Appl Biochem Biotechnol (2011) 164:841–850
DOI 10.1007/s12010-011-9178-2

Fucoidans from Brown Seaweeds *Sargassum hornery*, *Eclonia cava*, *Costaria costata*: Structural Characteristics and Anticancer Activity

Svetlana Ermakova · Roza Sokolova · Sang-Min Kim · Byung-Hun Um · Vladimir Isakov · Tatyana Zvyagintseva

Received: 18 October 2010 / Accepted: 18 January 2011 /
Published online: 8 February 2011

Abstract Fucoidans were isolated by water extraction and ion-exchange chromatography from brown algae *Eclonia cava*, *Sargassum hornery*, and *Costaria costata* collected near of Korean coasts. The structures of fucoidans were investigated. Fucoidan from *E. cava* was mixture of sulfated rhamnogalactofucan and galactofucan. Fucoidan from *C. costata* was a sulfated galactofucan. Fucoidan isolated from *S. hornery* was separated into three fractions: a homofucan sulfate, a homofucan but without sulfate groups, and a sulfated rhamnofucan. The results clearly showed that fucoidans play an inhibitory role in colony formation in human melanoma and colon cancer cells and may be effective antitumor agents.

Keywords Fucoidans · Structure · Anticancer activity

Introduction

Brown seaweeds find practical application as a source of structurally and functionally unique polysaccharides: alginic acids, laminarans, and fucoidans. They are the most abundant polysaccharides of brown seaweeds: the amounts vary from 40% to 80% of dry defatted seaweed biomass. The content of water-soluble polysaccharides from different sources and their structures were shown to depend on the area and season of harvesting [1]. Alginic acids and their salts (alginates) are widely used in the food-processing industry, biotechnology, and medicine [2]. Other components of the biomass, to which belong polysaccharides of another chemical nature (laminarans and fucoidans) and low molecular metabolites: mannitol, free amino acids, polyphenols, iodine-containing compounds, vitamins, and lipids [3], could also easily be obtained at a complex industrial processing

S. Ermakova (✉) · R. Sokolova · V. Isakov · T. Zvyagintseva
Pacific Institute of Bioorganic Chemistry of Far Eastern Branch of the Russian Academy of Sciences, Vladivostok 690022 Russia
e-mail: svetlana_ermakova@hotmail.com

S.-M. Kim · B.-H. Um
Korea Institute of Science and Technology, Gangneung 210-340 South Korea

of seaweed. These substances, first of all, fucoidans, are of interest mainly as biologically active compounds [4, 5]. Brown seaweeds fucoidans are highly branched, heterogeneous in monosaccharide composition and have a high molecular weight. They are comprised of long chains of linked sugar molecules, decorated with sulfate groups which make them negatively charged. Each spice of brown seaweed has a characteristic fucoidan. Very few fucoidans have been fully characterized. The brown seaweeds *Eclonia cava*, *Sargassum hornery*, and *Costaria costata* are widely spread in the seas of the South Korea and convenient for practical utilization. In this work, we described the purification of fucoidans from seaweeds *E. cava*, *S. hornery*, and *C. costata*, their characterization and anticancer activity investigation.

Materials and Methods

Reagents and Materials

The mediums Roswell Park Memorial Institute medium (RPMI), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin–streptomycin, and gentamicin were purchased from Biolot (Russia). CellTiter 96 nonradioactive cell proliferation assay kit was purchased from Promega (Madison, WI, USA). The solvents used for extraction were analytical grade and purchased from Daejung Chemical (Gyunggi, Korea).

Polysaccharide Extraction

A sample of the seaweed *E. cava* was collected from Hyunpo, Ulleung Island, and *S. hornery* and *C. costata* were collected from Jumunjin, Gangneung, Korea in April and in May 2009, respectively. The isolation and separation of water-soluble polysaccharides were carried out by modified methods [6, 7]. Fresh or deep-frozen seaweed biomass (1 kg) was treated subsequently with ethanol, acetone, and chloroform. Samples of defatted, dried, and powdered algal fronds (200 g) were extracted twice with 0.1 M HCl (2.5 L) for 2 h at 60 °C. The extracts were combined, centrifugated, dialyzed, concentrated, and polysaccharides were precipitated with four volumes of 96% ethanol. The precipitates were washed with 96% ethanol and acetone and air-dried.

Anion Exchange Chromatography

A solution of polysaccharide in 0.1 M NaCl (0.5 g in 10 ml) was applied onto a Macro-prep DEAE (Bio-Rad, USA) column (Cl^- form, 2.5×9 cm) equilibrated with 0.1 M NaCl. The column was then successively eluted with linear gradient of NaCl (from 0 to 2 M). Eluates obtained were analyzed by the phenol–sulfuric acid method [8]. The polysaccharide fractions were concentrated by ultrafiltration (1-kDa cutoff), dialyzed, and lyophilized.

Analytical Procedures

Total carbohydrates were quantified by the phenol–sulfuric acid method [8]. Monosaccharide composition was determined by HPLC with column ISA-07/S2504 (0.4×25 cm, Shimadzu), bicinhoninate assay, and a C-R2 AX integrating system (Shimadzu, Kyoto, Japan) after hydrolysis by 2 M trifluoroacetic acid (6 h, 100 °C). The content of proteins was determined by the method of Lowry et al. [9]. Sulfate group determination was carried out using the $BaCl_2$ gelatin method [10].

^{13}C-Nuclear Magnetic Resonance

Spectra of ^{13}C-nuclear magnetic resonance (NMR) for solutions of substances in D_2O were obtained on an Avance DPX-500 NMR spectrometer (Bruker, Berlin, Germany) with a working frequency of 75.5 MHz at 60 °C.

Cell Culture

The SK-MEL-28 (ATCC no. HTB-72™) human skin melanoma cell line was grown in monolayer in DMEM supplemented with 10% (*v*/*v*) heat-inactivated FBS, 2 mM L-glutamine, and 1% penicillin–streptomycin a humidified atmosphere containing 5% CO_2. The DLD-1 (ATCC no. CCL-221™) human colon cancer cell line was grown in monolayer in RPMI-1640 supplemented with 10% (*v*/*v*) heat-inactivated FBS, 2 mM L-glutamine, and 1% penicillin–streptomycin in a humidified atmosphere containing 5% CO_2.

Cell Proliferation Assay

To estimate cell cytotoxicity, cells (SK-MEL-28 and DLD-1) were seeded (3×10^4/well) in 96-well plates in 200 μl of 10% FBS McCoy 5a at 37 °C in a 5% CO_2 incubator. After 24 h, the medium was removed and replaced by a fresh medium containing the different concentrations (50, 100, 200, and 400 μg/mL) of the polysaccharides for additional 24 h at 37 °C in a 5% CO_2 incubator. After incubation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS reagent) (10 μL) was added to each well, and cells were then incubated for 4 h at 37 °C in a 5% CO_2. Absorbance was measured at 490/630 nm.

Soft Agar Clonogenic Assay

Soft agar assay was performed on human skin melanoma and colon cancer cells, SK-MEL-28 and DLD-1. In brief, cells (2.4×10^4/mL) were grown in 1 ml of 0.3% basal medium Eagle's (BME's) agar containing 10% FBS. The culture was maintained at 37 °C in 5% CO_2 incubator for 3 weeks, and the cell colonies were scored using a microscope and the ImageJ computer software program as described by Colburn et al. [11].

Data Analysis

All figures shown in this manuscript are representative of at least three independent experiments with similar results. Statistical differences were evaluated using the Student's *t* test and considered significant at $p\leq0.05$.

Results and Discussion

Purification and Structural Characteristics of Fucoidans from Brown Seaweeds *E. cava*, *S. hornery*, and *C. costata*

Polysaccharides were isolated from the seaweeds *E. cava* and *S. hornery* and *C. costata* collected from Hyunpo and Jumunjin, respectively, in Korea as mentioned in Materials and methods. The extraction of polysaccharides from the fresh or deep-frozen seaweeds was

carried out by modified method [7]. Seaweeds were initially defatted with ethanol (three times) to remove pigments and other low molecular weight compounds. Water-soluble polysaccharides were then extracted from defatted biomass with 0.1 M HCl at 60 °C. The extracts were combined, concentrated, and dialyzed, and polysaccharides were precipitated with ethanol. The resulting crude polysaccharides were purified and fractionated by ion-exchange chromatography on Macro-prep DEAE using linear gradient of NaCl from 0 to 2 M.

Ion-exchange chromatography on Macro-prep DEAE separated crude polysaccharides from extract into three peaks for *S. hornery* (ShF1, ShF2, ShF3), into two peaks for *E. cava* (EcF1, EcF2), and into one peak for *C. costata* (CcF) (Fig. 1). The chemical composition of the six obtained polysaccharides fractions are presented in Table 1.

Fraction ShF1 was a homofucan sulfate containing fucose residues and sulfate (14.9%), and only traces of other monosaccharide constituents. It is interesting to note that fraction ShF2 also had fucose residues and trace of rhamnose residues but without sulfate groups. Fraction ShF3 was a sulfated (16.9% of sulfate) rhamnofucan.

Two fucoidans were purified from brown seaweed *E. cava* by ion-exchange chromatography. Fucoidans EcF1 and EcF2 were sulfated rhamnogalactofucan and galactoglucofucan, respectively, with corresponding containing of sulfate groups 19% and 22%. Fucoidan from *C. costata* CcF was a sulfated galactofucan and contained almost 19% of sulfate groups. This fucoidan has a high content of sulfates compare with fucoidan purified from *C. costata* (5.3% of sulfate) collected in the Trinity Bay (Sea of Japan, Russia) [12]. It is well known that polysaccharides structure is dependent on seaweeds species, area, and season of harvesting [1].

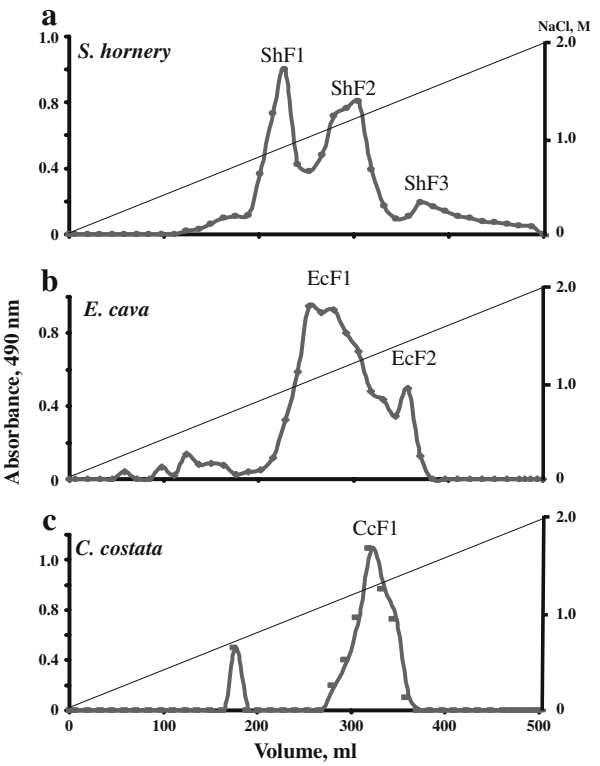

Fig. 1 Chromatography on Macro-prep DEAE (Cl^- form, 2.5×9 cm) column of fucoidans from brown seaweeds. **a** *S. hornery* (ShF1, ShF2, ShF3). **b** *E. cava* (EcF1, EcF2). **c** *C. costata* (CcF)

Table 1 Composition of fucoidans from brown seaweeds *S. hornery*, *E. cava*, and *C. costata* obtained by ion-exchange chromatography

Fraction	SO_3Na, %	Neutral monosaccharides, mol%						Notes
		Fuc	Gal	Man	Rha	Xyl	Glc	
ShF1	14.9	1	0.1	0	0.09	0.04	0	Sulfated fucan
ShF2	0	1	0	0	0.11	0	0	Nonsulfated fucan
ShF3	16.9	1	0	0	0.44	0	0	Sulfated rhamnofucan
EcF1	19.1	1	0.21	0.05	0.16	0	0	Sulfated rhamnogalactofucan
EcF2	22.2	1	0.28	0	0.08	0	0.4	Sulfated galactoglucofucan
CcF	18.9	1	0.83	0.01	0.05	0.06	0	Sulfated galactofucan

All purified fucoidans were subjected to structural analysis. Fucoidans presented in brown seaweeds are usually very complex nonregular polysaccharides (Fig. 2), which may be even mixtures of molecules of different structural types. As a result, their structural analysis is extremely difficult. The structure of isolated polysaccharides was elucidated by combination of chemical methods of structural analysis (Table 1) and NMR spectroscopy (Fig. 2).

According to NMR spectroscopy data, fucoidan from *S. hornery* ShF1 has a linear chain of (1,3)-linked α-L-fucopyranose residues (100.29 and 99.69 ppm) with sulfate groups at positions 2 (76.07, 74.34, 69.46, and 17.03 ppm)(Fig. 2a).

Fucoidan from *S. hornery* ShF2 has a linear chain of alternating (1,3)- and (1,4)-linked α-L-fucopyranose residues (100.3, 99.11, and 94.8 ppm) without sulfate groups (Fig. 2b).

Fucoidan from *S. hornery* ShF3 is presumably a linear chain of (1,4)-linked α-L-fucopyranose residues (98.8 and 82.06 ppm) with sulfate groups at positions 2 (74.81 and 76.88 ppm) (Fig. 2c). We did not find a signals in NMR spectra related to (1,2)-linked α-L-fucopyranose residues for fucoidans from *S. hornery* as published Preeprame S. base of methylation analysis of sulfated polysaccharides. According to his data, one fifth of the fucose residues were estimated to be branched residues, and sulfate groups were found to be substituted at C2 or C4 in 1,3-linked residue and C3 of 1,2- and 1,4-linked residues.

Fucoidan from *E. cava* EcF1 is probably high molecular weight α-L-fucan because the area of C1 signals is difficult to resolve (Fig. 2d). EcF1 has some amount of rhamnose residues (17.9 ppm).

Fucoidan EcF2 has a linear chain of alternating (1,3)- or (1,6)-, and (1,4)-linked α-L-fucopyranose residues (102.86, 98.47, 96.77, and 96.37 ppm) with sulfate groups at positions 2 (83.28 ppm). It is likely that fucoidan EcF2 contains fragment buildup of β-1,3- and β-1,6-linked glucose residues (103.2 and 103.47 ppm) (Fig. 2e). It was reported that fucoidan from *Saccharina latissima* (formerly *Laminaria saccharina*) was very heterogenic in monosaccharide composition and contains sustention amount of glucose residues in its backbone also [13].

The NMR spectra of fucoidan from *C. costata* CcF are complex, showing a number of peaks in anomeric region. The NMR spectra showed peaks corresponding the acetyl groups (20.98, 173.85, and 173.70 ppm), methyl groups of fucopyranose residue (16.01–16.98 ppm), and galactopyranose residues with nonsubstituted CH_2OH groups (61.6–61.89 ppm) (Fig. 2f). Anomeric resonances are equal by intensity of signal of NMR spectra because of regular structure of polysaccharide. So, our data indicate that fucoidan CcF is galactofucan sulfated and acetylated at different position of galactose and fucose residues except for C6 of galactose residues which are nonsubstituted.

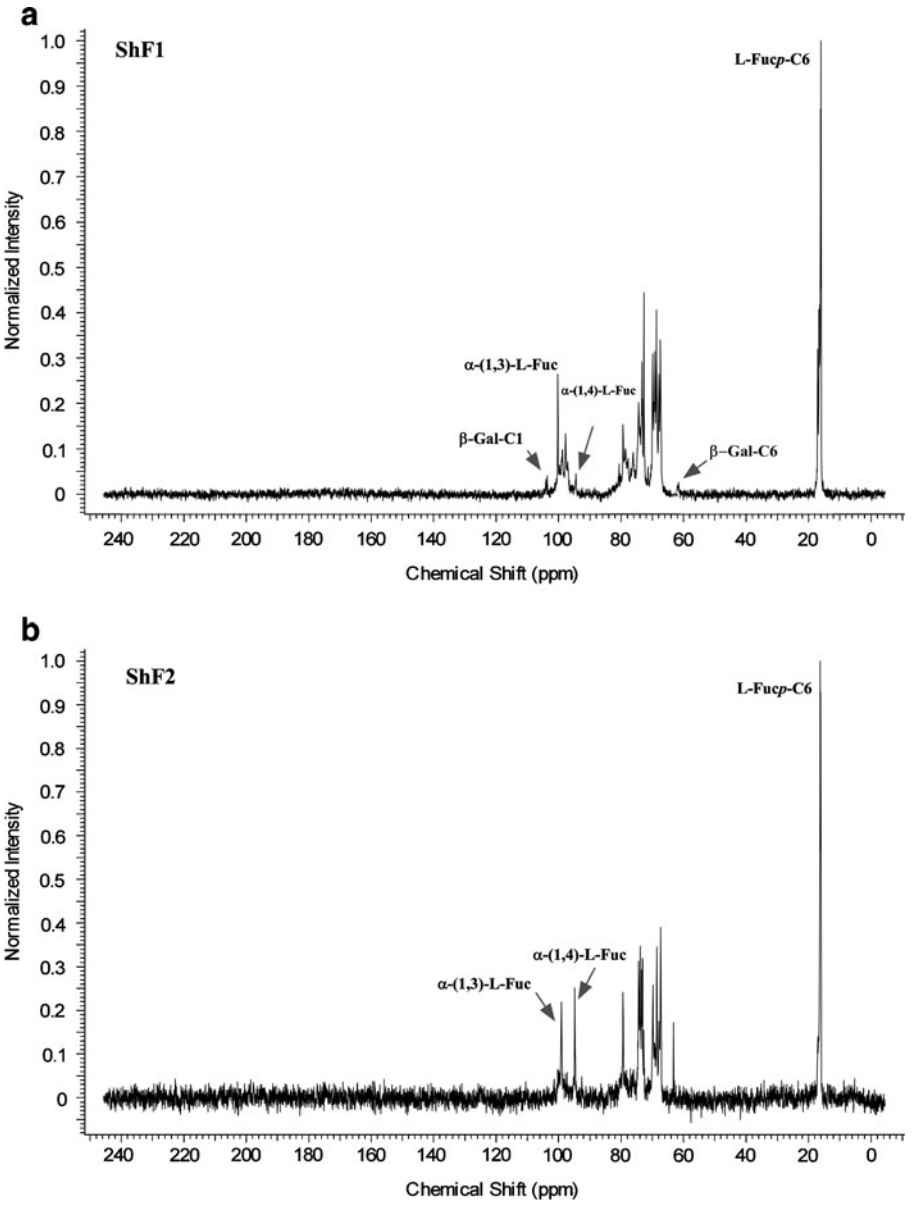

Fig. 2 ^{13}C-NMR spectra of fucoidans from brown seaweeds *S. hornery* (ShF1, ShF2, ShF3), *E. cava* (EcF1, EcF2), and *C. costata* (CcF). **a** *S. hornery* (ShF1). **b** *S. hornery* (ShF2). **c** *S. hornery* (ShF3). **d** *E. cava* (EcF1). **e** *E. cava* (EcF2). **f** *C. costata* (CcF)

From three species of brown seaweeds collected from Hyunpo and Jumunjin three known types of fucoidans were isolated namely, sulfated 1,3-α-L-fucan; sulfated 1,3;1,4-α-L-fucan; and sulfated fucogalactan and moreover two new types of fucoidans: nonsulfated 1,3;1,4-α-L-fucan from *S. hornery* and sulfated 1,4-α-L-fucan that contained fragment buildup of β-1,3- and β-1,6-linked glucose residues.

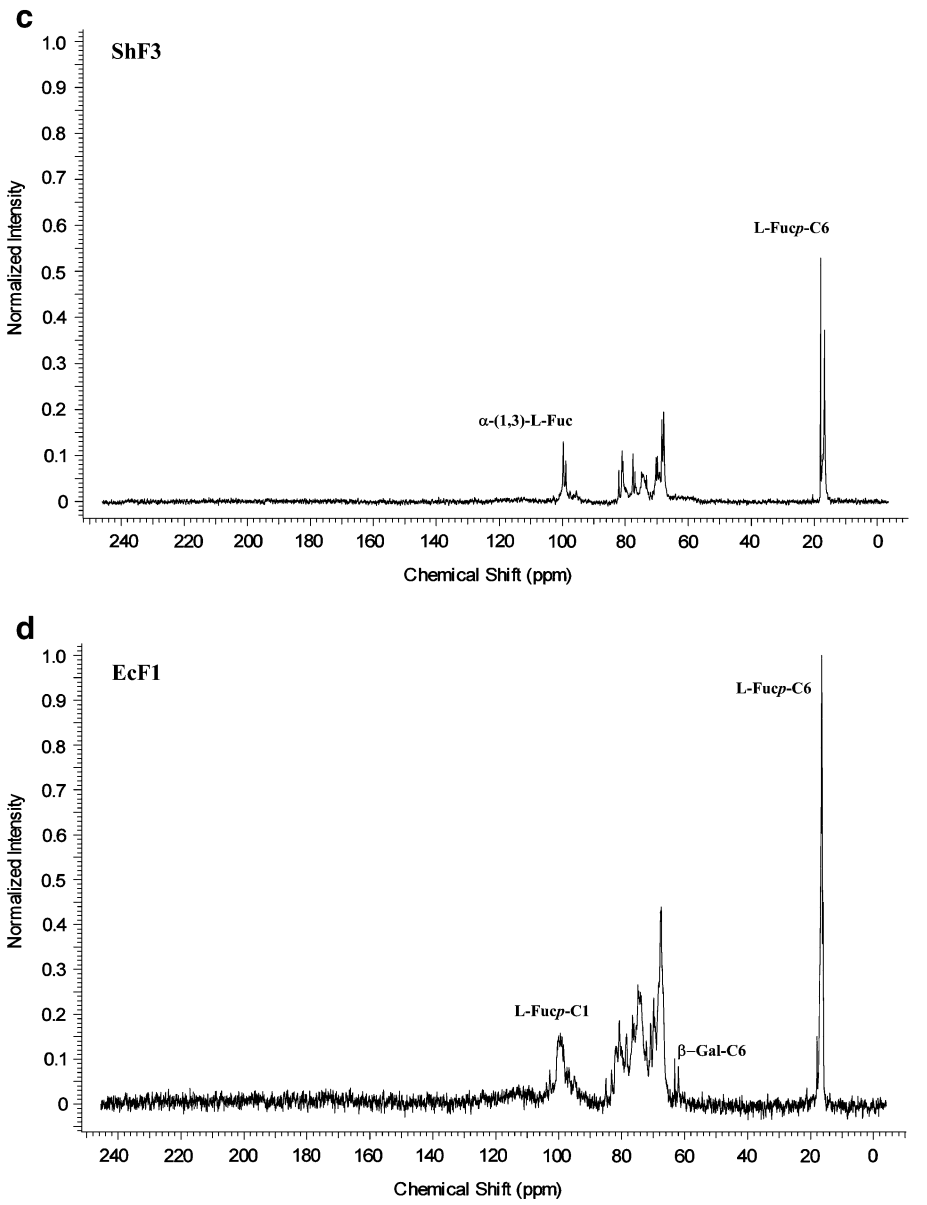

Fig. 2 continued.

Cytotoxicity and Anticancer Activity of Purified Fucoidans

We examined the effect of fucoidans from *E. cava*, *S. hornery*, and *C. costata* on the cytotoxicity of SK-MEL-28 human melanoma and DLD-1 human colon cancer cells with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS assay). Fucoidans did not show significantly cytotoxicity after treatment during 24 h at concentration from 1 to 200 μg/mL and did not inhibit cell proliferation of SK-MEL-28

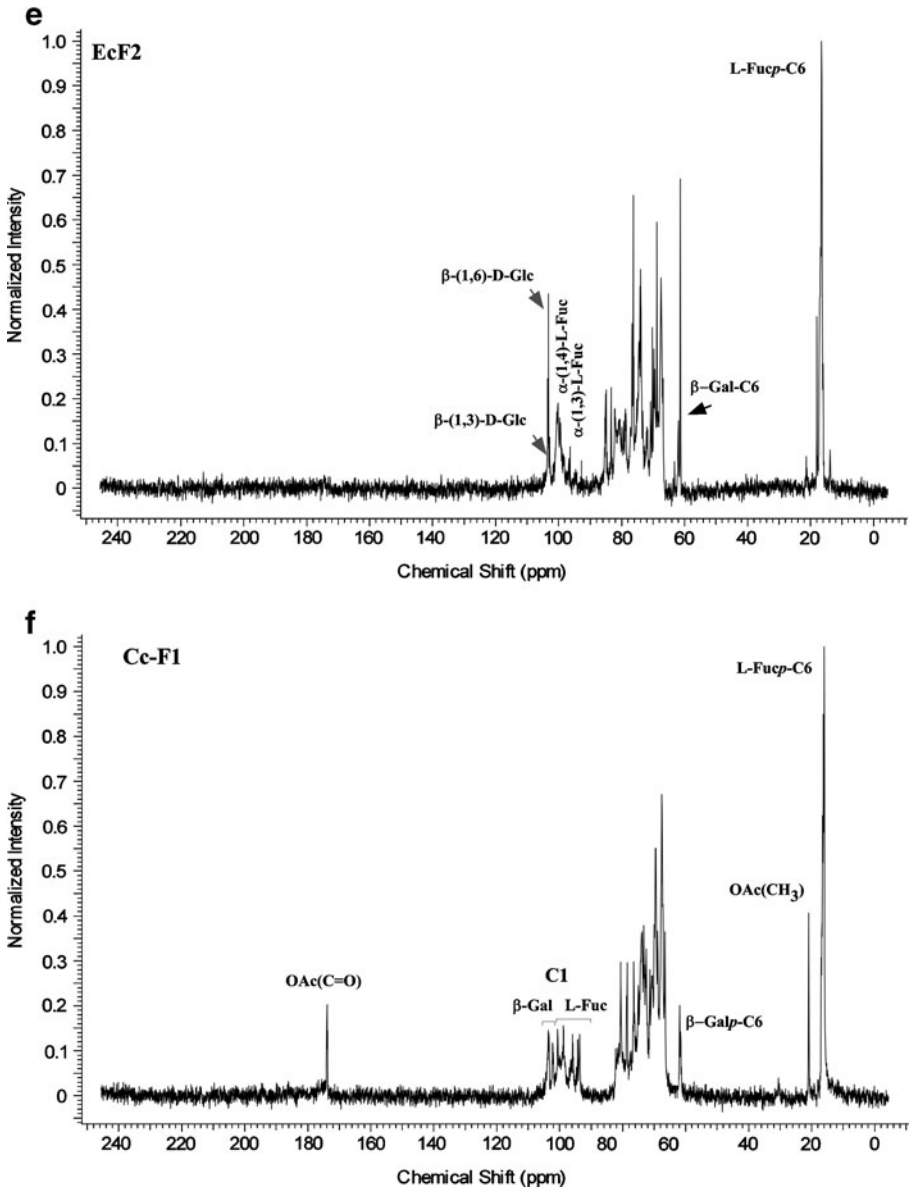

Fig. 2 continued.

and DLD-1 cells at those concentrations during 24, 48, 72, and 96 h (data not shown). These results indicated that fucoidans were less cytotoxic to SK-MEL-28 and DLD-1 human melanoma and colon cancer cells.

Anticancer activity of fucoidans ShF1, ShF2, ShF3, EcF1, EcF2, and CcF1 were assayed with soft agar clonogenic assay. It is a well-developed model for studying a potential of antitumor agents. SK-MEL-28 and DLD-1 human melanoma and colon cancer cells were treated or not with 100 μg/mL of fucoidan in a soft agar matrix and

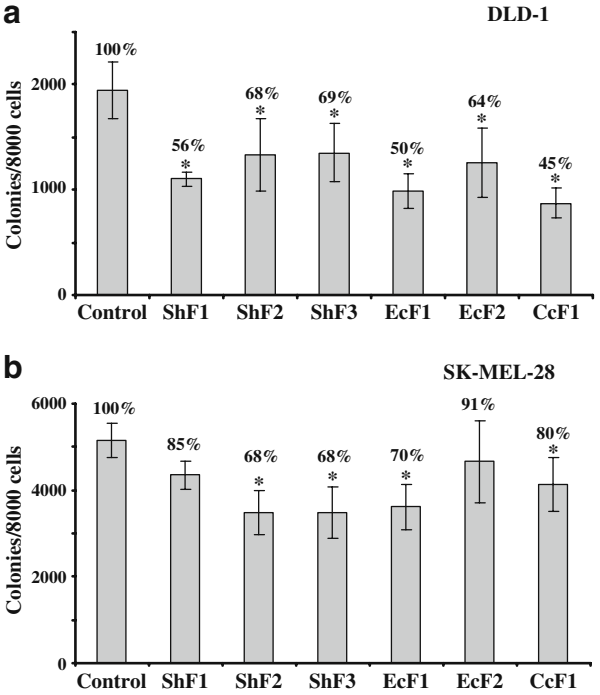

Fig. 3 The inhibitory effects of fucoidans from brown seaweeds *S. hornery*, *E. cava*, and *C. costata* on colony formation in human colon cancer cells DLD-1 (**a**) and human skin melanoma cell line SK-MEL-28 (**b**) comparing untreated control cells. Cells (2.4×10^4/mL) treated with/without fucoidans (100 μg/ml) were exposed in 1 mL of 0.3% BME's agar containing 10% FBS. The culture was maintained at 37 °C in a 5% CO_2 atmosphere for 3 weeks. The colonies were counted under a microscope with the aid of the ImageJ soft ware program. Data are represented as the means. SDs of the number of colonies are determined from three independent experiments

incubated at 37 °C in a 5% CO_2 incubator for 15 days for SK-MEL-28 cells and 20 days for DLD-1 cells. We determined whether purified fucoidans could have an inhibitory effect on colony formation of SK-MEL-28 and DLD-1 human melanoma and colon cancer cells, respectively. Results indicated that fucoidans ShF1, ShF2, ShF3, EcF1, EcF2, and CcF1 well inhibited colony formation of SK-MEL-28 and DLD-1 cells, except fucoidan EcF2 which has not significantly inhibited colony formation of SK-MEL-28 cells, only for 8% (Fig. 3).

Fucoidans ShF2 and ShF3 significantly inhibited colony formation of DLD-1 cells as well as SK-MEL-28 for 32% and 33%, respectively. The potency of inhibition of colony formation of DLD-1 cells by fucoidans ShF1, EcF1, and CcF1 was highest, being 44%, 50%, and 55%, respectively. For inhibition of colony formation of SK-MEL-28, those fucoidans showed only 15%, 30%, and 20% of inhibition, accordantly.

Fucoidans ShF2 and ShF3 have been shown to exert anticancer activities in both cell cultures. In contrast, fucoidan EcF2 has no significant effect at 100 μg/mL for SK-MEL-28 cells and showed an inhibition of colony formation of DLD-1 cells. Compared with the antitumor activity of fucoidans ShF2 and ShF3, the fucoidans ShF1, EcF1, and CcF1 exhibited a more potent inhibitory effect on DLD-1 colony formation compared with SK-MEL-28 cells.

It is difficult to make a correlation between anticancer activity of purified fucoidans and their structures. The amount of sulfate groups has been reported to play role for anticoagulant activity of fucoidans. There are very few reports [14–17, 4] in the literature on the relationship between chemical properties and anticancer activity of different fucoidans. In our case, we did not find a correlation between amount of sulfate groups and anticancer activity, as fucoidan ShF2 does not have sulfate groups, but its anticancer activity is the same as for fucoidan ShF3 with 16.9% of sulfate. The anticancer activity of tested fucoidans is not a function of a single factor but a combination a many factors such as amount of sulfate groups, monosaccharide residues ratio, and type of sugar residues bounding.

The results clearly showed that fucoidans play an inhibitory role in colony formation in human melanoma and colon cancer cells and may be effective antitumor agents.

This work was supported by the grants of REBR (09-04-00761), FEB RAS, the Program "Molecular and Cellular Biology" of the Presidium of Russian Academy of Sciences, and grant no. RTI05-01-02 from the Regional Technology Innovation Program of the Ministry of Knowledge Economy (MKE).

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