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Extraction, characterization and its antioxidant efficacy of polysaccharides from *Sepia aculeata* (Orbigny, 1848) cuttlebone

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Ocean is the source of large group of structurally unique natural products that are mainly found in invertebrates such as sponges, tunicates, bryozoans and mollusks. In the present study, polysaccharide was extracted from the cuttlebone of *Sepia aculeata* and the structure was determined through Fourier transform infra red spectroscopy. Then, the antioxidant efficacy was evaluated through various antioxidant assays such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, hydroxyl radical scavenging assay, superoxide radical scavenging assay and chelating ability on ferrous ions. The total sugar content of *S. aculeata* polysaccharides was 86.2%. The polysaccharide shows good antioxidant activity when compared with standard commercial antioxidants butylated hydroxyanisole (BHA) and ascorbic acid. The DPPH, superoxide and hydroxyl radical scavenging effect of polysaccharides was found to be 36.27% at 10 mg/ml, 59.57% at 0.5 mg/ml and 45.86% at 3.2 mg/ml, respectively. The chelating effect of polysaccharides was 48.61% at 10 mg/ml.

Key words: Polysaccharide, cuttlebone, Fourier transform infrared (FT-IR), antioxidant.

INTRODUCTION

A vast amount of circumstantial evidence implicates oxygen-derived free radicals as mediators of inflammation, shock and ischemia/reperfusion injury. Furthermore, the radicals also play a role in the process of ageing and carcinogenesis (Cuzzocrea et al., 2001). The harmful action of the free radicals can, however, be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism. Current research in free radicals has confirmed that food items rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases, as well as inflammation and problems caused by cell and cutaneous aging (Li et al., 2007).

There are two basic categories of antioxidants namely synthetic and natural ones. In order to reduce damage to the human body and prolong the storage stability of food, synthetic antioxidants are used for industrial processing. Restriction on the use of synthetic antioxidants is being imposed because of their carcinogenicity (Guyton et al., 1991). Therefore, the development and utilization of more effective antioxidants of natural origin are desired.

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Abbreviations: HIV, Human immunodeficiency virus; **MAP,** *Misgurnus anguillicaudatus* polysaccharide; **EDTA,** ethylene diaminetetraacetic acid; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **FT-IR,** Fourier transform infrared; **BHA,** butylated hydroxyanisole.

In the search of new antioxidants, exploration of aquatic habitats has led to the discovery that marine plants and invertebrates also contain antioxidants. Among the invertebrates, the discovered bioactive compounds in mollusks were identified essentially as peptides, depsipeptides, sterols, sesquiterpenes, terpenes, polypropinates, nitrogenous compounds, macrolides, prostaglandins and fatty acid derivatives and alkaloids which presented specific types of activities (Balcazar et al., 2006). Polysaccharides are common structural and storage polymers in living organisms. Polysaccharides are potentially useful, and are biologically active ingredients for pharmaceutical uses due to a variety of biological activities, such as immunological, anti-radiation, anti-blood coagulation, anti-cancer, anti-human immunodeficiency virus (HIV) and hypoglycemic activities (Yoon et al., 2003; Yang et al., 2005). The activity of polysaccharides is closely related to their structure-related aspects such as molecular mass, degree of substitution, degree of branching, sugar component and the structure of main chain and branches (Bohn and BeMiller, 1995).

Although antioxidant polysaccharides have mostly been found in plants, microorganisms and algae, animal sources of antioxidant polysaccharides recently have been found. Qin (1993) found that *Misgurnus anguillicaudatus* polysaccharide (MAP) may play an important role in the prevention of oxidative damage in living systems. Polysaccharide isolated from the cuttlebone of *Sepia aculeata* and *Sepia brevimana* using 10 mM ethylene diaminetetraacetic acid (EDTA) were studied for their antibacterial and antifungal activity (Shanmugam et al., 2008). In the present study, polysaccharide was isolated from cuttlebone of cephalopod mollusk *S. aculeata* using EDTA and characterized through Fourier transform infrared (FT-IR) spectroscopy. Its possible antioxidant activity was then investigated using various *in vitro* assay systems such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, superoxide radical scavenging, hydroxyl radical scavenging, and ferrous ion chelating ability, in order to understand the potential usefulness of this polysaccharide as an ingredient in functional food.

MATERIALS AND METHODS

Chemicals and reagents

Ascorbic acid, butylated hydroxyanisole (BHA), DPPH, 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.

Collection of animals

The cuttlefish *S. aculeata* was collected from Mudasalodai landing centre (Lat. 11°29' N; Long. 79°46' E), located along the east coast near Parangipettai (South East coast of India).

Isolation of polysaccharides

The polysaccharide extract was obtained from the internal shell of *S. aculeata* by following the method of Okutani and Morikawa (1978). The air-dried shell powder was pulverized and washed with acetone. The powder was extracted with hot 10 mM EDTA solution and filtered (Whatmann No.1) with hyflosuper cel. Then, saturated barium hydroxide solution was added to the filtrate and allowed to stand overnight. Then, the precipitate was collected on a filter paper (Whatmann No.1) with hyflosuper cel and washed with distilled water. The dialysate present in the dialysis membrane was then freeze-dried and white colour powder was obtained. The lyophilized polysaccharide powder thus obtained was used for further analysis.

Total sugar

The total sugar content of polysaccharides from *S. aculeata* cuttlebone was determined by following the method of Dubois et al. (1956) using glucose as a standard.

Fourier transform infrared (FT-IR) spectral analysis

FT-IR spectroscopy of solid sample of polysaccharide from *S. aculeata* relied on an AVATAR 330 Spectrometer. Sample (10 µg) was mixed with 100 µg of dried potassium bromide (KBr) and compressed to prepare a salt discs (10 mm diameter) for reading the spectrum.

DPPH radical scavenging activity

The scavenging ability of DPPH radicals was determined according to the method of Shimada et al. (1992). Polysaccharides (0.1-10 mg/ml) in distilled water were mixed with 1 ml of methanolic solution containing DPPH radicals, resulting in a final concentration of 10 mM/l DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark and the absorbance was then measured at 517 nm against a blank. BHA and α- tocopherol were used for comparison.

Superoxide radical scavenging activity

The superoxide scavenging ability of polysaccharides was assessed by the method of Nishikimi et al. (1972). The reaction mixture, containing polysaccharides (0.05 to 0.5 mg/ml), PMS (30 mM), NADH (338 mM) and NBT (72 mM) 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:

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

Hydroxyl radicals scavenging assay

The reaction mixture containing polysaccharides (0.1 to 3.2 mg/ml), was incubated with deoxyribose (3.75 mM), H_2O_2 (1 mM), $FeCl_3$ (100 mM), EDTA (100 mM) and ascorbic acid (100 mM) in potassium

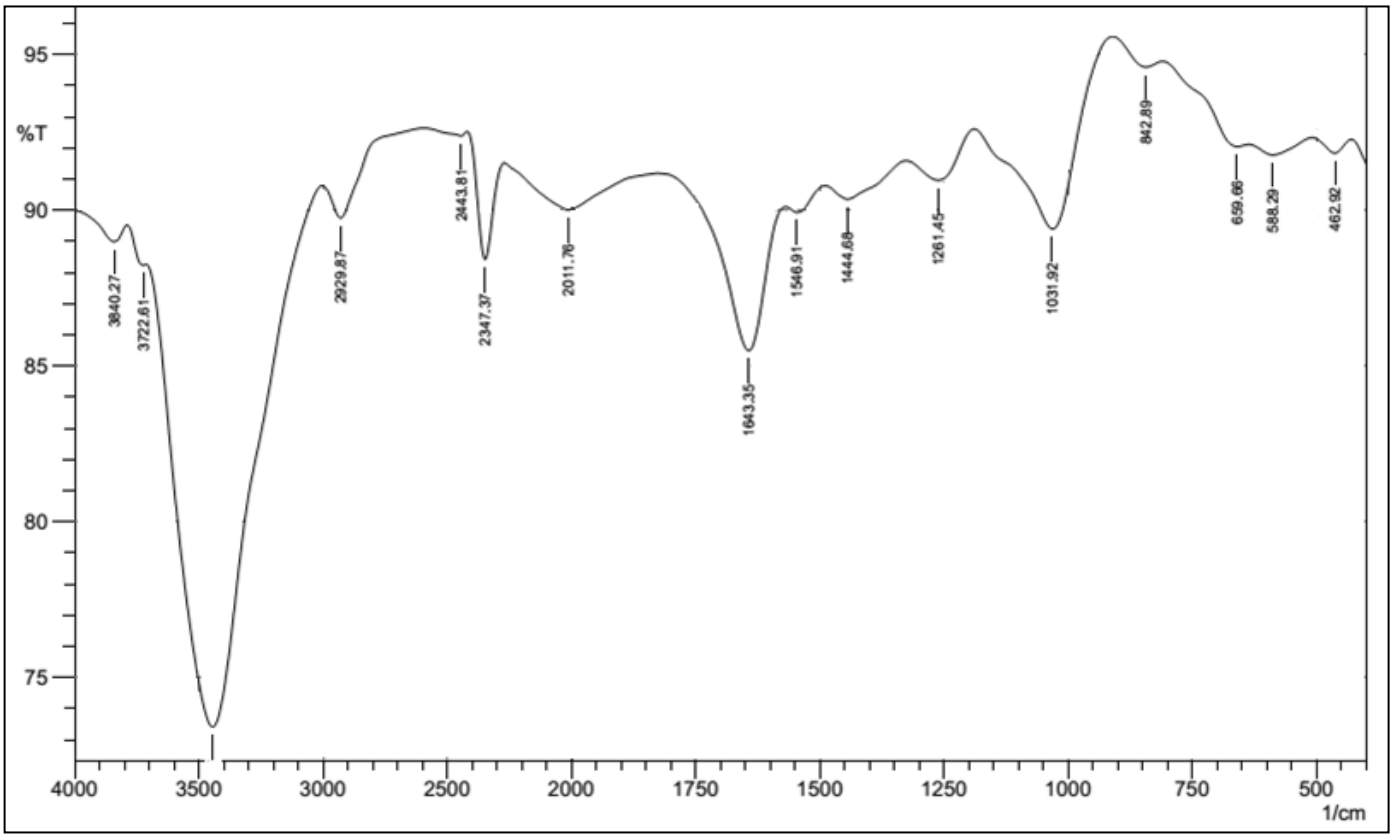

Figure 1. FT-IR spectrum of polysaccharides from *S. aculeata* cuttlebone.

phosphate buffer (20 mM, pH 7.4) for 60 min at 37°C (Halliwell et al., 1987). The reaction was terminated by adding 1 ml of thiobarbituric acid (TBA) (1%, w/v) and 1 ml of trichloroacetic acid (TCA) (2%, w/v) and then heating the tubes in a boiling water bath for 15 min. The test tubes 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.

Chelating ability

The ferrous ion-chelating potential of polysaccharides was investigated according to the method of Decker and Welch (1990), wherein the Fe^{2+} chelating ability of polysaccharide from *S. aculeata* cuttlebone was monitored by measuring the ferrous iron-ferrozine complex at 562 nm. EDTA was used as a positive control.

RESULTS AND DISCUSSION

Total sugar content of polysaccharides

The amount of total sugar content of *S. aculeata* polysaccharides was found to be 86.2%. The amount of total sugar present in the polysaccharides from rhizome and aerial part of *Athyrium multidentatum* was reported as 81.04 and 84.72% respectively (Liu et al., 2011). The total sugar content of polysaccharide from shoots of *Phyllostachys edulis* extracted with water, sulfuric acid and sodium hydroxide was 88.4, 81.7 and 93.9%, respectively (Zhang et al., 2011).

FT-IR spectral analysis

The FT-IR spectrum of *S. aculeata* polysaccharides showed a characteristic peaks in the range of 3840.27 to 462.92 cm^{-1} (Figure 1). The broad peak at 3444.87 cm^{-1} indicates the hydroxyl stretching vibration and the sharp peak at 2929.87 cm^{-1} represents the characteristic -CH- stretching vibrations. Similarly, Jin (2012) found the characteristic peak of polysaccharide from fruit shell of *Camellia oleifera* at 3463 and 2933 cm^{-1} representing the hydroxyl stretching vibration and CH stretching vibration, respectively. In the FT-IR spectrum of *S. aculeata* polysaccharides, the absorption band at 842.89 cm^{-1} represents the glycosidic linkages (Table 1).

DPPH radical scavenging activity

The free radical-scavenging activity of the polysaccharides was assayed through $DPPH^{\cdot}$ method and the results were compared with those of BHA and α-tocopherol

Table 1. Wave number, nature and possible assignment of absorption band of polysaccharides.

Wave number (cm^{-1})	Nature of peak	Possible assignment of absorption band
3840.27	Sharp	H-bonded OH stretching
3444.87	Broad	Hydroxyl stretching vibrations
2929.87	Sharp	Aliphatic -CH- stretching vibrations
2443.81	Shoulder	Aliphatic -CH- stretching vibrations
1643.35	Sharp	NH-bending
1444.68	Very Sharp	NH-bending
1031.92	Broad	C-O-C stretching
842.89	Shoulder	Glycosidic linkages

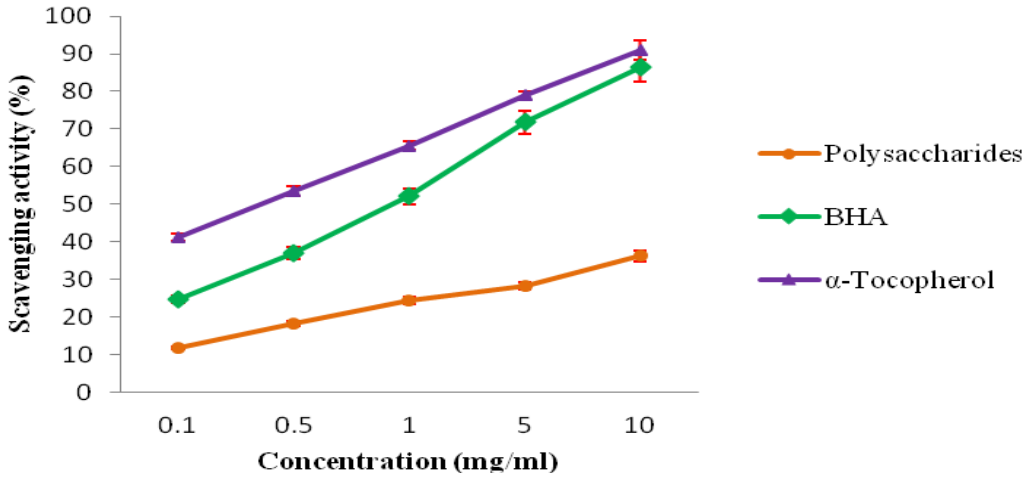

Figure 2. DPPH radical scavenging activity of polysaccharides.

(Figure 2). DPPH is usually used as a substrate to evaluate antioxidant activity of antioxidants. The method is based on the reduction of methanolic $DPPH^{\cdot}$ solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable radical $DPPH^{\cdot}$ to the yellow-coloured diphenylpicryl-hydrazine (Li et al., 2007).

Polysaccharides exhibited the concentration dependent antioxidant activity. BHA and α-tocopherol showed higher degree of free radical-scavenging activity than that of the *S. aculeata* polysaccharides at each concentration points. At 10 mg/ml concentration, BHA and α- tocopherol showed higher (86.38 and 90.96%) free radical-scavenging activity than that of the polysaccharides (36.27%), suggesting that the polysaccharides have weaker DPPH radical scavenging activity, but the polysaccharides have higher scavenging ability than the glycosaminoglycans from the cuttlebone of *S. brevimana* which showed scavenging ability against DPPH radicals of 19.3% at 10 mg/ml (Barwinvino, 2010). Li et al. (2007) found that the polysaccharides from *Lycium barbarum* scavenged DPPH radicals of 46.7% at 250 µg/ml.

Superoxide radical scavenging activity

The superoxide radical is highly toxic species that is generated by numerous biological and photochemical reactions (Banerjee et al., 2005). Although $O_2^{\cdot-}$ was a relatively weak oxidant, it decomposed to form stronger reactive oxidative species, such as singlet oxygen and $HO^{\cdot}$, which initiate peroxidation of lipids, further, $O_2^{\cdot-}$ was also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of $HO^{\cdot}$ (Meyer and Isaksen, 1995). Therefore, it is very important to study the scavenging of $O_2^{\cdot-}$. In the present study, the scavenging or preventive capacity of *S. aculeata* polysaccharides against the superoxide anion free radicals was investigated. Superoxide anion radical scavenging effects of the polysaccharides increased with increasing concentration in the range of 17.65 -59.57% (0.05 to 0.5 mg/ml); whereas BHA and α- tocopherol scavenged superoxide anion of 88.41 and 78.23%, respectively at 0.5 mg/ml (Figure 3). Water soluble and alkali soluble polysaccharides from Chinese truffle *Tuber sinense* showed superoxide anion scavenging activities of 45.9 and 35.98%, respectively at 2.5 mg/ml (Zhao et al.,

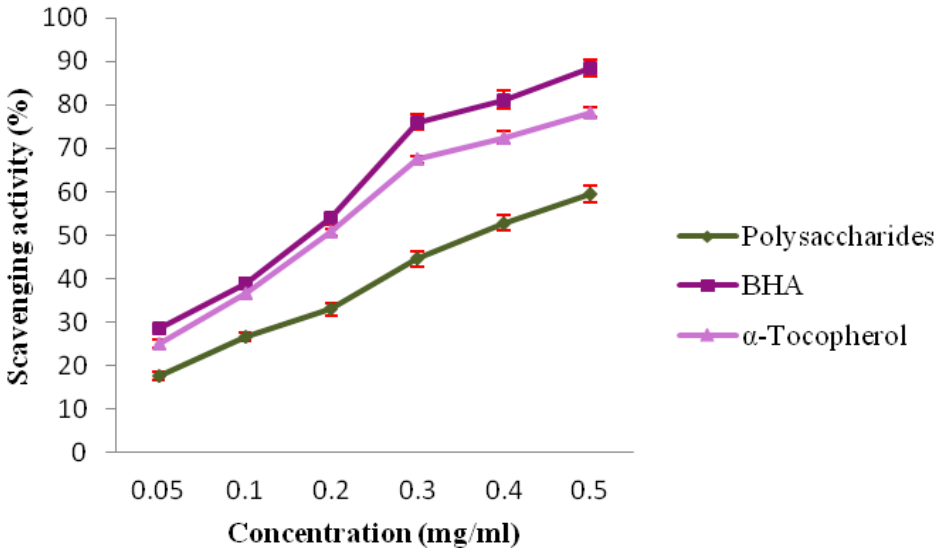

Figure 3. Superoxide radicals scavenging activity of polysaccharides.

2012). The superoxide radical scavenging activity of cuttlefish glycosaminoglycans was 88.23% at 0.5 mg/ml (Barwinvino, 2010).

Hydroxyl radical scavenging activity

H_2O_2 can be generated in biological and food systems. Being a non-radical oxygen-containing reactive agent, it can form $HO^{\cdot}$, the most highly reactive oxygen radical known, in the presence of transition metal ions and participate in free radical reaction (Halliwell et al., 1995). Hydroxyl radical scavenging mechanism was related to the transition metal ions. In the absence of transition metal ions, hydrogen peroxide was fairly stable. However, hydroxyl radicals acted in super-oxidation by hydrogen peroxide with metal ions, usually ferrous or copper. The molecules that could chelate iron and render them inactive in Fenton reaction might have scavenging ability on hydroxyl radical (Macdonald et al., 2003).

Hydroxyl radicals were generated by reaction of iron-EDTA complex with H_2O_2 in the presence of ascorbic acid, attack deoxyribose to form products upon heating with 2-thiobarbituaric acid under acid conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish tint formation (Cheng et al., 2002). The effect of ppolysaccharides on oxidative damage induced by hydroxyl radical at different concentrations (0.1 - 3.2 mg/ml) was found between 9.35 and 45.86%; whereas the BHA and α- tocopherol scavenged hydroxyl radical in the range of 26.33 - 84.36% and 20.99 - 72.40%, respectively (Figure 4). The scavenging effect of hydroxyl radical by litchi fruit polysaccharide I was 38.15% at 2 mg/ml. The effect of cuttlefish anticoagulant glycosaminoglycans on oxidative damage induced by Fe^{3+}/H_2O_2 on deoxyribose was 64.03% at 3.2 mg/ml (Barwinvino, 2010). The scavenging effect of *S. aculeata* polysaccharide on hydroxyl radical was higher than that of plant polysaccharide, and lower than the animal polysaccharide.

Chelating ability of polysaccharides on ferrous ion

Transition metals, in particular iron, have a major role in the generation of reactive oxygen species in living organisms. Iron exists in two distinct oxidation states - ferrous and the relatively biologically inactive form, ferric ion. Ferric ions, however, have been shown to be reduced to the active Fe^{2+}, depending on the conditions, particularly pH (Strlic et al., 2002) and oxidized back through Fenton-type reactions, with production of $OH^{\cdot}$ or Haber-Weiss cycle reactions with superoxide anions (Kehrer, 2000). Iron chelating agents are thus expected to inhibit the metal-dependent oxidative processes and have potential in combating reactive oxygen species mediated diseases (Finefrock et al., 2003).

Chelating agents may serve as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ions (Gordon, 1990). In the present investigation, the chelating ability of *S. aculeata* polysaccharides on ferrous ion was 48.61% at 10 mg/ml. However, EDTA showed high chelating ability of 95.52% at 10 mg/ml (Figure 5). Factors affecting the ion-chelating ability of polysaccharides and its derivatives are rather complex. Subhapradha et al. (2013) illustrated the chelating ability of phosphorylated chitosan on ferrous ion was 59.56% at 10 mg/ml. The chelating activity of sulfated polysaccharides, acetylated

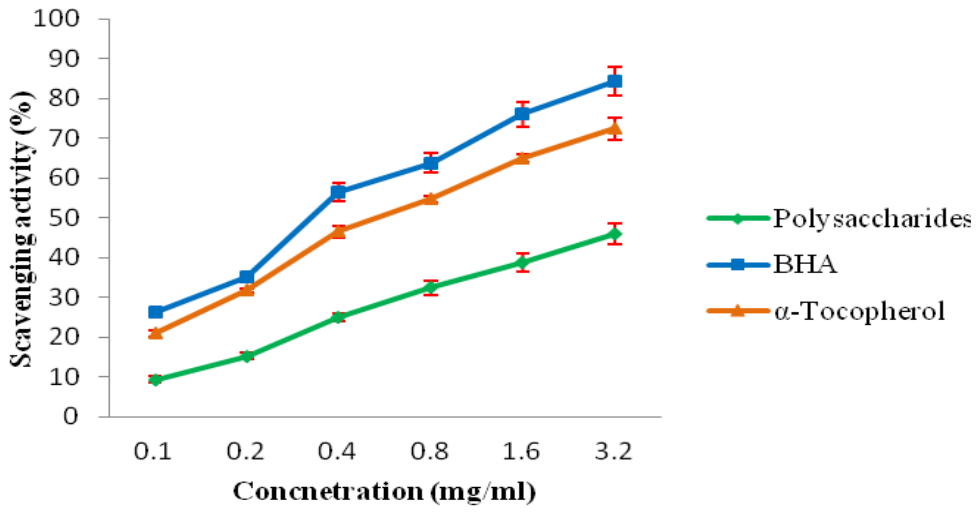

Figure 4. Hydroxyl radical scavenging activity of polysaccharides.

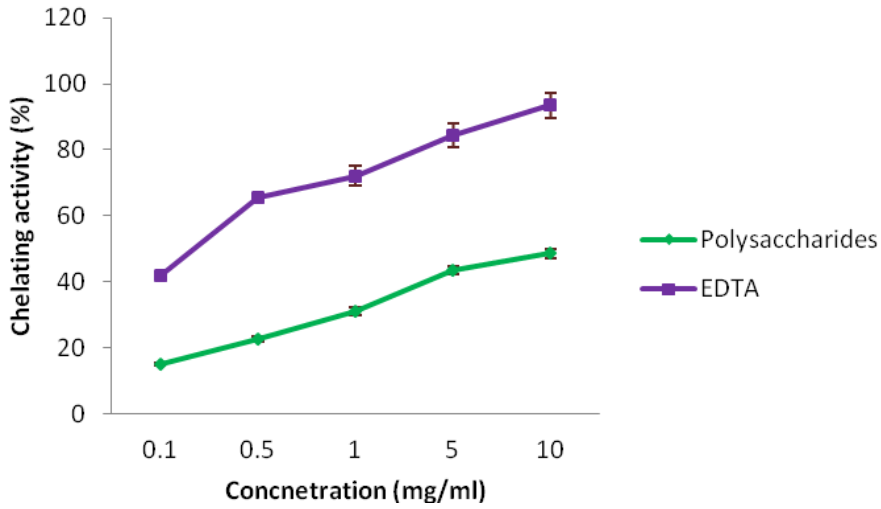

Figure 5. Chelating ability on ferrous ions.

polysaccharides and phosphorylated polysaccharides from *Ramulus mori* was found to be 74.5, 67.2 and 58.7% at 2.1 mg/ml, respectively (Zhang et al., 2008).

The results of the present findings depicts that polysaccharide from *S. aculeata* have good antioxidant and antiradical activities. So the polysaccharide should be used as a source of natural antioxidant or ingredient in the pharmaceutical or nutraceutical industries. However, the *in vivo* antioxidant activity and the antioxidant mechanism need to be analyzed in future.

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