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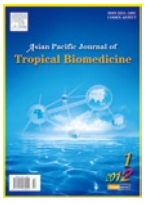

Document heading

Extraction, characterization and *in vitro* antioxidative potential of chitosan and sulfated chitosan from Cuttlebone of *Sepia aculeata* Orbigny, 1848

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ABSTRACT

Objective: To investigate antioxidant potency of chitosan and sulfated chitosan in various established *in vitro* systems, such as superoxide (O^{2-})/hydroxyl (–OH) radicals scavenging, reducing power, metal ion chelating. **Methods:** Chitosan was prepared from deacetylation of chitin from cuttlefish. FTIR, degree of acetylation and mineral studies was carried out from the chitosan. Chitosan was converted into sulfated chitosan using $DMF/HClSO_3$ and their antioxidant activity was tested. **Results:** Mineral content of chitosan was Ca – 30 ppm, Na– 0.092 ppm, Cu– 0.172 ppm, Mg– 3.601, Mn– 0.264 and Zn– 0.924 ppm, DA was 49.9% (IR spectroscopy) and 40.56% (UV spectrophotometer) recorded. Cuttlefish chitosan showed high level of superoxide radical scavenging activity (88.6%) and hydroxyl radical scavenging activity (72.1%), moderate activity was noted in chelating activity (62.6% at 100 μgm/mL). At the same time low level of reducing power was observed (0.300 Absorbance at 0.75 mg/mL) when compared to standard BHA and Ascorbic acid (2.305 and 2.05 Absorbance). **Conclusions:** Finally, the scavenging rate, reducing power, chelating and reducing power of sulfated chitosan increased with their increasing concentration.

1. Introduction

Naturally occurring sulfated biopolymers, such as heparin and chondroitin sulfate, are widely spread in nature and demonstrate important functions in the regulation of cellular proliferation and differentiation[1,2]. Even polysaccharides without sulfate groups exhibit biological activities after sulfation, e.g. cellulose sulfate shows anticoagulant and antiviral functions[3–5]. Among others, the application of sulfated polysaccharides or polyanions to influence the biological activity of growth factors has attracted broad attention in tissue engineering. Besides being used as part of scaffolds or for the encapsulation of proteins, the sulfated polysaccharides were also directly applied to control the binding and activity of growth factors in cells[6–9].

Chitosan is a deacetylated derivative of chitin representing naturally occurring polysaccharides. Chitosan has some beneficial properties, such as antimicrobial activity, excellent biocompatibility and low toxicity that promote its applications in many fields including food industry and pharmaceutics[10–12]. Moreover, chitosan is readily soluble in some inorganic and organic acids, including hydrochloric acid and acetic acid, while cellulose is only very poorly soluble in such acids[12,13].

Chemical modification of chitosan and chitin has been frequently carried out, in order to prepare their derivatives with advantageous properties, such as carboxy methylated, oxidized and sulfated chitosan/chitin[10,14,15]. These derivatives have wide applications, e.g. as additives in food and cosmetic products, as drug and gene delivery system or as chiral stationary phases for HPLC[10,14–18]. Among many derivatives of chitosan, chitosan sulfate have been proven to be anticoagulant, antiviral, antimicrobial, and antioxidant[19–22].

Cortizo[23] investigated the characterization of chitin from Illex argentinus squid pen. β–Chitin was isolated by using

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chemical methods and the pens composition determined. The structural characteristics of β-chitin were identified with infrared (IR). Sagheer[24] reported the extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. Two different forms of chitin α and the β have been extracted from different marine crustacean from the Arabian Gulf. Kucukgulmez[25] investigated the physicochemical characterization of chitosan extracted from Metapenaeus stebbingi shells. Recently, the physicochemical characterization of biopolymers chitin and chitosan extracted from squid pen Doryteuthis sibogae[26].

The antioxidant capacity of sulfated polysaccharides has been studied by different in vitro methods, including hydrogen peroxide, superoxide anion, and hydroxyl radical scavenging assays. DPPH radical scavenging assay is frequently used for the analysis of food and substances obtained from natural sources[27].

The aim of this work was preparation of chitosan from the cuttlebone of cephalopod molluscs Sepia acculeata. FT-IR spectrum of chitin, chitosan and sulfated chitosan was taken and degree of acetylation was calculated using IR stretching bands and UV spectrophotometer. Furthermore sulfated chitosan was prepared from cuttlefish chitosan and evaluate in vitro antioxidant capacity of sulfated chitosan on superoxide radical scavenging assay, hydroxyl radical scavenging assay, metal ion chelating assay and reducing power.

2. Materials and methods

2.1. Chemicals and reagents

The animal (Sepia aculeata) was purchased from the landing centre of Cuddalore (Lat.11^{0}42′ N; Long. 79^{0}46′E) Tamil Nadu, India. Cuttlebone were removed from the animal, and then washed and air dried. Standards (Chitin and Chitosan), DMF, $HClSO_3$, PMS, NADH, NBT, DR and all the other chemicals and reagents are purchased from sigma aldrich and dialysis membrane also was bought from Sigma Chemicals, molecular weight cut-off was 8000 Da.

2.2. Extraction of chitin

Chitin was prepared by following the method of Takiguchi[28] after removing the minerals and protein using 2NHCI and 1N NaOH.

2.3. Conversion of chitin into chitosan

Chitin was deacetylated following the method of Takiguchi[29] using 40% NaOH to obtain chitosan.

2.4. Mineral analysis of chitosan

Mineral study was carried out following the method of Topping[30] using Inductively Coupled Plasma spectrophotometer (ICP).

2.5. Fourier Transform - Infra Red spectroscopy (FT-IR)

The chitin, chitosan, standard chitin and chitosan were determined using FT-IR spectrometer (Bio-Rad FTIS-40 model, USA). Sample (10 μg) was mixed with 100 μg of dried Potassium Bromide (KBr) and compressed to prepare a salt disc (10 mm diameter).

2.6. Measurements of degree of N- acetylation

The DA of chitosan was calculated using two methods i.e. FT-IR spectrometer and UV spectrophotometer. The absorbance of IR spectrum at 1637 and 3430 cm^{-1} were used to calculate the DA according to the following equation:

$DA(\%) = 100 - (A_{1637}/ A_{3430}) \times 115$[31].

DA was calculated using UV - spectrophotometer (Hitachi, 220S) following the method of Liu et al[32]. DA was calculated according to the following equation:

DA = (161.1.A.V. - 0.0218 m / 3.3615 m - 42.1. A. V)

2.7. Preparation of sulfating reagent

Five milliliter of $HClSO_3$ was added drop wise with stirring to 30 mL of N, N-dimethyl formamide (DMF) which was previously cooled at 0-4 ℃. The reaction mixture was stirred without cooling until the solution ($DMF.SO_3$) reached room temperature.

2.8. Preparation of sulfated chitosan

Chitosan sulfate was prepared by following the method of Xing *et al.*[33]. Briefly $DMF.SO_3$ was added a 500 mL of flask containing chitosan solution in a mixture of DMF -formic acid with swirling to get gelatinous chitosan. The reaction was run at adequate temperature for 1-2.5 hrs and 95% of EtOH was added to the precipitate the product. The precipitate was washed with EtOH, and then redissolved in distilled water. The solution was dialyzed against distilled water. The product was then concentrated and lyophilized to give chitosan sulfates.

2.9. Determination of antioxidant activity

2.9.1. Super oxide radical scavenging assay

The superoxide scavenging ability of sulfated chitosan was assessed by the method of Nishikimi *et al.*[34]. The reaction mixture, containing sulfated chitosan (0.05 - 0.5 mg/mL), PMS (30 μM), NADH (338 μM) and NBT (72 μM) in phosphate buffer (0.1 M pH 7.4) was incubated at room temperature for 5 min and the absorbance was read at 560 nm against a blank. The capability of scavenging the superoxide radical was calculated using the following equation

Scavenging effect (%) = (1 - Asample 560 nm) / Acontrol 560 nm

2.9.2. Hydroxyl radical scavenging assay

The reaction mixture containing sulfated chitosan (0.1 - 3.2

mg/mL), was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$) (100 μM), EDTA (100 μM) and ascorbic acid (100 μM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37 ℃[35]. The reaction was terminated by adding 1ml of TBA (1%, w/v) and 1ml of TCA (2%, w/v) and then heating the tubes in a boiling water bath for 15 min. The contents were cooled, and the absorbance of the mixture was measured at 535 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

2.9.3. Measurement of reducing power

The reducing power of sulfated chitosan was quantified by the method described by Xing *et al.*[36]. Briefly, reaction mixture 1 mL, containing different concentration of sulfated chitosan in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1%, w/v) at 50 ℃ for 20 min. The reaction was terminated by adding TCA solution (10%. w/v) and the mixture was centrifuged at 3 000 rpm for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1%, w/v) solution and the absorbance of the reaction measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.9.4. Metal ion chelating assay

The ferrous ion-chelating potential of sulfated chitosan was investigated according to the method of Decker and Welch[37], wherein the Fe^{2+} -chelating ability of sulfated chitosan was monitored by measuring the ferrous iron-ferrozine complex at 562 nm. Briefly, 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 min at room temperature. The absorbance of the mixture was measured at 562 nm against blank. EDTA was used as a positive control. The ability of sulfated chitosan to chelate ferrous ion was calculated using the following equation:

Chelating effect (%) = (1 − Asample 562 nm) / Acontrol 562 nm

2.10. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) using SPSS Software followed by Duncun's multiple range test (DMRT). Results were expressed as mean±S.D. from triplicate estimates in each assays. P values <0.05 were considered as significant.

3. Results

The yield of chitin and chitosan from cuttlebone of S.

Table 1

Main bands observed in the FT IR spectra of standard chitin and cuttlefish (*S. aculeata*) chitin.

Vibration mode (Pearson et al., 1960)	Standard Chitin (α -chitin) (cm^{-1})	Chitin from S. aculeata (cm^{-1})
OH out -of Plane bending	690	699
NH-out of plane bending	752	712
Ring Stretching	896	855
CH3 wagging alone chain	952	948
CO-stretching	1026	1033
CO -stretching	1073	1082
Asymmetric in phase ring stretching	1116	1160
Asymmetric bridge O2 stretching	1156	–
Amide III band and CH2 wagging	1315	–
CH bending and symmetric CH3 deformation	1377	–
CH2 bending and CH3 deformation	1418	1447
Amide II band	1563	–
Amide I band	1661	1788
Symmetric CH3 stretching and asymmetric CH2 stretching	2930	2921
NH stretching	3108	–
OH stretching	3436	3404
CH stretching	2878	2853

Table 2

Wave length of the main bands obtained from the standard chitosan and Cuttlefish (S. aculeata) chitosan

Vibration mode Chitosan Shell Operculum	Std. chitosan	Chitosan	Sulfated chitosan
HPO4	891.41	873	883.62
(NH) Amide III	897.41	–	–
PO43-	1026.63	1029	1024.66
PO3 4-	1259.54	1155	1121.20
-OH group monomer	1422.73	1443	1416.45
(-NH2) Amide II	1587.94	–	1595.77
Structural unit	3377.95	3423	3428.70

acculeata was 21% and 49.71%, respectively. Chitin was prepared from cuttlebone of *S. aculeata* by using acid and alkaline treatments; the yield of chitin was 21% in the total weight of the dried cuttlebone, after N- acetylation, the yield of chitosans were in the range of 49.71%. Whereas in the case of sulfated chitosan obtained from the chitosan of S. acculeata was 90.3%

The higher amount of protein content was present in cuttlebone (30 - 32%). At the same time no protein content was found in chitin as indicated by no absorbance at 280 nm. The mineral content of chitin from cuttlefish was found to be Ca 30 ppm, Na 0.092 ppm, Mg 3.601 ppm, Mn 0.264 ppm and Zn 0.924 ppm. But in the case of K and Fe are absent in the above chitin.

IR spectrum of the cuttlefish chitin, chitosan, standard chitin and chitosan was given in Table 1 and 2 and Figure 1-5. In the present investigation, the DA of chitosan showed 49.9% (IR spectrum) and 40.56% (UV spectrophotometer).

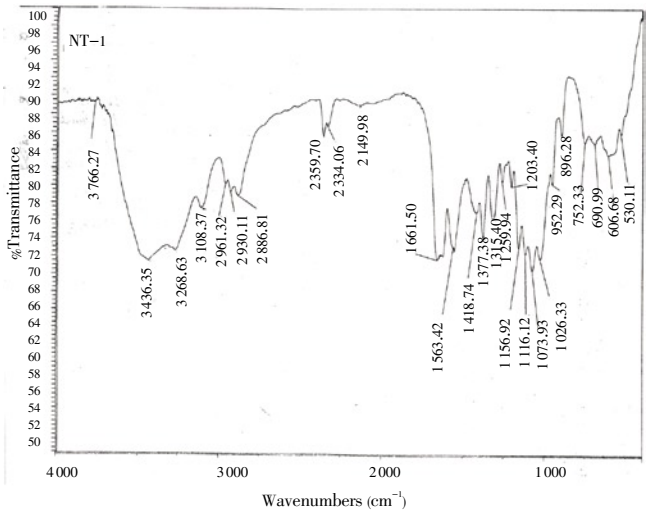

Figure 1. Showing the FT - IR spectrum of standard chitin.

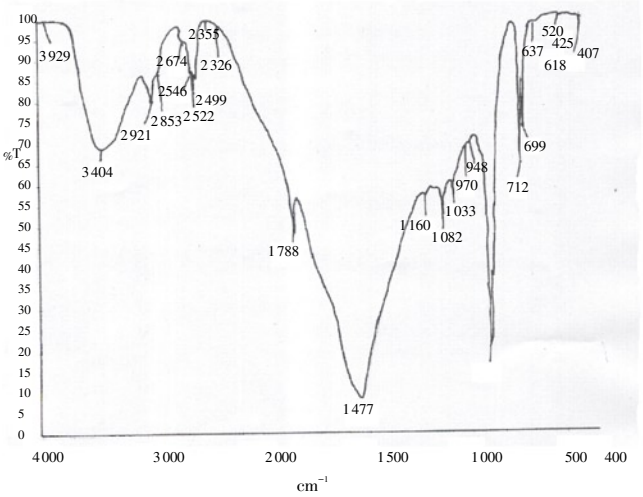

Figure 2. Showing the FT - IR spectrum of S. aculeata chitin.

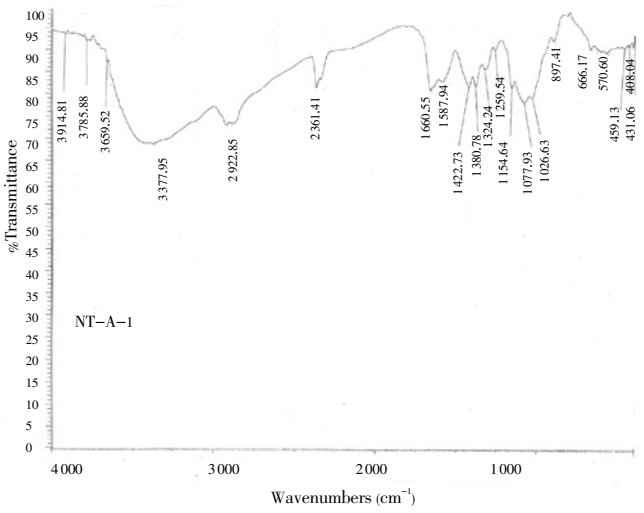

Figure 3. Showing the FT - IR spectrum of standard chitosan.

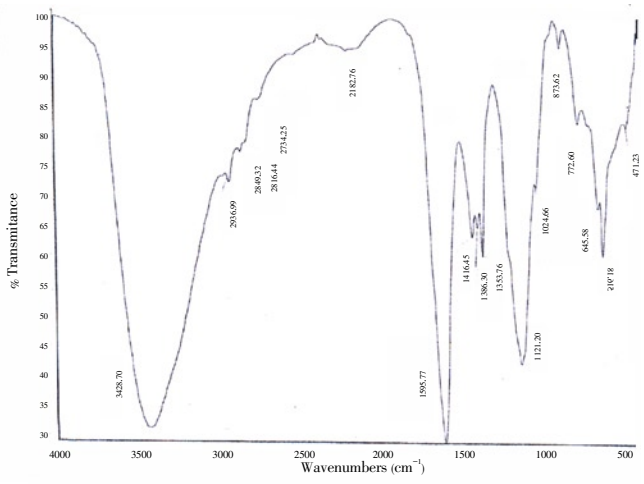

Figure 4. Showing the FT - IR spectrum of S. aculeata chitosan.

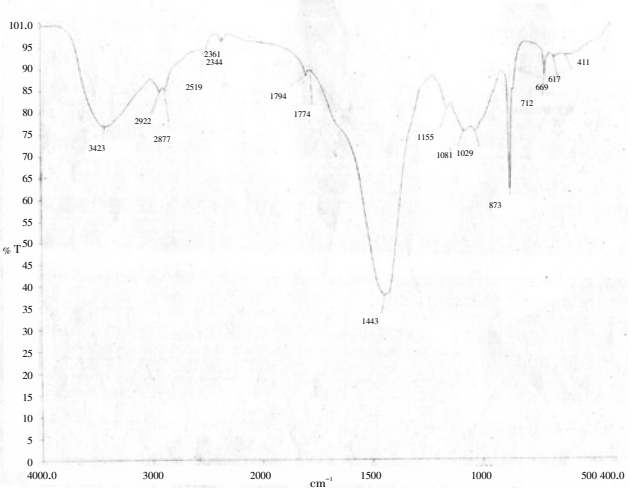

Figure 5. Showing the FT - IR spectrum of S. aculeata sulfated chitosan.

3.1. Antioxidant activity

The inhibitory effect of sulfated chitosan scavenging activity of superoxide radicals was marked and concentration related. A significant scavenging effect (20.4 - 88.6%) of super radicals was evident at all tested concentrations of sulfated

chitosan (Figure 6).

The effect of sulfated chitosan on oxidative damaged, induced by Fe^{3+}/ H_2O_2 on deoxyribose, is plotted in Figure 7. Nearly 72.1% inhibition was observed at the highest concentration (3.2 mg/mL), 75.9 and 88% was observed in Ascorbic acid and BHA respectively.

The ferrous ion-chelating effect of sulfated chitosan was concentrated related as shown in Figure 8. At 1 mg/mL, chelating ability of chitosan on ferrous ions was 62.6%. However, EDTA showed a 68% chelating ability.

The reducing power of chitosan and sulfated chitosan was increased with the increasing concentration. Figure 9 shows the reducing power increased with increasing chitosan and sulfated chitosan concentration.

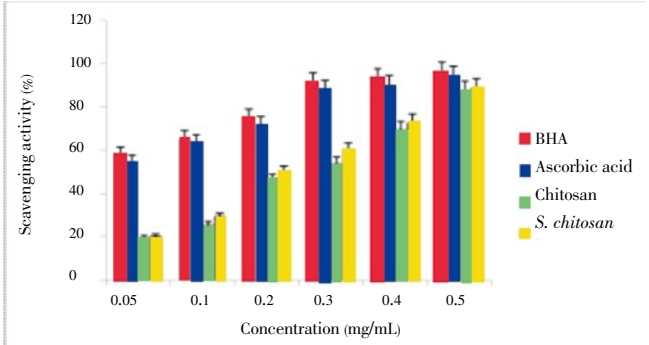

Figure 6. Scavenging effect of sulfated chitosan of cuttlebone of S. aculeata on superoxide radical.
Values are means±SD of three determinations.

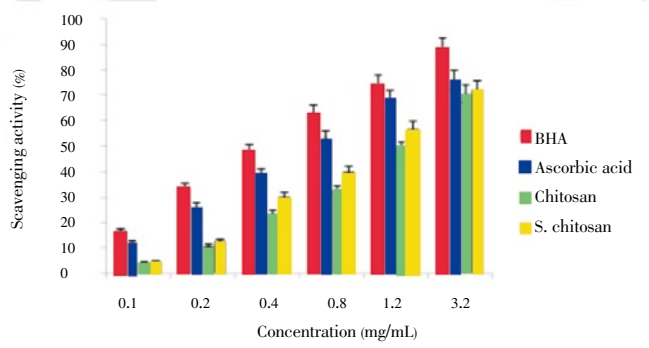

Figure 7. Inhibitory effect of sulfated chitosan of cuttlebone and standards of S. aculeata on deoxyribose oxidative damage.
Values are means±SD of three determinations.

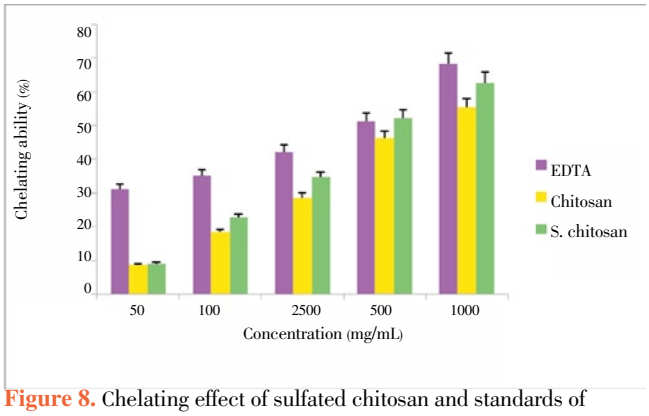

Figure 8. Chelating effect of sulfated chitosan and standards of cuttlebone of S. aculeata on ferrous ions. Values are means±SD of three determinations.

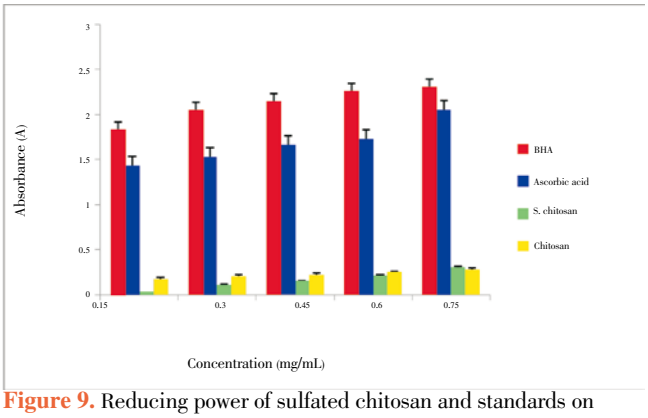

Figure 9. Reducing power of sulfated chitosan and standards on superoxide radical. Values are means±SD of three determinations.

4. Discussion

The cuttlebone of *Sanguisorba officinalis* was found to be 20% of chitin[38,39], whereas in general, the squid/ cuttlefish reported 3-20% of chitin[40]. One of the major problems related to the preparation of pure chitins is keeping a structure as close as possible than the native form is to minimize the partial deacetylation and chain degradation caused by demineralization and deproteinization applied during process of the raw materials. Squid chitin showed no color and odor. Chitin occurs naturally partially deacetylated (with a low content of glucosamine units), depending on the source[41]; nevertheless, both α - and β - forms are insoluble in all the usual solvent, despite natural variation in crystallinity. The insolubility is a major problem that confronts the development mechanisms and uses of chitin. The β- chitin is more reactive than the α- form, an important property with regard to enzymatic and chemical transformations of chitin[42].

Cuttlefish chitin which is associated to organic and inorganic matter. Doryteuthis sibogae Squid pen found the mineral content, Ca 21 ppm, Mg 11.01 ppm, Mn 0.260 ppm, Cu 0.905 ppm and this was absent in Na, K, Fe and Zn Barwin Vino[43]. *D. sibogae* pen chitosan contain only very small less amount of mineral content (1.50 and 1.37%). The similar less amount of mineral content were recorded in S. aculeata chitin. Generally the mineral salts content in squid pen and cuttlebone of cuttlefish (1%w/w) is much lower than shrimp shell (14% w/w). Consequently, in the preparation of chitin from squid pen, it is not need to be demineralized by acid treatment as preparation from shrimp and crab shells[44].

The properties of chitosan depend on various intrinsic parameters such as percentage of degree of deacetylation (DDA) and molecular weight and thereby on the uses of chitosan[45,46]. But in the case of quality parameters of chitosan for chitin and chitosan is lacking[46,47]. Rinaudo and Domard[45] analysed the above parameters for different commercially available chitins and chitosans and found that they differed with the products. Besides this, numerous methods are being used for the estimation of the same

parameters leading to discrepancies. Hence there exists a need for standard analytical parameters and methodologies that are recognized by all.

The yield of high molecular weight sulfated chitosan was recorded as 173% at 50 ℃[33] and Vikhoreva *et al*.[48] reported the low molecular weight chitosan yield was 0.95 to 1.20g/g. But in the present study only 90.3% of sulfated chitosan was obtained.

In the FT - IR spectrum of sulfated chitosan obtained from Ulva pertusa[49] the peaks at 847 cm^{-1}, 1052 cm^{-1}, 1056 cm^{-1}, 1641 cm^{-1} and 3446 cm^{-1} were reported to be caused by the bending vibration of C-O-S of sulfate in axial position, stretching vibration of C-O, S-O of sulfate, C-O of uronic acids and O-H respectively. In the present study also the observed peaks (873.62 cm^{-1}, 1024.66 cm^{-1}, 1353.78 cm^{-1}, 1595.77 cm^{-1} and 3428.70 cm^{-1}) represented the same structural features.

The inhibitory effect of sulfated chitosan on superoxide radical scavenging effect (20.4 - 88.6%) of superoxide radicals was evident at all concentration of sulfated chitosan (0.05 - 0.5 mg/mL). Compared with low molecular weight chitosan and parent chitosan, their scavenging activity for superoxide radical was 80.3% and 13% at 0.5 mg/mL, respectively[50]. High molecular weight chitosan sulfate (HCTS) shows the superoxide scavenging effect was 27.93 - 97.21 in the concentration of 0.005 - 0.4mg/ml[33]. Although superoxide is a relatively weak oxidant, it decomposes to from stronger reactive species, such as single oxygen and hydroxyl radicals, which initiate peroxidation of lipids[51]. In the present study, chitosan sulfate effectively scavenged superoxide in a concentration - dependent manner. Further, superoxides are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals[52]. These results showed that the chitosan sulfate had strong scavenging activity of superoxide radical and clearly suggested that the antioxidant activity of sulfated chitosan was also related to its ability scavenge superoxide radical.

Hydroxyl radical scavenging activity of sulfated chitosan was obtained in the deoxyribose system. In this system, sulfated chitosan exhibited a concentration dependent inhibition of deoxyribose oxidation. Earlier Halliwell *et al*.[35] have employed this system to assess the biological activity of various natural plant - derived biomolecules. Smith *et al*.[52] reported that molecules that can inhibit deoxyribose degradation are those that can chelate iron and render them inactive or poorly active in a fenton reaction. In the present study, in another assay system, we found that the sulfated chitosan has considerably soft ferrous ion chelating power, so it is impossible that the chelating effect of sulfated chitosan on metal ions may be responsible that the inhibition of deoxyribose oxidation. Therefore, the mechanism of sulfated chitosan on scavenging hydroxyl radical needs to be further researched.

Xing *et al*.[33] found that the ferrous ion - chelating effect of all kinds of sulfated chitosan was concentration related with sulfated TSCTS being the most of effective (~72% at 0.25 mg/mL). Lin and Chou[52] reported the chelating abilities of N- alkylated disaccharide chitosan derivatives were less than the observed with EDTA. However, at 1mg/ml, EDTA chelated ~ 60% of cupric ions (Liu and Chou, 2004). Whereas Xing *et al*.[53] reported HCTS was ~30% in 1 mg/mL and GH show ~5.5%. But, at 1 mg/mL, chelating ability of chitosan B60 and C60 on ferrous ions was 88.7% and 90.3% respectively[54].

Transition metal ions can initiate lipid peroxidation and start a chain reaction, which lead to the deterioration of flavor and test in food[55]. It has also proposed that the catalysis of metal ions might correlate to cancer and arthritis[35]. Since ferrous ions are the most effective pre-oxidants in the food system. In the present show the chelating effect of chitosan and sulfated chitosan was low, especially compared to EDTA.

Mau *et al*.[56] reported reducing powers were 0.80, 0.89 and 0.92at 1.0 mg/mL for ascorbic acid, a-tocopherol and BHA, respectively. However, the reducing power of different molecular weight chitosans and sulfated chitosans was lower than that of ascorbic acid, a-tocopherol and BHA[33]. Pin-Der Duh *et al*.[54] observed a direct correlation between antioxidant activities and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductions which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[57]. Redactors are also reported to react with certain precursors of peroxide formation. Our data on the reducing power of sulfated chitosan suggested that it was likely to contribute significantly towards the observed antioxidant effect. Further research is needed to evaluate the in vivo antioxidative potential of chitosan.

Conflict of interest statement

We declare that we have no conflict of interest.

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