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Antioxidant activities of sulfated polysaccharides from brown and red seaweeds

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Abstract The *in vitro* antioxidant activities of the following six sulfated polysaccharides were investigated: iota, kappa and lambda carrageenans, which are widely used in the food industry, fucoidan (homofucan) from the edible seaweed *Fucus vesiculosus* and fucans (heterofucans) F0.5 and F1.1 from the seaweed *Padina gymnospora*. With respect to the inhibition of superoxide radical formation, fucoidan had an IC_{50} (the half maximal inhibitory concentration) of 0.058 mg·mL^{-1}, while the IC_{50} for the kappa, iota and lambda carrageenans were 0.112, 0.332 and 0.046 mg·mL^{-1}, respectively. All of the samples had an inhibitory effect on the formation of hydroxyl radicals. The results of peroxidation tests showed that fucoidan had an IC_{50} of 1.250 mg·mL^{-1} and that the kappa, iota and lambda carrageenans had an IC_{50} of 2.753 and 2.338 and 0.323 mg·mL^{-1}, respectively. Fucan fractions showed low antioxidant activity relative to fucoidan. These results clearly indicate the beneficial effect of algal polysaccharides as antioxidants.

Key words antioxidant activity · carrageenan · fucoidan · *Fucus vesiculosus* · *Padina gymnospora* · seaweed

Abbreviations

MDA	Malondialdehyde
TBA	2-Thiobarbituric acid
NADH	Nicotinamide adenine dinucleotide
F0.5	Fucan precipitated with 0.5 vol. of acetone
F1.1	Fucan precipitated with 1.1 vol. of acetone

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Introduction

Polysaccharides from some seaweeds have been reported to possess biological activity of potential medicinal value. These polysaccharides have become very important products in the food industry (Usov 1998; Usov et al. 2002).

Fucoidans which are homopolysaccharides, and the class of heteropolysaccharides known as fucans are metabolic products of sulfated fucose found in brown seaweed. Fucoidan is a complex sulfated

polysaccharide, derived from *Fucus vesiculosus*, that mediates a variety of significant biological effects (Patankar et al. 1993). Fucans can also contain galactose, glucuronic acid, mannose and xylose (Patankar et al. 1993; Leite et al. 1998; Berteau and Mulloy 2003; Rocha et al. 2005), and those from brown seaweeds are byproducts of the industrial processes involved in the preparation of alginates for the food and cosmetic industries (Boisson-Vidal et al. 1995; Rupérez et al. 2002)

Carrageenans is a generic name for a family of linear, sulfated galactans obtained from certain species of marine red algae. The backbone of the polysaccharide is composed of D-galactose units (G) linked alternately to α-(1→3) and β-(1→4) linkages. The β-linked residue always belongs to the D-series, while the α-linked residues may be either D- or L-galactose units that partially occur as 3,6-anhydrogalactopyranosyl moieties. Sulfated galactans, such as carrageenans, are used mainly in products that require gelling, suspension (Norziah et al. 2006), thickening or water-holding properties in the food industry.

Antioxidant activity has become a 'hot' topic and the subject of intensive investigations due to the ever-increasing demand by the food and pharmaceutical industries to develop natural bioactive anti-aging and anticarcinogenic compounds that demonstrate measurable health benefits. Antioxidative substances obtained from natural sources, such as seed oil, grains, beans, vegetables, fruits, leaf waxes, bark, roots, spices and hulls, have already been investigated (Fujimoto et al. 1985; Guiry and Blunden 1991; Gordon et al. 1993; Duh 1999). However, there are very few studies in the literature on antioxidant activity associated to sulfated polysaccharides from seaweeds.

Le Tutour (1990) reported that two extracts from the brown algae, *Laminaria digitata* and *Himanthalia elongata*, exhibited the highest activity in sunflower oil preservation and in the inhibition of methyl linoleate oxidation in addition to synergistically enhancing the antioxidant effect of vitamin E. In another study, Le Tutour et al. (1998) demonstrated the ability of several brown seaweed extracts to scavenge peroxyl radicals. Ruperéz et al. 2002 demonstrated that fucoidan from *Fucus vesiculosus* had the highest antioxidant activity in relation to the other fractions, with high levels of uronic acid. Several studies were subsequently performed to verify the antioxidant properties of algae (Zhang et al. 2003; Yuan et al. 2005). Recently, the antioxidant activity of polysaccharides from the chlorophyte *Ulva pertusa*, was also investigated. All of the compounds analyzed showed that molecular weight (MW) had a significant effect on antioxidant activity (Qi et al. 2005).

The aim of this study was to evaluate *in vitro* the antioxidant activity of sulfated polysaccharides, carrageenans and homofucans (fucoidans) from red and brown seaweeds, respectively. We also used a heterofucan from the alga *Padina gymnospora* for comparative studies with the fucoidan. These polysaccharides may represent a new approach for inhibiting the harm caused by excessive free radicals.

Materials and methods

Materials

The polysacchacarides fucoidan (*Fucus vesiculosus*) and lambda (*Gigartina acicularis*, *G. pisillata*), kappa (*Eucheuma cottonii*) and iota (*E. spinosa*) carrageenans were purchased from Sigma Aldrich, (St. Louis, Mo.), and fucans from *Padina gymnospora* (fraction F0.5 and F1.1) were extracted as described by Silva et al. (2005). The algae were stored in our laboratories and dried at 50°C under ventilation in an oven, ground in a blender and incubated with acetone to eliminate lipids and pigments. Approximately 50 g of powdered algae was suspended with 5 vol. of 0.25 *M* NaCl, and the pH was adjusted to 8.0 with NaOH. Ten milligrams of maxataze, an alkaline protease from *Esporobacillus* (BioBrás, Montes Claros, MG, Brazil), was then added to the mixture for proteolytic digestion. After incubation for 24 h at 60°C under shaking and periodical pH adjustments, the mixture was filtered through cheesecloth and precipitated with 0.3 vol. of ice-cold acetone calculated from the initial solution, which was maintained at 4°C for 24 h. The precipitate formed was collected by centrifugation at 10,000 *g* for 20 min, dried under vacuum, resuspended in distilled water and analyzed. To each resulting supernatant was added 0.5 and 1.1 vol. of acetone, using the same procedures described above. Three fractions were then obtained and named according to the volumes of acetone used. The F0.3 fraction was discarded because of contamination with several compounds.

Chemical analysis

Total sugar content was analyzed by the phenol sulfuric acid method (Dubois et al. 1956) using L-fucose as the standard. Sulfate content was determined according to the gelatin-barium method (Dodgson and Price 1962) using sodium sulfate (1 mg·mL^{-1}) as standard and after acid hydrolysis of the polysaccharides (6 *N* HCl, 100°C, 6 h). Protein content was measured by Spector's method (1978). Fucose, xylose and uronic acid content of the polymers was also estimated by the methods described by Dische (1962a, b, c). The polysaccharides were hydrolyzed with 2.0 *M* HCl for 1 h at 100°C.

Molecular weight determination

The polysaccharides carrageenans, fucoidan, F0.5 and F1.1 were subjected to gel-permeation chromatography on Sepharose CL-4B (140×1.8 cm) using 0.2 *M* acetic acid as eluent. The elution was monitored for total sugar (Dubois et al. 1956). To estimate the MW of the polysaccharides, we used dextrans of different sizes as standards (Pharmacia). The eluted polysaccharides were dialyzed against water, freeze-dried and used in the antioxidant assays.

Antioxidant assays

Superoxide anion scavenging activity

Superoxide radicals are a highly toxic species generated by numerous biological and photochemical reactions (Yuan et al. 2005). These radicals were generated in the phenazin methosulfate-NADH system which contained 3 mL Tris-HCl buffer (16 m*M*, pH 8.0), 78 μ*M* NADH (reduced form), 50 μ*M* nitroblue tetrazolium, 10 μ*M* phenazin methosulfate and varying concentrations of polysaccharides (0.067–0.267 mg·mL^{-1}). The color reaction of the superoxide radicals and nitroblue tetrazolium was detected by monitoring absorbance at 560 nm. In the control, NADH was substituted with Tris-HCl buffer (Nishikimi et al. 1972; Zhou and Zheng 1991). IC_{50} values (concentration of samples required to scavenge 50% of free radicals or to prevent lipid peroxidation by 50%) were calculated from the regression equations prepared from the concentration of samples and percentage inhibition of each system.

Hydroxyl radical scavenging activity

The scavenging activity of seaweed polysaccharides against the hydroxyl radical was investigated using Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$). These results were expressed as an inhibition rate. Hydroxyl radicals exhibit a small diffusion capacity and are most reactive in the induction of injuries to cellular molecules and, accordingly, deserve special attention. Hydroxyl radicals were generated using an modified Smirnoff and Cumbes' method (1989) in 3 mL sodium phosphate buffer (150 m*M*, pH 7.4), which contained 10 m*M* FeSO4.7H_2O, 10 m*M* EDTA, 2 m*M* sodium salicylate, 30% H_2O_2 (200 μL) and varying concentrations of polysaccharides (0.067–0.267 mg/ml). In the control, sodium phosphate buffer replaced H_2O_2. The solutions were incubated at 37°C for 1 h, and presence of the hydroxyl radical was detected by monitoring absorbance at 510 nm.

Liver microsomal lipid peroxidation

Lipid peroxidation is a complex process and when induced by free radicals is the main cause of cellular damage. This process involves the formation and propagation of lipid peroxides, and the eventual destruction of the lipid membranes, producing secondary products such as the malondialdehyde (MDA) in microsomes (Zhang et al. 2003).

Liver microsomes were prepared from Wistar rats, and the effects of polysaccharides on lipid peroxidation were determined according to Liu et al. (1997).

Table 1 Chemical component of the sulfated polysaccharide (g/100 g dry weight)[a]

Polysaccharides	Total sugar[b]	Sulfate[c]	Proteins[d]
Fucoidan	55.20 ± 1.43	44.10 ± 0.16	0.80 ± 0.39
F 0.5	80.24 ± 3.23	18.40 ± 0.28	0.90 ± 0.83
F 1.1	70.10 ± 2.02	27.57 ± 0.18	2.56 ± 0.24
Kappa carrageenan	72.00 ± 3.66	17.90 ± 0.05	1.1 ± 0.31
Iota carrageenan	65.98 ± 0.52	27.60 ± 0.12	1.5 ± 0.55
Lambda carrageenan	64.26 ± 2.36	33.38 ± 0.06	2.0 ± 0.45

[a] Data are mean value of triplicate determinations ± standard deviation.

[b] Dubois et al. (1956)

[c] Turbidimetric method (Dodgson and Price 1962)

[d] Spector (1978)

Table 2 Molecular weight, sugar and sulfate molar ratio in sulfated polysaccharides

Polysaccharides	M.W[a] (kDa)	Molar ratios					
		Fucose	Galactose	Glucose	Xylose	Uronic Acid	Sulfate
Fucoidan	170	1	–	–	–	–	1.8
F 0.5	200	1	0.6	0.2	0.5	3.1	0.5
F 1.1	18	1	0.3	–	0.6	1.5	1.5

[a] The molecular weight was estimated by gel filtration in Sepharose -CL4B, followed by elution with 0.02 *M* acetic acid.

The liver was removed and rapidly homogenized in ice-cold 0.25 *M* sucrose and then centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant obtained was centrifuged at 105,000 *g* for 60 min at 4°C. The microsomes were washed using ice-cold 0.15 *M* KCl, and then stored at −20°C. The lipid peroxidation assay was performed in a Fe2+/vitamin C system. The microsomes (300 $\mu g \cdot mL^{-1}$) were incubated at 37°C for 60 min with varying concentrations of polysaccharide (0.286–1.144 $mg \cdot mL^{-1}$), 10 μ*M* $FeS0_4.7H_2O$ and 0.1 m*M* ascorbic acid in 1.0 mL potassium phosphate buffer (0.2 *M*, pH 7.4). The reaction was stopped by the addition of 20% (wt/vol) trichloroacetic acid (1.0 mL) and 0.67% (wt/vol) 2-thiobarbituric acid (TBA) (1.5 ml) in succession, and the solution was then heated at 100°C for 15 min (Bueg and Aust 1978). The condensation reaction occurring between the MDA and TBA produces a pink compound, which has a strong absorption at 532 nm (Wei et al. 2003).

The percentage of antioxidant activity of the samples was evaluated according to the following formula (Zhang et al. 2003): Inhibition rate (%) = $(A0 - A)/(A0 - Ae) \times 100\%$, where A0 is the absorbance of the free radical generation system, A is the absorbance of the test sample and Ae is the absorbance of the essential control.

Table 3 The IC_{50} for superoxide radicals, hydroxyl radicals and lipid peroxidation (*n.d.* not determined)

	Superoxide radicals	Hydroxyl radicals	Lipid peroxidation
Fucoidan	0.058 ± 0.011	0.157 ± 0.005	1.250 ± 0.174
F 0.5	0.243 ± 0.014	n.d.	2.753 ± 0.051
F 1.1	0.243 ± 0.013	0.353 ± 0.036	23.887 ± 5.975
Lambda-carrageenan	0.046 ± 0.001	0.357 ± 0.120	2.697 ± 0.267
Iota-carrageenan	0.332 ± 0.080	0.281 ± 0.072	0.830 ± 0.063
Kappa-carrageenan	0.112 ± 0.003	0.335 ± 0.016	0.323 ± 0.011

[a] The IC_{50} was calculated by linear regression. Values given are means of triplicate determinations ± SD

Statistical analyses

All of the data were expressed as means ± standard deviation (SD) of three replications, and the ANOVA test was used for statistical analysis. The values were considered to be significantly different when the *p* value was less than 0.05.

Results and discussion

Chemical constitutions of polysaccharides

The chemical constitutions of the polysaccharides are shown in Table 1. In this study we used polysaccharide fractions (fucans) from the brown seaweed *P. gymnospora* fractioned with acetone (0.5 and 1.1 vol.) as previously described by Silva et al. (2005) and Rocha et al. (2005), fucoidan from *Fucus vesiculosus* and carrageenans. The two fucans differ from fucoidan because they are heterogenous polysaccharides, even though both classes of substances are sulfated. Fucoidan (44.1%) and lambda carrageenan (33.38%) were found to have a high sulfate content, while the fucan (F) 0.5 fraction (18.40%) and F1.1 fraction (27.57%) had a relatively low sulfate content – 18.40 and 27.57%, respectively (Table 1). The results of these analyses demonstrate that all of the samples analyzed contained low levels of contamination with protein (0.8–2.5%) and high levels of polysaccharides (55.20–80.24%) (Table 1). Table 2 shows the molar ratio of the sugars of these fucans and fucoidan and the MW of the polysaccharides. Fraction F0.5 is rich in uronic acid, as previously demonstrated by electrophoresis and chem-

ical methods (Dietrich et al. 1995; Silva et al. 2005). The F1.1 fraction contains a small amount of xylose and residual galactose. The sugars of these polysaccharides were previously characterized by gas-liquid chromatography (GLC) and colorimetric methods. The MW of fraction F1.1 was very low (18 kDa) when compared to that of the other polysaccharides studied, such as fucoidan from *F. vesiculosus* (MW: 170,000 kDa), as also described by Patankar et al. (1993) and Santos et al. (2004). The partial chemical analysis of fucoidan showed that this compound contains only sulfated fucose, as suggested by Patankar et al. (1993). Sulfated galactans are classified according to the presence of the 3,6-anhydro bridge on the 4-linked galactose residue and the position and number of sulfate groups. The iota polysaccharide was found to have an average MW above >100 kDa, kappa polysaccharide, 400–600 kDa and lambda polysaccharide, 500–900 kDa; all showed a high polidispersivity. These results coincide with the experimental data obtained by Usov et al. (2002). We observed different levels of sulfate – 17.90, 27.60 and 33.38 for kappa, iota and lambda carrageenans, respectively. The results of these studies are in agreement with values reported in the literature.

Superoxide radical

The fucoidan from *F. vesiculosus* and the F1.1 fraction from *P. gymnospora* showed an IC_{50} of 0.058 and 0.243 mg·mL^{-1} while the F0.5 fraction showed the same value as F1.1. The IC_{50} of the lambda carrageenan (0.046 mg·mL^{-1}) showed a high inhibitory effect ($p < 0.001$) on kappa and iota carrageenans of 0.112 and 0.332 mg·mL^{-1}, respectively (Table 3). Figure 1a and b shows that the fucoidan and lambda carrageenan were more active in inhibiting superoxide radicals. In studies performed by Zhang et al. (2003) with porphyran, a polysaccharide extracted from the red seaweed *Porphyra haitanesis*, the F3 fraction, with a high sulfate content, exhibited strong superoxide radical scavenging activity, while the F1 and F2 fractions displayed weak activity. This demonstrates that there is a degree of variability in the action of these compounds and that the sulfate content affects their antioxidant action. Zhao et al. (2004) also demonstrated that low-MW sulfated polysaccharides (8,000–10,000 Da) from *Laminaria japonica*, a brown seaweed, have the potential ability to stop free radical chain reactions.

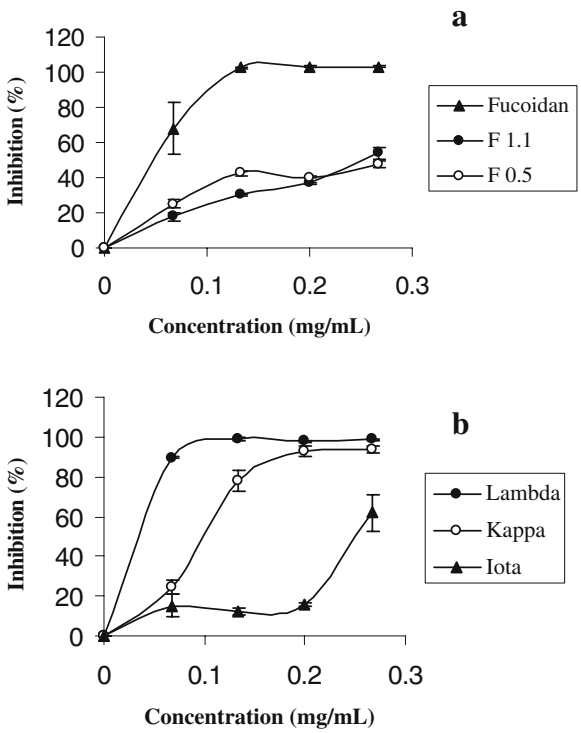

Figure 1 Inhibition of superoxide radicals by sulfated polysaccharides from: fucans of *Padina gymnospora* (fractions F 0.5 and F 1.1) and fucoidan (a) and carrageenans lambda, iota and kappa (b). The standard deviation was 8–12% for three measurements for each sample.

Hydroxyl (OH^-) radical scavenging activity

The results obtained for the inhibition of hydroxyl radical formation demonstrated that all the samples, with the exception of fucoidan, had a moderate effect on inhibiting the formation of these radicals (Figure 2a, b); fucoidan reached an IC_{50} with 0.157 mg·mL^{-1} (Table 3). Surprisingly, the F0.5 fraction exhibited a descending curve at 0.2 and 0.3 mg/ml, suggesting that free radical generation, and not inhibition, probably occurs at these concentrations. This fucan fraction was rich in alginates and exhibited a poor antioxidant activity at low concentrations (Figure 2a). These results are in accordance with those of Zhou and Zheng (1991). Figure 2b shows that the iota carrageenan had a high inhibitory effect on hydroxyl radicals in relation to the lambda and kappa carrageenans.

Hydrogen peroxide scavenging activity

The scavenging effects of various samples on hydrogen peroxide are shown in Figure 3a and b. The free

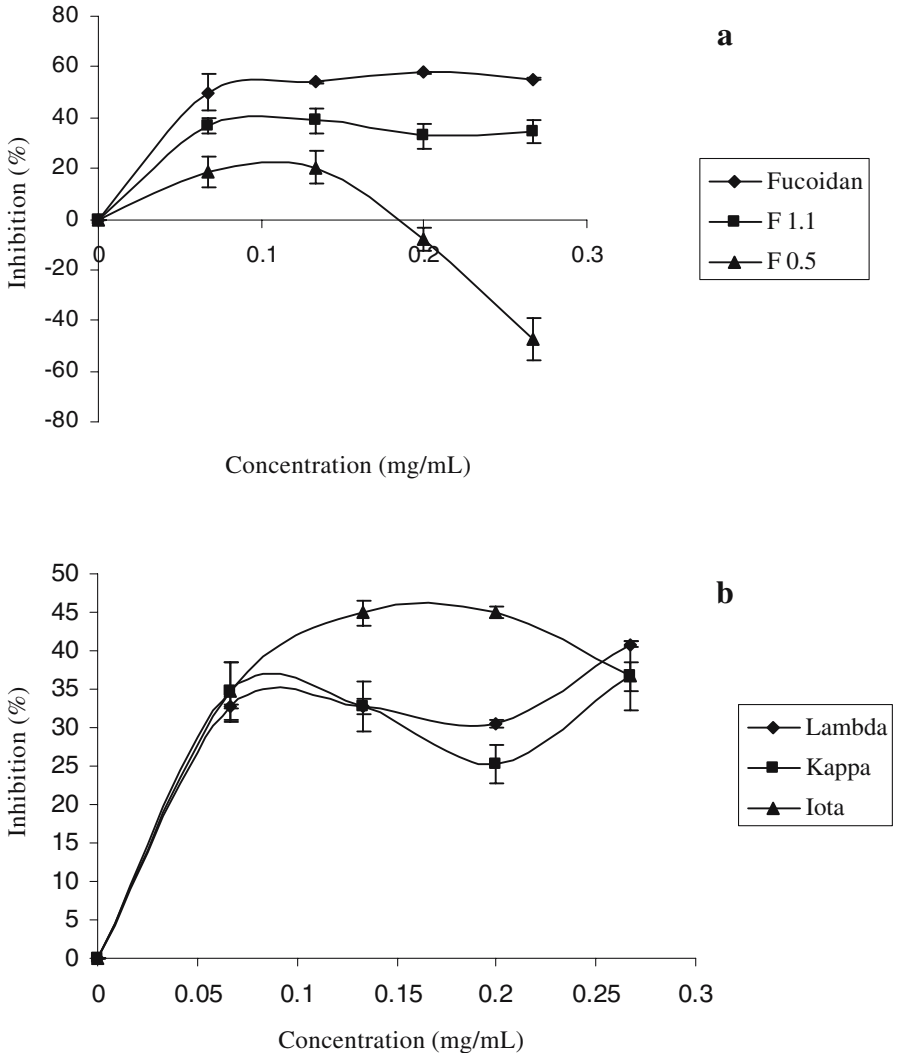

Figure 2 Inhibition of hydroxyl radicals by sulfated polysaccharides from:) fucans of *P. gymnospora* (fractions F0.5 and F1.1) and fucoidan (a) and lambda, kappa and iota carrageenans (b). The standard deviation was 8–12% for three measurements for each sample.

radical formation in this system was inhibited by all of the polysaccharide samples, with IC_{50} values of 1.250, 2.753 and 2.341 mg·mL^{-1} for fucoidan, F0.5 and F1.1, respectively ($p < 0.001$) (Table 3). However, the values obtained for kappa, iota and lambda carrageenans were 2.697, 0.830 and 0.323 mg·mL^{-1} respectively (Table 3). The results found for inhibition are in agreement with those found by Zhang et al. (2003), who observed strong inhibition of MDA production *in vitro* by porphyran from *Porphyra haitanesis* using the same Fe2+/vitamin C system. The relation between polysaccharide structure and function was also analyzed by Yuan et al. (2005). The results of this study, in which oversulfated, acetylated and phosphorylated derivative polymers were used, suggest that the scavenging actions of these compounds are different, with acetylated and oversulfated polymers being effective in scavenging the superoxide radical. The authors further suggest that this action is independent of the MW. Our results showing that fucoidan and lambda carrageenan are better inhibitors of superoxide radical formation are in agreement with those obtained by Zhang et al. (2003) and Yuan et al. (2005). Rupérez et al. (2002) obtained results similar to ours with fucoidan from *F. vesiculosus*\. The potential antioxidant of this polysaccharide was higher than that of the agar-like sulfated galactans. These results are in agreement with those of Matsukawa et al.

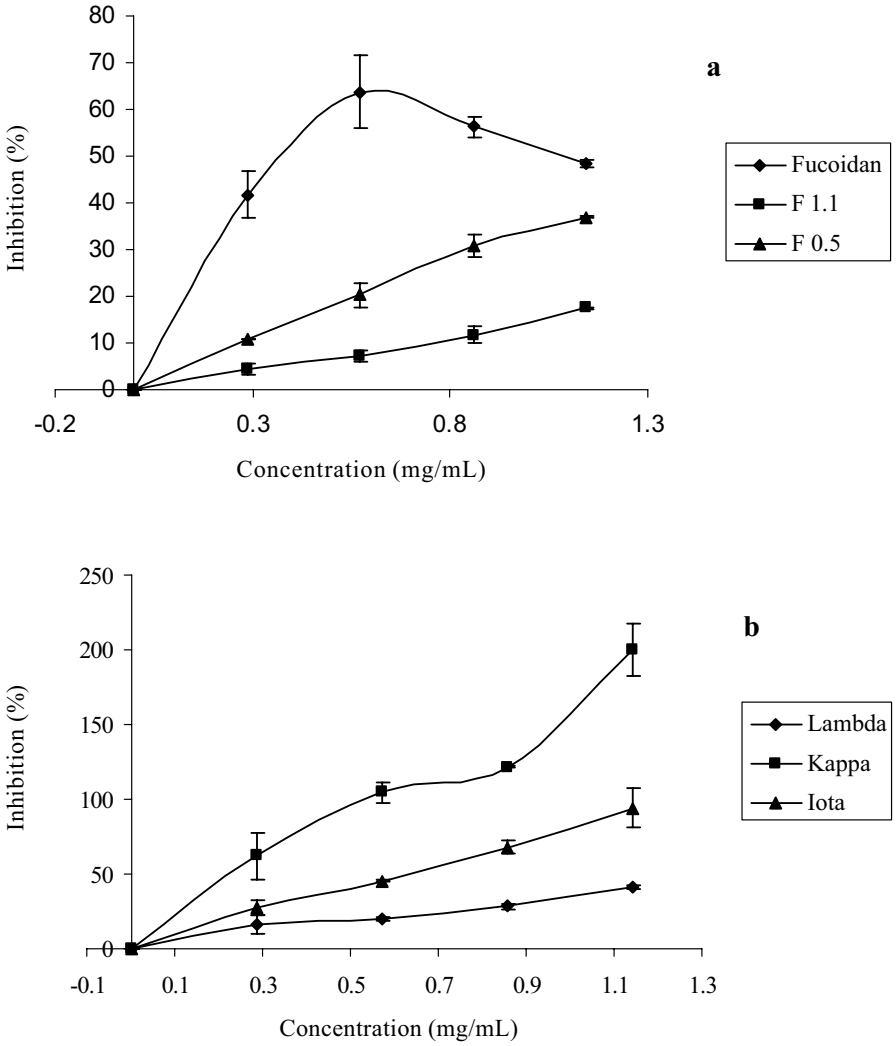

Figure 3 Inhibition of lipid peroxidation of rat liver microsome by sulfated polysaccharides from fucans of *P. gymnospora* (fractions F0.5 and F1.1) and fucoidan (a) and by sulfated polysacchacarides lambda, kappa and iota carrageenans (b). The standard deviation was 8–12% for three measurements for each sample.

(1997) who demonstrated that antioxidant activity of brown seaweed was superior to that of red algae.

Conclusion

The results of the present study indicate that among the different polysaccharides derived from brown and red seaweeds, fucoidan and lambda carrageenan exhibit the highest antioxidant activity and free radical scavenging activity. We found a positive correlation between sulfate content and antioxidant activity. The present findings provide a basis for further experiments on the identification and characterization of specific compounds with relatively high antioxidant activities. Fucoidan has several biological properties, and carrageenans are used in the food industry. Our results also indicate that inclusion of antioxidant-rich polysaccharides or their fractions will probably prevent the oxidative deterioration of food.

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