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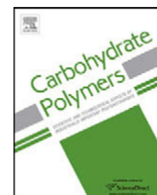

Molecular characteristics and anti-inflammatory activity of the fucoidan extracted from *Ecklonia cava*

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ABSTRACT

Enzymatic extraction has been successfully used for extracting numerous biologically active compounds from a wide variety of seaweeds. In this study, we found that enzymatic extraction of the fucoidan from *Ecklonia cava* may be more advantageous than water extraction. Therefore, we studied the *E. cava* fucoidans extracted by the enzymatic extraction technique and used ion-exchange chromatography to determine their molecular characteristics and anti-inflammatory activities. The crude and fractionated fucoidans (F_1, F_2, and F_3) consisted mostly of carbohydrates (47.1–57.1%), uronic acids (9.0–15.8%), and sulfates (16.5–39.1%), as well as varying levels of proteins (1.3–8.7%). The monosaccharide levels significantly differed, and the composition included fucose (53.1–77.9%) and galactose (10.1–32.8%), with a small amount of rhamnose (2.3–4.5%), xylose (4.0–8.2%), and glucose (0.8–2.2%). These fucoidans contained one or two subfractions with an average molecular weight (M_w) ranging from 18 to 359 × 10^3 g/mol. These fucoidans significantly inhibited NO production in lipopolysaccharide (LPS)-induced Raw 264.7 macrophage cells by down-regulating the expression of iNOS, COX-2, and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. Thus, the present results suggest that *E. cava* fucoidan may be a potentially useful therapeutic approach for various inflammatory diseases.

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1. Introduction

Naturally occurring fucoidans are commonly found in brown seaweeds and some marine invertebrates such as sea cucumbers and sea urchins (Chevolot et al., 1999; Vieira & Mourao, 1988). The structural and compositional properties of fucoidans from different brown algae vary from species to species, but they mainly consist of fucose and sulfate with small amounts of galactose, xylose, mannose, and uronic acids (Bilan et al., 2002; Chizhov, Dell, Morris, Haslam, & McDowell, 1999; Partankar, Oehninger, Barnett, Williams, & Clark, 1993). Fucoidans isolated from brown algae have been extensively studied because of their diverse biological activities such as anticoagulant, antitumor, immunomodulatory, and anti-inflammatory activities (Li, Lu, Wei, & Zhao, 2008). Because of these activities, fucoidans have been investigated in the recent years to be developed as drugs and functional foods.

Several techniques to extract a bioactive compound from algal biomass are available; of these, using enzymatic hydrolysate of the algal biomass is more advantageous over other conventional techniques. Enzymes can convert water-insoluble materials to water-soluble materials, and this method does not use any toxic chemicals. Importantly, this technique results in a high yield of bioactive compound that has enhanced biological activity compared with the compounds isolated from water or by organic extraction techniques (Heo, Lee, Song, & Jeon, 2003).

In our previous study, we extracted a highly sulfated polysaccharide (fucoidan) from an enzymatic hydrolysate of the brown alga *Ecklonia cava* (*E. cava*), which was mainly composed of fucose, with small amounts of galactose, xylose, and mannose. Furthermore, we demonstrated that the sulfated polysaccharide extracted from *E. cava* had anticoagulant, and antitumor activities, immunomodulatory effects, and was protective against high glucose-induced cell

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damage (Ahn et al., 2008; Athukorala et al., 2009; Athukorala, Jung, Vasanthan, & Jeon, 2006; Lee, Heo, Hwang, Han, & Jeon, 2010). However, the chemical and molecular characteristics of the fucoidan extracted from *E. cava* are yet to be determined.

In the present study, the fucoidan from *E. cava* was extracted using an enzymatic extraction technique, and subsequently fractionated by ion-exchange chromatography. The purpose of this study was to investigate the chemical and molecular characteristics of the fractionated fucoidan and to evaluate their anti-inflammatory activities.

2. Materials and methods

2.1. Materials

The brown alga *E. cava* was collected from the coast of Jeju Island, South Korea. Salt, sand and epiphytes were removed with tap water. The samples were then rinsed carefully with fresh water and freeze-dried. The dried alga sample was ground and sifted through a 50-mesh standard testing sieve. All chemicals and reagents used were of analytical grade and obtained from commercial sources.

2.2. Hot water extraction

For hot water extraction using dried *E. cava*, 1 g of the ground *E. cava* powder was mixed with 100 mL of water, and placed in shaking incubator for 24 h at 70 °C. The mixtures were centrifuged at 3000 × *g* for 20 min at 4 °C and filtered with Whatman filter paper to remove the residues.

2.3. Enzymatic extraction and fractionation of fucoidan

Fucoidan was extracted and fractionated using a slight modification of the previously reported methods (Athukorala et al., 2006; Lee et al., 2010). A 10 g sample of the ground, dried *E. cava* powder was homogenized with 1 L of distilled water (dH_2O) and mixed with 100 μL of Celluclast (Novo Nordisk, Bagsvaerd, Denmark). This reaction continued for 24 h at 50 °C, and then, the digest was boiled for 10 min at 100 °C to inactivate the enzyme. The product was clarified by centrifugation (3000 × *g* for 20 min) to remove the unhydrolyzed residue. Then, the enzymatic hydrolysate was adjusted to pH 7.0 and precipitated with 3 volumes of ethanol. After centrifugation at 10,000 × *g* for 20 min at 4 °C, the precipitate was re-dissolved in dH_2O and sequentially treated with 4 M $CaCl_2$. The resulting precipitate was removed by centrifugation and the supernatant was treated with cetylpyridinium chloride. The pyridinium salts were solubilized with 3 M $CaCl_2$ and reprecipitated with ethanol. The precipitate was re-dissolved in dH_2O, dialyzed (M_WCO, 10–12 kDa) against water at 4 °C for 72 h, and then lyophilized; the lyophilized sample was used as crude fucoidan sample.

Crude fucoidan (300 mg) from *E. cava* was dissolved in dH_2O (10 mL) and fractionated using ion-exchange chromatography on DEAE-cellulose column (17 cm × 25 cm) equilibrated in 50 mM sodium acetate (pH 5.0) and washed with the same buffer containing 0.2 M NaCl. Elution was carried out at a flow rate of 15 mL/h with a linear gradient of 0.2–1.2 M NaCl containing 50 mM sodium acetate (pH 5.0). The three fractions obtained (F_1, F_2, and F_3) were dialyzed against dH_2O for three days, and then lyophilized. The carbohydrate elution profile was determined by the phenol-H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) by measuring the absorbance at 490 nm.

2.4. Chemical composition

The sulfate content of the fucoidan was determined by the $BaCl_2$ gelatin method using K_2SO_4 as a standard (Dodgson & Price, 1962), after hydrolyzing the polysaccharide in 0.5 M HCl at 105 °C for 5 h. The total carbohydrate and protein contents were determined by the phenol-sulfuric acid method using glucose as a standard (Dubois et al., 1956) and the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using a commercial assay kit (DC Protein assay kit, Bio-Rad, USA). The uronic acid content of the polysaccharide was determined by the sulfamate/*m*-hydroxydiphenyl assay using glucuronic acid as a standard (Filisetti-Cozzi & Carpita, 1991).

2.5. Determination of monosaccharide composition

The fucoidan was hydrolyzed in a sealed glass tube with 4 M of trifluoroacetic acid for 4 h at 100 °C to analyze neutral sugars. To analyze the monosaccharides, the samples were digested using 6 N HCl for 4 h. Then, fucoidan fractions were separately applied to CarboPac PA1 (4.5 mm × 250 mm; Dionex, Sunnyvale, CA, USA) with a CarboPac PA1 cartridge (4.5 mm × 50 mm) column to analyze neutral and amino sugars, respectively. The column was eluted using 16 mM NaOH at flow late of 1.0 mL/min. Each sugar in the sample was detected using an ED50 Dionex electrochemical detector, and the data were analyzed by Peak Net on-line software.

2.6. Determination of the average molecular weight

To measure the average molecular weight (M_w) and radius of gyration (R_g), the fucoidan (2 mg) was dissolved in dH_2O (1 mL). The solution was heated in a microwave oven (RE-552 W; SamSung, Seoul, Korea) using a microwave bomb (#4872; Parr Instrument Co., Moline, IL, USA) for 30 s. The heated fucoidan solution was centrifuged for 10 s at 12,000 × g before injection into a high-performance size-exclusion chromatography column coupled to a UV, multi-angle laser light scattering, and refractive index detection (HPSEC-UV-MALLS-RI) system. The HPSEC-UV-MALLS-RI system consisted of a pump (model 321; Gilson, Middleton, WI, USA), an injector valve with a 200 μL sample loop (model 7072, Rheodyne), SEC columns (TSK G5000 PW, 7.5 mm × 600 mm; Toso-Biosep, Mongomeryville, PA, USA), a UV detector at 280 nm (Waters 2487), a MALLS detector (HELEOS; Wyatt Technology Corp., Santa Barbara, CA, USA), and a RI detector (Waters 2414). An aqueous solution of 0.15 M $NaNO_3$ and 0.02% NaN_3 was used as the mobile phase at a flow rate of 0.4 mL/min. The MALLS detector as well as the determination of volume delays among the UV, MALLS, and RI detectors was normalized with bovine serum albumin (BSA). The M_w and R_g values were calculated using ASTRA 5.3 software (Wyatt Technology Corp.).

2.7. Macrophage proliferation and nitric oxide production assay

Raw 264.7 cells were plated in a 96-well microplate (1×10^4 cells/well, in a volume of 100 μL; obtained from ATCC) and incubated in RPMI-1640 medium containing 10% FBS. Cells were plated in triplicate and cultured for 4 h at 37 °C in the presence of 5% CO_2. Fucoidan solutions (100 μL), at a concentration of 12.5, 25, 50, and 100 μg/mL, were added to the wells, and the cells were cultured for 72 h. After incubation, 20 μL of the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) solution was added to the well, and the solution was further incubated for 4 h at 37 °C. Absorbance at 450 nm was determined by spectrophotometry (EL-800; BioTek Instruments, Winooski, VT, USA). The absorbance (*A*) was translated into the macrophage proliferation ratio (%) = $A_t/A_c \times 100$, where A_t and A_c are the absorbance values of the test and control groups, respectively.

The anti-inflammatory activity of the fucoidans was determined on the basis of nitric oxide (NO) production in macrophage culture supernatants, and the nitrite concentration was measured using the

Griess reaction, as described by Green et al. (1982). Raw 264.7 cells were plated in a 96-well microplate (1×10^5 cells/well, in a volume of 100 μL) and incubated for 24 h at 37 °C in a 5% CO_2 incubator. The cultured cells were treated in triplicate with lipopolysaccharide solution (LPS, 1 μg/mL) plus fucoidan solution (100 μL), at concentrations ranging from 12.5 to 100 μg/mL. After incubating for 24 h at 37 °C, the cultured cell supernatant (100 μL) was mixed with an equal volume of Griess reagent (1% [w/v] sulfanilamide and 0.1% [w/v] N-[1-naphthyl] ethylenediamine hydrochloride in 2.5% [v/v] phosphoric acid) and incubated at room temperature for 10 min. The absorbance was measured at 540 nm by using a microplate reader. NO production from the Raw 264.7 cells was calculated with reference to a standard curve obtained with $NaNO_2$ (1–200 μM in the culture medium).

2.8. Reverse transcription-polymerase chain reaction (RT-PCR) for transcriptional analysis of iNOS, COX-2, and pro-inflammatory cytokines

To investigate the levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, Raw 264.7 cells (1 mL, 1×10^5 cells/well) were treated with LPS (1 μg/mL) and different concentrations of fucoidan (12.5, 50, and 100 μg/mL) at 37 °C in the presence of 5% CO_2 for 18 h. RNA was extracted from the cells by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and stored at −80 °C until use. The concentration of the extracted RNA was measured using a spectrophotometer before constructing cDNA with an oligo-$(dT)_{20}$ primer and Superscript III RT (Invitrogen). The resulting cDNA was amplified by PCR using GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). Reverse transcriptase amplification was conducted with an initial denaturation at 94 °C for 3 min and 30 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 40 s), and extension (72 °C for 1 min), followed by a final extension step at 72 °C for 10 min. The products of RT-PCR were separated by gel electrophoresis using 1% agarose gel stained with ethidium bromide, and the gels were viewed under UV transilluminator. The sequences of the primers used in this experiment are indicated in Table 1.

2.9. Statistical analysis

All experiments were performed in triplicate ($n = 3$). Data are presented as the mean value with standard deviation. All statistical analyses were performed using SAS (SAS Institute, Cary, NC, USA). Statistical differences were tested by the Student's t-test, one-way analysis of variance (ANOVA), and Duncan's multiple-range test. The critical p value was set at 0.05; a probability value of $p < 0.05$ was considered statistically significant.

Table 1
Sequence of primers of the investigated genes in a RT-PCR analysis.

Gene	Primer	Sequence
iNOS	Sense	5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′
	Antisense	5′GGCTGTCAGAGCCTCGTGGCTTTGG-3′
COX-2	Sense	5′-CACTACATCCTGACCCACTT-3′
	Antisense	5′-ATGCTCCTGCTTGACTATGT-3′
IL-1β	Sense	5′-CAGGATGAGGACATGAGCACC-3′
	Antisense	5′-CTCTGCAGACTCAAACTCCAC-3′
IL-6	Sense	5′-GTACTCCAGAAGACCAGAGG-3′
	Antisense	5′-TGCTGGTGACAACCACGGCC-3′
TNF-α	Sense	5′-TTGACCTCAGCGCTGAGTTG-3′
	Antisense	5′-CCTGTAGCCCACGTCGTAGC-3′
β-actin	Sense	5′-GTGGGCCGCCCTAGGCACCAG-3′
	Antisense	5′-GGAGGAAGAGGATGCGGCAGT-3′

3. Results and discussion

3.1. Chemical composition analysis

Fucoidan is generally extractable with hot water, diluted acid, or diluted alkali, but the extraction technique possesses disadvantages such as lower extraction efficiency and environmental pollution/toxicity. In the last decade, enzymatic extraction has been successfully applied for the extraction of numerous biologically active compounds from a wide variety of seaweeds. The enzymes work primarily by macerating the tissues of the algae and breaking down the algal cell walls and complex interior storage materials like polysaccharides to release the internal compounds. Breakdown of such barriers can enhance the extraction of desired bioactive materials in the tissues and cells. This technique results in high yields of bioactive compound, which show enhanced biological activity compared with that observed with the use of water or in case of organic extraction (Ahn et al., 2008; Athukorala et al., 2009; Athukorala et al., 2006; Heo et al., 2003; Kim et al., 2006; Lee et al., 2010).

Several brown algal species have been enzymatically extracted using several carbohydrases and proteases to investigate their potential bioactivities (Heo et al., 2003; Kim et al., 2006). In that study, the Celluclast extract of *E. cava* displayed a high extraction yield and the highest bioactivities among the tested extracts. Therefore, we used an enzymatic extraction technique by using the carbohydrase Celluclast to extract the fucoidan from *E. cava* in the present study. As shown in Table 2, extraction yield and the carbohydrate, sulfate, and fucose contents were significantly higher in the enzymatic extract compared to the hot water extract. Taken together, these data indicate that enzymatic extraction techniques for fucoidan extraction may be more advantageous than water extraction. Therefore, in the present study, a fucoidan from *E. cava* was extracted using the enzymatic extraction technique, and subsequently fractionated using ion-exchange chromatography.

The chemical composition of the fucoidan from *E. cava* is shown in Table 3. The yield (1.8%) of the crude fucoidan extracted in this study was similar to those of the fucoidans isolated from *Laminaria japonica* (2.3%), but was much larger than those from other brown seaweeds (0.4%) (Durate, Cardoso, Noseda, & Cerezo, 2001; Wang, Zhang, Zhang, & Li, 2008). Most fucoidans from brown seaweeds have a complex composition. In this study, the crude fucoidan extracted from *E. cava* consisted of mostly carbohydrates (51.8%), uronic acid (11.3%), sulfate (20.1%), and small amounts of protein (8.7%), which is in good agreement with the constituents of the fucoidan from *Sargassum stenophyllum* (Durate et al., 2001). Monosaccharide composition analysis revealed that fucose (61.1%)

Table 2
Comparison of chemical composition of hot water and enzymatic extracts from *E. cava*.

Component	Sample	
	Hot water extract	Enzymatic extract
Yield (%)	30.3 ± 2.8	40.6 ± 1.2
Total carbohydrate (%)	32.4 ± 2.3	45.5 ± 1.6
Sulfate content (%)	7.5 ± 0.7	12.5 ± 1.0
Proportion of monosaccharide (%)		
Fucose	45.2 ± 1.6	55.8 ± 2.4
Rhamnose	1.8 ± 0.4	1.9 ± 0.2
Arabinose	0.2 ± 0.5	nd[a]
Galactose	18.2 ± 0.3	21.8 ± 0.5
Glucose	10.2 ± 0.1	6.7 ± 0.1
Mannose	8.1 ± 0.8	7.3 ± 0.6
Xylose	14.4 ± 1.2	6.4 ± 1.9

Experiments were performed in triplicate and the data are expressed as mean ± SE.
[a] Not determined.

and galactose (27.2%) were the major sugars in the crude fucoidan, with minor amounts of xylose (7.0%), rhamnose (3.9%), and glucose (0.8%). Fucoidans from other brown seaweeds, including *S. stenophyllum* and *L. japonica*, also contained fucose (67.8% and 62.1%, respectively) as the main sugar, but the respective amounts of xylose (16.1% and not detected), galactose (13.6% and 24.33%), mannose (1.2% and 6.1%), and glucose (not detected and 1.93%) varied (Durate et al., 2001; Wang et al., 2008). Other monosaccharides such as mannose and arabinose were not detected in the present study. In general, the chemical compositions of the fucoidans from seaweeds significantly differed depending on the species, extraction procedure, and analytical method (Yang et al., 2008).

The crude fucoidan was fractionated on a DEAE-cellulose anion-exchange chromatography column, which yielded three fractions: F_1 (eluted with 0.2 M NaCl), F_2 (eluted with 0.6 M NaCl), and F_3 (eluted with 1.0 M NaCl). The yields of F_1, F_2, and F_3 were 25.3, 17.6, and 32.6%, respectively (Table 3). F_1 and F_2 contained mainly carbohydrates (57.1% and 55.7%, respectively), sulfates (20.0% and 16.5%, respectively), and uronic acid (15.8% and 13.8%, respectively), with minor amount of proteins (7.2% and 5.3%, respectively), whereas F_3 contained significant amounts of sulfates (39.1%) and carbohydrates (47.1%), with minor amounts of uronic acid (9.0%) and proteins (1.3%). Fucose was the major sugar in F_1 (53.1%) and F_2 (59.7%) fractions with a considerable amount of galactose (32.8% and 30.9%, respectively) (Table 3). On the other hand, the major sugar in F_3 was (77.9%), with a small amount of galactose (10.1%). These results suggest that it is possible to obtain fucoidans with varying chemical compositions by fractionating the crude fucoidan preparation by using ion-exchange chromatography. It has been reported (Durate et al., 2001) that the fucoidans from *S. stenophyllum* were fractionated into six fractions with varying amounts of carbohydrates, sulfates, monosaccharides, and uronic acid, supporting our views regarding significant differences in the chemical compositions of fucoidan fractions. These considerable differences in fucoidan composition may be attributed to the differences in the growing conditions of brown seaweeds, extraction procedures, and analytical methods.

3.2. Molecular characteristics of fractionated fucoidans

The UV and RI superimposed chromatograms for the fractionated fucoidans are shown in Fig. 1. As shown in the RI chromatogram, F_1 and F_2 were eluted from the SEC column with elution times of 55–90 min (F_1) and 59–90 min (F_2), with two distinct peaks, indicating two different polymer distributions (Fig. 1A and B). On the other hand, the RI chromatogram of F_3 (Fig. 1C) showed one major peak at an elution times of 54–80 min. The UV chromatogram of all the fractions showed a weak UV response at peak II, indicating the presence of small amounts of proteins. A weak UV response was observed at peak II of the F_1 and F_2 fractions, and almost no UV response was noted in the F_3 fraction. These findings are in good agreement with the chemical composition of these fractions, showing that most proteins of the crude fucoidan formed fractionated fucoidans by ion-exchange chromatography (Table 3). The average M_w values of first peaks obtained by the MALLS technique were 359 g/mol (F_1), 178 g/mol (F_2), and 88 g/mol (F_3). The average M_w values of the second peaks obtained were 18 g/mol (F_1)

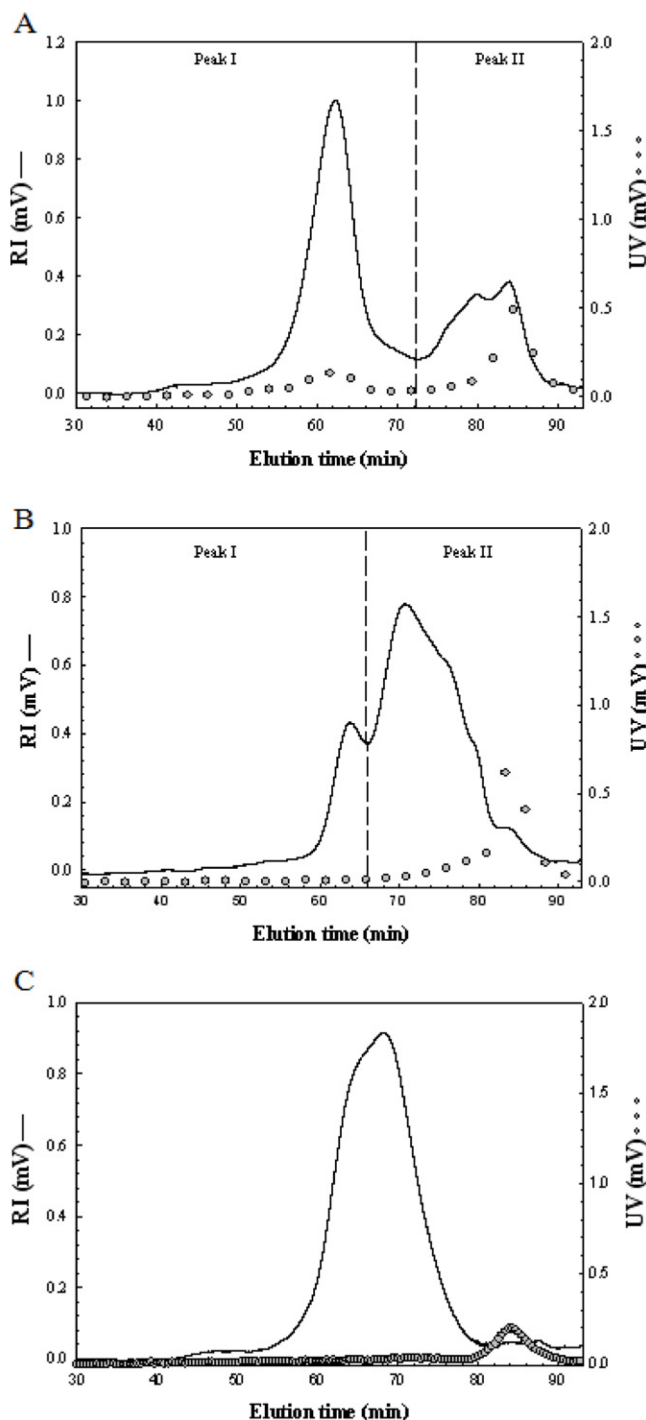

Fig. 1. RI and UV chromatograms of the fucoidans (F_1 (A), F_2 (B), and F_3 (C)) fractionated from *E. cava*.

Table 3
Chemical composition of crude and fractioned fucoidan (F_1, F_2, and F_3) extracted from *E. cava*.

Component	Fucoidan			
	Crude	F_1	F_2	F_3
Yield (%)	1.8 ± 0.8[a]	25.3 ± 0.2[b]	17.6 ± 0.3	32.6 ± 0.4
Total carbohydrate (%)	51.8 ± 1.3	57.1 ± 1.6	55.7 ± 2.3	47.1 ± 1.9
Sulfate content (%)	20.1 ± 0.7	20.0 ± 1.0	16.5 ± 0.5	39.1 ± 1.0
Uronic acid (%)	11.3 ± 0.5	15.8 ± 0.1	13.8 ± 0.2	9.0 ± 0.2
Protein (%)	8.7 ± 0.3	7.2 ± 0.4	5.3 ± 0.2	1.3 ± 0.1
Proportion of monosaccharide (%)				
Fucose	61.1 ± 1.6	53.1 ± 2.4	59.7 ± 3.9	77.9 ± 3.6
Rhamnose	3.9 ± 0.4	4.1 ± 0.2	4.5 ± 0.1	2.3 ± 0.1
Galactose	27.2 ± 1.2	32.8 ± 1.9	30.9 ± 1.1	10.1 ± 0.9
Glucose	0.8 ± 0.1	1.8 ± 0.1	0.91 ± 0.3	2.2 ± 0.5
Xylose	7.0 ± 0.3	8.2 ± 0.5	4.0 ± 0.3	7.5 ± 0.3

Experiments were performed in triplicate and the data are expressed as mean ± SE.
[a] Yield (weight of crude fucoidan/weight of seaweed powder) × 100.
[b] Yield (weight of fractionated fucoidan/weight of crude fucoidan injected into ion-exchange chromatography) × 100.

Table 4
Weight average molecular weights (M_w), radius of gyration (R_g) and specific volume for gyration (SV_g) of fractioned fucoidan (F_1, F_2, and F_3) extracted from *E. cava*.

Sample	$M_w \times 10^3$ (g/mol)		R_g (nm)		SV_g (cm^3/g)	
	Peak I	Peak II	Peak I	Peak II	Peak I	Peak II
F_1	359.6 ± 13.8	18.0 ± 0.5	27.6 ± 0.1	27.7 ± 0.2	0.1474 ± 0.0073	2.9713 ± 0.1163
F_2	178.7 ± 2.2	27.7 ± 1.1	17.1 ± 0.9	4.5 ± 0.1	0.0706 ± 0.0003	0.0083 ± 0.0002
F_3	88.3 ± 3.6	–	16.6 ± 0.2	–	0.1295 ± 0.0063	–

Experiments were performed in triplicate and the data are expressed as mean ± SE.

and 27 g/mol (F_2) (Table 4). In studies of other brown seaweeds, the M_w values of fucoidans ranged from 2000 to 1.6×10^3 g/mol, exhibiting significant variations (Pereira, Mulloys, & Mourao, 1999; Shanmugam & Mody, 2000). These considerable differences in M_w may be due to the differences in the species of brown seaweed and in the extraction and analysis methods. In particularly, proper dissolution of the water-soluble fucoidan such as with the 30-s microwave heating used in this study is important for obtaining an accurate M_w of the polysaccharide because the polymer chains may aggregate by secondary interactions through hydrogen bonding (Yang et al., 2008). The radius of gyration (R_g) of the fractionated fucoidans was also calculated from the peaks to estimate the approximate size of the fucoidan (Table 4). The R_g values of the first peaks from the fractionated fucoidans ranged from 16.6 to 27.6 nm, while the second peak ranged from 4.5 to 27.7 nm. The M_w and R_g values of the fractionated fucoidans varied and were significantly different. From the values of M_w and R_g, the specific volume for gyration (SV_g) of the polysaccharides could be calculated, as described by You and Lim (2000), by using the following equation:

$$SV_g = \frac{4/3\pi(R_g \times 10^8)^3}{M_w/N} = 2.522\frac{R_g^3}{M_w}$$

in which the units for SV_g, M_w and R_g are cm^3/g, g/mol, and nm, respectively, and N is Avogadro's number (6.02×10^{23}/mol). SV_g is inversely proportional to the degree of molecular compactness, providing the theoretical gyration volume per unit of molar mass, which gives mass-based information on the density of the fucoidans. The SV_g values of the peaks from the fractionated fucoidans ranged from 0.008 to 0.14 cm^3/g, but that of peak II (2.97 cm^3/g) from the F_1 fraction was significantly higher, suggesting that the polysaccharides in peak II had a less compact and more expanded conformational structure than the other peaks (Table 4). The above results indicate that fractionating the crude polysaccharide preparation by ion-exchange chromatography resulted in the production of fucoidans with considerable variations in their M_w and molecular compactness.

3.3. Anti-inflammatory activities of fractionated fucoidans

Raw 264.7 murine macrophage cell line releases cytokines in response to LPS stimulation. This system has been used to determine the anti-inflammatory activities of compounds by studying cytokine production. In this study, the anti-inflammatory effects of the fractionated fucoidans (F_1, F_2, and F_3) were observed using Raw 264.7 macrophages and investigating the released amount of NO. Fig. 2A shows the cytotoxic effect of the F_1, F_2, and F_3 fractions at concentrations of 12.5–100 μg/mL, as measured by Raw 264.7 macrophages proliferation. Proliferation was not influenced by the presence of F_1, F_2, and F_3 fractions at the concentrations tested. This result suggests that fractionated fucoidans were not toxic to the cells over the concentration range tested. To evaluate the effect of fractionated fucoidans on NO production, Raw 264.7 macrophages were stimulated with LPS (1 μg/mL) for 24 h to evoke NO generation, and the accumulation of its metabolite, nitrite, in the culture medium was measured. NO was produced by treatment with LPS, which was significantly inhibited by the addition of F_1, F_2, and F_3 fractions at 50–100 μg/mL and LPS-induced NO production was decreased by the fractionated fucoidans in a dose-dependent manner (Fig. 2B). In particular, the F_3 fraction exhibited maximum inhibitory effect on NO production. NO is an important inflammatory mediator that is synthesized from arginine by nitric oxide synthase (NOS). Generally, NO plays an important role as a vasodilator, neurotransmitter, and in the immunological system as a defense against tumor cells, parasites, and bacteria (Nakagawa & Yokozawa, 2002). However, under pathological conditions, NO production is increased by inducible NOS (iNOS), which further causes cytotoxicity and tissue damage (Kim, Cheon, Kim, Kim, & Kim, 1999). Therefore, the considerable inhibition of NO release from murine macrophages by the fractionated polysaccharides suggests that these fucoidans could act as inhibitors of the inflammatory response. Hwang et al. (2011) reported that sulfate-enriched polysaccharides from *Sargassum hemiphyllum*, a type of

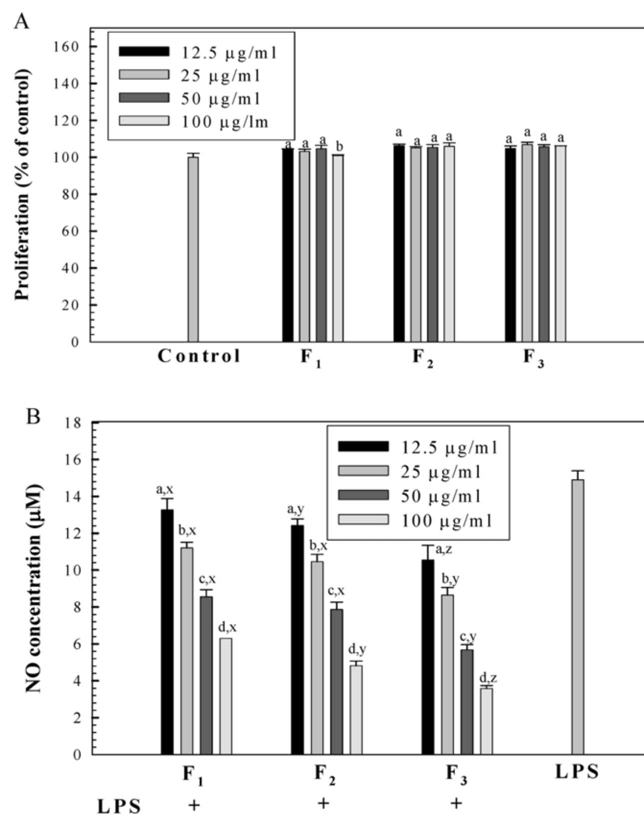

Fig. 2. Effect of fractionated fucoidans treatment on proliferation (A) and LPS-induced NO production (B) in Raw 264.7 macrophages. Proliferation was assayed in Raw 264.7 macrophages incubated for 72 h with different concentrations of fucoidans. NO production was measured in the culture medium of macrophages stimulated with LPS for 24 h in the presence of fucoidans. Experiments were performed in triplicate and the data are expressed as mean ± SE. [x,y,z] indicate a significant difference ($p < 0.05$) between the concentrations of the fractions; [a,b,c,d] indicate a significant difference ($p < 0.05$) between the fractions at each concentration.

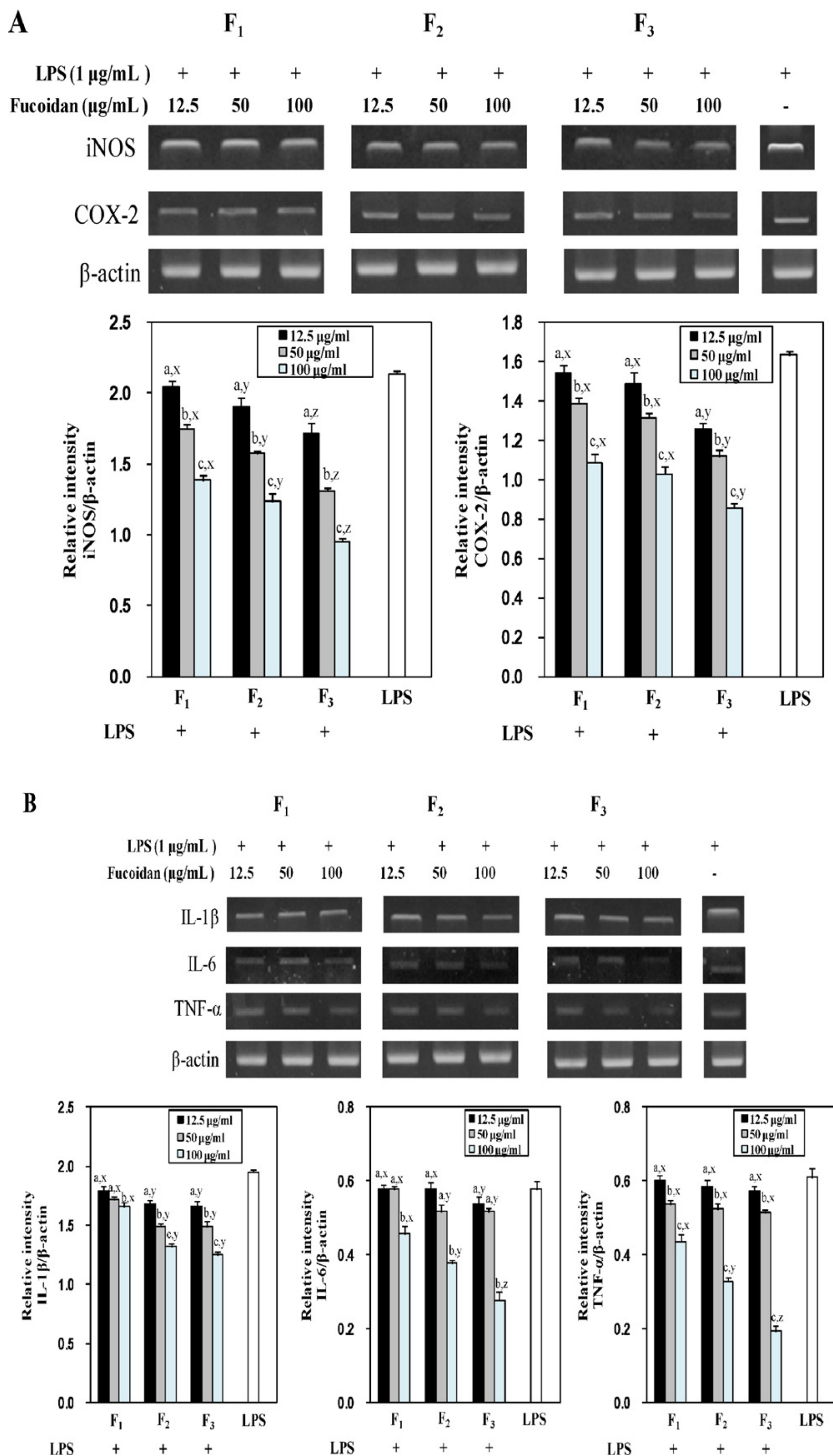

Fig. 3. Effect of LPS-induced iNOS and COX-2 mRNA (A) and IL-1β, IL-6, and TNF-α mRNA (B) expression by fractionated fucoidans treatment in Raw 264.7 macrophages. After LPS treatment, total RNA was prepared from Raw 264.7 macrophages, and RT-PCR was performed for the iNOS, COX-2, IL-1β, IL-6, and TNF-α genes. Experiments were performed in triplicate and the data are expressed as mean ± SE. [x,y,z] indicate a significant difference ($p < 0.05$) between the concentrations of the fractions; [a,b,c] indicate a significant difference ($p < 0.05$) between the fractions at each concentration.

brown seaweed, showed a very strong anti-inflammatory effect. In the present study, the F_3 fraction, which had the highest sulfate content, inhibited NO production from Raw264.7 macrophages, suggesting that the level of NO released from macrophages was proportionally related to the sulfate content of the fucoidan. In a study of other polysaccharides, a relationship between M_w and NO production from macrophages was observed (Schepetkin et al., 2008). They found that low-M_w polysaccharides induced low NO production from Raw 264.7 macrophages. In this study, the low-M_w F_3 fraction also decreased NO production from Raw 264.7 macrophages, and a correlation between fucoidan Mw and NO production was observed. These results suggest that the sulfate content and M_w of fucoidan may contribute to the inhibition of NO production. However, further research is necessary to obtain a better understanding of their considerable composition and structural heterogeneity and bioactivities.

Proinflammatory enzymes, including inducible iNOS and COX-2, produce a large amount of NO, which influences many chronic diseases associated with inflammation. Thus, reducing the levels of iNOS and COX-2 may be an effective strategy for preventing inflammatory diseases. Therefore, the expression of iNOS and COX-2 by fractionated fucoidans was determined by agarose gel analysis of the RT-PCR products obtained by using primers for iNOS and COX-2 mRNA. As shown in Fig. 3A, Raw 264.7 macrophages incubated with the F_1, F_2, and F_3 fractions for 18 h exhibited significantly decreased expression of iNOS and COX-2 mRNA in a dose-dependent manner, and their levels of the expression were considerably reduced in the F_3 fraction. Therefore, we hypothesize that the low amount of NO productions can be attributed to the decreased expression of iNOS and COX-2, because the murine macrophages get inactivated in the presence of fractionated fucoidans.

An abnormality in the production or function of cytokines such as TNF-α, IL-6, and IL-1β plays a role in many inflammatory lesions (De Nardin, 2001). TNF-α is a potent activator of macrophages and can stimulate the production or expression of IL-6, IL-1β, PGE_2, collagenase, and adhesion molecules. TNF-α elicits a number of physiological effects, including septic shock, inflammation, and cytotoxicity (Aggarwal & Natarajan, 1996). IL-6 is a well-known pro-inflammatory cytokine and is regarded as an endogenous mediator of LPS-induced fever (Kim et al., 2008). IL-1β is also considered a pivotal pro-inflammatory cytokine, primarily released by macrophages, which is believed to play an important role in the pathophysiology of rheumatoid arthritis (Jung et al., 2008).

Inflammatory stimuli such as LPS, induce cytokines in the process of macrophage activation, and this, in turn, mediates tissue responses in different phases of inflammation (Hseu et al., 2005; Laskin & Pendino, 1995). Thus, inhibition of cytokine production or function is a key mechanism to control inflammation. To determine the effects of fractionated fucoidans on the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, Raw 264.7 macrophages were incubated with the fractionated fucoidans in the presence or absence of LPS (1 μg/mL) for 18 h, and the cytokine levels were measured by RT-PCR. As shown in Fig. 3B, all mRNA levels were increased by treatment with LPS; these increases were significantly decreased in a concentration-dependent manner by treatment with the fractionated fucoidans. Our results suggest that the fractionated fucoidans significantly reduced the expression of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β mRNA in LPS-stimulated Raw 264.7, suggesting that the inhibition of the iNOS/NO pathway by fractionated fucoidans may be associated the with the attenuation of TNF-α, IL-6, and IL-1β expression.

Overall, the above results suggest that the fractionated fucoidans (F_1, F_2, and F_3 fractions) from *E. cava* could act as strong inhibitors of NO by down-regulating the expression of iNOS, COX-2, and pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β in murine macrophage cells. This outcome could explain the potential anti-inflammatory activity of these fractionated fucoidans, which might have a beneficial effect during the treatment of inflammatory diseases.

4. Conclusion

The molecular characteristics and anti-inflammatory activities of fucoidans that were extracted from *E. cava* and fractionated by ion-exchange chromatography were investigated. The extracted fucoidan consisted of mostly carbohydrates (51.8%), uronic acid (11.3%), and sulfates (20.1%), with small amounts of protein (8.7%). Ion-exchange chromatography led to the identification of three fucoidans with different chemical compositions and molecular weights. These fucoidans from *E. cava* exhibited a significant ability to reduce NO production from Raw 264.7 macrophages, suggesting that they might be strong anti-inflammatory agents. The current study demonstrates the possible applications of these fucoidans as medicinal, pharmacological, and functional food ingredients. Further studies regarding the structure and bioactivities of fucoidans are in progress to determine correlation between the molecular structures and biological activities.

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References

Aggarwal, B. B., & Natarajan, K. (1996). Tumor necrosis factors: Developments during the last decade. *European Cytokine Network*, *7*, 93–124.

Ahn, G., Hwang, I., Park, E., Kim, J., Jeon, Y. J., Lee, J., et al. (2008). Immunomodulatory effects of an enzymatic extract from *Ecklonia cava* on murine splenocytes. *Marine Biotechnology*, *10*, 278–289.

Athukorala, Y., Ahn, G. N., Jee, Y. H., Kim, G. Y., Kim, S. H., Ha, J. H., et al. (2009). Antiproliferative activity of sulfated polysaccharide isolated from an enzymatic digest of *Ecklonia cava* on the U-937 cell line. *Journal of Applied Phycology*, *21*, 307–314.

Athukorala, Y., Jung, W. K., Vasanthan, T., & Jeon, Y. J. (2006). An anticoagulative polysaccharide from an enzymatic hydrolysate of *Ecklonia cava*. *Carbohydrate Polymers*, *66*, 184–191.

Bilan, M. I., Grachev, A. A., Ustuzhanina, N. E., Shashkov, A. S., Nifantiev, N. E., & Usov, A. I. (2002). Structure of a fucoidan from the brown seaweed *Fucus evanescens* C.Ag. *Carbohydrate Research*, *337*, 719–730.

Chevolot, L., Foucault, A., Chaubet, F., Kervarec, N., Sinquin, C., Fisher, A. M., et al. (1999). Further data on the structure of brown seaweed fucans: Relationships with anticoagulant activity. *Carbohydrate Research*, *319*, 154–165.

Chizhov, A. O., Dell, A., Morris, H. R., Haslam, S. M., & McDowell, A. (1999). A study of fucoidan from the brown seaweed *Chorda filum*. *Carbohydrate Research*, *320*, 108–119.

De Nardin, E. (2001). The role of inflammatory and immunological mediators in periodontitis and cardiovascular disease. *Annals of Periodontology*, *6*, 30–40.

Dodgson, K. S., & Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, *84*, 106–110.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Durate, M. E. R., Cardoso, M. A., Noseda, M. D., & Cerezo, A. S. (2001). Structural studies on fucoidans from the brown seaweed *Sargassum stenophyllum*. *Carbohydrate Research*, *333*, 281–293.

Filisetti-Cozzi, T. M., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry*, *197*, 157–162.

Green, L. C., Wanger, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Analytical Biochemistry*, *126*, 131–138.

Heo, S. J., Lee, K. W., Song, C. B., & Jeon, Y. J. (2003). Antioxidant activity of enzymatic extracts from brown seaweeds. *Algae*, *18*, 71–81.

Hseu, Y. C., Wu, F. Y., Wu, J. J., Chen, J. Y., Chang, W. H., Lu, F. J., et al. (2005). Anti-inflammatory potential of *Antrodia camphorate* through inhibition of iNOS, COX-2 and cytokines via the NF-κB pathway. *International Immunopharmacology*, *5*, 1914–1925.

Hwang, P. A., Chein, S. Y., Chan, Y. L., Lu, M. K., Wu, C. H., Kong, Z. L., et al. (2011). Inhibition of lipopolysaccharide (LPS)-induced inflammatory responses by *Sargassum hemiphyllum* sulfated polysaccharide extract in RAW 264.7 macrophage cells. *Journal of Agricultural and Food Chemistry*, *59*, 2062–2068.

Jung, W. K., Choi, I., Lee, D. Y., Yea, S. S., Choi, Y. H., Kim, M. M., et al. (2008). Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-kB pathways. *The International Journal of Biochemistry and Cell Biology*, *40*, 2572–2582.

Kim, H. K., Cheon, B. S., Kim, Y. H., Kim, S. Y., & Kim, H. P. (1999). Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure–activity relationships. *Biochemical Pharmacology*, *58*, 759–765.

Kim, J. Y., Park, S. J., Yun, K. J., Cho, Y. W., Park, H. J., & Lee, K. T. (2008). Isoliquiritigenin isolated from the roots of *Glycyrrhiza uralensis* inhibits LPS-induced iNOS and COX-2 expression via the attenuation of NF-κB in RAW 264.7 macrophages. *European Journal of Pharmacology*, *584*, 175–184.

Kim, K. N., Heo, S. J., Song, C. B., Lee, J. H., Heo, M. S., Yeo, I. K., et al. (2006). Protective effect of *Ecklonia cava* enzymatic extracts on hydrogen peroxide-induced cell damage. *Process Biochemistry*, *41*, 2393–2401.

Laskin, D. L., & Pendino, K. J. (1995). Macrophages and inflammatory mediators in tissue injury. *Annual Review of Pharmacology and Toxicology*, *35*, 655–677.

Lee, S. H., Heo, S. J., Hwang, J. Y., Han, J. S., & Jeon, Y. J. (2010). Protective effects of enzymatic digest from *Ecklonia cava* against high glucose-induced oxidative stress in human umbilical vein endothelial cells. *Journal of the Science Food Agriculture*, *90*, 349–356.

Li, B., Lu, F., Wei, X., & Zhao, R. (2008). Fucoidan: Structure and bioactivity. *Molecules*, *13*, 1671–1695.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*, 265–275.

Nakagawa, T., & Yokozawa, T. (2002). Direct scavenging of nitric oxide and superoxide by green tea. *Food and Chemical Toxicology*, *40*, 1745–1750.

Partankar, M. S., Oehninger, S., Barnett, T., Williams, R. L., & Clark, G. F. (1993). A revised structure for fucoidan may explain some of its biological activities. *The Journal of Biological Chemistry*, *268*, 21770–21776.

Pereira, M. S., Mulloys, B., & Mourao, A. S. (1999). Structure and anticoagulant activity of sulfated fucans. *The Journal of Biological Chemistry*, *274*, 7656–7667.

Schepetkin, I. A., Xie, G., Kirpotina, L. N., Klein, R. A., Jutila, M. A., & Quinn, M. T. (2008). Macrophage immunomodulatory activity of polysaccharides isolated from *Opuntia polyacantha*. *International Immunopharmacology*, *8*, 1455–1466.

Shanmugam, S., & Mody, K. H. (2000). Heparonid active sulfated polysaccharides from marine algae as potential blood coagulant agents. *Currant Sciences*, *79*, 1672–1683.

Vieira, R. P., & Mourao, P. A. (1988). Occurrence of a unique fucose-branched chondroitin sulfate in the body wall of a sea cucumber. *The Journal of Biological Chemistry*, *263*, 18176–18183.

Wang, J., Zhang, Q., Zhang, Z., & Li, Z. (2008). Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *International Journal of Biological Macromolecules*, *42*, 127–132.

Yang, C., Chung, D., Shin, I. S., Lee, H. Y., Kim, J. C., Lee, Y. J., et al. (2008). Effects of molecular weight and hydrolysis conditions on anticancer activity of fucoidans from sporophyll of *Undaria pinnatifida*. *International Journal of Biological Macromolecules*, *43*, 433–437.

You, S. G., & Lim, S. T. (2000). Molecular characterization of corn starch using an aqueous HPSEC–MALLS–RI system under various dissolution and analytical conditions. *Cereal Chemistry*, *77*, 303–308.