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Antioxidant Capacities of Phlorotannins Extracted from the Brown Algae *Fucus vesiculosus*

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ABSTRACT: A process for the effective extraction and fractionation of phlorotannins from *Fucus vesiculosus* with high antioxidant potentials was investigated. The antioxidant activity of *F. vesiculosus* extract/fractions was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, reducing power, and ferrous ion-chelating assays. Among the crude extract and different polarity fractions, the phlorotannin-enriched ethyl acetate fraction possessed the highest DPPH scavenging activity and reducing power. This fraction was further fractionated by Sephadex LH-20 column chromatography or ultrafiltration. The antioxidant properties were evaluated by both the above chemical antioxidant tests and a mononuclear cell-based bioassay. Sephadex subfractions LH-2 and LH-3 with high total phlorotannin content exhibited strong DPPH quenching activity, comparable to those of ascorbic acid and butylated hydroxytoluene and significantly higher than that of α-tocopherol. Polyphenols in *F. vesiculosus* were found to consist mainly of high molecular weight phlorotannin polymers. There were no clear relationships between the degree of polymerization, molecular size, and antioxidant activity. All the subfractions separated by Sephadex LH-20 column chromatography and ultrafiltration showed a high ability to scavenge reactive oxygen species generated by mononuclear cells. Further characterization of the phlorotannin compounds was performed on six Sephadex subfractions. Several phlorotannin oligomers were tentatively identified on the basis of HPLC−ESI-MS^n analyses.

KEYWORDS: *Fucus vesiculosus, phlorotannins, antioxidant activity, reactive oxygen species, mononuclear cells*

INTRODUCTION

Currently there is an increased awareness and safety concern toward synthetic antioxidants and a worldwide trend to apply natural antioxidants to replace synthetic compounds as additives in foods or as nutraceuticals and functional food ingredients.[1,2] Natural antioxidants derived from various plants and marine algae not only show great potential for improving the oxidative stability of food products, but also have a broad array of additional health-promoting benefits. An appropriate intake of dietary antioxidants has been suggested to play an important role in enhancing the body's defense system and preventing reactive oxygen species (ROS)-related diseases.[3,4]

Marine macroalgae have been an important part of the human diet in Asia since ancient times and are traditionally well renowned for their versatile health benefits. Recent studies have revealed that marine algae not only are a good source of dietary fiber, proteins, vitamins, and minerals, but also contain a large array of secondary metabolites with valuable biological activities which cannot be found in terrestrial plants.[5] A number of potent antioxidant compounds have been isolated and identified from different types of seaweeds, including phlorotannins, sulfated polysaccharides, carotenoid pigments such as fucoxanthin and astaxanthin, sterols, catechins, and mycosporine-like amino acids.[6−8] Phlorotannins, the dominant polyphenolic secondary metabolites found only in brown algae (Phaeophyta), have recently attracted considerable research interest because of their superior antioxidant activities. Phlorotannins are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) monomer units and biosynthesized via the acetate−malonate pathway, also known as the polyketide pathway.[9] On the basis of the type of linkage between the phloroglucinol subunits as well as the number of additional hydroxyl groups on the aromatic skeletons, phlorotannins can be classified into six major subclasses: phlorethols, fucols, fuhalols, fucophlorethols, isofuhalols, and eckols.[9,10] Several species of brown algae, such as *Ecklonia cava*, *Ecklonia kurome*, *Fucus vesiculosus*, *Hizikia fusiformis*, and *Sargassum ringgoldianum*, have been found to possess remarkably high antioxidant activity in vitro, which is well correlated with their total phlorotannin content (TPC).[11,12] The superior antioxidant properties have also been reported for individual phlorotannin compounds, including eckol, dieckol, phlorofucofuroeckol A, and 8,8′-bieckol.[13] The scavenging activities of these phlorotannin compounds on 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide anion radicals were found to be around 2−10 times higher than those of catechin, α-tocopherol, and ascorbic acid.

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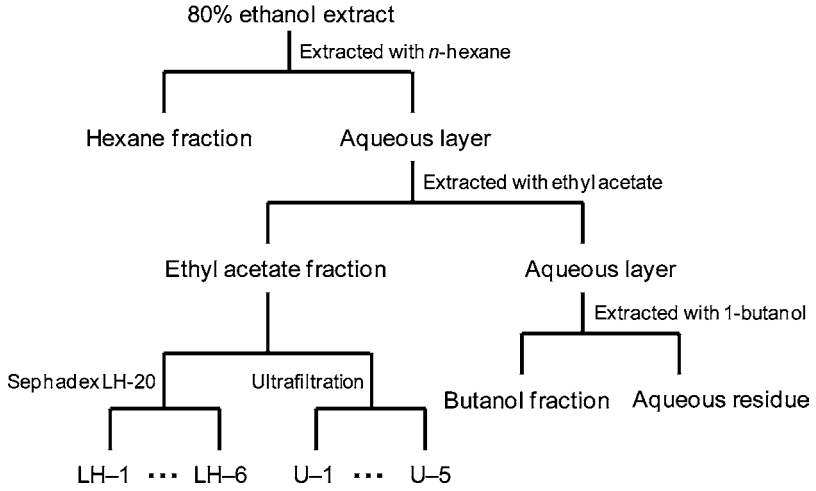

Figure 1. Flowchart for extraction and fractionation of phlorotannins from *F. vesiculosus*. LH-1 to LH-6 represent six subfractions obtained from Sephadex LH-20 column chromatography with solvent systems of decreasing polarity. U-1 to U-5 represent five subfractions obtained from centrifugal ultrafiltration of the ethyl acetate fraction: U-1, MW > 100000; U-2, MW = 30000−100000; U-3, MW = 10000−30000; U-4, MW = 5000−10000; U-5, MW < 5000.

Earlier experiments in our laboratory on the antioxidant potentials of 10 species of common Icelandic seaweeds indicated that *F. vesiculosus* had the highest TPC, the strongest scavenging activities against DPPH and peroxyl radicals, and a moderate ferrous ion-chelating ability. [14] A subsequent study suggested that phlorotannins were the active components in *F. vesiculosus* extract responsible for the inhibition of lipid oxidation in fish model systems. [15] However, no comprehensive study has been performed on the extraction and characterization of antioxidant properties of phlorotannins from *F. vesiculosus* harvested in Iceland. Little information is available on the relationships between the molecular size, degree of polymerization, and antioxidant activity of phlorotannins. Therefore, the objective of the present work was to develop a process for effective extraction and fractionation of phlorotannins from *F. vesiculosus*. The antioxidant activity of different extracts/fractions was characterized using a multiple-method approach which included well-documented in vitro antioxidant assays and a mononuclear cell-based bioassay. HPLC-DAD−ESI-MS^n analyses were used to establish the polyphenolic profile in different Sephadex subfractions and to tentatively identify the major phlorotannin components. The information gained will help to understand how the molecular size and degree of polymerization may influence the antioxidant activity and to promote the potential application of phlorotannin-rich extract and fractions as natural antioxidants in food products and in health-promoting functional food formulations.

MATERIALS AND METHODS

Algal Materials. The brown algae *F. vesiculosus* (Linnaeus) was collected in the Hvassahraun coastal area near Hafnarfjordur, southwestern Iceland, on Oct 15, 2008. The algae were washed with clean seawater to remove epiphytes and sand attached to the surface and transported to the laboratory. The samples were carefully rinsed with tap water. Small pieces were cut and then freeze-dried, pulverized into fine powder, and stored in tightly sealed polystyrene containers at −20 °C prior to extraction.

Chemicals. Folin−Ciocalteu's phenol reagent, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (Ferrozine), and ferric chloride ($FeCl_3$) were purchased from Fluka (Buchs, Switzerland). Iron(II) chloride was obtained from Sigma−Aldrich (Steinheim, Germany). DPPH, potassium ferricyanide ($K_3Fe(CN)_6$), phorbol 12-myristate 13-acetate (PMA), horse radish peroxidase (HRP), and 4-aminophthalhydrazide (isoluminol) were from Sigma-Aldrich (St. Louis, MO). All the solvents used were of HPLC grade. All other chemicals and reagents were of analytical grade.

Preparation of Seaweed Extracts and Fractions. The solvent extracts were prepared according to the method described by Wang et al.[14] Briefly, 2 g of dried algal powder was dispersed in 50 mL of extraction solvent, i.e., methanol/water (80:20, v/v), ethyl acetate/water (80:20, v/v), acetone/water (70:30, v/v), ethanol/water (80:20, v/v), and ethanol/water (50:50, v/v), and incubated in a platform shaker (Innova 2300, New Brunswick Scientific, Edison, NJ) for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at 2500*g* for 10 min at 4 °C and filtered with Whatman no. 4 filter paper. The solvent was removed by rotary evaporation. The residue was lyophilized, weighed, and stored at −20 °C until further analysis. The extraction yield (amount of total extracted substances, TESs) was expressed as grams of dried extract per 100 grams of dried algal powder. Each extraction was conducted in triplicate. Cold and hot water extracts were also prepared (2 g of algal powder extracted with 50 mL of distilled water for 24 h at 20 and 70 °C, respectively) and tested in parallel.

The crude 80% ethanol extract was subjected to solvent fractionation. The dried algal powder (40 g) was extracted with 200 mL of 80% ethanol by the same procedure described above. The extract was concentrated in vacuo to a small volume, and the residue was suspended in a mixture of methanol and water (40:30, v/v) and partitioned three times with *n*-hexane, ethyl acetate, and 1-butanol successively (Figure 1). After the solvent was removed and freeze-dried, four fractions were obtained, the *n*-hexane, ethyl acetate, and 1-butanol soluble fractions and an aqueous residue.

The ethyl acetate-soluble fraction, which showed the highest TPC, DPPH scavenging activity, and reducing power, was further subjected to gel filtration on a Sephadex LH-20 column and eluted stepwise with solvent systems of decreasing polarity, yielding six subfractions: LH-1 (50% aqueous methanol, v/v), LH-2 (75% aqueous methanol, v/v), LH-3 (pure methanol), LH-4 (methanol/acetone, 5:1, v/v), LH-5 (methanol/acetone, 3:1, v/v), and LH-6 (methanol/acetone, 1:1, v/v). The solvent in each subfraction was evaporated under reduced pressure and then lyophilized (Figure 1).

Preparation of Different Molecular Weight Subfractions from the EtOAc-Soluble Fraction. To characterize the MW distribution, the ethyl acetate-soluble fraction was also subfractionated into five fractions, U-1 (MW > 100000), U-2 (MW = 30000−100000), U-3 (MW = 10000−30000), U-4 (MW = 5000−10000), and U-5 (MW < 5000), by using a series of Centricon filters (Centricon Plus-70 centrifugal filter units, nominal molecular weight limits of 100000,

30000, 10000, and 5000, Millipore Corp., Billerica, MA) (Figure 1). A 2 g sample of dried ethyl acetate-soluble fraction from 80% ethanol crude extract was redissolved in Milli-Q water, first filtered at 15 °C through the Centricon-100, and the fraction with MW > 100000 was saved. The flow-through was then centrifuged successively over Centricon-30, -10, and -5 filters. All the retentates and the MW < 5000 filtrate were collected separately and freeze-dried.

Determination of Total Phlorotannin Content. The TPC of the extracts/fractions was quantified according to the method of Turkmen et al.[16] and Koivikko et al.[17] with modifications. One milliliter aliquot of sample solution (concentration range from 0.1 to 8 mg/mL) was mixed with 5 mL of Folin−Ciocalteu reagent (10% in distilled water). After 5 min, 4 mL of sodium carbonate (7.5% in distilled water) was added. The samples were incubated for 2 h at room temperature in the dark. The absorbance was measured at 725 nm with a UV−vis spectrophotometer (Ultrospec 3000 pro, Amersham Pharmacia Biotech, Ltd., Cambridge, U.K.). A standard curve with serial phloroglucinol solutions (20−100 μg/mL) was used for calibration. The results are expressed as grams of phloroglucinol equivalents (PGEs) per 100 g of extract.

DPPH Radical Scavenging Activity. The DPPH radical scavenging activity was estimated according to Sánchez-Moreno et al.[18] with slight modifications. Briefly, 0.1 mL of the sample solution (at least five different concentrations were prepared for each extract/fraction) was mixed with 3.9 mL of DPPH$^{\bullet}$ solution (64 μM or 2.5×10^{-2} g/L in methanol, prepared daily). After incubation for 2 h at room temperature, the absorbance was measured at 515 nm with the Ultrospec 3000 pro UV−vis spectrophotometer. L-Ascorbic acid, butylated hydroxytoluene (BHT), and α-tocopherol were used as reference standards. The calibration curve made with DPPH$^{\bullet}$ between 4.15×10^{-3} and 4.15×10^{-2} g/L was used to calculate the remaining concentration of DPPH$^{\bullet}$ in the reaction medium. The EC_{50} value was calculated as the concentration of sample or standard antioxidant (μg/mL) required to scavenge 50% of the DPPH$^{\bullet}$ in the reaction mixture.

Reducing Power. The reducing power was measured as described by Benjakul et al.[19] A 50 μL volume of seaweed extract solution (0.1 mg/mL) was mixed with 250 μL of phosphate buffer (0.2 M, pH 6.6) and 250 μL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. After 30 min of incubation at 50 °C, 250 μL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 4500*g* for 10 min. The supernatant (200 μL) was mixed with 40 μL of ferric chloride solution (0.1%), and the absorbance was measured at 700 nm. The relative activity of the sample was calculated in relation to the activity of ascorbic acid standards (0−200 μg/mL), and the results are expressed as milligrams of ascorbic acid equivalents (ASEs) per gram of extract.

Ferrous Ion-Chelating Ability. The ferrous ion-chelating ability was determined according to the method of Decker et al.[20] with minor modifications. A 100 μL volume of sample solution (5 mg/mL) was mixed with 135 μL of distilled water and 5 μL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 10 μL of 5 mM ferrozine. After incubation for 10 min at room temperature, the absorbance was measured at 562 nm with a Tecan Sunrise microplate reader (Tecan Austria Gesellschaft, Salzburg, Austria). Distilled water (100 μL) instead of sample solution was used as a control. Distilled water (10 μL) instead of ferrozine solution was used as a blank. L-Ascorbic acid, citric acid, and EDTA-Na_2 were used as reference standards. The ferrous ion-chelating ability was calculated as follows:

$$\text{ferrous ion-chelating ability (\%)} = [(A_0 - (A_1 - A_2)]/A_0 \times 100$$

where A_0 is the absorbance of the control, A_1 is the absorbance of the sample or standard, and A_2 is the absorbance of the blank.

Isolation of Mononuclear Cells from Whole Blood. Mononuclear cells were isolated from the blood of human volunteers. Whole blood was collected in BD Vacutainer CPT cell preparation tubes containing sodium heparin (Becton, Dickinson and Co., Franklin Lakes, NJ) and was immediately mixed by inverting the tubes 8−10 times. The blood samples were then centrifuged at 1500*g* for 15 min at room temperature (18−25 °C) to separate the mononuclear cells as a white layer beneath the blood plasma. The cells were washed twice with cold phosphate-buffered saline solution containing calcium and magnesium salts and suspended in Krebs−Ringer bicarbonate buffer supplemented with glucose, pH 7.3. The number of cells was counted using a hematocytometer, and the concentration was adjusted to 5×10^5 cells/mL. The percentage of various cells obtained using the BD Vacutainer method was reported as 79% lymphocytes and 12% monocytes (data on file, report no. R-88-99-QC-195, BD Vacutainer Systems, Franklin Lakes, NJ).

Chemiluminescence Method. The chemiluminescence (CL) technique[21] was used for detecting ROS produced by PMA-stimulated human mononuclear cells. In this method, the reaction mixture contained 200 μL of 51.2 mM isoluminol, 100 μL of 5 units of HRP, 2.0 mL of Krebs−Ringer bicarbonate buffer, and 200 μL of mononuclear cells (5×10^5 cells/mL). The reaction was initiated using 200 μL of PMA (10^{-5} M). Subfractions obtained from Sephadex LH-20 chromatography and ultrafiltration were dissolved and diluted in water and added to the reaction mixture at 100 μL to obtain final concentrations of the reaction mixture of 1.5 and 2.4 μg/mL, respectively. The ability of the seaweed samples to quench ROS was measured as CL using an LS-45 luminescence spectrometer (Perkin-Elmer Instruments, Waltham, MA) at 25 °C. The area under the CL curve was calculated and compared with a control containing only Krebs−Ringer bicarbonate buffer. The ROS scavenging abilities of various subfractions were measured by their ability to reduce the area under the CL curve.

HPLC-DAD−ESI-MS^n Analyses. Chromatographic analyses were performed on an Agilent 1200 series HPLC system (Agilent, Palo Alto, CA) equipped with an autosampler/injector, a binary pump, a column compartment, and a diode array detector. A Zorbax Stablebond Analytical SB-C18 column (4.6 mm × 250 mm, 5 μm, Agilent Technologies, Palo Alto, CA) was used for separation. Mobile phases consisted of 0.1% formic acid aqueous solution (A) and 0.1% formic acid in acetonitrile (B). UV−vis spectra were scanned from 220 to 600 nm with a recording wavelength of 280 nm. The flow rate was 1 mL/min. The linear gradient was as follows: 10% B from 0 to 5 min, 10−26% B from 5 to 15 min, 26−30% B from 15 to 30 min, 30−44% B from 30 to 32 min, 44−60% B from 32 to 42 min, and 60−10% B from 42 to 45 min. Electrospray mass spectrometry was performed on an HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The column effluent was monitored in the positive ion mode of the instrument. Other experimental conditions for the mass spectrometer were as follows: nebulizer, 45 psi; dry gas, 10.0 L/min; dry temperature, 350 °C; ion trap, scan from m/z 100 to m/z 2000; smart parameter setting (SPS); compound stability, 50%; trap drive level, 60%. The mass spectrometer was operated in Auto MS^2 mode. MS^2 was used to capture and fragment the most abundant ion in full-scan mass spectra.

Statistical Analysis. Each extraction and fractionation experiment was replicated three times, and analyses were performed at least two times. Analysis of variance (ANOVA) was applied to the data using Number Cruncher Statistical Software, NCSS 2000 (NCSS, Kaysville, UT). Significant differences were determined by one way ANOVA, and the Tukey−Kramer multiple-comparison test was used to determine the statistical difference between sample groups. The significance of differences was defined at the 5% level ($p < 0.05$).

RESULTS AND DISCUSSION

Effect of Solvent Type on the Extraction of Phlorotannins from *F. vesiculosus*. On the basis of our preliminary experiment (data not shown) and other studies, polar solvents are more efficient at extracting phlorotannins than water and apolar solvents.[17] Therefore, the focus of the present study was to compare the extraction efficiency of various polar solvent systems.

The type of solvent had an impact on the amount of total extracted substances (Table 1). In general, the yield decreased with decreasing polarity of the solvent in the order water, 50% ethanol, 80% methanol, 80% ethanol, 70% acetone, and 80% ethyl acetate. The highest extraction yield was recorded for the

Table 1. TESs, TPCs, and DPPH EC_{50} (μg/mL) of *F. vesiculosus* Extracts[a]

extractant	TESs[b] (g/100 g)	TPC[c] (g of PGEs/100 g)	DPPH EC_{50}[d] (μg/mL)
water (20 °C)	24.4 ± 0.6 b	26.3 ± 0.2 e	11.61 ± 0.59 b
water (70 °C)	37.8 ± 0.5 a	18.0 ± 0.3 f	24.04 ± 0.70 a
80% methanol	22.9 ± 0.4 b	37.4 ± 0.6 b	7.54 ± 0.36 d
80% ethyl acetate	12.0 ± 0.3 d	36.5 ± 0.7 bc	8.28 ± 0.39 cd
80% ethanol	21.3 ± 0.5 c	35.4 ± 0.2 c	7.92 ± 0.11 cd
50% ethanol	23.1 ± 0.3 b	32.2 ± 0.3 d	8.76 ± 0.29 c
70% acetone	20.2 ± 0.9 c	39.3 ± 0.5 a	7.32 ± 0.17 d

[a]Each value is expressed as the mean ± SD ($n = 3$). Values in the same column followed by different on-line letters are significantly different ($P < 0.05$). [b]Grams of dried extract/100 g of dried algal powder. [c]Grams of phloroglucinol equivalents/100 g of extract. [d]Concentration of extract (μg/mL) required to scavenge 50% of the DPPH• in the reaction mixture.

hot water extract (37.8 ± 0.5 g/100 g), whereas the lowest yield was recorded for the 80% ethyl acetate extract (12.0 ± 0.3 g/100 g).

The influence of the solvent type on the extraction efficiency and selectivity of phlorotannins was examined. In general, all the polar solvents were more efficient than water, and the highest recovery was obtained by using 70% aqueous acetone (Table 1). This is in agreement with the findings of Koivikko et al.,[17] who reported that 70% aqueous acetone was the most efficient solvent for the extraction of phlorotannin compounds from *F. vesiculosus* among eight extractants with different polarities. Hot water extraction resulted in a significantly lower level of TPC than did cold water extraction, which might be due to the thermal decomposition of some phlorotannin compounds at elevated temperatures as well as a significant increase in the extraction of undesired concomitant components. There was no clear relationship between the amount of total extracted substances and the TPC of the extracts.

The TPC of all solvent extracts of *F. vesiculosus* in the present study were higher than those reported in the literature, including the 70% acetone extract of *F. vesiculosus* from the Finnish Archipelago Sea (30 g of PGEs/100 g of extract on dry weight basis)[22] and methanol extract (23.21 g of PGEs/100 g of extract) from the Atlantic coast of Canada.[23] It was also noted that the TPC values of both cold water and the 70% acetone extract were much higher than those reported in our earlier study, in which the sample was collected in early spring.[14] Significant seasonal fluctuations of TPC in brown fucoid algae are well documented, and the variations appear to be related to several environmental factors, such as light intensity, ultraviolet radiation, and nutrient availability.[24,25] Higher TPC levels generally occur in summer and early autumn, whereas lower amounts are observed during winter, supporting the important photoprotective roles of phlorotannins.

DPPH radical scavenging activity of various extracts was in the following order: 70% acetone > 80% methanol > 80% ethanol > 80% ethyl acetate > 50% ethanol > cold water (20 °C) > hot water (70 °C) (Table 1). It was observed that the antioxidant activity of different extracts in the DPPH assay was linearly correlated with the TPC level (data not shown).

Since 80% ethanol also showed considerable ability to extract phlorotannins from *F. vesiculosus*, the use of ethanol would obviously be preferred over acetone and methanol for the extraction of food-grade natural antioxidants. The 80% ethanol extract was therefore chosen for further fractionation studies.

Total Phlorotannin Content and Antioxidant Activities of the 80% Ethanol Extract and Its Solvent Fractions. The 80% ethanol extract was sequentially separated into four fractions, *n*-hexane, ethyl acetate, and 1-butanol soluble fractions and the final aqueous residue, by liquid−liquid partitioning.

There were significant differences in TPC among the 80% ethanol extract and its subsequent fractions. The highest level of TPC was found in the ethyl acetate fraction with a value of 88.3 ± 2.2 g of PGEs/100 g of extract. The crude extract, 1-butanol fraction, and aqueous residue had TPC values of 35.4 ± 0.2, 62.8 ± 1.3, and 12.0 ± 0.7 g of PGEs/100 g of extract, respectively. The lowest TPC was observed for the *n*-hexane fraction (2.2 ± 0.2 g of PGEs/100 g of extract). Ethyl acetate has been widely used to selectively extract polyphenolic compounds of intermediate polarity from various plants.[26,27] The present study showed that it is also effective in concentrating/enriching phlorotannins from the crude extract of *F. vesiculosus*.

As shown in Table 2, four fractions with different polarities scavenged DPPH radicals in a dose-dependent manner over a concentration range of 2−20 μg/mL. The ethyl acetate-soluble fraction exhibited the highest scavenging activity, followed by the 1-butanol fraction, while the aqueous residue and hexane fraction were much less effective. The activity of the ethyl acetate and 1-butanol fractions was comparable to that of the commercial antioxidants α-tocopherol, BHT, and ascorbic acid. The reducing power of the extract/fractions decreased in the same order, ethyl acetate fraction (757.7 ± 38.2 mg of ASEs/g)

Table 2. DPPH Radical Scavenging Activity of Different Solvent Fractions of *F. vesiculosus* Compared with Standard Antioxidants[a]

	inhibition (%)					
fraction/std antioxidant	2 μg/mL	5 μg/mL	10 μg/mL	16 μg/mL	20 μg/mL	EC_{50} (μg/mL)
n-hexane	4.6 ± 0.3 f	11.5 ± 1.0 e	21.8 ± 0.6 f	33.4 ± 0.4 f	39.1 ± 1.4 d	nd[b]
ethyl acetate	36.1 ± 1.2 b	64.0 ± 0.5 b	84.4 ± 0.2 bc	94.2 ± 0.5 bc	95.3 ± 0.7 ab	3.76 ± 0.22 c
1-butanol	25.3 ± 0.8 d	52.7 ± 0.8 c	79.7 ± 1.4 d	89.7 ± 0.7 d	92.6 ± 0.6 b	4.77 ± 0.25 b
aqueous residue	5.2 ± 0.3 f	12.6 ± 0.9 e	23.9 ± 1.0 e	36.0 ± 0.8 e	43.9 ± 1.9 c	nd
BHT	31.0 ± 0.4 c	63.6 ± 0.2 b	85.4 ± 0.1 b	93.2 ± 0.3 c	95.4 ± 0.3 a	3.28 ± 0.09 c
α-tocopherol	15.3 ± 0.3 e	42.2 ± 0.6 d	82.4 ± 0.4 c	95.2 ± 0.3 b	95.7 ± 0.2 a	5.93 ± 0.04 a
L-ascorbic acid	38.0 ± 0.7 a	95.0 ± 0.3 a	96.8 ± 0.2 a	97.3 ± 0.4 a	97.6 ± 0.6 a	2.49 ± 0.06 d

[a]Each value is expressed as the mean ± SD ($n = 3$). Values in the same column followed by different on-line letters are significantly different ($P < 0.05$). [b]Not determined.

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Table 3. Relative Yields, TPCs, and Antioxidant Activities of Different Sephadex Subfractions from the Ethyl Acetate Fraction of *F. vesiculosus*[a]

subfraction	relative yield[b] (%)	TPC (g of PGEs/100 g)	DPPH EC_{50} (μg/mL)	Fe^{2+}-chelating ability (%)	reducing power[c] (mg of ASEs/g)
LH-1	7.3 ± 1.3 d	82.7 ± 1.5 d	4.23 ± 0.17 a	46.5 ± 1.4 a	760.3 ± 63.9 a
LH-2	7.5 ± 0.5 d	96.6 ± 0.9 a	2.79 ± 0.05 d	28.5 ± 0.4 e	790.3 ± 61.3a
LH-3	7.4 ± 0.9[d]	97.0 ± 0.7[a]	2.82 ± 0.03[d]	30.5 ± 0.8[de]	822.3 ± 29.2[a]
LH-4	12.7 ± 0.3[c]	85.0 ± 0.9[cd]	3.89 ± 0.07[b]	32.3 ± 1.1[d]	717.8 ± 56.4[a]
LH-5	46.2 ± 2.4 a	91.3 ± 2.4 b	3.50 ± 0.13 c	38.0 ± 0.5c	785.8 ± 77.1 a
LH-6	18.9 ± 2.0 b	87.5 ± 1.2 bc	3.71 ± 0.07 bc	41.9 ± 1.8 b	780.5 ± 82.1 a
total recovery[d] (%)	75.4 ± 1.3				

[a]Each value is expressed as the mean ± SD (n = 3). Values in the same column followed by different on-line letters are significantly different (P < 0.05). [b]Relative yields are expressed as percentage by weight of individual freeze-dried subfractions. [c]ASEs = ascorbic acid equivalents. [d]Total percent recovery from 300 mg of ethyl acetate fraction loaded onto the Sephadex LH-20 column.

Table 4. Relative Yields, TPCs, and Antioxidant Activities of Different MW Subfractions from the Ethyl Acetate Fraction of *F. vesiculosus*[a]

MW subfraction	relative yield[b] (%)	TPC (g of PGEs/100 g)	DPPH EC_{50} (μg/mL)	Fe^{2+}-chelating ability (%)	reducing power (mg of ASEs/g)
>100000	73.9 ± 1.6 a	87.8 ± 2.3 b	3.80 ± 0.13 a	45.1 ± 1.0 a	881.3 ± 40.2 a
100000−30000	9.6 ± 0.6 b	95.4 ± 1.3 a	3.58 ± 0.07 a	47.6 ± 1.3 a	899.0 ± 38.0 a
30000−10000	3.3 ± 0.3 c	97.0 ± 1.5 a	3.07 ± 0.04 b	36.6 ± 1.8 b	910.7 ± 27.5 a
10000−5000	2.7 ± 0.2 c	94.8 ± 0.5 a	2.91 ± 0.09b	33.7 ± 0.6b	822.0 ± 40.3 ab
<5000	10.4 ± 0.9 b	83.1 ± 1.8 b	3.62 ± 0.11a	25.1 ± 0.2c	734.0 ± 43.0 b

[a]Each value is expressed as the mean ± SD (n = 3). Values in the same column followed by different on-line letters are significantly different (P < 0.05). [b]Relative yields are expressed as percentage by weight of individual freeze-dried subfractions.

> 1-butanol fraction (487.7 ± 12.6 mg of ASEs/g) > 80% ethanol extract (324.8 ± 8.9 mg of ASEs/g) > aqueous residue (109.3 ± 0.6 mg of ASEs/g). However, the extract and all the fractions showed relatively similar chelating abilities of 34.7 ± 1.9%, 34.2 ± 1.3%, 30.9 ± 1.6%, and 35.1 ± 2.3% for the 80% ethanol extract, ethyl acetate and 1-butanol fractions, and aqueous residue, respectively.

In addition, positive correlations between TPC, DPPH radical scavenging activity, and reducing power were observed for the 80% ethanol extract and its solvent fractions on the basis of Pearson correlation analysis (data not shown), supporting our previous finding that phlorotannins are the major antioxidant components present in *F. vesiculosus*. However, there was no relationship between TPC and ferrous ion-chelating capacity, and phlorotannins did not appear to be very effective metal chelators.[14]

Total Phlorotannin Content and Antioxidant Activities of Sephadex LH-20 Chromatographic Subfractions. The ethyl acetate-soluble fraction, which possessed the highest TPC as well as the strongest DPPH scavenging activity and reducing power, was further separated by Sephadex LH-20 column chromatography to give six subfractions (LH-1 to LH-6).

Sephadex LH-20 gel has been widely used for the fractionation and purification of various groups of phenolic compounds that occur in plants and marine algae.[28−30] The separation is mainly based on hydrogen bonding between phenolic −OH groups and the ether oxygen atoms of the cross-linking chain of the gel.[29,31] The gel thus functions as a hydrogen bond acceptor, and the strength of adsorption depends on the number of phenolic hydrogens per molecule. Polymeric polyphenols containing many phenolic hydroxyl groups are retained more strongly on the column than monomers and oligomers. Using an appropriate eluent system allows separation of nonpolymeric and polymeric phenols based on hydrogen-bonding properties.[29,32] In this study, phlorotannin oligomers were desorbed first from the gel by aqueous methanol. Gradual desorption of polymeric phlorotannins was achieved by a stepwise increase in the acetone concentration of the acetone−methanol elution mixture. The carbonyl oxygen of acetone has been reported to serve as a strong hydrogen bond acceptor and enables displacement of complexed polymeric phenols from the Sephadex LH-20 column.[29,33]

The relative yields of different Sephadex subfractions are shown in Table 3. Approximately 75.4% of the algal material could be recovered after Sephadex LH-20 chromatographic separation. The majority of the phlorotannin components in the ethyl acetate-soluble fraction were eluted from the column in the last three subfractions, and the recovery was 12.7%, 46.2%, and 18.9%, respectively. LH-5 was found to be the most abundant fraction in mass, while LH-1 was the least abundant. It can therefore be assumed that phlorotannins in *F. vesiculosus* comprise mainly high molecular weight (HMW) polymers.

Subfractions LH-2 and LH-3 with higher TPC were found to possess better DPPH scavenging activity (the EC_{50} value was 2.79 and 2.82 μg/mL, respectively) than the other subfractions and the ethyl acetate fraction itself (Table 3). The activity was comparable to those of the positive controls, ascorbic acid (EC_{50} = 2.49 μg/mL) and BHT (EC_{50} = 3.28 μg/mL), and was significantly higher than that of α-tocopherol (EC_{50} = 5.93 μg/mL) (Table 2). The TPC and DPPH quenching activity of subfractions LH-4 to LH-6 were similar to those of the original ethyl acetate fraction, whereas subfraction LH-1 had significantly lower TPC and weaker scavenging activity. Similar trends were observed with reducing power, and no direct relationship was found between TPC and metal-chelating capacity. In general, there was no clear trend in antioxidant activity among oligomeric and polymeric subfractions, but the differences in the reducing and DPPH scavenging activities

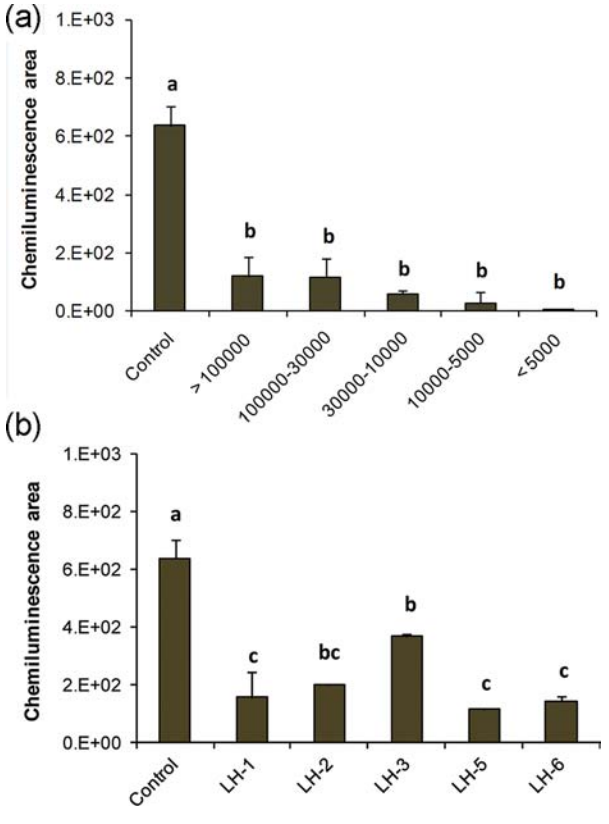

Figure 2. (a) Effect of ultrafiltered subfractions of *F. vesiculosus* on PMA-stimulated chemiluminescent emission of freshly prepared human mononuclear cells. The concentration of cells was 5×10^5 cells/mL. The reaction was started using 5 units of HRP. The samples were added at a final concentration of 2.4 μg/mL. The ROS scavenging activity of the samples was measured by their ability to reduce the area under the chemiluminescence curve. (b) Effect of Sephadex subfractions of *F. vesiculosus* on PMA-stimulated chemiluminescent emission from human mononuclear cells. The concentration was 5×10^5 cells/mL. The reaction was started using 5 units of HRP. Phlorotannin subfractions LH-2 to LH-6 were added at a final concentration of 1.5 μg/mL, and LH-1 was tested at 3.0 μg/mL. The ROS scavenging activity of the samples was measured by their ability to reduce the area under the chemiluminescence curve.

appeared to be explained only by their relative TPC levels. Phlorotannin oligomers and polymers did not seem to differ markedly in antioxidant activity.

Unlike plant-derived polyphenols, the relationship between the molecular structure and antioxidant activity of brown algal phlorotannins is still poorly understood. Several lines of evidence indicate that oligomeric and polymeric phlorotannins are more potent antioxidants than the monomer phloroglucinol.[11,34,35] Oligomerization of phloroglucinol appears to be crucial for the radical scavenging activity. However, research exploring the correlation between the antioxidant capacity and specific structural features of phlorotannins has yielded contradictory findings. Cerantola et al.[34] isolated two types of structurally distinct phlorotannins from *Fucus spiralis* extracts and compared their DPPH radical scavenging activities. The first polymer had a relatively simple structure and belonged to the fucol subclass, whereas the second one was more complex and linked through aryl−aryl and aryl−ether bonds, thus belonging to the fucophlorethol subclass. Both phlorotannin compounds showed equally high scavenging activity in comparison to ascorbic acid and phloroglucinol monomer, indicating that there was no clear association between the type of structural linkages and radical scavenging activity of phlorotannins. On the other hand, several other studies showed that the presence of O-bridge linkages (ether linkages) and the number and arrangement of phenolic hydroxyl groups in the phlorotannin skeleton have a profound influence on the antioxidant property. The extraordinary antioxidant potential of specific phlorotannin compounds extracted from *Ecklonia* species such as *E. cava* and *E. stolonifera* has been ascribed to their unique dibenzo-1,4-dioxin unit in the molecular skeleton.[30,36] In another study, several oligomeric phlorotannins such as eckol, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol were found to possess varying degrees of scavenging capacity against DPPH and superoxide anion radicals, which was assumed to be related to the number of hydroxyl groups attached to the eckol skeleton.[13]

Total Phlorotannin Content and Antioxidant Activities of Different Molecular Weight Subfractions. Currently, little information is available concerning the MW distribution of phlorotannins in *F. vesiculosus* as well as the relationship between MW and antioxidant activity. In this study, the ethyl acetate-soluble fraction was sequentially fractionated through ultrafiltration membranes to yield five MW subfractions: >100000, 100000−30000, 30000−10000, 10000−5000, and <5000 Da. The antioxidant activity of different subfractions was evaluated by the methods described above.

The subfraction with MW > 100000 was found to be the most abundant and represented about 73.9% of the total amount recovered, indicating that highly polymerized phlorotannins are the largest pool of phenolic compounds in *F. vesiculosus*. The yields of MW subfractions 5000−10000 and 10000−30000 were significantly lower than those of the other subfractions (Table 4). This is consistent with a previous study showing that the ethanol extract of *F. vesiculosus* was composed of mainly HMW phlorotannin polymers, around 83% of which having a MW of more than 50000.[37]

As summarized in Table 4, MW subfractions containing high TPC, such as 10000−30000, 30000−100000, and 5000−10000, generally exhibited strong DPPH radical scavenging activity and reducing power. It is worth noting that the low MW fraction (<5000) exhibited a DPPH radical scavenging activity similar to that of the 30000−100000 fraction, although it had a lower TPC value. This could be due to the presence of other coextracted antioxidant compounds, such as L-ascorbate and glutathione (GSH), in this fraction. There also seemed to be no clear relationship between the molecular size of phlorotannin compounds and in vitro antioxidant activity. Similarly, Audibert et al.[38] reported that the antioxidant activity of different MW fractions of *Ascophyllum nodosum* was correlated mainly with their phenolic content, rather than the chemical structure or the molecular size (or degree of polymerization) of phlorotannins.

Chemiluminescence Assay for the Detection of Reactive Oxygen Species. The ability of different subfractions obtained from sequential ultrafiltration and Sephadex LH-20 gel chromatography to scavenge ROS produced by stimulated human mononuclear cells was studied using an isoluminol-enhanced CL method. The antioxidant activity of the algal samples was measured by their ability to quench ROS (or chromogenic radicals) as reflected by a decrease in the chemiluminescence signal. At a concentration of 2.4 μg/mL, all the ultrafiltered subfractions showed a significantly high ($P < 0.05$) ability to scavenge ROS compared to the control (Figure

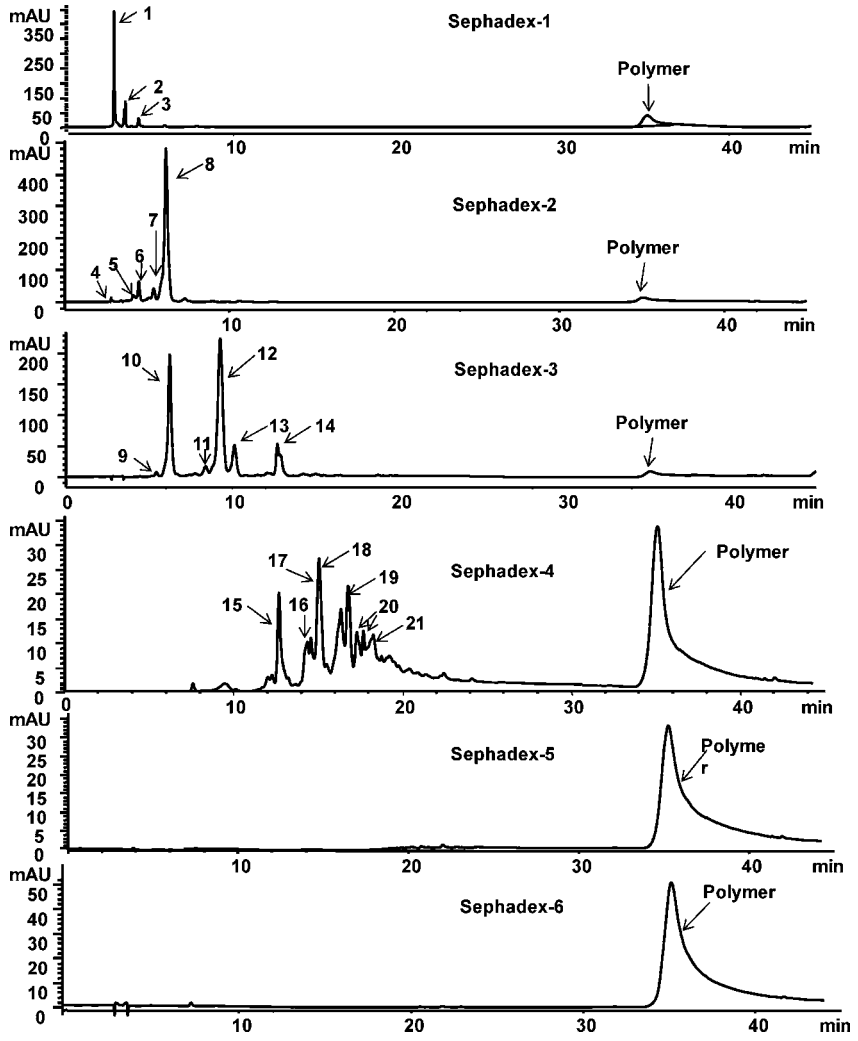

Figure 3. HPLC-DAD chromatograms of Sephadex subfractions of *F. vesiculosus* (peaks marked with numbers were tentatively identified using HPLC−ESI-MS^n).

2a). The average ROS quenching ability appeared to increase with a decrease in MW, but the difference was not statistically significant ($P > 0.05$). To our best knowledge, no previous study has been performed to assess the ROS scavenging ability of algal phlorotannins in a mononuclear cell-based bioassay system. Only studies using fish protein hydrolysates have been reported. In a recent investigation, Raghavan et al.[39] reported a higher potential for 25% hydrolyzed proteins (fractions with lower MW) compared to 15% and 7.5% hydrolyzed proteins (high MW proteins) to inhibit PMA-induced CL. It appears that lower MW compounds possess a higher ability to quench PMA-induced ROS. Furthermore, it is noted that the chemiluminescence quenching ability of different MW subfractions was not reflected in their DPPH radical scavenging activity, reducing power, and iron-chelating ability (Table 4).

All the gel filtration subfractions also exhibited a significantly higher ROS scavenging efficiency than the control group, although no obvious differences could be detected among them (Figure 2b). It needs to be mentioned that a large deviation was observed for LH-4, indicating that it was probably an outlier and was therefore eliminated from further analysis. In accordance with the chemical antioxidant tests, oligomeric and polymeric subfractions did not seem to differ markedly with respect to ROS scavenging ability in mononuclear cells, although these subfractions exhibited different reactivity patterns in different antioxidant assays.

HPLC-DAD−ESI-MS^n Analyses. HPLC-DAD−ESI-MS^n analyses were performed to characterize the major phlorotannin compounds in six Sephadex subfractions of the ethyl acetate fraction from *F. vesiculosus*. Subfractions LH-1, LH-2, and LH-3 were composed mainly of low MW phlorotannin oligomers with small amounts of polymers, whereas phlorotannin polymers were the dominant components in LH-5 and LH-6. Subfraction LH-4 contained a noticeable amount of both oligomers with higher MW as well as polymers. The results further demonstrated that Sephadex LH-20 was effective to separate the phlorotannin compounds with different degrees of polymerization.

Several phlorotannin compounds (peaks marked with numbers in Figure 3) were tentatively identified using mass spectrometry as well as literature data.[9,40,41] As an example, peaks 3, 5, 8, and 10 had the same $[M + H]^+$ at m/z 499 but slightly different fragmentation patterns (Table 5). They would be isomers of phlorotannin tetramers. The most intensive mass peak (base peak) of all these tetramers was at m/z 355, corresponding to the elimination of phloroglucinol (126 amu) and water. The product ions of m/z 373, 233/232, and 231 might be due to the loss of phloroglucinol, bifuhalol (266 amu),

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Table 5. Retention Times and Mass Spectrometric Data of Phlorotannins in *F. vesiculosus* Determined by HPLC−ESI-MS^n [a]

peak	phlorotannin compd	retention time (min)	MW	MS^1 (m/z)	MS^2 (m/z)
1*	trimer	2.8	374	375 $[M + H]^+$	**357**, 287, 232, 231, 163
2*	trimer	3.4	374	375 $[M + H]^+$	**357**,232,231
3*	tetramer	4.2	498	499 $[M + H]^+$	481, **355**, 327, 285, 231
4*	trimer	2.8	374	375 $[M + H]^+$	**357**, 232, 231
5*	tetramer	4.1	498	499 $[M + H]^+$	481, **355**, 327, 285, 232, 231, 207
6	trimer	4.6	374	375 $[M + H]^+$	**357**, 232, 231
7	pentamer	5.4	622	623 $[M + H]^+$	**605**, 480, 479, 409, 356, 231
8	tetramer	6.2	498	499 $[M + H]^+$	481, 463, 395, 374, 373, 358, 357, 356, **355**, 285, 234, 233, 231, 179
9*	pentamer	5.4	622	623 $[M + H]^+$	605, 480, 479, 356, 340, **231**
10*	tetramer	6.2	498	499 $[M + H]^+$	374, 373, 358, 357, 356, **355**, 234, 233, 232, 231
11	hexamer	8.4	746	747 $[M + H]^+$	**729**, 622, 603, 479, 462, 356, 231
12	pentamer	9.2	622	623 $[M + H]^+$	605, 540, 497, 434, 356, 340, **231**, 179
13	pentamer	10.1	622	623 $[M + H]^+$	605, 565, 479, 371, 337, **231**, 205, 179
14	hexamer	12.8	746	747 $[M + H]^+$	**729**, 622, 621, 462, 356, 338, 231
15	hexamer	12.8	746	747 $[M + H]^+$	**729**, 622, 462, 356, 338, 231
16	heptamer	14.3	870	871 $[M + H]^+$	**853**, 727, 710, 623, 462, 355
17	heptamer	15.1	870	871 $[M+ H]^+$	**853**, 710, 586, 462
18	nonamer	15.1	1118	1119 $[M + H]^+$	1100, 957, **871**, 692, 569, 461, 338
19	decamer	16.8	1242	1243 $[M + H]^+$	1225, **995**, 835, 729, 603
20	nonamer	17.7	1118	1119 $[M + H]^+$	975, **871**, 727, 585, 462
21	nonamer	18.3	1118	1119 $[M + H]^+$	956, **871**, 727, 603, 481

[a]Ions in bold indicate the most intense product ion.

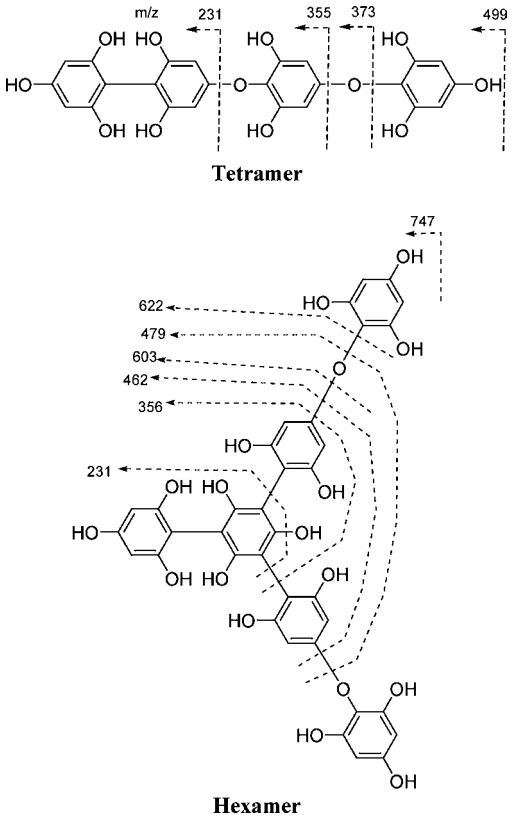

Figure 4. Structure of phlorotannin compounds tentatively identified in *F. vesiculosus* extract and proposed fragmentation pathway.

and diphlorethol (250 amu) and successive loss of water, respectively. Several other low-intensity product ions (such as m/z 327, 285, and 207 ions) were probably generated via the elimination of methylene, water, or a combination of methylene and water as a result of the cleavage of benzene ring structures. One of the proposed chemical structures of these phlorotannin tetramers is shown in Figure 4, corresponding to fucodiphlorethol A as reported by Sandsdalen et al.[40] However, the exact bonding patterns and sequence of phloroglucinol units remain uncertain in the present study because of the unavailability of authentic standards of phlorotannins (except phloroglucinol monomer) as well as the limitation of mass spectrometry. In a similar manner, peaks 14 and 15 with $[M + H]^+$ at m/z 747 and product ion peaks observed at m/z 729, 622/621, and 462 led to possible structures of trifucodiphlorethol isomers (Figure 4 shows one of the proposed structures),[41] which is probably due to the loss of water, one molecule of phloroglucinol, and two molecules of phloroglucinol and successive loss of water, respectively.

This study provides evidence of antioxidant activities of *F. vesiculosus* extracts/fractions based on in vitro tests and their possible health benefits as reflected by the ability to scavenge ROS generated by mononuclear cells. The phlorotannin-enriched ethyl acetate fraction showed the highest reducing and DPPH scavenging capacities among the crude extract and different polarity fractions. High correlations were observed between TPC and DPPH scavenging and reducing capacities (R^2 = 0.98 and 0.96, respectively), suggesting that phlorotannins are the major antioxidant principles in *F. vesiculosus*. Subfractions rich in oligomeric and polymeric phlorotannins exhibited potent DPPH scavenging activity, comparable to or even higher than that of several commercial antioxidants. There were no clear correlations among degree of polymerization, molecular size, and chemical antioxidant activity. Moreover, all the subfractions exhibited similarly high scavenging activity toward ROS produced by stimulated human mononuclear cells. Several phlorotannin components were tentatively identified primarily through the HPLC-DAD−ESI-MS^n technique. Further isolation and structure elucidation of phlorotannin compounds are needed for a deeper understanding of the mechanisms behind the high antioxidant effects observed herein.

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Notes

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