antioxidants is under strict regulation due to the potential health hazards caused by such compounds[10]. Hence, it has been given more importance for the study of natural antioxidant because it is safe and non-toxic to offer protection from free radicals, thus retarding the progress of numerous chronic diseases in humans[11]. However, natural antioxidants are not limited to terrestrial sources. In the search of new antioxidants, exploration of aquatic habitats has led to the discovery that marine plants and invertebrates also contain antioxidants. Marine plants and invertebrates characteristically contain sulfated polysaccharides which are not found in land plants and which may have specific functions in ionic regulation. The objective of this study was to assess the antioxidant properties of chitosan from *D. scortum* shell.

MATERIALS AND METHODS

Chemicals

Ascorbic acid, butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), ferrozine, linoleic acid, potassium ferricyanide and potassium permanganate were purchased from Sigma Chemical Co. (St. Louis, MO). Ferrous chloride and H_2O_2 were obtained from Merck Co. (Darmstadt, Germany). Other chemicals used were of analytical grade.

Animal Collection

D. scortum species was collected from Porto Novo coastal region (11.49°N 79.76°E); east coast of India. The shell obtained after the removal of mantle tissue was washed, air-dried and pulverised using pestle and mortar.

Extraction of chitin and chitosan

Chitin was extracted from the shells of a bivalve, *D. scortum* by demineralization and deproteinization. The pulverised shell was treated with 2N HCl for 24 hours to remove the mineral content and then treated with 1N NaOH at 80˚Cfor 24 hours to remove protein[12]. The extracted chitin was converted into chitosan through deacetylation process[13].Chitin was deacetylated in 40% aqueous NaOH by heating under reflux for 6 hours at 110˚C and was cooled at room temperature. The obtained precipitate was washed with distilled water and stood for 12 hours at room temperature with constant stirring in 10% acetic acid solution. The pH was adjusted to 10 with 40% NaOH solution. The solution was dialyzed against deionized water for 24 hours. The product was centrifuged at 10,000 rpm for ten minutes and lyophilized to give chitosan.

UV-*vis* spectroscopy

0.1M acetic acid solution of chitosan was used to scan at the wavelength ranging from 200 to 600 nm in a UV-*Vis* Spectrophotometer.

Fourier Transform – Infra Red (FT-IR) Spectral Analysis

FT-IR spectroscopy of solid sample of chitin and chitosan from *D. scortum* shell was relied on an AVATAR 330 Spectrometer. Sample (10µg) was mixed with 100µg of dried Potassium Bromide (KBr) and compressed to prepare a salt discs (10mm diameter) for reading the spectrum.

Determination of the deacetylation degree (DDA) by potentiometric titration

250 mg of chitosan was dissolved in 10 ml of 0.3 M HCl and after being diluted to 50ml with Millipore water, it was titrated with 0.1 M NaOH. The consumed volume of NaOH solution, which corresponds to the amount of amine groups in chitosan, is obtained from the difference between two inflection points of acid-base titration[14].

Molecular weight determination

The viscosity average molecular weight of chitosan was calculated using Mark-Houwink equation, $[\eta] = K (M_v)^{\alpha}$ Where, [η] is the intrinsic viscosity of the depolymerised chitosan, K and α are constants for given solute system and temperature; $K = 1.38 \times 10^{-5}$ and $\alpha = 0.85$[15].

Differential Scanning Calorimetry (DSC) of chitosan

Thermograms were obtained using a Shimadzu **(**TA instruments Q 20-DSC). Samples were lyophilized; 2.0 mg of lyophilized chitosan powder was crimped in a standard aluminium pan and heated from 20 to 200°C at a heating constant rate of 10°C/min under constant purging of nitrogen at 20 ml/min.

***In vitro* Antioxidant Activity of chitosan**

Antioxidant activity by the conjugated diene method

The antioxidant activity was determined by the conjugated diene method[16]. Chitosan sample (0.1–10 mg/ml) in 2 g/l acetic acid solution was mixed with 2ml of 10 mmol/l linoleic acid emulsions in 200 mmol/l sodium phosphate buffer (pH 6.5) in test tubes and placed in darkness at 37°C to accelerate oxidation. After incubation for 15 h, 6ml of 600 g/l methanol in deionized water was added and the absorbance of the mixture was measured at 234nm against a blank in a UV-*Vis* spectrophotometer. A control consisted of methanol and the reagent solution without chitosan. Ascorbic acid and butylated hydroxyanisole (BHA) were used for comparison.

Scavenging ability on 1, 1-diphenyl-2-picrylhydrazyl radicals

The Scavenging ability of DPPH radicals was determined according to the method of Shimada[17]. Chitosan sample (0.1–10 mg/ml) in 2 g/l acetic acid solution was mixed with 1ml of methanolic solution containing DPPH radicals, resulting in a final concentration of 10mmol/l DPPH. The mixture was shaken vigorously and left to stand for 30 minutes in the dark and the absorbance was then measured at 517nm against a blank. Ascorbic acid and BHA were used for comparison.

Reducing power

The reducing power was determined according to the method of Oyaizu[18]. Each chitosan sample (0.1–10 mg/ml) in 2 g/l acetic acid solution (2.5 ml) was mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 10 g/l potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. Thereafter 2.5 ml of 100 g/l trichloroacetic acid was added and the mixture was centrifuged at 200 rpm for 10 min. The upper layer (5 ml) was mixed with 5ml of deionized water and 1ml of 1 g/l ferric chloride and the absorbance was measured at 700nm against a blank. A higher absorbance indicates a higher reducing power. Ascorbic acid and BHA were used for comparison.

Chelating ability on ferrous ions

The ferrous ion-chelating potential of chitosan was investigated according to the method of Decker and Welch[19], wherein the Fe^{2+}-chelating ability of sulfated chitosan was monitored by measuring the ferrous iron–ferrozine complex at 562 nm. The reaction mixture containing sulfated chitosan of different concentrations, $FeCl_2$ (2 mM), and ferrozine (5 mM) was adjusted to a total volume of 0.8 ml with water, shaken well and incubated for 10 minutes at room temperature. The absorbance of the mixture was measured at 562 nm against blank. EDTA was used as a positive control.

Statistical Analysis

The data were subjected to ONE-WAY ANOVA followed by Duncan's multiple range tests using statistics software package (SPSS, ver. 16) and the mean value of six replicates is presented.

RESULTS AND DISCUSSION

The present study represents the first attempt to investigate various physiochemical properties of chitosan extracted from *D. scortum* shell. The yield of chitin and chitosan is varying in different animals of the same group or different groups. In the present study the yield of chitin and chitosan from the *D. scortum* shell was found as 11.96% and 18.8% respectively. Whereas the carapace of *Penaeus monodon*[20] and *Metapenaeus stebbingi* [21] reported 14.6% and 17.5% of chitosan, which is lower than that of the present study. However, the yield of chitosan was reported so high (57.14%) in the squid *Sepioteuthis lessoniana*[22]. Palpandi *et al* isolated chitin from shell and operculum of gastropod *Nerita crepidularia* was found to be 23.91 and 35.43% respectively[23]. The yield of chitin from *D. scortum* was low when compared to that of other molluscs and crustaceans, which may be due to the high calcium carbonate content of the shell.

UV-*vis* and FT-IR spectroscopy

Chitosan was analyzed by UV-Vis spectroscopy and the results are shown in Fig. 1. The absorbance was obtained at 249 nm. FT-IR spectrum was used to determine the structure of chitin and chitosan. The infrared spectrum of chitin and chitosan were depicted in Fig 2 & 3. Chitin showed the absorbance bands of 3419.67 cm^{-1}, 2922.87 cm^{-1}, 1747.95 cm^{-1}, 1642.04 cm^{-1}, 1471.87 cm^{-1}, 1128.77 cm^{-1} and 858.98 cm^{-1} indicating the symmetric CH_3 & asymmetric CH_2 stretching, C–H stretching, amide I band, CH_2 bending & CH_3 deformation (amide II band), amide III band, CH_2 wagging & stretching and ring skeletal vibrations respectively. The spectrum of chitosan showed the absorbance bands at 3431.29 cm^{-1}, 2923.60 cm^{1}, 2849.32 cm^{-1}, 1789.69 cm^{-1}, 1633.44 cm^{-1}, 1475.50 cm^{-1}, 1125.37 cm^{-1} and 608.22 cm^{-1}. The region between 3000 cm^{-1} and 3500 cm^{-1} shows the stretching of OH groups. This band is broad because of the hydrogen bonds. The OH band overlaps the stretching band of NH. Another significant change is observed in the region from 1000 cm^{-1} to 1200cm^{-1}. In this region chitosan presents a broad band centered at 1084.93 cm^{-1} associated with the stretching of C=O. According to the study of Li *et al.* the peak at 1415 cm^{-1} indicates the C–H bending vibrations of –CH2. The C–O stretching of structure is observed at 1075 cm^{-1} according to the literature[24]. The broad band around 3300 is usually attributed to intermolecular –H bands. The out plane bending –OH vibrations are observed at 608 cm^{-1}. Strong amide II bands could not be observed, which is due to the high deacetylation degree of the produced chitosan. The studies in the literature about FTIR spectroscopy related with chitosan showed some characteristic peaks, which are at 2940 cm^{-1} (–CH3, –CH2), 1655 cm^{-1} (C=O stretch vibration of secondary amide I band), 1555 cm^{-1} (N–H bending vibration of amide II band), 1570 cm^{-1} (N–H bending vibration of primary amides) and 1070 cm^{-1} (C–O stretching)[25].

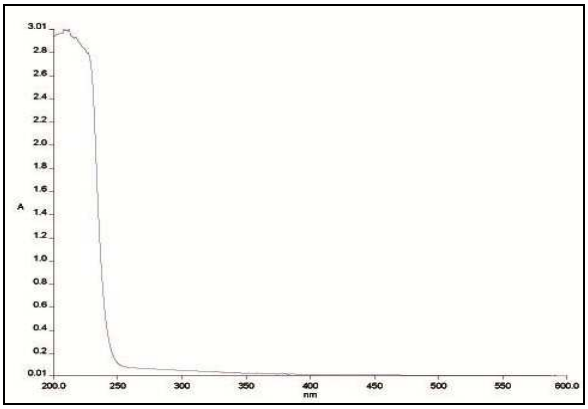

Fig. 1: UV- spectrum of *D. scortum* chitosan.

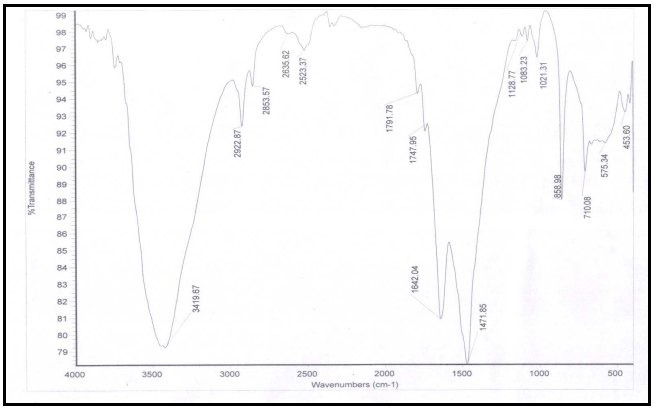

Fig. 2: FT-IR spectrum of chitin from *D. scortum* shell

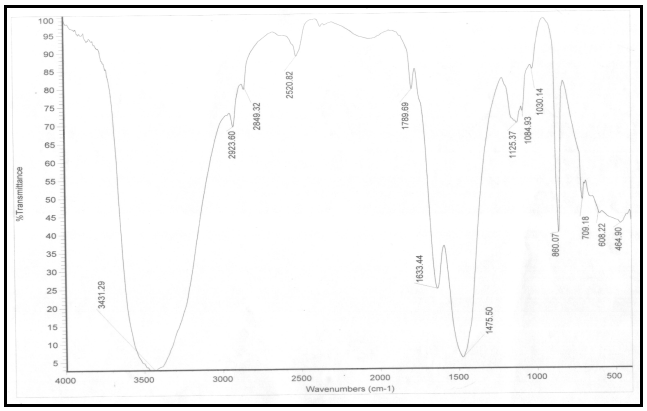

Fig. 3: FT-IR spectrum of chitosan from *D. scortum* shell

Table 1: Showing wave number, possible assignment of absorption band and their nature of peaks of chitin from *D. scortum* shell.

S. No	Wave number (cm^{-1})	Possible Assignment of absorption band	Nature of Peaks
1	3419.67	H-bonded OH stretching	Broad
2	2922.87	Aliphatic CH stretching	Sharp
3	2853.57	Aliphatic CH stretching	Sharp
4	1642.04	Amide C=O stretching	Very sharp
5	1471.85	NH bending	Sharp
6	1128.77-1021.31	C-O-C stretching	Medium
7	858.98-575.34	Ring skeletal vibrations	Medium

Table 2: Showing wave number, possible assignment of absorption band and their nature of peaks of chitosan from *D. scortum* shell.

S. No	Wave number (cm^{-1})	Possible Assignment of absorption band	Nature of Peaks
1	3431.29	H-bonded NH_2 & OH stretching	Broad
2	2923.60	Aliphatic CH stretching	Sharp
3	2849.32	Aliphatic CH stretching	Shoulder
4	1633.44	Amide C=O stretching	Sharp
5	1475.50	NH bending	Sharp
6	1125.37	C-O-C stretching	Shoulder
7	1084.93	C-O-C stretching	Sharp
8	860.07-608.22	Pyranose bending vibrations	Medium

Degree of deacetylation and molecular weight of chitosan

The deacetylation degree, molecular weight and the order of its repetitive units are important parameters for chitosan, as they affect its properties[26]. Further, the deacetylation degree of chitosan is an important property for its use in food industry. In the present study, degree of deacetylation was determined as 74.35% by potentiometric titration that is based on the deprotonation (by NaOH) or protonation (by HCl) of amino group. The molecular weight of chitosan is on of the most important properties as it considerably affects the physiochemical and functional properties. The molecular weight of chitosan from *D. scortum* was calculated as 373.80 kDa. The average molecular weight of chitosan from crab shell was in the range of 483-526 kDa and decreased with the prolonged reaction time[27].

The variation in molecular weight is due to the difference in deacetylation degree and the source of chitosan. In addition, several factors in the production of chitosan, such as the high temperature, concentration of alkali, reaction time, previous treatment of the chitin, particle size, chitin concentration, dissolved oxygen concentration and shear stress may also influence the molecular weight of chitosan[28].

Generally, chitosan needed high thermal energy for dissociation of its structure, i.e., thermal decomposition[29]. Crab chitosan with varying degree of DDA such as 83.3 – 93.3 % showed a melting endothermic peak at 152.3-159.2°C[27]. But in the present study DSC of the bivalve chitosan (Fig. 4) showed an endothermic peak at 151.6°C.

***In vitro* antioxidant activity**

Recently antioxidant activity of chitosan and its derivatives has attracted the most attention due to their non-toxic nature and natural abundance. Antioxidant activity of chitosan and its derivatives mainly related to the content of active hydroxyl and amino groups in the polymer chains. And with the decrease of molecular weights, the antioxidant activity of chitosan and its derivatives will be enhanced due to the partly destroying of intermolecular and intramolecular hydrogen bonds[30].

Antioxidant activity by conjugated diene method

Linoleic acid, an unsaturated fatty acid is usually used as a model compound in lipid oxidation and antioxidation-related assays in which carbon-centered, peroxyl radicals and hydroperoxides, etc., are involved in the oxidation process. During the linoleic acid oxidation, peroxides are formed. These compounds oxidize Fe^{2+} to Fe^{3+}. The Fe^{3+} ions form a complex with SCN^-, which has a maximum absorbance at 500nm. Therefore, high absorbance indicates high linoleic acid oxidation[31]. While using the conjugated diene methods for chitosan derived from the *D. scortum* shell showed, consistent antioxidant activity with chitosan showed moderate to high antioxidant activities of 48.34% at 1mg/ml and 72.69% at 10mg/ml by the conjugated diene method (Fig. 4); whereas BHA and ascorbic acid reported 85.34% and 77.43% of antioxidant activity respectively at the highest concentration.

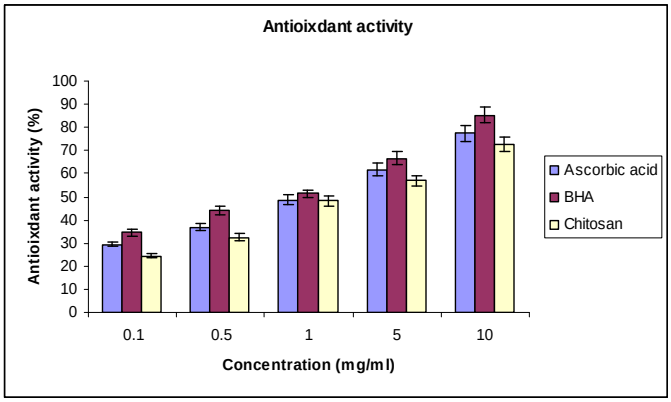

Fig.4: Antioxidant activity of chitosan.

Scavenging ability on 1, 1-diphenyl-2-picrylhydrazyl radicals

DPPH is one of the compounds that possessed a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers. Further, it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen-donating ability. Thus, scavenging of DPPH free radical was directly affected by the amount of attractable atoms in chitosan molecules. Yin observed that the chitosan derivatives having the same disaccharide and possessing a DS of 20–30% exhibited the highest radical-scavenging activity, followed by the derivatives with DS 40–50% and 60–70%[32]. In the present study, scavenging ability of chitosan on DPPH radicals was reported as 38.92% at 10 mg/ml (Fig.5).

The DPPH radical-scavenging effect increased as the concentration of the chitosan derivatives increased to a certain extent, and then levelled off even with further increase in the concentration.

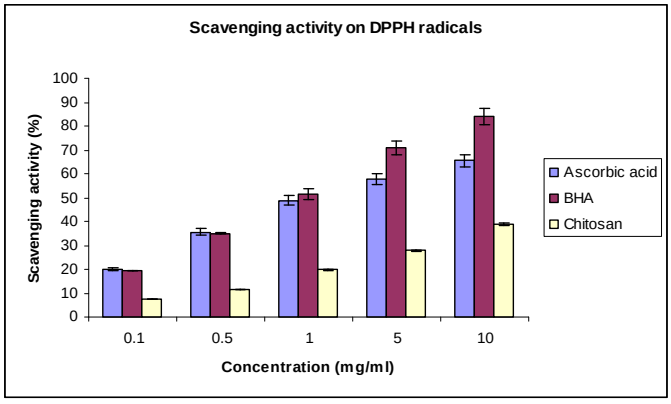

Fig. 5: Scavenging activity of DPPH radical by chitosan.

Reducing power

Reducing power assay has also been used to evaluate the ability of natural antioxidants to donate electrons. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[33]. The reducing ability of chitosan extracted from *D. scortum* shell was assessed based on the measurement of Fe^{3+}-Fe^{2+} transformation (Fig.6). At 1mg/ml, chitosan showed slight reducing power of 0.27%. The reducing power of chitosan was correlating well with increasing concentration but values remained lower than those for BHA and ascorbic acid (0.89% and 0.46% at 0.1 mg/ml respectively). Yen *et al* mentioned that fungal chitosans showed slight (0.13–0.29) at 1 mg/ml and moderate reducing power (0.42% - 0.57%) at 10 mg/ml[34].

It seems that chitosan from crab shells and shiitake stipes was not effective in its reducing power. The reducing power of chitosan also increased with increasing concentration. At different concentrations of 0.15 – 0.75 mg/ml, the reducing power was found ranging from 0.019 – 0.578%.

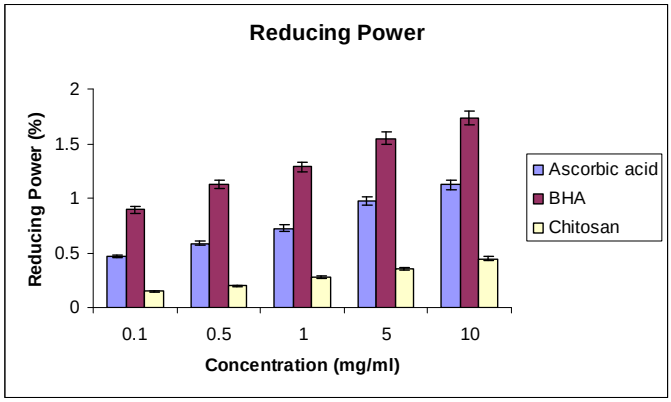

Fig.6: Reducing power of chitosan.

Chelating ability on ferrous ions

Iron chelating agents are thus expected to inhibit the metal-dependent oxidative processes and have potential in combating reactive oxygen species mediated diseases[35]. Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions[36]. Yen *et al* reported that at 1 mg/ml, chelating abilities of all crab chitosans on ferrous ions were in the range of 82.9–96.5%[37]. At 10 mg/ml, ferrous ions were all chelated.

The chelating ability of chitosan was seen concentration related. In the present study the chelating ability of chitosan on ferrous ions was in the range of 49.38-71.06% (1 mg/ml-10 mg/ml) (Fig.7). However, EDTA showed a high chelating ability of 85.22% at the concentration of 10mg/ml.

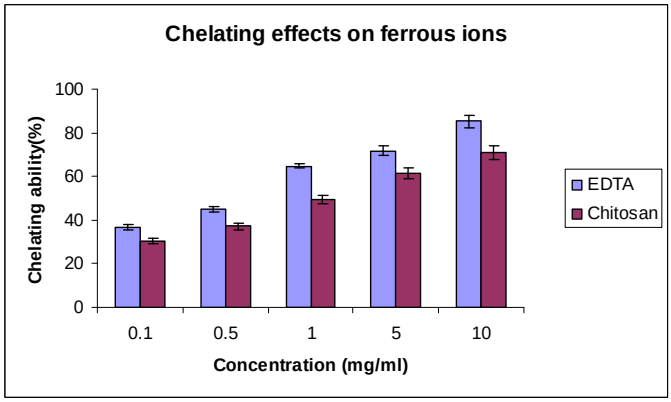

Fig. 7: Chelating effects of chitosan on ferrous ion.

CONCLUSION

This study investigated the extraction of chitin and preparation of its derivative, chitosan apart from studying the physicochemical characteristics and antioxidant property of chitosan from the shells of bivalve mollusk *D. scortum*. Based on the results it is suggested that there is good potential for the production of chitosan from the bivalve shell also and the chitosan shows good antioxidant activity. Based on the results obtained the bivalve chitosan with remarkable antioxidant activity could be used as an antioxidant in the food and pharmaceutical industry.

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