JOURNAL OF FOOD AND DRUG ANALYSIS 23 (2015) 766–777

Available online at www.sciencedirect.com

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journal homepage: www.jfda-online.com

Original Article

Antioxidant activity and growth inhibition of human colon cancer cells by crude and purified fucoidan preparations extracted from *Sargassum cristaefolium*

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ARTICLE INFO

Article history:
Received 23 March 2015
Received in revised form
25 June 2015
Accepted 3 July 2015
Available online 26 July 2015

Keywords:
antioxidant
colon cancer cells
extraction
fucoidan
Sargassum cristaefolium

ABSTRACT

Fucose-containing sulfated polysaccharides, also termed "fucoidans", which are known to possess antioxidant, anticoagulant, anticancer, antiviral, and immunomodulating properties, are normally isolated from brown algae via various extraction techniques. In the present study, two methods (SC1 and SC2) for isolation of fucoidan from *Sargassum cristaefolium* were compared, with regard to the extraction yields, antioxidant activity, and inhibition of growth of human colon cancer cells exhibited by the respective extracts. SC1 and SC2 differ in the number of extraction steps and concentration of ethanol used, as well as the obtained sulfated polysaccharide extracts, namely, crude fucoidan preparation (CFP) and purified fucoidan preparation (PFP), respectively. Thin layer chromatography, Fourier transform infrared analysis, and measurements of fucose and sulfate contents revealed that the extracts were fucoidan. There was a higher extraction yield for CFP, which contained less fucose and sulfate but more uronic acid, and had weaker antioxidant activity and inhibition of growth in human colon cancer cells. In contrast, there was a lower extraction yield for PFP, which contained more fucose and sulfate but less uronic acid, and had stronger antioxidant activity and inhibition of growth in human colon cancer cells. Thus, since the difference in bioactive activities between CFP and PFP was not remarkable, the high extraction yield of SC1 might be favored as a method in industrial usage for extracting fucoidan.

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http://dx.doi.org/10.1016/j.jfda.2015.07.002

1. Introduction

Sulfated polysaccharides comprise a complex group of bioactive macromolecules which are widely distributed in nature and occur in a great variety of organisms including mammals and invertebrates [1]. Marine algae are the most important source of non-animal sulfated polysaccharides. The structure of algal sulfated polysaccharides varies according to the species of algae [2,3]. To date, each newly discovered sulfated polysaccharide purified from a marine alga is a new compound with unique structures and, thus may have novel biological activities [4]. Sulfated polysaccharides can be found in varying amounts in three major divisions of marine algal groups; Rhodophyta, Chlorophyta, and Phaeophyta. The polysaccharides found in Rhodophyta are mainly galactans consisting entirely of galactose or modified galactose units [5,6]. The major polysaccharides in Chlorophyta are polydisperse heteropolysaccharides and homopolysaccharides [7–9]. The sulfated polysaccharides of Phaeophyta are called fucose-containing sulfated polysaccharides, or "fucoidans", which comprise families of polydisperse molecules based on sulfated L-fucose [10]. Fucoidans exhibit a wide range of important pharmacological activities such as anticoagulant, antioxidant, antiproliferative, antitumoral, anti-inflammatory, antiviral, and antiadhesive activities [11–13] and thus there is considerable research interest in the isolation of fucoidan from brown seaweed, identification of the bioactive components in fucoidan, and elucidation of the molecular mechanisms involved.

Reactive oxygen species (ROS) in the forms of superoxide anion ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) are metabolic products which may also be present in the environment. ROS interact with one another in biological systems. The uncontrolled generation of ROS often correlates directly with molecular markers of many diseases [14]. Furthermore, the formation of cancer cells in the human body can be directly induced by free radicals. Natural anticancer drugs such as chemopreventive agents in the treatment of cancer have gained in popularity. Studies demonstrated that fucoidan extracted from brown seaweed is a potential ROS scavenger and an important free-radical scavenger which is also capable of preventing oxidative damage [15,16], and is therefore an important effector in the prevention of cancer. Radical scavenging compounds such as fucoidan from seaweeds can thus be used indirectly to reduce cancer formation in the human body. Several studies have reported that fucoidan has antiproliferative activity in cancer cell lines *in vitro*, as well as inhibitory activity in mice with tumors [17,18]. The anticancer activity of fucoidan has been reported to be closely related to their sulfate content and molecular weight [19]. Owing to the antioxidant and anticancer properties of fucoidans, they are considered to have potential as novel chemopreventative agents for cancer therapy. In Taiwan, there has been a gradual switch to a western style diet over the past three decades. Colon cancer has leapt forward to have the third highest death rate of cancers [20]. Therefore, finding a novel chemopreventive agent such as fucoidan with minimal or no side effects for colon cancer therapy is urgently needed.

Fucoidan can be extracted and purified from brown algae via various multi-step processes involving hot water, dilute acid, dilute alkali, physical and/or enzymatic treatments and different purification and fractionation steps using a large volume of chemicals and long extraction times [15,21,22]. These steps are generally complicated, time-, energy-, reactant-, and cost-consuming, and probably harmful to the environment. Therefore, a simple, highly productive, and reactant-saving method for the isolation of bioactive fucoidan from brown algae would be highly desirable. Taiwan is an island with plenty of marine resources such as a variety of algae. The production of brown algae is sufficient in Taiwan for extraction of fucoidan on a commercial scale. In the present study, two methods (SC1 and SC2) for extraction of fucoidan from *Sargassum cristaefolium* were compared with regard to the extraction yields, antioxidant activity and inhibition of growth of human colon cancer cells of the respective extracts. SC1 has one water extraction step and one ethanol extraction step, and yields crude fucoidan preparation (CFP). In contrast, the SC2 has one water extraction step and three ethanol extraction steps, and yields purified fucoidan preparation (PFP). Generally, SC1 is simple, fast, and reactant-saving as compared to SC2. This is the first report to compare the antioxidant activity and inhibition of growth of human colon cancer cells of crude and purified fucoidan preparations from *S. cristaefolium*. This study might be useful in the selection of a method for isolation of bioactive fucoidan from brown algae for industrial application.

2. Methods

2.1. Materials

A sample of *S. cristaefolium*, collected from a coastal area of Penghu Islands, Taiwan, was washed with fresh water soon after collection in order to remove salt and sand. It was then dried at 50°C and kept in plastic bags at 4°C until use.

2.2. Chemicals

Potassium bromide (KBr), potassium persulphate, sodium sulphite, and ferrous chloride were purchased from Merck (Darmstadt, Germany). 2, 2, 2-trifluoroacetic acid (TFA) was obtained from Panreac (Barcelona, Spain). Fucose, galactouronic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 3-(2-pyridyl)-5, 6-bis (4-phenylsulfonic acid)-1, 2, 4-triazine (ferrozine), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Calbiochem (San Diego, CA, USA). RPMI-1640 medium, trypsin/EDTA, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco

Laboratories (Grand Island, NY, USA). All other chemicals and reagents used were of analytical grade.

2.3. Chemical composition analyses

Determination of crude protein, fat, moisture, and ash content was carried out using the Association of Analytical Communities (AOAC) procedures [23]. To determine the percentage of moisture (%), samples were dried in an oven at 103°C for 8 hours; crude fat content (%) was determined gravimetrically after Soxhlet extraction with petroleum ether. Crude ash content (%) was determined by incineration in a muffle furnace at 580°C for 8 hours, and crude protein content ($N \times 6.25$; %) was evaluated by the Kjeldahl method after acid digestion. Protein, fat, and ash content were calculated on a dry basis.

2.4. Extraction of polysaccharide

Two extraction methods (SC1 and SC2) were utilized in this study. SC1 was carried out as follows. The dried alga sample was ground and mixed with double-distilled water (w/v = 1:10) and placed in a water bath maintained at 40°C for 15 minutes with shaking. The mixture was centrifuged at 3870*g* for 10 minutes and the supernatant was collected. Ethanol (95%) was added to the supernatant to give a final ethanol concentration of 71.25% and shaken. The ethanol-precipitated polysaccharides were then recovered by centrifugation at 9170*g* for 30 minutes, dried at 40°C and milled, which yielded the CFP. The SC2 method was performed as follows. The dried alga sample was ground and mixed with 95% ethanol (w/v = 1:10), shaken for 1 hour at room temperature to remove pigments, proteins, and lipid [18], then centrifuged at 970*g* for 10 minutes. The residue was collected, mixed with double-distilled water (w/v = 1:10) and placed in a water bath maintained at 40°C for 15 minutes with shaking. The mixture was centrifuged at 3870*g* for 10 minutes and the supernatant was collected. Ethanol (95%) was added into the supernatant to give a final ethanol concentration of 20% in order to precipitate alginic acid and pigment [24]. The mixture was centrifuged at 9170*g* for 30 minutes, the supernatant was collected, and 95% ethanol was added until a final ethanol concentration of 50% was reached in order to obtain fucoidan [24]. The final ethanol-precipitated fucoidan was then recovered by centrifugation at 9170*g* for 30 minutes, dried at 40°C, and milled, which yielded purified fucoidan preparation (PFP). A detailed presentation of the extraction processes in SC1 and SC2 is provided in Fig. 1. Extraction yield was calculated using the following equation: Extraction yield (%) = (gA/gB)×100, where gA represents the weight of the extracted solid, and gB is the weight of the sample on a dry basis.

2.5. Analyses of fucose, uronic acids, and sulfate content

The fucose content was determined according to the method of Gibbons [25] using fucose as the standard. Uronic acids were estimated by the colorimetric method using galacturonic acid as the standard [26]. For the determination of sulfate content, the sample was hydrolyzed with 1N HCl solution for 5 hours at 105°C. The percentage sulfate composition in the hydrolysate was then quantified using Dionex ICS-1500 Ion Chromatography with IonPac AS9-HC column (4 × 250 mm) at a flow rate of 1 mL/minute at 30°C with conductometric detection. A solution of 9mM Na_2CO_3 was used as the eluent, and K_2SO_4 was used as the standard.

2.6. Thin layer chromatography

Polysaccharide sample (30 mg/mL) was hydrolyzed with 6M trifluoroacetic acid (TFA) at 100°C for 24 hours. The hydrolysate was centrifuged at 7380*g* for 10 minutes and the supernatant was used for the analyses by thin layer chromatography (TLC). Samples (50 μL) were applied to the TLC plate with standardized micropipettes. An aliquot of each sample was spotted three times with a developing solvent system of n-butyl alcohol/acetic acid/water (6:3:1, v/v). The monosaccharides were visualized on the plate after dipping into sulfuric acid/methanol (1:1, v/v) and heating until they appeared as dark spots. D-Glucose and D-fucose were used as standard monosaccharides. Identification of monosaccharides was made when the Rf value (the ratio of the distance traveled by the monosaccharides to the distance traveled by the solvent front) and color of the spot were the same as a known standard monosaccharide.

2.7. Fourier transform infrared spectroscopy

Sample (2 mg) was ground evenly with approximately 100 mg KBr until particles measured <2.5 μm in size. The transparent KBr pieces were made at 500 kg/cm^2. The Fourier transform infrared spectroscopy (FTIR) spectra were obtained using a FT-730 spectrometer (Horiba, Japan). The signals were automatically collected using 60 scans over the range of 4,000–400 cm^{-1} at a resolution of 16 cm^{-1} and were compared to a background spectrum collected from the KBr alone at room temperature.

2.8. 1, 1-diphenyl-2-picrylhydrazyl radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was determined by the method of Shimada et al [27]. Sample (50μL) was added to 200 μL 0.1mM DPPH solution (in methanol). The mixture was vortexed for 1 minute and left in the dark for 30 minutes at room temperature. The absorbance of all sample solutions was measured at 517 nm using an ELISA reader (PowerWave 340; Bio-Tek Instruments, Winooski, VT, USA). The scavenging activity of DPPH radicals was calculated as follows:

$$\text{Scavenging activity}\ (\%) = \left(1 - A_{\text{sample}}/A_{\text{control}}\right) \times 100$$

where A_{control} represents absorbance of the methanol solution of DPPH without the sample, and A_{sample} is absorbance of the methanol solution of DPPH with tested samples.

2.9. 2, 2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging activity

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity was

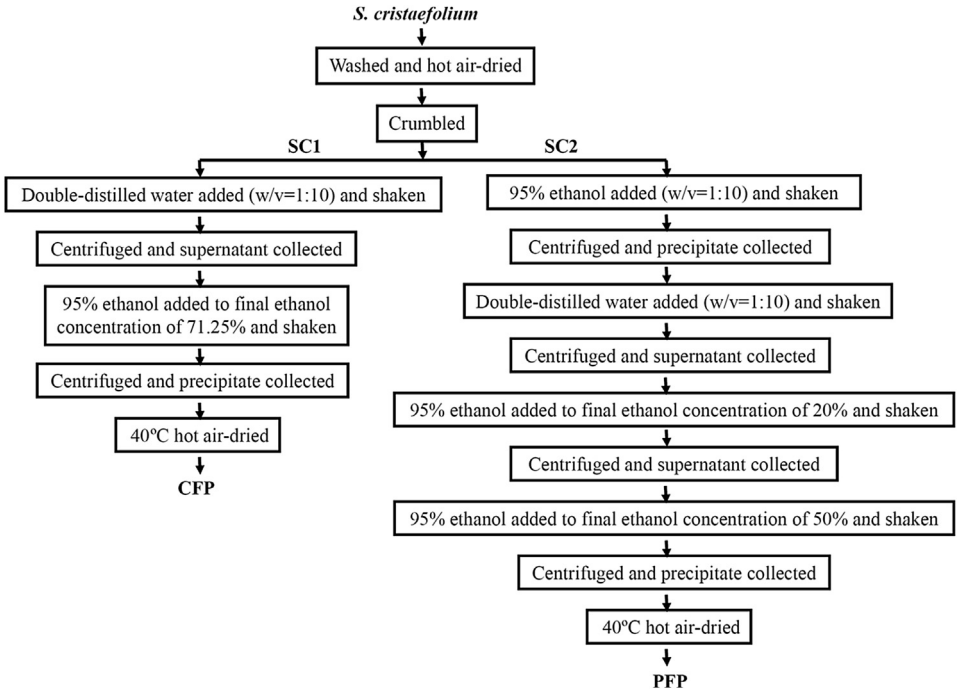

Fig. 1 – Flowchart of the two methods (SC1 and SC2) for extraction of fucoidan from *Sargassum cristaefolium*.

determined according to the method of Binsan et al [28]. The ABTS reagent was generated by mixing 5 mL of 7mM ABTS solution with 88 μL of 140mM potassium persulfate in the dark at room temperature for 16 hours to allow the completion of radical generation. The solution was diluted with 95% ethanol so that its absorbance at 734 nm was adjusted to 0.70 ± 0.05. To determine the scavenging activity, 100 μL ABTS reagent was mixed with 100 μL of various sample solutions. The mixture was allowed to react at room temperature for 6 minutes and absorbance was measured at 734 nm using an ELISA reader. The blank was prepared in the same manner, except that distilled water was used instead of the sample. The scavenging activity of ABTS radicals was calculated as follows:

$$\text{Scavenging activity}\ (\%) = \left(1 - A_{sample}/A_{control}\right) \times 100$$

where $A_{control}$ represents absorbance of the ABTS without the sample, and A_{sample} is absorbance of the ABTS with tested samples. Trolox was prepared as a standard, at different concentrations from 0 ppm to 50 ppm. A calibration curve of scavenging percentage against different concentrations of Trolox standard was prepared. The ABTS radical scavenging activity of samples was expressed as Trolox equivalent antioxidant capacity (TEAC) which represents the concentration (ppm) of Trolox, and exhibiting the same activity as 1 mg of sample.

2.10. *Ferrous ion-chelating activity*

The ferrous ion-chelating activity of polysaccharides was measured using the method of Wang et al [15]. Briefly, 200 μL of sample, 740 μL of methanol, and 20 μL of $FeCl_2$ solution (2mM) were mixed. The mixture was incubated for 30 seconds followed by the addition of 5mM ferrozine (40 μL). After allowing the reaction to continue for 10 minutes at room temperature, the absorbance of the mixture was measured at 562 nm using an ELISA reader. The chelating activity of ferrous ion was calculated as follows:

$$\text{Chelating activity}\ (\%) = \left(1 - A_{sample}/A_{control}\right) \times 100$$

where $A_{control}$ represents absorbance without the sample, and A_{sample} is absorbance with tested samples.

2.11. *Reducing power assay*

Reducing power was measured according to the method of Wang et al [15]. Briefly, 0.5 mL of the sample was mixed with 0.5 mL of phosphate buffer (0.2M; pH 6.6) and 0.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 minutes, and 0.5 mL of trichloroacetic acid (10%) was added to the reaction followed by a centrifugation step (970*g* for 10 min). Finally, 0.5 mL of the supernatant solution was mixed with 0.5 mL of double-distilled water and 0.1 mL of $FeCl_3$ (0.1%), and then let stand for 10 minutes. The absorbance was measured at 700 nm using an ELISA reader.

2.12. *Cell line and culture*

HT-29 (human colon cancer cell line) cells were obtained from the Food Industry Research and Development Institute (FIRDI; Hsinchu, Taiwan). HT29 cells were cultured in RPMI-1640 medium supplemented with 10% of fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2mM

glutamine and incubated at 37°C in a humidified chamber with 5% CO_2.

2.13. Cytotoxicity assay

The cytotoxicity of the samples was determined by 3-(4, 5-dimethylthiazole-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. In brief, human cancer cells were plated at 1×10^4 cells per well in 96-well microtiter plates (Costar 3599; Corning, New York, USA) with 100 μL RPMI-1640 growth medium and incubated for 24 hours at 37°C, with 5% CO_2 in a humidified atmosphere. Thereafter, the medium was removed and fresh serum-free medium containing different concentrations (0, 20, 40, 60, 80, 100, 200, 300, 400, and 500 ppm) of the tested compound was added. After 2 days of incubation at 37°C, with 5% CO_2, the MTT reagent (0.45 mg/mL) was added. After incubating at 37°C for 2 hours, the MTT reagent was removed and dimethyl sulphoxide (DMSO; 75 μL) was added to each well and thoroughly mixed by pipetting. The absorbance was then determined by an ELISA reader at a wavelength of 540 nm. The cell number (% of control) was determined as follows:

$$\text{Cell number (\% of control)} = (T/C) \times 100$$

where T is the absorbance in the test, and C is for the control.

2.14. 4′, 6-diamidino-2-phenylindole-staining analysis

To visualize DNA condensation in nuclei, the control and treated HT-29 cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI) according to the manufacturer's instructions. In brief, the cells were washed with phosphate buffered saline (PBS) and stained with 0.5 μg/ml DAPI solution for 10 minutes at room temperature. Cells were then washed twice with PBS and analyzed using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.15. Cell cycle analysis

Cells were treated with tested compound at 500 μg/ml for 48 hours. Floating and adherent cells were then collected. The cells in suspension were fixed with 70% ice-cold methanol and then stored in a freezer until use. After washing with PBS, cells were stained with 50 μg/ml propidium iodide (PI) in the presence of 25 μg/ml RNase A at 37°C for 30 minutes. A minimum of 10,000 cells per sample were collected. To estimate the percentage of cells in each phase of the cell cycle, the DNA histograms were analyzed by Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

2.16. Statistical analysis

All data were shown as mean ± standard deviation (SD). Statistical evaluation of data was performed by Student t-test or one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range tests. The differences were considered to be statistically significant if $p < 0.05$.

3. Results

3.1. Compositional characteristics of brown algae

A sample of *S. cristaefolium* was collected from a coastal area of the Penghu Islands, Taiwan. The chemical composition of *S. cristaefolium* was found to be 3.83 ± 0.81% protein, 1.51 ± 0.14% lipid, 24.80 ± 0.08% ash, and 69.87 ± 0.83% carbohydrate (Table 1). The chemical compositions of two other previously reported species of brown algae, namely *S. horneri*, and *S. naozhouense*, are also shown in Table 1 [29,30].

3.2. Extraction yield of fucoidan in SC1 and SC2

A comparison of the extraction parameters of SC1 and SC2 is listed in Table 2. SC1 has one water extraction step and one ethanol extraction step; whereas the SC2 has one water extraction step and three ethanol extraction steps. In SC1, the polysaccharides are extracted by hot water, and then precipitated by 71.25% ethanol. Since alginic acid could be precipitated by 20% ethanol, fucoidan could be precipitated by 50% ethanol, and laminaran could be precipitated by ethanol over 65% [24], it was postulated the extract of SC1 might be a mixture of alginic acid, fucoidan, and laminaran, and is termed crude fucoidan preparation (CFP). In SC2, the brown alga powder is first mixed with 95% ethanol in order to remove pigments, proteins, and lipid [18]. Polysaccharides are extracted from the precipitates using hot water, and alginic acid in polysaccharides is precipitated by 20% ethanol and discarded. Fucoidan in polysaccharides is precipitated by 50% ethanol and collected. The recovered fucoidan in SC2 is called purified fucoidan preparation (PFP). Table 2 also illustrates SC1 possesses a significantly higher extraction yield (3.75 ± 0.44 g/100g; dry basis) compared with that of SC2 (0.73 ± 0.02 g/100g; dry basis; $p < 0.001$), and the fold increase of extraction yield between SC1 and SC2 is approximately 5.14 (3.75/0.73 = 5.14).

3.3. Characterization of the CFP and PFP

The compositions of CFP and PFP were characterized with respect to the qualitative and quantitative analyses of fucose, sulfate groups, and uronic acids. The TLC chromatogram (Fig. 2A) showed that both CFP and PFP contained fucose by comparing the Rf values and spot color between monosaccharide standards and samples. Since the PFP is a purified form of fucoidan, it was found that the PFP had a relatively high fucose content (27.38 ± 1.45%) as compared to CFP (21.41 ± 0.60%; $p < 0.001$; Table 2). The presence of sulfated groups in CFP and PFP was confirmed by FTIR analysis and determination of sulfate, respectively. Fig. 2B of FTIR spectra in CFP and PFP showed characteristic absorption bands for sulfated polysaccharides as well as the presence of sulfate groups. The peak at 1249.7 cm^{-1} indicates the presence of sulfate esters (SO) [31], and the peaks at 817.7 cm^{-1} were caused by the bending vibrations of C-O-S of sulfate [32]. The sulfate contents for CFP and PFP were found to be 11.58 ± 0.59 and 13.58 ± 0.62% ($p < 0.05$), respectively (Table 2). In addition, Table 2 shows that CFP has a significantly higher amount of uronic acids (52.10 ± 2.92%) than PFP (33.90 ± 1.00%; $p < 0.001$).

Table 1 – Comparison of chemical compositions of *Sargassum cristaefolium*, *S. horneri*, and *S. naozhouense*.

Brown algae	g/100 g (dry weight)			
	Protein	Lipid	Ash	Total carbohydrate
S. cristaefolium[a,d]	3.83 ± 0.81	1.51 ± 0.14	24.80 ± 0.08	69.87 ± 0.83
S. horneri[b]	10.58	0.99	26.69	61.82
S. naozhouense[c]	11.20	1.06	35.18	47.73

[a] Sample of *S. cristaefolium* was collected from Penghu County, Taiwan.
[b] The values are quoted from Murakami et al [29].
[c] The values (average of four analyses) are quoted from Peng et al [30]. The values were calculated by weight of chemical composition (g)/dry weight of sample (100 g). Total carbohydrate was calculated as weight differences between the total weight and the sum of the amounts of moisture, protein, lipid, and ash.
[d] Values are mean ± SD ($n = 3$).

Table 2 – Comparison of extraction parameters, extraction yield, fucose, sulfate, and uronic acids of fucoidans in SC1 and SC2.

	Extraction methods		
	SC1[a]	SC2	*p*
Number of water extraction steps	1	1	
Number of ethanol extraction steps	1	3	
Concentration of ethanol used (%)	71.25	95, 20, 50	
Extraction yield (%)[b,d]	3.75 ± 0.44	0.73 ± 0.02	0.0003
Fucose (%)[c,d]	21.41 ± 0.60	27.38 ± 1.45	0.0001
Sulfate (%)[c,d]	11.58 ± 0.59	13.58 ± 0.62	0.029
Uronic acids (%)[c,d]	52.10 ± 2.92	33.90 ± 1.00	< 0.001

[a] The conditions for SC1 and SC2 are illustrated in Fig. 1.
[b] Extraction yield (%) = ($g_{solid\ extract}/g_{sample,dry\ basis}$) × 100
[c] Fucose (%), sulfate (%), and uronic acid (%) = ($g/g_{extracted\ polysaccharide,dry\ basis}$) × 100
[d] Values are mean ± SD ($n = 3$). A p value < 0.05 was considered significant.

3.4. *Effects of CFP and PFP on antioxidant activities*

The antioxidant activities of CFP and PFP were evaluated by measuring DPPH scavenging activity, ABTS radical scavenging activity, ferrous ion-chelating activity, and reducing power, and the results are presented in Fig. 3. The scavenging effects of CFP and PFP on DPPH free radicals are shown in Fig. 3A. Both CFP and PFP exhibited DPPH scavenging properties in a dose-dependent manner. The IC_{50} values (concentration of fucoidan capable of scavenging 50% of DPPH) of CFP and PFP on DPPH radical scavenging activity were 6833 ppm and 3533 ppm, respectively (Fig. 3A). PFP had an approximately 1.93-fold (6833/3533 = 1.93) greater DPPH radical scavenging activity as compared to CFP. The TEAC of CFP and PFP on ABTS free radicals is shown in Fig. 3B. Both CFP and PFP showed ABTS radical scavenging activities dose-dependently. The highest TEAC value for both CFP and PFP was approximately 20 ppm. When the concentration of fucoidans was fixed at 400 ppm, the TEAC values for CFP and PFP were 17.82 ppm and 19.99 ppm, respectively, suggesting PFP had higher ABTS radical scavenging activity than CFP (Fig. 3B). The ferrous ion-chelating activities for CFP and PFP are shown in Fig. 3C. Both CFP and PFP exhibited ferrous ion-chelating activities in a concentration-dependent manner. The IC_{50} values (concentration of fucoidan required to chelate 50% of the ferrous ion activity) of CFP and PFP on ferrous ion-chelating activities were 3167 ppm and 883 ppm, respectively (Fig. 3C). Reducing power of CFP and PFP was monitored in Fig. 3D, and it exhibited a reducing power in a dose-dependent manner. Moreover the PFP was found to exhibit a better reducing power than CFP.

3.5. *Effects of CFP and PFP on the inhibition of growth in human colon cancer cells*

The inhibitory activity of CFP and PFP was investigated using a tetrazolium-based colorimetric assay (MTT test) on human colon cancer cell line HT-29. Fig. 4A shows that both CFP and PFP dose-dependently inhibited HT-29 cancer cell growth *in vitro*. PFP possessed a higher inhibition of cancer cell growth as compared to CFP. The treatments of HT-29 cells with 500 μg/mL of CFP and PFP for 48 hours resulted in the induction of chromatin condensation, which could be visualized as an intense, highly condensed, blue fluorescence (Fig. 4B) within the cell nuclei. When HT-29 cells were treated with 500 μg/mL of CFP and PFP for 48 hours, PFP possessed a significantly higher percentage of cells in the sub-G_1 phase (7.04 ± 0.72%), followed by CFP (5.78 ± 0.28%) and then untreated cells (2.65 ± 0.16%; Fig. 4C), indicating enriched DNA fragmentation and apoptosis in the cells. In addition, the cell cycle phase distribution of HT-29 cells after treatments with 500 μg/mL of CFP and PFP for 48 hours is also shown in Fig. 4C, and a significant increase ($p < 0.05$) of the cell population in the G_0/G_1 phase after treatment with CFP and PFP (untreated, 57.83 ± 0.29%; CFP, 64.9 ± 0.24%; PFP, 65.27 ± 1.45%) is observed. The results indicate that the accumulation of cells in the G_0/G_1 phase might account for the growth inhibition of HT-29 cells by CFP and PFP.

4. Discussion

A comparison of the chemical compositions of *S. cristaefolium* with two other previously reported species of brown algae, namely *S. horneri* and *S. naozhouense*, is shown in Table 1. It reveals that *S. cristaefolium* possesses the least amount of ash (24.80 ± 0.08%; dry basis) and protein (3.83 ± 0.81%; dry basis), and the largest amount of lipid (1.51 ± 0.14%; dry basis) and total carbohydrate (69.87 ± 0.83%; dry basis). It has been

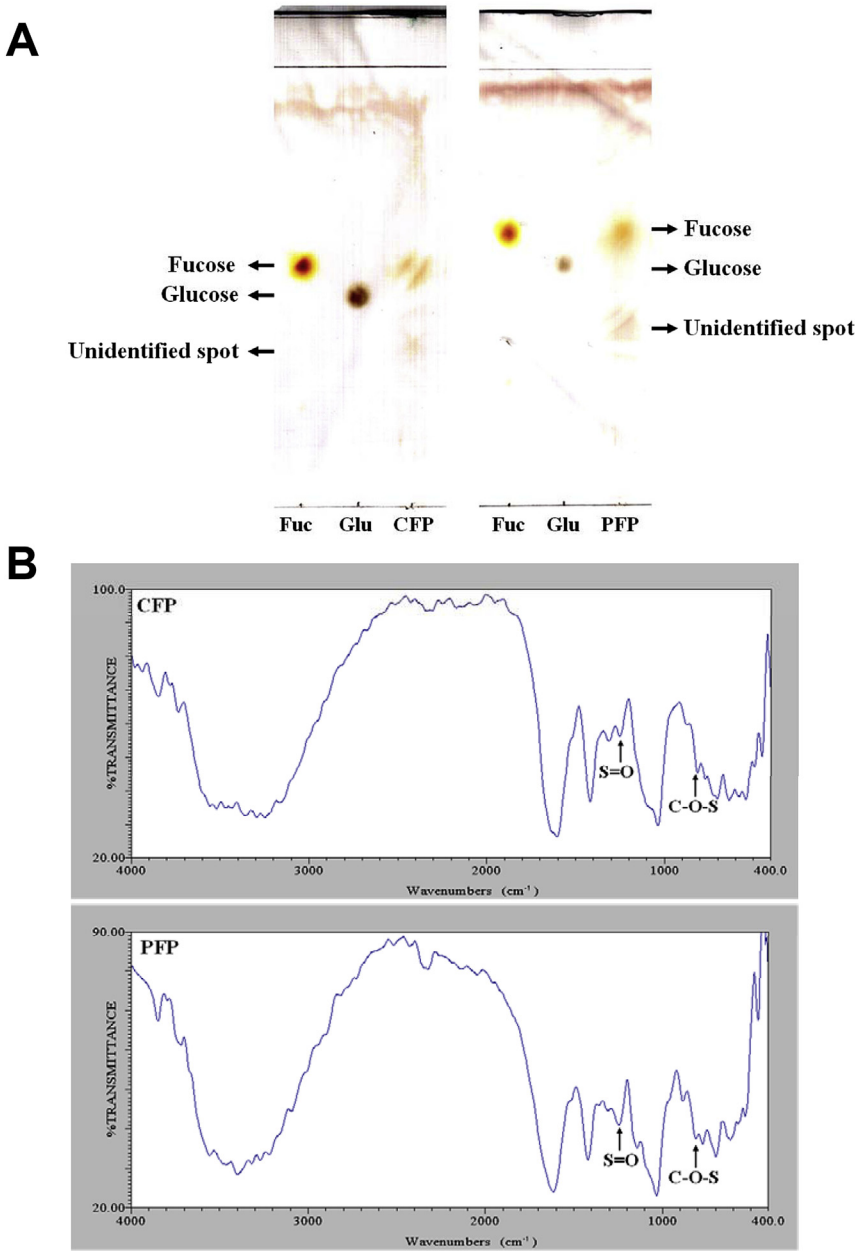

Fig. 2 – Thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR) spectra of crude fucoidan preparation (CFP) and purified fucoidan preparation (PFP). (A) TLC spectrum for CFP and PFP. Fucose and glucose were used as the reference standards. Position of standards are indicated. (B) FTIR spectra for CFP and PFP. Absorption bands for SO and C-S-O are indicated. CFP = crude fucoidan preparation; fuc = fucose; glu = glucose; PFP = purified fucoidan preparation.

suggested that the major component of brown algae besides moisture is carbohydrate [33]. Studies also showed that, in brown algae, approximately 25.9% of the carbohydrate composition is fucoidan [33,34]. Thus, *S. cristaefolium*, which was shown to have a high average carbohydrate content, appeared to be a good source for extracting fucoidan.

The comparison of the extraction parameters in SC1 and SC2 is shown in Table 2. Generally, SC1 is simple, fast, and reactant-saving as compared to SC2. Moreover SC1 possessed a significantly higher extraction yield compared with that of SC2 (Table 2). Thus, SC1 has simple steps, is reactant-saving, and offers a high extraction yield, which taken together suggest that it may be the method of choice for extraction of fucoidan from brown seaweed for industrial applications. However, differences in the precise composition and bioactive activities of CFP and PFP need to be further elucidated.

A previous study suggested fucose-containing sulfated polysaccharides could be extracted from brown seaweed species such as *Fucus*, *Laminaria*, and *Sargassum* by various extraction techniques, and the extracts also displayed distinct compositional characteristics [2]. We thus characterized the compositions of CFP and PFP with respect to the qualitative and quantitative analyses of fucose, sulfate groups, and uronic acids. The monosaccharides could be separated and

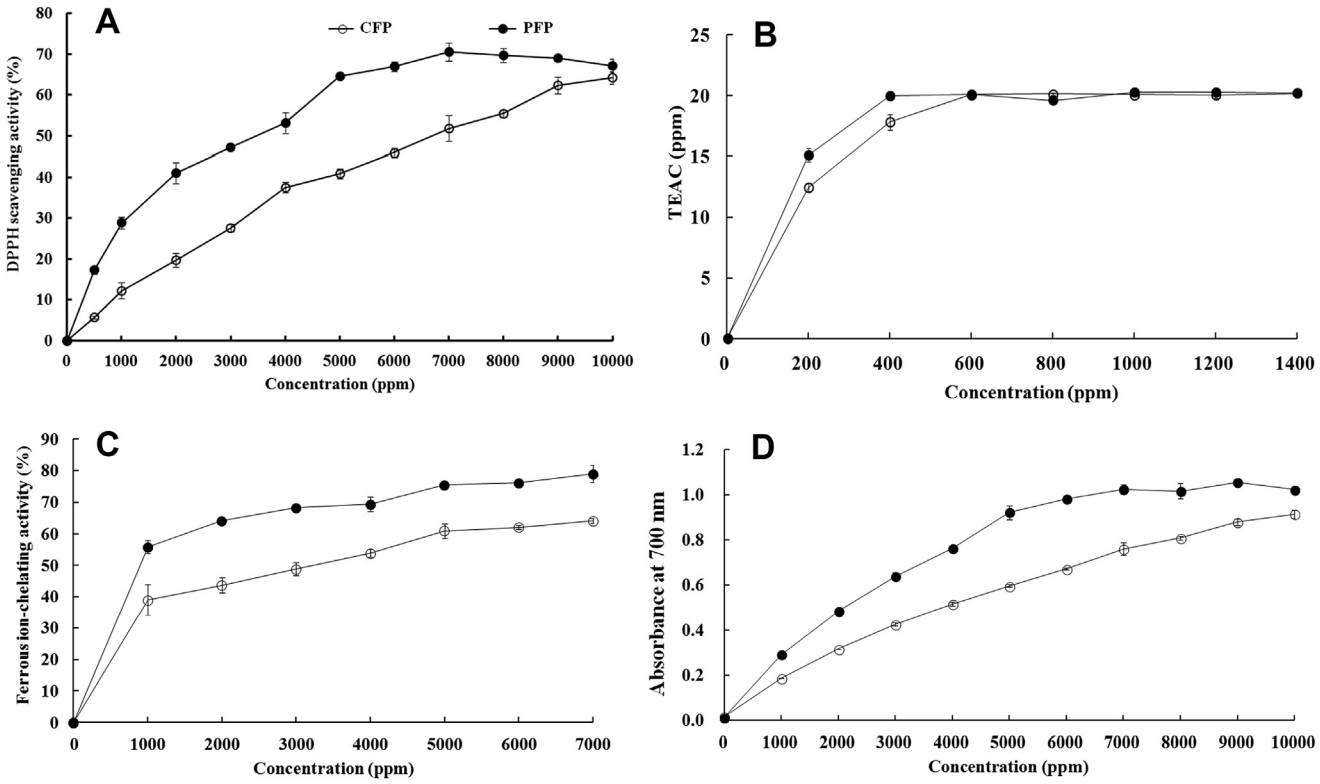

Fig. 3 – Antioxidant activities of crude fucoidan preparation (CFP) and purified fucoidan preparation (PFP). (A) DPPH scavenging activity for CFP and PFP. (B) ABTS radical scavenging activity for CFP and PFP. (C) Ferrous ion-chelating activity for CFP and PFP. (D) Reducing power for CFP and PFP. Each value represents the mean ± standard deviation of three determinations. ABTS = 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; CFP = crude fucoidan preparation; DPPH = 2, 2-diphenyl-1-picrylhydrazyl; PFP = purified fucoidan preparation; TEAC = Trolox equivalent antioxidant capacity.

identified by TLC relatively simply and quickly with a small amount of sample, using inexpensive semimicro equipment and materials [35]. The TLC chromatogram showed that both CFP and PFP contained fucose (Fig. 2A). Since the PFP is a purified form of fucoidan, it was found that the PFP had a relatively high fucose content as compared to CFP (Table 2). A previous study suggested that the high fucose content of alga polysaccharide might be positively correlated with the high biological activities as compared to other polysaccharides [36], indicating that PFP might have better biological functions. The presence of sulfated groups in CFP and PFP was confirmed by FTIR analysis (Fig. 2B) and determination of sulfate (Table 2), respectively. From Table 2, it was found the purified fucoidan PFP had a significantly higher amount of sulfate than the crude fucoidan CFP. Several investigators suggested that the degree of sulfation of fucoidan was positively related to biological functions such as antioxidant, anticoagulant, and anticancer activities [2,31]. Consequently, PFP which has a higher level of sulfate content, may possess superior biological activities. These results warrant further study. In addition, Table 2 shows that CFP has a significantly higher amount of uronic acids than PFP. This may be explained by the fact that CFP is a mixture of polysaccharides containing alginic acid, which is mainly composed of n-mannuronic and L-guluronic acids. Thus, CFP contains a remarkably high uronic acid content. Taken together, we confirmed that CFP and PFP contain fucose, sulfate groups, and uronic acids, and both CFP and PFP appear to possess biological functions.

DPPH has been widely used as a free radical to examine reducing substances and is a useful reagent for investigating the free radical scavenging activities of compounds [37]. Fig. 3A showed the scavenging effects of CFP and PFP on DPPH free radicals. Both CFP and PFP exhibited DPPH scavenging properties in a dose-dependent manner. However PFP had an approximately 1.93-fold greater DPPH radical scavenging activity as compared to CFP (Fig. 3A). Investigators reported that the IC_{50} of the DPPH radical scavenging activity for low molecular weight fucoidan extracted from *Laminaria japonica* and fucoidan extracted from *S. wightii* were about 3700 ppm and 8250 ppm, respectively [38,39], and the DPPH radical scavenging activity of PFP was found to be higher than those of low molecular weight fucoidan extracted from *L. japonica* and fucoidan extracted from *S. wightii*. Moreover, the DPPH radical scavenging activity of CFP was also higher than that of the fucoidan extracted from *S. wightii*. ABTS assay is a remarkable tool for determining the antioxidant activity of hydrogen-donating compounds (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavenger of lipid peroxyl radicals) [28]. With their high ABTS radical scavenging activity, antioxidative compounds were considered to most likely be hydrophilic [40]. Ferrozine can quantitatively form complexes with Fe^{2+}, which has absorbance at 562 nm. In the

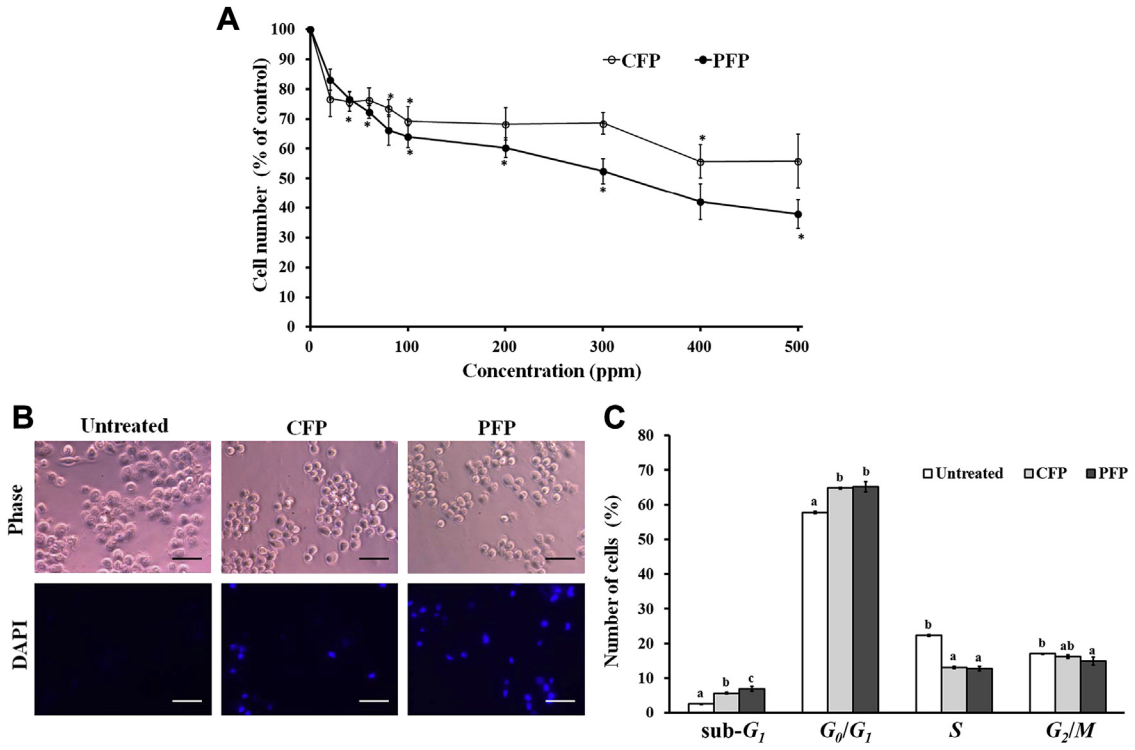

Fig. 4 – Inhibition of growth in human colon cancer cell line HT-29 by crude fucoidan preparation (CFP) and purified fucoidan preparation (PFP). (A) Cytotoxic effects of CFP and PFP on HT-29 cancer cells. The cell number (% of control) was determined by MTT assay. Each value represents the mean ± standard deviation of three determinations. *Significantly different from control, $p < 0.001$. (B) Morphological assessments of CFP and PFP treated HT-29 cells for 48 hours. Nuclei were stained with DAPI and observed under a fluorescence microscope (scale bar, 50 μm). (C) The cell cycle pattern of HT-29 cells after treatment of CFP and PFP at 500 μg/ml for 48 hours. Percentages of cells in the sub-G_1, G_0/G_1, S, and G_2/M phases were determined using established Multicycle software. Means at a group without a common letter differ, $p < 0.05$. CFP = crude fucoidan preparation; DAPI = 4′,6-diamidino-2-phenylindole; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PFP = purified fucoidan preparation.

presence of chelating agent, the complexes are disrupted resulting in a reduction of the red color of the complexes. The ferrous ion-chelating effect is estimated by measurement of color reduction. Fig. 3C showed the ferrous ion-chelating activities for CFP and PFP. Ferrous ions were revealed to stimulate lipid peroxidation and are recognized as an effective prooxidant in food systems [41]. Both CFP and PFP exhibited ferrous ion-chelating activities in a concentration-dependent manner, and PFP had greater ferrous ion-chelating activity than CFP as revealed by the IC_{50} values of ferrous ion-chelating (Fig. 3C). A previous study suggested the maximum ferrous ion-chelating activity of fucoidan extracted from *L. japonica* was about 25% at a concentration of 1200 ppm [15]. Our results showed that the ferrous ion-chelating activities of CFP and PFP were 40% and 57.5%, respectively, at a concentration of 1200 ppm (Fig. 3C), indicating both CFP and PFP possessed a higher metal ion-binding ability than that of fucoidan extracted from *L. japonica*. Reducing power has been reported to have a direct and positive correlation with antioxidant activity [38]. In the reaction system, antioxidant components in samples cause a reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} form, and Fe^{2+} can be monitored by measuring the formation of Prussian blue at 700 nm. Fig. 3D shows that both CFP and PFP exhibited a reducing power in a dose-dependent manner, and the PFP exhibited a better reducing power than that of CFP. In a previous study it was shown that the absorbance at 700 nm for the reducing power of fucoidan extracted from *L. japonica* was about 0.12 at a concentration of 2000 ppm [15]. Our data suggest that the absorbance values at 700 nm for the reducing power of CFP and PFP were 0.32 and 0.48, respectively, at a concentration of 2000 ppm. Thus, the reducing power in CFP and PFP surpassed that of fucoidan extracted from *L. japonica*. Research suggests that the high fucose content of alga polysaccharide might be positively correlated with high biological activities as compared to those observed in other polysaccharides [36]. Investigators have also suggested that the degree of sulfation of fucoidan was positively related to biological functions such as antioxidant, anticoagulant, and anticancer activities [2,31]. Our data confirmed that the purified fucoidan PFP, which had higher fucose and sulfate contents, possessed elevated antioxidant activities as compared to those of crude fucoidan CFP (Table 2 and Fig. 3). In summary, both CFP and PFP possess antioxidant activities and some of these activities were superior to those of fucoidans reported in other studies. The data also provide evidence that both CFP and PFP may serve as

natural antioxidants, and thus they may have potential applications in the food and pharmaceutical industries.

In Taiwan, due to the gradually switch to a westem style diet, the prevalence of colon cancer appears to rise. The anticolon cancer activity of fucoidan was reported by several investigators [42,43]. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring and converts MTT to an insoluble purple formazan and the amount of formazan produced is directly proportional to the number of viable cells [44]. In the present study, the cytotoxicity of CFP and PFP were investigated using an MTT test on the human colon cancer cell line HT-29. Fig. 4A shows that both CFP and PFP dose-dependently inhibited HT-29 cancer cell growth, and PFP possessed a higher inhibition of cancer cell growth as compared to CFP. PFP showed significantly higher sulfate content compared with that of CFP (Table 2), suggesting that the higher cytotoxic effect of PFP was possibly due to the higher amount of sulfate in PFP. A similar observation was found in three sulfated polysaccharides WPS (crude polysaccharide), WPS-2 (second fraction of WPS), WPS-2-1 (purified polysaccharide from WPS-2) purified from aqueous extracts of *L. japonica* [31]. Moreover, it has been revealed that fucose or fucan were found to be a critical factor in algal polysaccharide bioactivities [45]. The amount of fucose content in CFP and PFP might contribute to their bioactivities. Table 2 showed that the fucose content of CFP and PFP was 21.41 ± 0.60% and 27.38 ± 1.45%, respectively, indicating that the higher cytotoxic activity of PFP might also attribute to the higher amount of fucose. The inhibitory activity of CFP and PFP in human colon cancer cells was further confirmed by DAPI-staining and cell cycle analysis, respectively. DAPI staining is a tool for identification of chromatin condensation and nuclear fragmentation in apoptotic cells [46]. The morphological evaluations of chromatin condensation and/or fragmentation induced by CFP and PFP on HT-29 cells are shown in Fig. 4B. Both CFP and PFP induced greater chromatin condensation in the HT-29 cells as compared with that in the untreated cells. Flow cytometry is a rapid technique for analyzing the cell cycle. After treatment of cells with an apoptosis-inducing agent, DNA fragmentation can take place. The small fragments of the DNA can be eluted by washing with PBS. The remaining DNA quantitatively binds with the binding dye (PI) [47]. Cells that have lost DNA take up less stain and will appear to the left of the G_1 peak (so-called sub-G_1 peak). Hence, the cell population in the sub-G_1 phase is directly proportional to the induced DNA fragmentation and apoptosis of a given treatment. Fig. 4C shows the treatment with CFP and PFP significantly induced pronounced cell numbers of sub-G_1 cells in HT-29 cells as compared to untreated cells. This phenomenon clearly showed that the CFP and PFP induced DNA fragmentation and apoptosis. Additional functional characterization of the CFP and PFP by cell cycle analysis of HT-29 cells (Fig. 4C) revealed that the CFP and PFP significantly caused arrest or delay of G_0/G_1, suggesting the growth inhibition of HT-29 cells by CFP and PFP. Moreover, as shown in Fig. 4, the difference in cytotoxicity, DAPI-staining, and cell cycle arrest between CFP and PFP was unremarkable, and thus it appears that CFP could also serve as an agent for inhibition of growth in human colon cancer cells. In summary, CFP and PFP had inhibitory effect on human colon cancer cells and both are potential candidates for chemopreventive agents in colon cancer therapy.

5. Conclusion

In this paper, we compared the extraction yield as well as antioxidant activity and inhibition of growth in human colon cancer cells of fucoidans extracted from *S. cristaefolium* using two different methods (SC1 and SC2). SC1 and SC2 differed in the number of extraction steps, concentration of ethanol used, and obtained sulfated polysaccharide extracts, namely crude fucoidan preparation (CFP) and purified fucoidan preparation (PFP), respectively. CFP possessed a higher extraction yield, contained less fucose and sulfate, but more uronic acid. In contrast, PFP had a lower extraction yield, contained more fucose and sulfate, but less uronic acid. Both CFP and PFP exhibited antioxidant activity and inhibition of growth in human colon cancer cells. Certain biological functions in CFP and PFP were superior to those of fucoidans reported in other studies, and since there was little difference in the bioactive activities between CFP and PFP, the high extraction yield of SC1 would make it a more favorable choice as a method of industrial extraction of fucoidan. Further investigation is needed to elucidate the antioxidant and colon cancer preventive mechanism *in vivo* and to establish the safety of fucoidan from *S. cristaefolium* for human consumption.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

This work was supported by funding from the National Science Council of the Republic of China NSC 101-2321-B-022-001-MY2 (to C-Y H) and Kaohsiung Medical University Hospital, Kaohsiung Medical University (to C-Y W).

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