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Article

Anticancer Activity of Sulfated Polysaccharides Isolated from the Antarctic Red Seaweed *Iridaea cordata*

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Abstract : This study aimed to isolate and characterize sulfated polysaccharides (SPs) from *Iridaea cordata* and evaluate their anticancer activity. SPs of the Antarctic red seaweed were obtained by $CaCl_2$ (SP1) and ethanol precipitations (SP2) following diluted acid extraction at room temperature. Yields of SP1 and SP2 were approximately 14% and 23%, respectively, of the dry weight of red seaweed. The average molecular mass of the SP1 and SP2 was estimated about 1.84×10^3 and 1.42×10^3 kDa, respectively, by size-fractionation High-Performance Liquid Chromatography (HPLC). From the High-Performance Anion-Exchange Chromatography-Pulsed Amperometric Detection (HPAEC-PAD) analysis, the main monosaccharide was galactose with glucose and fucose as minor components. The sulfate content of SP2 (40.4%) was slightly higher than that of SP1 (33.8%). The FT-IR spectra also showed characteristic band of carrageenan-like sulfated polysaccharides. Taken together the SPs are thought to be carrageenan-like sulfated galactan. The polysaccharides (SPs) from *I. cordata* exhibited weak antitumor activity against PC-3 (prostate cancer), HeLa (cervical cancer), and HT-29 (human colon adenocarcinoma). To our knowledge, this is the first data on biological activity of the Antarctic red seaweed *I. cordata*.

Key words : antarctic red seaweed, *Iridaea cordata*, sulfated polysaccharides, antitumor activity

1. Introduction

Red algae contain unique sulfated galactans such as agars and carrageenans (Campo et al. 2009; Usov 2011). Typically the sulfated galactans are linear polymers of alternating 3-linked β-D-galactopyranose and 4-linked α-galactopyranose units. The latter residue is found in the L-configuration in the agars but in the D-configuration in carrageenans (Jiao et al. 2011). The hydroxyl groups in the polysaccharides are methylated, sulfated, or replaced by single monosaccharides. According to sulfation patterns and the presence of 3,6-anhydro-α-galactopyranose in place of α-galactopyranose, carrageenans can be classified into 15 different classes (Lahaye 2001; Usov 2011). These classes of polysaccharides have been used widely in the food industry as gelling and thickening agents (Dhargalkar and Verlecar 2009; Jiao et al. 2011; Usov 2011). In addition, they display a wide range of biological activities, which may be useful for medical application. Antitumor and immunomodulating activities were reported for λ-carrageenan (Tsuji et al. 2003), κ-carrageenan oligosaccharides from the red alga *Kappaphycus striatum* (Hu et al. 2006), low molecular λ-carrageenan from *Chondrus ocellatus* (Yuan et al. 2006; Zhou et al. 2004, 2005), and sulfated polysaccharide (SP) from *Champia feldmannii* (Lins et al. 2009). SPs have also displayed antiviral activities. SPs from *Gymnogongrus griffithsiae*

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and *Cryptonemia crenulata* inhibited dengue virus multiplication in Vero cells (Talarico et al. 2005, 2007; Talarico and Damonte 2007). SPs from *Sebdenia polydactyla* also inhibited the propagation of HSV-1 in the same cells (Ghosh et al. 2009). Superoxide radical scavenging activity of red algal sulfated polysaccharide has also been demonstrated (Souza et al. 2007). Recently, red algal SPs showed anti-diarrheal activity (Sousa et al. 2016), and also improved colitis (Brito et al. 2014) in rat models. The information on biological activities of red algal polysaccharides have been reviewed and compiled (Jiao et al. 2011; Usov 2011).

Iridaea cordata (Turner) Bory, an Antarctic red seaweed, is abundant in upper sublittoral zone, which is also colonized with *Palmaria decipiens* (Amsler et al. 1998; Clayton et al. 1997; Dhargalkar 1990; Dhargalkar and Verlecar 2009; Wiencke et al. 2002; Wiencke and Amsler 2012; Zacher et al. 2009). To our knowledge, this species has only been investigated for its population biomass (Cormaci et al. 1996), physiology (Zacher et al. 2009), life cycle (Wiencke et al. 2002), endophyte-host relationship (Schoenrock et al. 2015), and nutritional composition analysis (Peters et al. 2005). Compared to other temperate red seaweed, studies on polysaccharide of cell walls and the intercellular matrix of Antarctic seaweed are very limited. To our knowledge, only acidic polysaccharide extracted from *P. decipiens*, the most abundant Rhodophyta in the Antarctic Peninsula has been reported (Matsuhiro and Urzua 1996; Jerez et al. 1997). Further investigation into SPs from the Antarctic macroalgae and their biological properties will facilitate the exploitation for potential health benefits. In this study, we extracted and characterized SP from *I. cordata* (Turner) Bory and examined its antitumor activity.

2. Materials and Methods

Extraction of acidic polysaccharide from *I. cordata*

I. cordata was collected in austral summer 2012 at the King Sejong Station, King George Island, Antarctica. Polysaccharides from *I. cordata* were extracted as described by Kim et al. (2004) with slight modification. Briefly, 10 g of the dried *I. cordata* was cut into small pieces ($\leq 3 \times 3$ cm), ground, and placed in 0.5 L of 0.1 N HCl for 24 h at ambient room temperature. The extract was then filtered through a nylon cloth. The filtrate was neutralized with 1 N NaOH, precipitated with 3 volumes of ethanol, and centrifuged for 30 min at $6,000 \times g$. The precipitate was dissolved in water and then pH of the solution was adjusted to 2.0 with 1 N HCl. To this suspension, $CaCl_2$ was added to obtain a final concentration of 4 M. The resulting precipitate (SP1) was collected by centrifugation and the supernatant was further treated with 3 volumes of ethanol. Precipitation by ethanol was repeated twice. The precipitate (SP2) was dissolved in water, dialyzed (MWCO 14,000) at 4°C against water for 48 h, and then freeze-dried (Fig. 1). The SP1 and SP2 were further purified by anion-exchange chromatography. A total of 50 milligrams of SP1 and SP2 in 10 mL of distilled water (dH_2O) were loaded to a DEAE-cellulose column (3 Å, ~45 cm) pre-equilibrated with water, and eluted with a linear NaCl gradient from 0 to 3 M until no carbohydrate is detected by the phenol-sulfuric acid method (Dubois et al. 1956). The carbohydrate-positive fraction was pooled, dialyzed (MWCO 14,000) for 24 h against dH_2O, and lyophilized.

General methods

The total neutral carbohydrate content of the SPs was determined by the phenol-sulfuric acid method (Dubois et al. 1956) using D-glucose as a standard. The amount of sulfate residues was determined by the Loui's method (Silvestri et al. 1982) using Na_2SO_4 as a standard. Uronic acid content was quantified by the carbazole reaction (Bitter and Muir 1962) using D-glucuronic acid as a standard. Protein was quantified by the Bradford method (Bradford 1976).

Acid hydrolysis of SP1 and SP2, and monosaccharide analysis by HPAEC-PAD

To determine the monosaccharide composition of SP1 and SP2, 10 mg of freeze-dried polysaccharides was dissolved in 1 mL of dH_2O and equal volume of 4 M trifluoroacetic acid (TFA) was added. The mixture was gently stirred for 2 h at 100°C then filtered through 0.45 μm syringe filter and vacuum-dried using a Speed-Vac. The dried material was dissolved in 0.1 mL of dH_2O and dried. The monosaccharide analysis of TFA-hydrolyzed compounds was carried out by high-performance anion-exchange chromatography (HPAEC) using Bio-LC DX 500 Chromatography System (Dionex Co., USA) equipped with a pulsed amperometric detector (ED 50, Dionex Co., USA) (Lee et al. 2006).

Estimation of the molecular weight of SP1 and SP2

The relative molecular masses of the SP1 and SP2 was estimated by size-exclusion chromatography (SEC) using a Shodex OHpak column (SB-806HQ, 8.0×300 mm,

Showa Denko Co., Japan). A solution of 10 μL of 0.5% SP1 and SP2 in water was injected and eluted with water at the flow rate of 0.8 mL/min at 60°C, and detected with evaporative light scattering detector (ELSD) (Alltech). Pullulans of 2,000, 710, 106, 45, and 11.2 kDa were purchased from Sigma (USA) and used as the relative molecular mass standards.

Vibration spectroscopy

FT-IR spectra (spectral region 4000–400 cm^{-1}, resolution 2 cm^{-1}) of the solid samples in the form of KBr tablets were measured on a Nicolet 6700 spectrophotometer (Thermo Scientific, USA). Vibration spectra were 10-point filtered and baseline corrected using Origin 6.0 (Microcal Origin) software. The second derivatives of the spectra were used for wavenumber determination of overlapped bands.

Cell culture and antitumor activity

Cancer cells including HeLa (cervical cancer cells, ATCC No. CCL-2™), PC-3 (prostate cancer cells, ATCC No. CRL-1435™), and HT-29 (human colon adenocarcinoma cells, ATCC No. HTB-38™) cells, were grown in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO, USA). The cells were maintained at 37°C under 5% CO_2 and subcultured twice a week. To determine the antitumor activity of SP1 and SP2, cells were subcultured in 96-well plates at a density of 5×10^4 cells per well. After monolayer cultivation for 24 h in 5% CO_2 at 37°C, the medium was removed and replaced with 100 μL of the maintenance medium (MM) containing 2% FBS. Cells were then incubated for 24 h with different concentrations (0–1 mg/mL) of SPs. The cultures were reincubated for an additional 4 h with 20 μL of 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. After removal of the supernatant, 100 μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the crystals completely and then the absorbance was measured at 570 nm using an ELISA Reader.

3. Results and Discussion

Extraction, purification, and molecular weight estimation of sulfated polysaccharides

SPs were extracted from the dried powder of the *I. cordata* after treatment with a dilute HCl solution at room temperature as shown in Fig. 1. The first ethanol precipitates were dissolved in water, and treated with $CaCl_2$ solution

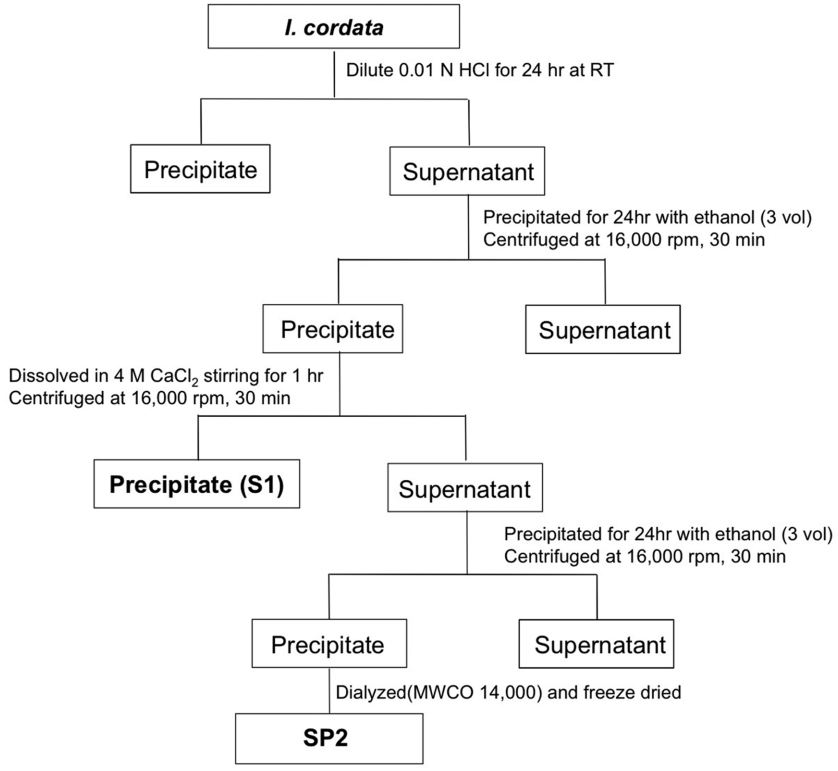

Fig. 1. Scheme of isolation of polysaccharide from the *I. cordata*

Table 1. Yields of the polysaccharides extracts from *I. cordata*

Species	SP1	SP2
	% in mass (10 g)	% in mass (10 g)
I. cordata	14 (1.4 g)	23 (2.3 g)

to separate gelling polysaccharides (SP1) and the soluble fraction (SP2) was further precipitated by ethanol and harvested. The yields of SP1 and SP2 were approximately 15 and 23% of the mass of the red seaweed dry weight, respectively (Table 1). The yield of SP2 is comparable to that of hot water extraction followed by cetrimide precipitation of *P. deipiens* (26%) (Matsuhiro and Urzua 1996), and that of red seaweed *Hypnea musciformis* (25%) using the same dilute acid extraction method (Knutsen et al. 1995). To further purify the crude SP1 and SP2, samples were loaded onto a DEAE-cellulose column and a single symmetrical peak was eluted at approximately 1.4 M NaCl in all cases (data not shown). The molecular weight (MW) of SP1 and SP2 was analyzed in dH_2O at 60°C using SEC along with an ELSD. The average molecular mass of SP1 and SP2 was estimated to be 1.81×10^3 and 1.42×10^3 kDa, respectively. The DEAE and SEC chromatogram data support that SPs purified in this study are homogeneous (data not shown).

Chemical composition of SPs

SP1 and SP2 contained 35% and 50% neutral sugars, 2.6% and 6.3% uronic acid, 1.7% and 2.5% protein, and 33.8% and 40.4% sulfate esters, respectively. This data demonstrates that these polysaccharides are highly sulfated like carrageenans κ and λ, which are usually 25% and 40% sulfated, respectively (Witvrouw and De Clercq 1997). It is also supported by data that show polysaccharides from *Gigartina skottsbergii, Tichocarpus crinitus, Gloiopeltis furcate, Chondrus crispus,* and *Halymenia durvillei, Geogiella confluens,* and *Cryptonemia seminervis* are sulfated 15–42% (Barabanova et al. 2005; Fenoradosoa et al. 2009; Kolender and Matulewicz 2002; Mendes et al. 2014; McCandless et al. 1973; Piriz and Cerezo 1991; Yu et al. 2010). In contrast, low sulfate content was detected

Table 2. Monosaccharide composition of the SPs isolated from *I. cordata*

Monomer	Molar ratio[1)]		Relative area (%)	
	SP1	SP2	SP1	SP2
L-fucose	0.03	0.08	1.42	4.79
D-galactose	1	1	71.95	94.53
D-glucose	0.35	0.01	26.6	0.67

The values were obtained from the area of each peak on the HPAEC-PAD chromatogram of acid hydrolysate of the isolated SPs

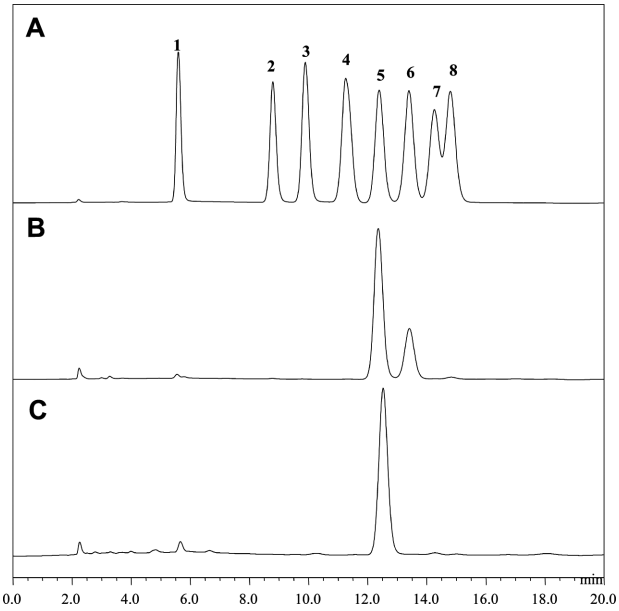

Fig. 2. HPAEC-PAD analysis for monosaccharide composition of the SPs. A, Chromatogram of standard monosaccharides (Sigma); B, Chromatogram of acid hydrolysates of SP1; C, Chromatogram of acid hydrolysates of SP2; 1; Fuc, 2; Rha, 3; Ara, 4; GlcN, 5; Gal, 6; Glc, 7; Man, 8; Xyl

in acidic polysaccharides from other red seaweed: 2.8% from *P. decipiens* (Matsuhiro and Urzua 1996), 8.4% from *Grarcilaria birdiae* (Souza et al. 2012), 4.8% from *Gracilaria cornea* (Melo et al. 2002), 1.2% from *Gracilaria dura* (Marinho-Soriano and Bourret 2005), and 5.08% from *H. musciformis* (Sousa et al. 2016). The monosaccharide composition of SP1 and SP2 analyzed by

Table 3. Chemical composition of the SPs

Algal source	Uronic acid (mass %)	Protein (mass %)	Neutral sugar (mass %)	Sulfate (mass %)	Proportion of monosaccharide (mole %)[1)]		
					Fuc	Gal	Glc
SP1	5.1	1.7	44	33.8	2.3	72.9	24.8
SP2	6.3	2.5	50	40.4	7.4	92.0	0.6

[1)]Values were obtained by setting the sum of each mole number at 100%

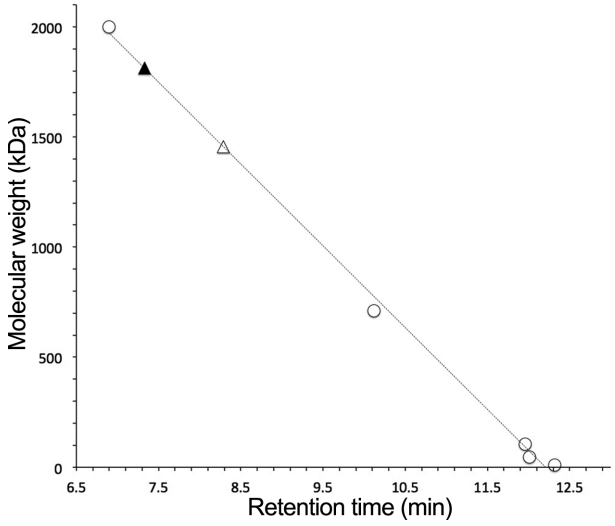

Fig. 3. Estimation of the molecular weight of SPs by size-exclusion HPLC. Molecular weights of SP1 (filled triangle) and SP2 (empty triangle) are estimated based on the pullulan standard size markers (empty circles) of 2000, 710, 106, 45.9, and 11.2 kDa

HPAEC-PAD revealed that galactose is the major monosaccharide (92.9% and 91.3% in mole %), while fucose (6.5% and 7.4%), glucose (1.8% and 0.6%), and xylose (0.9% and 0.7%) are present as minor components (Table 3 and Fig. 2). The detection of fucose in red seaweed is not common but is occasionally reported. The antioxidant SPs extracted from red seaweed *Gloiopeltis tenax* contained 20.03% fucose which forms α(1→3)-linked branches (Lim and Ryu 2009). A sulfated galactan extracted from *H. durvillei* also contained arabinose and fucose in minor amounts (Fenoradosoa et al. 2009). Considering high sulfate content and monosaccharide composition, SP1 and SP2 are thought to be carrageenan-like sulfated galactans.

Vibration spectroscopy

The FT-IR spectra of SP1 and SP2 were shown in Fig. 4. The assignments of FT-IR spectra on SP1 and SP2 are shown in Table 4 and were made according to the literature (Pereira et al. 2003; Qiu et al. 2006; Sekkal and Legrand 1993; Sekkal et al. 1993; Synytsya et al. 2003). The data revealed the presence of characteristic bands of SP in red seaweeds; the very intense and broad IR bands at 1219 and 1211 cm^{-1} were assigned to asymmetric O=S=O stretching vibration of sulfate esters with some contribution from vibration of COH, CC and CO. The intensity of these bands is proportional to the degree of sulfation (Pereira et al. 2009). Additionally the medium

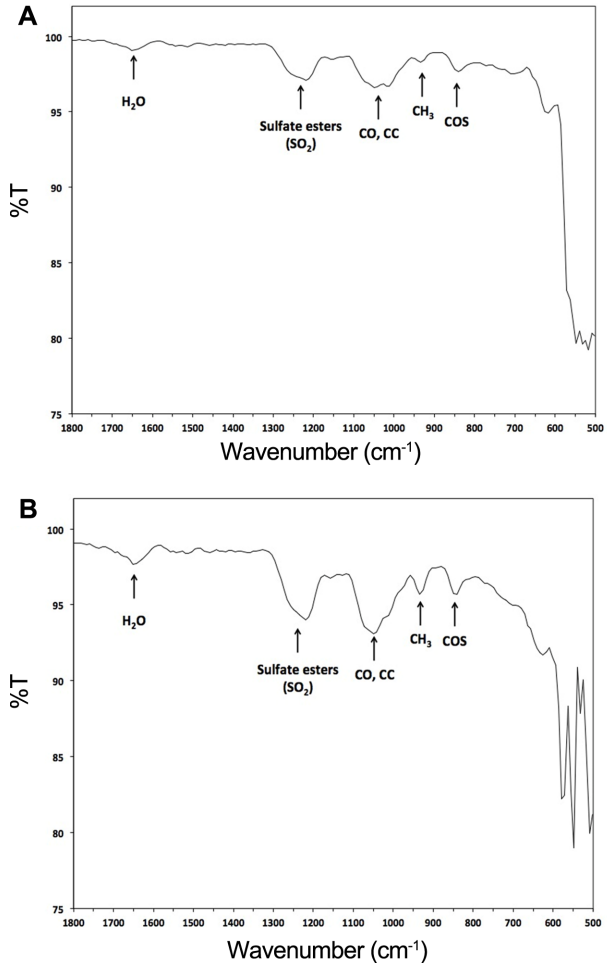

Fig. 4. FT-IR spectra of SP1 (A) and SP2 (B) from *I. cordata*. Spectral range of 1800−500 (1/cm) displayed

Table 4. IR band assignment for SPs SPs

Wavenumber (cm^{-1})		Assignment
SP1	SP2	
1735	1743	ν(C=O) − Ac
1643	1651	δ(H_2O)
1450	1469	δ(CH_2) − Gal; δ_{as}(CH_3) − Fuc, Ac
1219	1211	ν_{as}(SO_2)
1134	1149	ν(COC)
1041	1033	ν(CO),(CC)
933	933	Gal
840	840	ν_s(COS)
586	578	δ_s(SO_2)
		γ(CCO), γ(CCC)
		ρ(SO_3), γ(CCO), γ(CCC)
		γ(CCO), τ(CC)
		τ(CO), γ(CCO)
		τ(CC), τ(CO)

strength signal at 840 cm^{-1} indicated that the sulfate ester is predominantly an axial 4-sulfate of galactopyranosyl residue (Qiu et al. 2006). However we cannot rule out that there exists 6-sulfate on galactose unit and 2-sulfate ester group on a 3,6-anhydrogalactosyl unit due to the broad band around 840 cm^{-1} and its shoulder (Falshaw et al. 2005). The IR features at 586 and 578 cm^{-1} of SP1 and SP2, respectively, were attributed to the asymmetric and symmetric O=S=O deformation of sulfates (Sekkal and Legrand 1993). The strong to medium IR bands at 1200–970 cm^{-1} are caused mainly by CC and CO stretching in pyranoid ring and COC stretching of glycosidic bonds. Intense absorption at this region is common for all polysaccharides (Synytsya et al. 2003). The presence of abosorption bands characteristic of carrageenans at 1140, 1040, and 580 cm^{-1} also supported that SP1 and SP2 are carrageenan-like SPs.

Antitumor activity

The antitumor activity of SP1 and SP2 against HeLa, HT-29, and PC-3 cell lines was investigated (Fig. 5A–C). Overall, SP1 displayed higher antitumor activity than SP2 against HeLa and HT-29 cells, but similar antitumor activity to SP2 against PC-3 cells. SP1 and SP2 displayed significant antitumor activity at 1000 μg/mL: against HeLa cells (68.4% and 30.1% (Fig. 5A)), HT-29 cells (59.9% and 48.7% (Fig. 5B)), and PC-3 cells (59.8% and 56.4% (Fig. 5C)). No cytotoxicity in either SP was detected when non-tumorigenic Vero cell, which are African green monkey kidney cells (ATCC, USA), were incubated for 24 h in the presence of up to 1000 μg/mL of the SPs (data not shown). These results demonstrated that the SPs obtained from *I. cordata* possess a slight antitumor activity. Previous research showed that biological activity of SPs from red alga seem to strongly relate with sulfate content (Coombe et al. 1987) and molecular mass (Zhou et al. 2004). Yamada et al. (1997) showed that the sulfation of κ-carrageenan increased its biological effects. In addition 15 kDa λ-carrageenan with 28% sulfation content from *C. ocellatus* demonstrated higher antitumor activity (68.97%) than higher molecular weight counterparts did. Considering the high molecular weights of SP1 and SP2, the antitumor activity of acidic polysaccharides is thought to be moderately weak.

In conclusion, SPs extracted from *I. cordata* have high molecular weights and high degrees of sulfation. Based on the monosaccharide composition and FT-IR analysis, they are likely to be sulfated galactans containing glucose and fucose. Their antitumor activities, even though weak, make them attractive for medical application. Our current data are too preliminary to elucidate the relationship between the structures of these two polysaccharides and

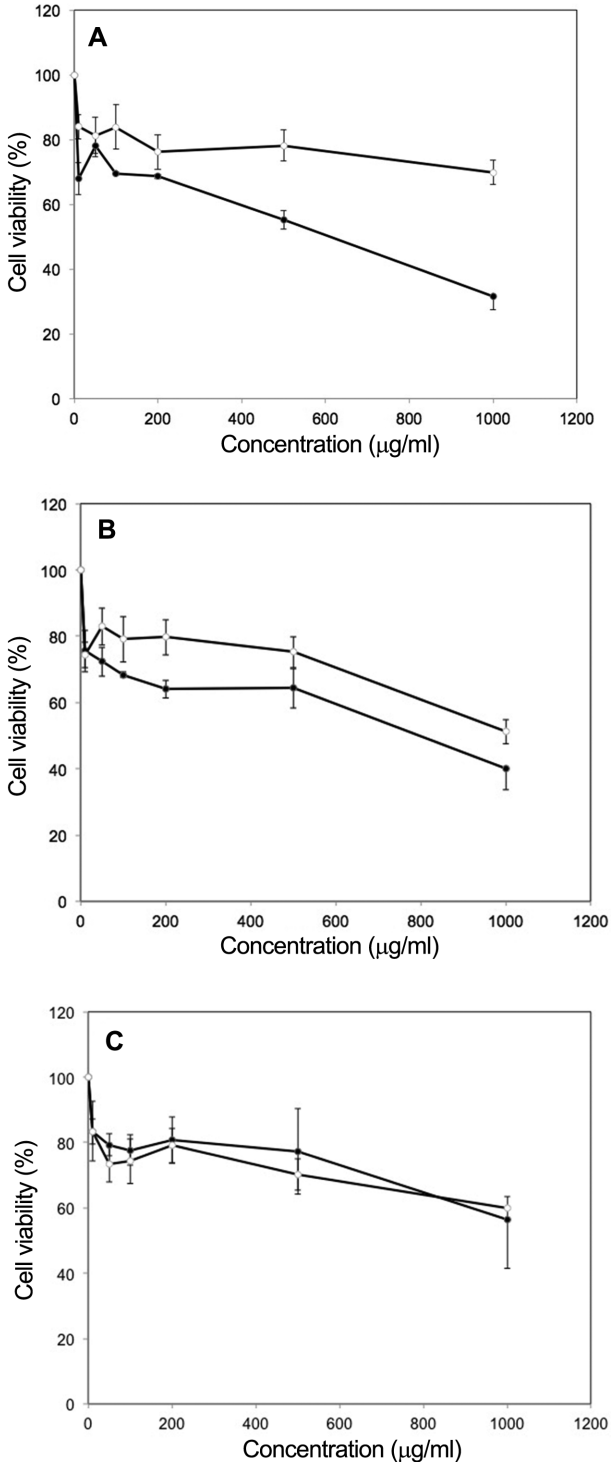

Fig. 5. Antitumor activity of the SPs. S1 (closed circle) and SPs S2 (open circle); HeLa (A), HT-29 (B), and PC-3 (C) cells

their biological activities completely; however, we believe this result will fuel further investigation of the biological properties of underexplored polysaccharides from the Antarctic macroalgae.

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