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Antitumor Activities of Sea Staghorn (*Codium fragile*) against CT-26 Cells

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Abstract The 10 species of marine green algae was collected from Jejudo(Island) in Korea. Methanolic and aqueous extracts were prepared and screened for inhibition activities against tumor cell growth. Of the tested samples, the sea staghorn (*Codium fragile*) aqueous extract (CFAE) showed the highest activity on CT-26 cell growth. Therefore, CFAE was selected for further experiments and the possibility to induce apoptosis by the CFAE was investigated. Flow cytometric analysis revealed that it dose-dependently increased apoptotic cells with hypodiploid DNA contents in CT-26 cell line. These results indicated that CFAE can suppress the growth of CT-26 cells through apoptosis. The CFAE decreased the protein expression of anti-apoptotic Bcl-xL and led to the activation of caspase-3 and -7. A crude polysaccharide was separated from CFAE and it mainly constituted with 61.2% galactose and 30.5% arabinose as analyzed by high performance liquid chromatography (HPLC).

Keywords: antitumor, marine green algae, sea staghorn (*Codium fragile*), polysaccharide, apoptosis

Introduction

Colon carcinoma is the leading cause of cancer-related death in the world, with increasing incidence in many developed countries (1-3). High prevalence, high death rate, and ineffective therapy have spurred the search for novel strategies in the treatment of colon cancer (4-6). Scientific studies can broadly be separated into those aimed at improving the therapeutic index of currently available compounds and those discovering new agents or approaches (7,8).

Apoptosis is a selective process of physiological cell deletion that regulates the balance between cell proliferation and cell death. The failure of apoptosis is considered to contribute generally to the development of human malignancies (9). Because it was recently suggested that cancer chemotherapeutics exert part of their pharmacological effects by triggering apoptotic cell death, the induction of apoptosis in cancer cells has become a target of cancer treatment (10,11).

Marine bioresources are known to be attractive as they sometimes contain new compounds showing several kinds of different bioactivities which are not possible in land plants. Screening of algal extracts for biologically active compounds began in the 1950s with simple antibiotic assays and expanded to include testing for products with antiviral, antibacterial, antifungal, anti-mitotic, and anti-tumorigenic activities (12-15). Studies on antitumor effects of algal species have been reported by a number of researchers (16-19). Sea staghorn (*Codium fragile*) is a siphonous marine green alga belonging to the family Codiaceae (order Siphonales, class Chlorophyceae). In China, it is eaten as food by some of the people along the coastlines and is used as anticancer, antiviral, antipyretic, and helminthic agents in Chinese traditional medicine (20). However, there is yet no report regarding sea staghorn inducing apoptosis in colon cancer cells. Understanding the underlying mechanism related to the induction of apoptosis by sea staghorn will benefit the development of chemopreventives and/or chemotherapeutics for colon cancer.

The present study was undertaken to perform the screening of antitumor properties from 10 species of marine green algae, and sea staghorn was selected for investigating the effect of morphological changes using a Hoechst 33342 dye, sub-G_1 peak by a flow cytometric analysis, and expressions of apoptosis-related gene proteins, including caspase-3, -7, Bax, and Bcl-xL in CT-26 cells by Western blot analysis.

Materials and Methods

Materials RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco/BRL (Burlington, ON, Canada). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), dimethyl sulfoxide (DMSO), and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other chemicals and reagents used were of analytical grade.

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Preparation of aqueous and methanolic extracts from marine green algae Ten species of marine green algae were collected from Jejudo(Island) coast in Korea during a period from February 2004 to March 2005. Salt, epiphytes, and sand were removed using tap water. Finally, the green algae were rinsed carefully with freshwater and stored in a medical refrigerator at –20°C. The frozen samples were lyophilized and homogenized with a grinder before extraction. The powdered samples were then extracted for 24 hr with water and 70% methanol under continuous shaking at 20°C. Finally, sample was clarified via 20 min of centrifugation at 1,900×g in order to separate the residues. The aqueous- and methanolic-extracts from marine green algae were obtained after filtration of the supernatant. The methanolic extracts were first subjected to evaporation and then dissolved in DMSO and used for experiments adjusting the final concentration of DMSO in culture medium to <0.01%. Respective aqueous extracts were freeze dried and a known amount of the powder was again dissolved in water.

Cell culture U-937 (human promonocytic leukemia cell line), CT-26 (mouse colon carcinoma cell line), and B-16 (mouse melanoma cell line) cells were grown in RPMI-1640 medium, HeLa (woman cervical carcinoma cell line), and V79-4 (Chinese hamster lung fibroblast cell line) cells were grown in DMEM supplemented with 10%(v/v) heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Cultures were maintained at 37°C in 5% CO_2 incubator.

Cell growth inhibitory assay The cytotoxicity of marine green algae against the tumor cells (U-937, CT-26, B-16, and HeLa cells) was determined by a colorimetric MTT assay. Suspension cells (U-937 cells) were seeded (4×10^4 cells/mL) together with the extracts and incubated up to 24 or 72 hr before MTT treatment. Attached cells (B-16, CT-26, HeLa, and V79-4 cells) were seeded in a 96-well plate at a concentration of 2×10^4 cells/mL. Sixteen hr after seeding, the cells were treated with the extract samples. The cells were then incubated for an additional 24 or 72 hr at 37°C. MTT stock solution (50 μL; 2 mg/mL in phosphate buffered saline, PBS) was then added to each well for a total reaction volume of 250 μL. After incubating for 4 hr, the plate was centrifuged at 900×g for 10 min and the supernatant was aspirated. The formazan crystals in each well were dissolved in DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm.

Nuclear staining with Hoechst 33342 The nuclear morphology of cells was studied by using cell-permeable DNA dye Hoechst 33342. Cells with homogeneously stained nuclei were considered to be viable, whereas the presence of chromatin condensation and/or fragmentation was indicative of apoptosis (21,22). The CT-26 cells were placed in 24-well plate at a concentration of 1.0×10^5 cells/mL. The cells were treated with various concentrations (25, 50, and 100 μg/mL) of the fraction samples. After 24 hr, 1.5 μL of Hoechst 33342 (stock 10 mg/mL) and a DNA-specific fluorescent dye were added to each well (1.5 mL), followed by incubation for 10 min at 37°C. The stained cells were then observed under a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

Flow cytometry analysis Flow cytometry analysis was performed to determine the proportion of apoptotic sub-G_1 hypodiploid cells (23). The CT-26 cells were placed in a 6-well plate at a concentration of 1.0×10^5 cells/mL. The cells were treated with various concentrations of the extract (25, 50, and 100 μg/mL). After 24 hr, the cells were harvested at the indicated time and fixed in 1 mL of 70% ethanol for 30 min at 4°C. The cells were washed twice with PBS and incubated in the dark in 1 mL of PBS containing 100 μg PI and 100 μg RNase A for 30 min at 37°C. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The effect on cell cycle was determined by changes in the percentage of cell distribution at each phase of the cell cycle and assessed by histograms generated by the computer program Cell Quest and Mod-Fit (24).

Western blot analysis Cells (2×10^5 cell/mL) were treated with various different concentrations of the extract and harvested. The cell lysates were prepared with lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 1 mmol/L ethylenediamide tetraacetic acid (EDTA)]. Cell lysate were washed by centrifugation, and protein concentrations were determined by using BCA™ protein assay kit. The lysate containing 40 μg of protein were subjected to electrophoresis on 12% SDS-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were incubated with primary antibody against Bax, Bcl-xL, cleaved caspase-3, -7, and β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA) in TTBS (25 mmol/L Tris-HCl, 137 mmol/L NaCl, 0.1% Tween 20, pH 7.4) containing 0.5% nonfat dry milk at 1 hr. Membranes were washed with TTBS and incubated with secondary antibodies. Signals were developed using an enhanced chemiluminescence (ECL) Western blotting detection kit and exposed to X-ray films.

Crude polysaccharide separation The aqueous extract was mixed well with 2 volume of 99.5% ethanol. Then, the mixture was allowed to stand for 30 min at a room temperature and then the polysaccharide-protein complexes were collected by centrifugation at 10,000×g for 20 min at 4°C (25,26). Hereafter, the collected precipitate was referred to as polysaccharide-protein complexes. Then the crude polysaccharides (CPS) were extracted by Sevag method to remove the dissociative protein (27). Then, to analyze the monosaccharide contents, the CPS was hydrolyzed in a sealed glass tube with 2 M of trifluoroacetic acid for 4 hr at 100°C. After the sample was digested using 6 N of HCl for 4 hr, 0.055 and 2.75 μg of the sample were separately applied to CarboPacc PA1 (4.5×250 mm, Dionex, Sunnyvale, CA, USA) with CarboPac PA1 cartridge (4.5×50 mm) column to analyze neutral and amino sugar, respectively. The column was eluted using 16 mM of NaOH at 1.0 mL/min flow rate. Each sugar of the sample was detected by

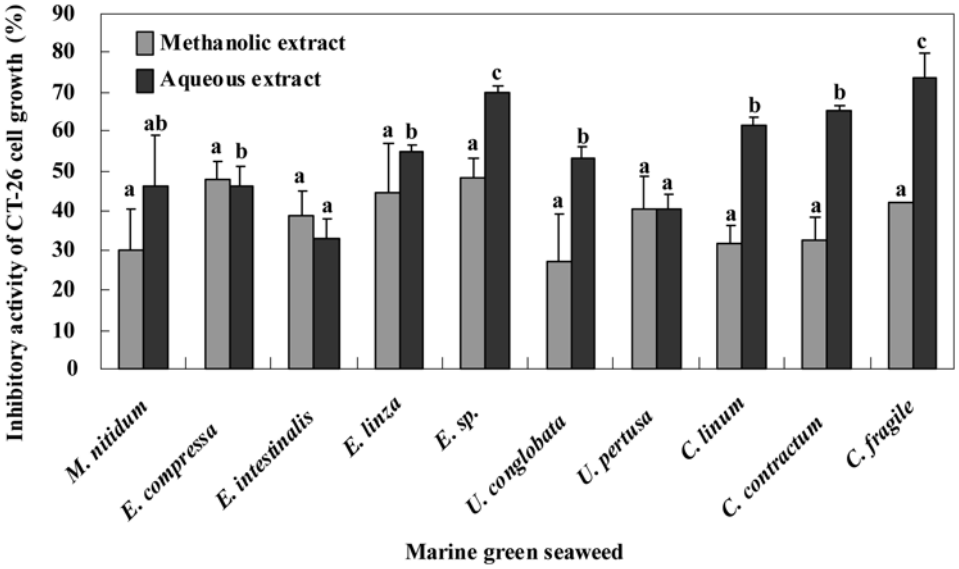

Fig. 1. Inhibitory effect of aqueous and methanolic extracts from the 10 marine green algae against growth of CT-26 tumor cell. Means with different letters are significantly different ($p<0.05$).

using ED50 Dionex electrochemical detector and data were analyzed by Peack Net on-line software. The sugar contents of the CPS were determined using the phenol/sulfonic acid methods. CPS was concentrated separately under vacuum at 40°C, and then samples were dissolved in water for further experiments.

Statistical analysis Data were analyzed using the SPSS package for Windows (Version 10). Values were expressed as mean±standard error (SE). The mean values of the tail intensity from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A p-value of less than 0.05 was considered significant.

Results and Discussion

Inhibitory effect of marine green algae extracts against the growth of tumor cells The CT-26 cell growth inhibition activities of methanolic and aqueous extracts of marine green algae were shown in Fig. 1. As shown in this figure, all the methanolic extracts of green algae showed poor growth inhibition activities (less than 50%) on CT-26 cells. On the other hand, the aqueous extracts from 6 marine green algae (*Eenteromopha linza, Enteromopha* sp., *Ulva conglobata, Chaetomorpha linum*, *Codium contractum*, and *C. fragile*) on CT-26 cells exceeded over 50% inhibitory activity against CT-26 cell growth. The highest inhibitory activities (about 73%) in this assay were observed in the aqueous extract from sea staghorn (CFAE). Therefore, the CFAE was selected for use in further experiments. In this study, 4 tumor cell lines including U-937, B-16, HeLa, and CT-26 cells were adapted to determine cell growth inhibitory activity of CFAE. The CFAE significantly suppressed proliferation of CT-26 and B-16 cells with inhibitory activities of 84 and 55%, respectively, but was less effective for U-937 and HeLa cells at the highest dosage of 50 μg/mL (Fig. 2). The IC_{50} values of CFAE on growth inhibition activity of CT-26 and B-16 cells were 28 and 36 μg/mL, respectively. The CFAE showed clear dose- and time-dependent antiproliferative activity on CT-26 cells and the highest activity (about 83%) was recorded at 50 μg/mL after 72 hr incubation time (Fig. 3).

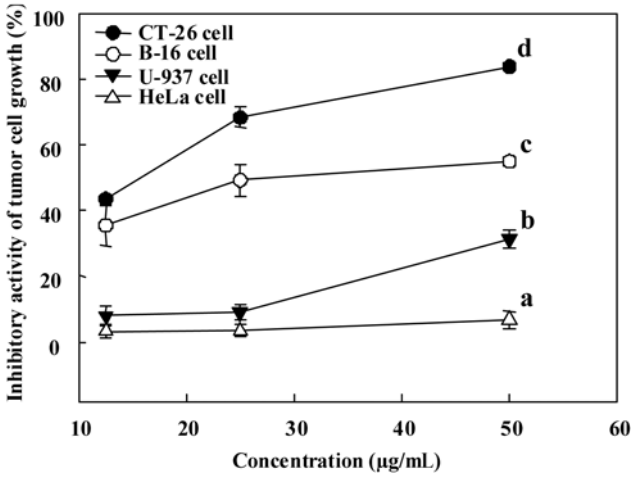

Fig. 2. Inhibitory effect of CFAE against growth of the tumor cells (CT-26, B-16, U-937, and HeLa cells). Means with different letters are significantly different ($p<0.05$).

Cytotoxic effect of CFAE in normal cells Cycytotoxic effect of CFAE between tumor (CT-26 cells) and normal (V79-4 cells) cells was compared in Table 1. In CT-26 cells, a dose-dependent loss of cell viability was observed in Table 2. On the other hand, normal cells such as V79-4 showed around 90% cell viability at even 50 μg/mL of the extract concentration which is the highest concentration tested. Much lower cytotoxicity in normal cells compared to tumor cells is a prerequisite for any chemo-preventive

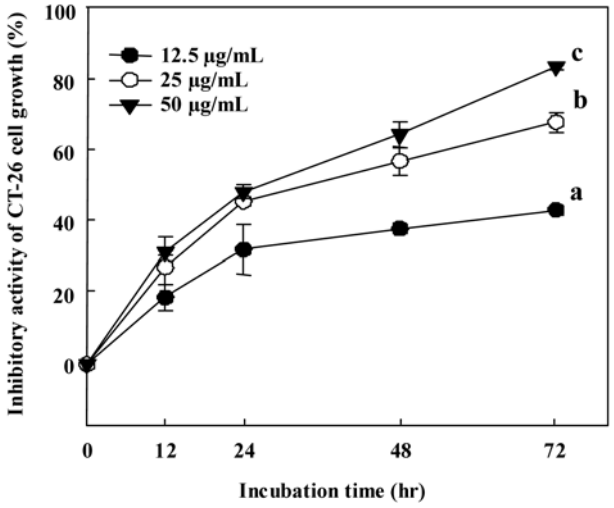

Fig. 3. Inhibitory effect of CFAE on difference incubation times against growth of CT-26 cells. Means with different letters are significantly different ($p<0.05$).

Table 1. Comparison with cell viability by CFAE in tumor and normal cells

Treatment (μg/mL)	Cell viability (%)	
	CT-26	V79-4
-	100.0±4.2[a1)]	100.0±2.4[a]
12.5	56.5±1.5[b]	103.4±4.3[a]
25	31.7±3.7[c]	102.3±5.9[a]
50	16.2±0.9[d]	89.2±3.5[b]

[1)]Means with different letters within a column are significantly different ($p<0.05$).

agent. There have been some previous reports related to high inhibitory activities for tumor cell growth in algae (28-30). In particular, green algae have been shown previously to exhibit profound tumor cell growth inhibition activity (31,32).

Induction effect of apoptosis in CT-26 cells by CFAE Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death (33-35). Apoptosis is a cellular suicide or a programmed cell death that is mediated by the activation of an evolutionary conserved intracellular pathway (36). Therefore, induction of apoptosis in cancer cells is one of the useful strategies for anticancer drug development (37). Apoptosis includes cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, nuclear membrane blebbing, chromatin condensation, and formation of apoptotic bodies (38). These apoptotic bodies are rapidly cleaned from the local tissue by macrophages (39). The nuclear morphological changes of CT-26 cells by treatment of CFAE were investigated by using the cell-permeable DNA dye Hoechst 33342. The control, without the CFAE showed clear image and exhibited no DNA damage (Fig. 4A). However, obvious cell damage was observed in the cells treated with CFAE. Cells treated with CFAE at different concentrations (25, 50, and 100 μg/mL) dramatically increased apoptotic bodies (Fig. 4B, 4C, and 4D). The

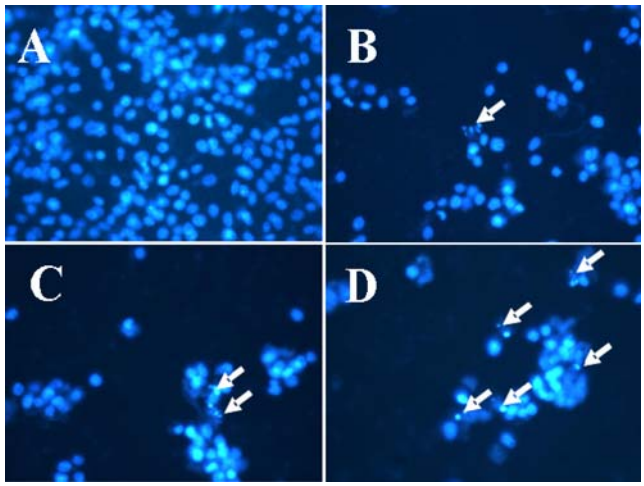

Fig. 4. Effect of CFAE on morphological changes in CT-26 cells. (A) Untreated cells; (B) treated with 25 μg/mL of sample; (C) with 50 μg/mL of sample; (D) with 100 μg/mL of sample. Arrows (B, C, and D) indicate a typical apoptotic cell with apoptotic body.

cytotoxicity of CFAE on CT-26 cells was due to the induction of apoptosis, so cell cycle analysis was performed. CFAE dose-dependently increased the apoptotic portion of sub-G_1 peaks to 18.88, 19.36, and 23.66% at the concentrations of 25, 50, and 100 μg/mL, respectively, compared to the untreated control (Fig. 5). Our present results strongly suggest that the cytotoxic mechanism of CFAE may be attributable to induction of apoptosis in CT-26 cells.

Effects of CFAE on apoptosis-related protein levels of CT-26 cells Apoptosis is a tightly regulated process, which involves changes in the expression of distinct genes. Members of the Bcl-2 family (such as Bcl-xL) of proteins are critical regulators of the apoptotic pathway (40). Bcl-2 and Bcl-xL are upstream molecule in the apoptotic pathway and is identified as a potent suppressor of apoptosis (41). In this study, a decrease in Bcl-xL protein expression was observed in CT-26 cells. In addition, the cleavage of caspase-3 and -7 appears to be correlated with CFAE-induced apoptosis in CT-26 cells (Fig. 6). Caspase-3 is one of the key executioners of apoptosis, as it is cleavage of many key proteins such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (42). Caspase-7 has been identified as a major contributor to the execution of apoptosis (43-45). Caspase-7 is cleaved by many enzymes, including caspase-3, -6, -8, -9, and granzyme B. Once activated caspase-7 cleaves many of the same substrates as caspase-3 including PARP (46,47).

Inhibitory effect of CPS from CFAE against the growth of CT-26 cells Many algal polysaccharides have been used extensively in industry (48,49). In recent years, the potential of algal polysaccharides as medicine has drawn more and more attention. The biological activities of antitumor, antivirus, antihyperlipidemia, and anticoagulant have been found in algal polysaccharides, some of which are exploited as new drugs (50,51). Therefore, CFAE was subjected to ethanol precipitation to obtain the CPS. The

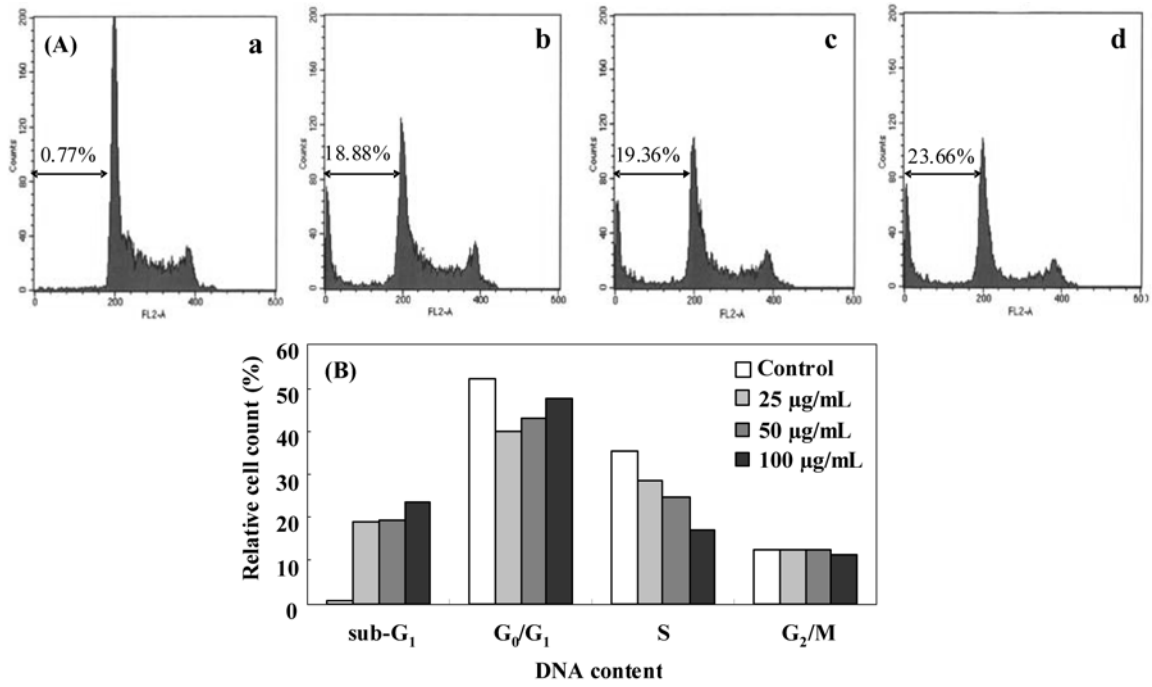

Fig. 5. Effect of CFAE on cell cycle pattern and apoptotic portion in CT-26 cells by flow cytometric analysis. (A) Histogram of cell cycle patterns of CT-26 cells (a) untreated; (b) threated with 25 μg/mL; (c) treated with 50 μg/mL; (d) treated with 100 μg/mL. (B) Bar graph of cell cycle patterns of CT-26 cells.

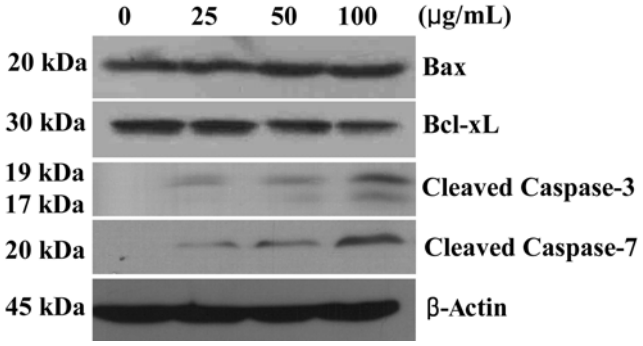

Fig. 6. Effect of CFAE on apoptosis-related proteins in CT-26 cells by Western blot analysis.

Table 2. The contents of composition of CPS from sea staghorn

Compound	Content (%)
Crude polysaccharides (CPS)	74.1
Galactose	61.21
Arabinose	30.53
Glucose	5.02
Mannose	1.99
Fucose	0.64
Rhamnose	-
Xylose	-

CPS mainly composed of galactose (61.2%), arabinose (30.5%), and trace amounts of glucose, mannose, and fucose (Table 2). Therefore, this compound belongs to the group of galactan/arabinan (or arabinogalactan). In the effect of CPS on inhibitory activities of CT-26 cell growth

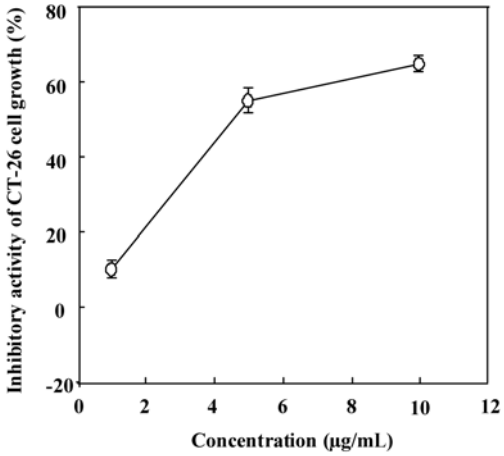

Fig. 7. Inhibitory effect of CPS against growth of CT-26 cells.

is shown in Fig. 7. The CPS had the highest growth inhibition activity of CT-26 cell, with an IC_{50} value of 4 μg/mL. This value was higher than of CFAE, with an IC_{50} value of 28 μg/mL. Love and Percival (52) and Ciancia *et al.* (53) previously reported the presence of galactan and arabinan (or arabinogalactan) in sea staghorn. Recently a considerable number of studies have been reported antioxidant and anticancer activities with polysaccharides isolated from marine alga (18,54-56). Therefore, the CPS of sea staghorn also may be a potential polysaccharide for high antitumor activity.

In conclusion, CFAE induced cytotoxicity and apoptosis of CT-26 cells. We further hypothesize that apoptosis was induced by a decrease in the level of Bcl-xL and a concomitant activation of caspase-3 and -7 in CT-26 cells

treated with CFAE. Therefore, sea staghorn is a possible candidate for anticancer drug discovery in the future. A CPS responsible for the activity was separated from CFAE and, in high performance liquid chromatography (HPLC) analysis, the crude polysaccharide fraction mainly constituted with 61.2% galactose and 30.5% of arabinose which are also known as galactan and arabinan, respectiveley (or arabinogalactan). No one has studied the anticancer activity of sea staghorn so far and we firstly report the anticancer effect of CPS from sea staghorn in this study.

Acknowledgments

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