

Extraction, Fractionation, and Chemical Characterisation of Fucoidans from the Brown Seaweed *Sargassum pallidum*

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Abstract

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The fucoidans were obtained by extraction with water and gradient precipitation with different concentrations of ethanol. The main structural characterisations and bioactivities were achieved. Four fractions of water-soluble crude fucoidans with different molecular weights – SPC60, SPC70, SPH60, SPH70 – were extracted from *S. pallidum* collected from the Yellow Sea, China, using cold water and hot water extraction, and fractional precipitation with gradient concentrations of ethanol. Chemical analysis demonstrated that all of these fucoidan fractions consisted of fucose, rhamnose, xylose, mannose, glucose, and galactose with different monosaccharide mole ratios. Fractions SPC60, SPC70, and SPH70 showed moderate cytotoxic activity against P388 murine leukaemia cells. Fucoidan is a kind of bioactive polysaccharide from the brown algae. This study provides a fast and feasible method to obtain fucoidans from *S. pallidum*, which can be used as pharmaceutical material and functional food.

Keywords: brown seaweed; *Sargassum pallidum*; fucoidan; extraction; cytotoxic activity

The brown seaweed *Sargassum pallidum*, distributed mainly in the Yellow Sea, China, has been used in folk medicine for a long history. Recorded in traditional Chinese medicine, *S. pallidum* is often used to eliminate phlegm and relieve asthma (GERASIMENKO *et al.* 2014). According to the traditional Chinese medicine theory, the active constituents in *S. pallidum* have multiple pharmacological effects, including antitumor and antioxidant activities, as well as they soften hardness to dissipate stagnation and expectorate to dispel phlegm and water. Previous phytochemical studies have shown that *S. pallidum* contains a variety of chemical constituents such as halogen compounds, tannins, sterioids, terpenoids, coumarins, and polysaccharides (LIU *et al.* 2009; YE *et al.* 2009, 2013; WANG *et al.* 2010). Among them, algal polysaccharides including alginates, laminarins, and

fucoidans are the main bioactive components with various biological activities. Specifically, fucoidans are of interest mainly as biologically active constituents. Fucoidans are unique algal polysaccharides mainly composed of fucose and sulphate groups, and usually found in the cell-wall matrix of various brown seaweed species. In the past decades, a great deal of attention has been paid to fucoidans due to their numerous biological activities with potential medicinal values, such as antitumor, anticoagulant, antioxidant, and hypolipidaemic effects (LI *et al.* 2008, SYNITSYA *et al.* 2010, HAHN *et al.* 2012). It should be noted that fucoidans from brown seaweeds are usually highly branched with high molecular weight (MW) and composed of hetero-monosaccharides decorated with sulphate groups which make them negatively charged. These properties make them

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difficult to be purified and identified. Despite of all the above-mentioned features, research on the extraction of fucoidans from *S. pallidum* is limited. The aim of the present study is to provide an effective and feasible method to obtain fucoidans from *S. pallidum*. Gradient precipitation with increasing concentration of ethanol was applied to fractionate the fucoidans from *S. pallidum*. The chemical properties including monosaccharide composition and MW values of different fractions were analysed and their cytotoxicities were also determined by a methyl thiazolyl tetrazolium (MTT) assay.

MATERIAL AND METHODS

The brown seaweed *S. pallidum* from the sea area of Weihai in the Yellow Sea, China, was collected in October 2012, and was identified by Prof SHUBEN QIAN, Ocean University of China. According to the 'Chinese Pharmacopoeia' (2010 Edition), *S. pallidum* sample used in this study has the following morphological characteristics: the seaweed sample has a dark brown frond, and its frond length is about 2 meters. It also has a cylindrical trunk with multiple branches and lanceolate or oval leaf. A dark brown spherical balloon grows in the bottom of the branch. *S. pallidum* grows on the rocks in the subtidal zone. Foetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco Industries (Grand Island, USA). The P388 murine leukaemia cell line was supplied by Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Dextran T-series standards (MW 800 000, 400 000, 100 000, 50 000, 10000, 6000 Da) and monosaccharide standards, including rhamnose, arabinose, fucose, xylose, mannose, glucose, galactose, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, USA). All the other reagents used in this study were of the highest quality available from commercial vendors.

Cold water extraction process. The seaweed was washed with fresh water to remove salt, sand, and impurities, dried at 40°C, and milled in a home blender. These pretreated samples were stored in a cool and dry environment for later use. The polysaccharides from the brown seaweed *S. pallidum* were extracted following the method of JIAO with modifications (JIAO *et al.* 2012). The dried frond of *S. pallidum* (500.0 g) was ground and sieved (10 mesh) to obtain a fine powder.

The seaweed powder was extracted with 80% ethanol at 80°C for 2 h to remove lipids and pigments. After ethanol extraction, the seaweed sample was drying in a cool and ventilated place. The air-dried residue was then extracted 3 times with 10 volumes of cold water at 25°C for 2 hours. The extraction solution was centrifuged at 4000 g for 15 min to collect the supernatant. The supernatant was incorporated and concentrated to one-fifth of the initial volume using a rotary evaporator at 60°C under vacuum. The resulting solution was mixed with 3/7, 3/2, and 7/3 volumes of dehydrated ethanol at final concentrations of 30, 60, and 70%, respectively. The precipitate was collected by centrifugation at 4000 g for 10 minutes. After being washed three times with acetone, the precipitate was dried at 60°C until its weight was constant. In this way, three precipitated fractions were obtained and designated as SPC30, SPC60, and SPC70, respectively. These fractions were deproteinised three times using the Sevag method (LIU *et al.* 2012). The polysaccharide solution and the Sevag reagent (chloroform/*n*-butanol 4 : 1, v/v) were mixed (polysaccharide solution/Sevag reagent 5 : 1, v/v) and shaken acutely for 30 min, then centrifuged for removing the denaturalised proteins, and the process was repeated 5 times. The precipitate was collected and lyophilised, and the yield was calculated as follows:

$$\text{polysaccharide extraction yield (\%, w/w)} = [\text{polysaccharide weight (g)/dried seaweed powder weight (g)}] \times 100\%$$

Hot water extraction process. The air-dried defatted seaweed residue was extracted 3 times with 10 volumes of distilled water at 85°C for 2 hours. The hot water extract was successively subfractionated by the same procedures as for the cold water extracts to provide other three polysaccharide fractions SPH30, SPH60, and SPH70 (Figure 1).

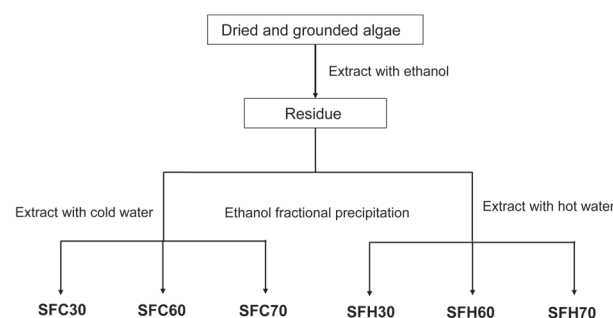


Figure 1. A flowchart of *Sargassum pallidum* crude polysaccharide extraction

Analytical techniques. Total sugar content was determined by the phenol-sulphuric acid method using glucose as the standard (DUBIOS *et al.* 1956). Protein content was measured by the method of BRADFORD (1976). Sulphate group content was determined using the BaCl₂-gelatin turbidimetry method (THERTO & HARTIALA 1971). Uronic acid content was determined by the carbazole-sulphuric acid method (BITTER & MUIR 1962). All analytical determinations were performed in triplicate, and the results are presented as mean ± standard deviation (SD).

Molecular weight (MW) measurement. The MW value was determined by high performance gel permeation chromatography (HPGPC) with a size-exclusion chromatography column (PL aquagel-OH column, 7.5 × 300 mm, 8 µm) on an Agilent 1260 LC (1260 Infinity; Agilent Technologies Co. Ltd, Palo Alto, USA) equipped with a refractive index detector (RID, Agilent 1362A). The mobile phase was 0.2 mol/l Na₂SO₄, and the flow rate was 0.5 ml/minutes. The sample was dissolved in the mobile phase (0.2%, w/v). A 20 µl sample solution was injected in each run, and the column temperature was 30°C. The average MW was measured according to the standard curve equation calculated from a Dextran T series standard of the known MW. The elution volumes were plotted against the logarithm of their respective MW. The Agilent GPC software was used to calculate the MW value. The calibration curve of Log MW of the standard dextrans against their elution time (ET) was obtained (Log MW = -0.6946ET + 15.007, R² = 0.9985) (LIU *et al.* 2014).

Fourier transform infrared spectrometry (FT-IR) analysis. The polysaccharide samples (2 mg) were dried in a P₂O₅ desiccator for 48 h, mixed with spectroscopic grade potassium bromide (KBr) powder, ground, and pressed into 1 mm pellets for FI-IR measurement (Nicolet Nexus 470; Thermo Electron Co., Waltham, USA). The FI-IR spectrum was determined using a Thermo Nicolet Nexus 470 instrument within the frequency range from 400 to 4000 cm⁻¹. The FT-IR represents an average of 256 scans with a frequency of 2 cm⁻¹ (CHEN *et al.* 2011).

Analysis of monosaccharide compositions. The polysaccharide samples (10 mg) were hydrolysed with 2 mol/l trifluoroacetic acid at 100°C for 12 hours. The sulphated fucoidans were hydrolysed entirely into monosaccharides. The excess acid was removed by co-distillation with methanol after the hydrolysis was completed. Sugars were converted to their alditol acetates by treatment with pyridine (2 ml) and acetic

anhydride (1.5 ml). Gas chromatography (GC) was performed on a gas chromatography (GC) instrument with a SE-54 fused silica capillary column (320 µm × 50 m) (HP6890; Agilent Technologies Co. Ltd., USA) equipped with a flame-ionisation detector. The operation was performed under the following conditions: H₂ – 1.5 ml/min; air – 200 ml/min; N₂ – 1.5 ml/min; injection temperature – 250°C; detector temperature – 250°C; column temperature – 212°C. Sugar identification was done by comparison with the reference sugars including rhamnose, fucose, arabinose, xylose, mannose, glucose, and galactose (FU *et al.* 2008).

In vitro cytotoxicity assay. The P388 murine leukaemia cell line was used to test the cytotoxic activity of the polysaccharide fractions SPC60, SPC70, SPH60, and SPH70 (MOSADDIK 2003). Cells were incubated into 96-cell plates at 2 × 10⁴ cells per well. RPMI-1640 containing antibiotics (100 µg/ml streptomycin and 100 U penicillin) and 10% FBS were used as the culture medium. After 4-h preincubation of the cells in a humidified 5% CO₂ incubator at 37°C, samples were added with the final concentration of 200 µg/ml, followed by further incubation for 24 hours. An MTT solution (5 mg/ml) dissolved in phosphate buffered saline (PBS) was added to each well by administering 10 µl per well. The cells were cultured for another 4 h, and then the culture medium was removed. Finally, 100 µl dimethyl sulfoxide (DMSO) was added to each well, mixed and measured at 570 nm. The inhibition rate (IR) was calculated according to the formula below:

$$\text{IR (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100]$$

where: A_{blank} – absorbance of blank control group; A_{sample} – absorbance of experimental group

Statistical analysis. All experiments were performed three times and the results are expressed as mean ± standard deviation (SD). All statistical comparisons were carried out using the one-way ANOVA (analysis of variance) test followed by Tukey's test. *P*-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Fucoidans from brown seaweeds usually contain the main structure composed of fucose and sulphate groups with other minor monosaccharides (PANG-

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Table 1. Extraction yield and chemical compositions of polysaccharide fractions obtained by different extraction methods and precipitation concentrations from *S. pallidum*; polysaccharide fraction extracted by cold water and precipitated at the final ethanol concentration of 30% (SPC30); 60% (SPC60), and 70% (SPC70); polysaccharide fraction extracted by hot water and precipitated at the final ethanol concentration of 30% (SPH30); 60% (SPH60), and 70% (SPH70)

Polysaccharide fractions	Extraction yield (% dry basis)	Molecular weight (MW, Da)	Component (%)			
			sugar	protein	sulphate	uronic acid
SPC30	0.31	–	10.23 ± 1.23	23.43 ± 2.11	nd	47.27 ± 3.12
SPC60	0.69	537 000	83.75 ± 4.96	9.62 ± 1.07	4.18 ± 0.63	32.53 ± 2.71
SPC70	0.44	512 000	84.10 ± 3.28	8.48 ± 0.97	6.84 ± 0.87	34.78 ± 3.22
SPH30	0.30	–	12.59 ± 1.67	27.61 ± 2.33	nd	42.82 ± 2.19
SPH60	0.96	432 000	82.47 ± 5.72	12.94 ± 1.73	3.84 ± 0.27	29.14 ± 3.27
SPH70	0.80	430 000	83.12 ± 6.03	10.37 ± 1.02	6.85 ± 0.71	20.05 ± 1.87

nd – not detected

ESTUTI & KIM 2013). The compositions of these polysaccharides may vary according to different extraction procedures (WUJESINHE & JEON 2012). In the present study, polysaccharides from the brown seaweed *S. pallidum* were extracted and separated by different extraction processes and ethanol fractional precipitation. Six polysaccharide fractions were obtained, of which SPC30, SPC60, and SPC70 were from cold water extraction and SPH30, SPH60, and SPH70 from hot water extraction. Among them, four fractions, SPC60, SPC70, SPH60, and SPH70 which were precipitated at higher ethanol concentrations, were identified as fucoidans. The other two fractions, SPC30 and SPH30, were found to have lower total sugar contents and higher uronic acid contents compared with those of SPC60, SPC70, SPH60, and SPH70, and no sulphate group was detected in either of them (Table 1). Moreover, SPC30 and SPH30 were

found to be insoluble in water, but well soluble in an alkaline solution. In the FT-IR spectra of SPC30 and SPH30 (Figure 2), the characteristic absorption bands for typical guluronic acids (780 cm⁻¹) were also observed, indicating that SPC30 and SPH30 were not fucoidans (FENORADOSA *et al.* 2010).

Chemical properties of fucoidan fractions. Contrasting to the fractions SPC30 and SPH30, the fucoidan fractions SPC60, SPC70, SPH60 and SPH70 were obtained with higher yields, while the sugar and sulphate group contents were high and similar in them (Table 1). It should be noted that the yields by hot water extraction were higher than those by cold water extraction. The increase in ethanol precipitation concentrations from 60 to 70% resulted in the increase of sulphate contents from 4.18% to 6.84% by cold water extraction and from 3.84% to 6.85% by hot water extraction. The sulphate contents of these

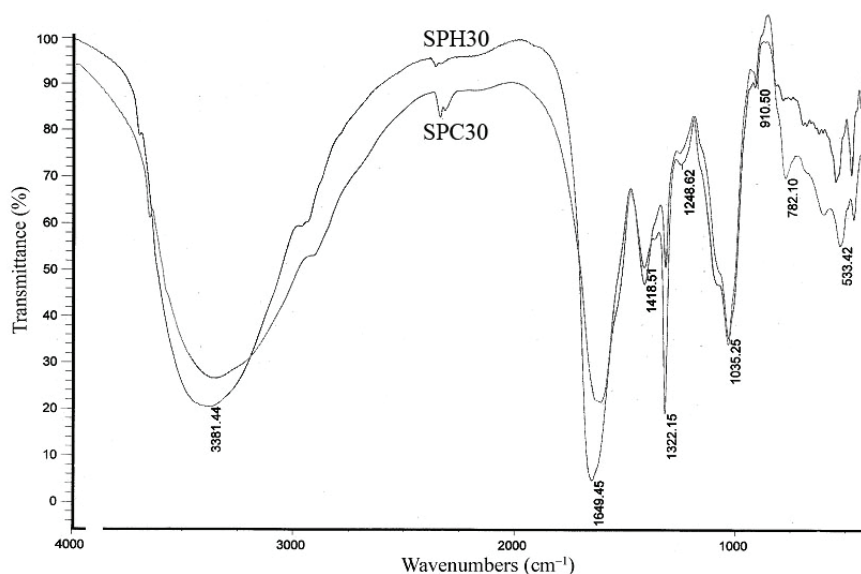


Figure 2. Fourier transform infrared (FT-IR) spectroscopy of two fractions extracted by different methods and precipitated at the final ethanol concentration of 30% (SPH30 and SPC30)

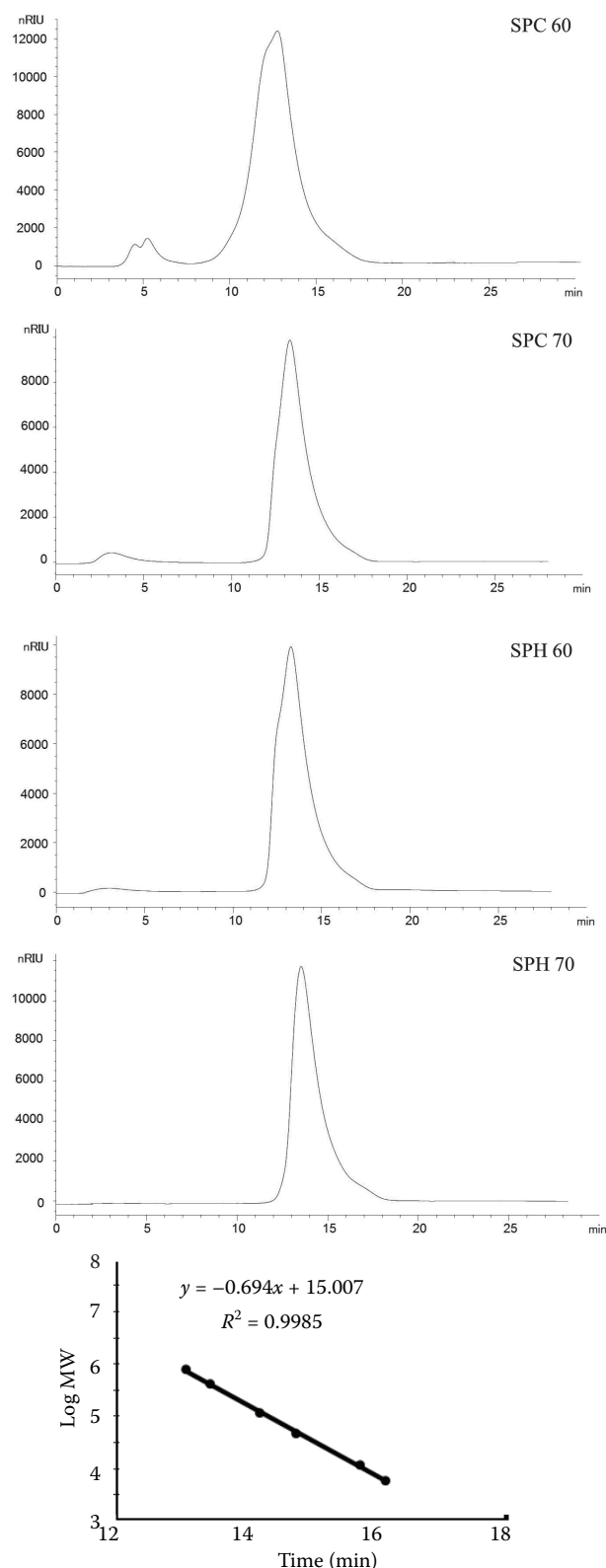


Figure 3. HPGPC chromatogram of four fractions extracted by different methods and precipitated at the final ethanol concentration of 60 and 70% (SPC60, SPC70, SPH60, and SPH70) on the PL aquagel-OH column and standard calibration curve

asymmetric, suggesting that these crude fucoidans are heterogeneous. This result may be attributed to two aspects. Firstly, crude fucoidans obtained in this study are a kind of heteropolysaccharides, and their molecular distribution was heterogeneous. On the other hand, some macromolecular substances (protein and some other compounds) existed in the crude fucoidans. Therefore, it is necessary to purify these fucoidans in further study.

FT-IR spectroscopy analysis of fucoidan fractions. The FT-IR spectra of SPC60, SPC70, SPH60, and SPH70 showed the typical absorption bands of fucoidans (Figure 4). The bands around 3400 and 2900 cm^{-1} were assigned to the deformation of O-H and C-H stretching vibrations, respectively. The absorptions at around 1600 and 1400 cm^{-1} indicated the presence of carbonyl groups. The fucoidan characteristic absorption bands at 1240~1260 cm^{-1} (S=O stretching vibration) confirmed the presence of sulphate groups, which were consistent with their sulphate contents. The absorption bands at 810~840 cm^{-1} were attributed to the C-O-S bending vibration of sulphate substituents, which suggested a complex pattern of substitution. An additional sulphate absorption band at 824 cm^{-1} and a relatively small shoulder at 845 cm^{-1} indicated that the majority of sulphate groups occupy positions 2 and/or 3, and only a minor part of sulphates is located at position 4 of fucopyranose residues (BILAN *et al.* 2002).

Analysis of monosaccharide compositions for fucoidan fractions. The monosaccharide compositions of the isolated polysaccharides were analysed by gas chromatography (GC), compared with seven monosaccharide standards (Figure 5). The results showed that all of the fucoidan fractions, SPC60, SPC70, SPH60, and SPH70, consisted of rhamnose,

Table 2. Monosaccharide compositions and their mole ratios in SPC60, SPC70, SPH60, and SPH70

Polysaccharide fraction	Monosaccharide composition and mole ratio
SPC60	Rha/Fuc/Xyl/Man/Glc/Gal = -3.98:0.46:2.58:1.00:1.62
SPC70	Rha/Fuc/Xyl/Man/Glc/Gal = 0.35:5.91:0.44:3.52:1.00:4.05
SPH60	Rha/Fuc/Xyl/Man/Glc/Gal = 0.09:1.27:0.17:0.92:1.00:0.58
SPH70	Rha/Fuc/Xyl/Man/Glc/Gal = 0.31:3.88:0.40:2.29:1.00:2.51

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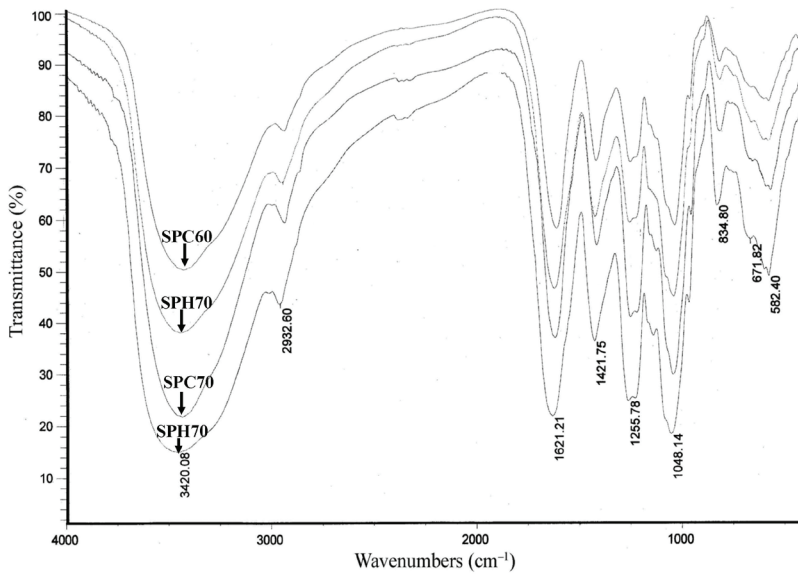


Figure 4. FT-IR spectroscopy of fucoidan fractions obtained by different extraction methods and precipitation at the final ethanol concentration of 60 and 70% (SPC60, SPH60, SPC70, and SPH70)

xylose, fucose, mannose, glucose, and galactose. Further analysis revealed that these fucoidans presented a heterogeneous structure mainly composed

of fucose, mannose, and galactose with different modes of monosaccharide mole ratios (Table 2). As shown in Figure 5 B–E, fucose was the predominant

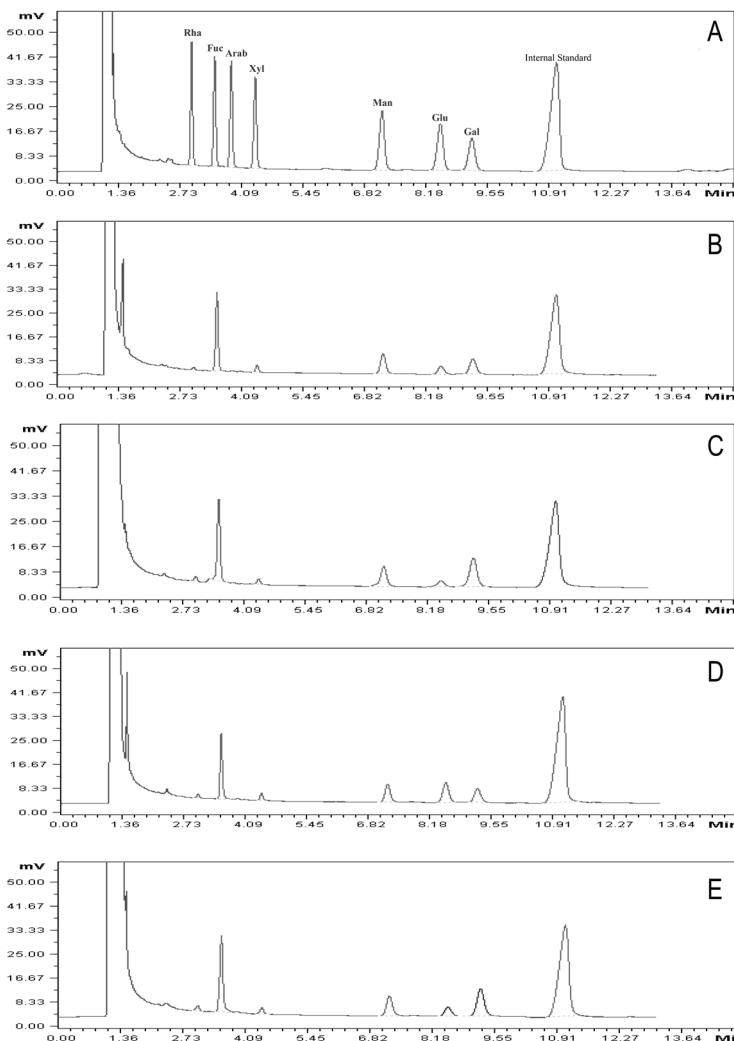


Figure 5. GC analysis of monosaccharide compositions in fucoidan fractions obtained by different extraction methods and precipitation at the final ethanol concentration of 60 and 70%; GC chromatograms of derivatives from standard monosaccharide (A), SPC60 (B), SPC70 (C), SPH60 (D), and SPH70 (E)

Rha – rhamnose; Fuc – fucose; Arab – arabinose; Xyl – xylose; Man – mannose; Glc – glucose; Gal – galactose

monosaccharide in the monosaccharide composition of these four fractions. The fucose contents in SPC60 and SPC70 were higher than those of SPH60 and SPH70, and this difference might be due to different extraction temperatures. The above analysis suggested that the different extraction processes and precipitation ethanol concentrations resulted in the fucoidan fractions with different monosaccharide compositions.

***In vitro* cytotoxicity assay for fucoidan fractions.** The cytotoxicities of the fucoidan fractions on P388 murine leukaemia cells *in vitro* were investigated using the MTT method. Fractions SPC60, SPC70, and SPH70 displayed moderate cytotoxic activities with the inhibitory rates of 11.09 ± 1.27 , 13.86 ± 1.67 , and $10.72 \pm 0.83\%$ at a concentration of 200 $\mu\text{g/ml}$, respectively. While SPH60 showed weak activity with an inhibitory rate less than 10%. It has been reported that the bioactivities of brown seaweed polysaccharides are closely related to their structural parameters, such as degree of sulphation, molecular weights, sulphation positions, monosaccharides, and glycosidic branching. Also, the sulphate content of fucoidan is one of the most important factors for its biological effects (Ye *et al.* 2008). Therefore, the mechanism for polysaccharide extraction and the relationships between structures and activities need to be further studied for the polysaccharides from *S. pallidum*.

CONCLUSIONS

Gradient ethanol precipitation was a simple and feasible method for the fractionation of crude fucoidans from different aqueous extractions of *S. pallidum*. Although these fucoidan fractions consisted of similar sugar contents with fucose as the main monosaccharide, their monosaccharide mole ratios, sulphate contents, as well as MW values were different. These differences may be attributed to different extraction temperatures and ethanol precipitation concentration. Chemical properties and MW distribution analysis demonstrated that these crude fractions may contain other ingredients such as protein, glycoprotein, and other compounds. Therefore, it is worthwhile to purify these fucoidan fractions, and elucidate the complete structures of the polysaccharides, including the configurations of glycosidic bonds, positions of glycosidic linkages, and sequences of monosaccharides. Three fractions (SPC60, SPC70, and SPH70) could

inhibit the P388 cell proliferation *in vitro*, and this bioactivity was probably generated by the sulphate groups in crude fucoidans. These findings need to be examined further to elucidate the underlying factors of fucoidan bioactivity.

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