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FOR THE DEGREE OF MASTER OF SCIENCE

# *In vivo* and *in vitro* Anti-inflammatory activities of sulfated polysaccharide, a fucoidan isolated from *Sargassum horneri*.

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However, I am the only person responsible for errors in the thesis.

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## Summary

*Sargassum horneri* China strain is an edible brown alga that grows in the subtidal zone as an annual species along the coasts of Korea, China and Japan. Recently, an extreme amount of *S. horneri* was moved into the coasts of Jeju, Korea from east coast of China and need to utilize this large biomass in order to protect environmental stability along the shores of Jeju Island. In addition, recent studies suggest that polysaccharide separated from *S. horneri* China strain has promising anti-inflammatory activities *in vitro* conditions. However, underlying mechanisms and or polysaccharide responsible for its anti-inflammatory activities not yet discovered. Therefore, the present study was designed to isolate the polysaccharide responsible for anti-inflammatory activities via enzyme assistance extraction from the brown seaweed S. horneri by using five food-grade enzymes (AMG, Celluclast, Viscozyme, Alcalase and Protamax) and FPLC system. Bioactivity-guided polysaccharide isolation lead to a separate fucose-rich sulfated polysaccharide (SH fucoidan) from cellular enzymatic extraction. The levels of nitric oxide (NO) and pro-inflammatory were measured by Gris sassy and enzyme-linked immunosorbent assay respectively. The levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) were measured using western blot in LPS-Stimulated RAW 264.7 cells. In addition image analysis software used to quantify cell death, NO and ROS production of LPS-exposed zebrafish embryo by using acridine orange, DFA-FM-DA and DCF-DA respectively.

SH fucoidan inhibited the NO production in LPS-stimulated RAW 264.7 cells with the IC50 value: 40.0 μg/m. in addition SH fucoidan down-regulated the LPS-stimulated iNOS and COX-2 protein levels as well as the production of inflammatory cytokine, tumor necrosis factor (TNF)-α in a dose-dependent manner. Furthermore, SH fucoidan

inhibited the activation of NF-κB p50, p65 and the phosphorylation of MAPKs, including p38 and extracellular signal-regulated kinase (ERK), in LPS-stimulated RAW 264.7 cells. In addition SH fucoidan also down-regulate the LPS-induced heart beating rate, cell death, NO production and ROS production in the LPS-exposed zebrafish embryo. A Fourier transform infrared spectroscopy (FT-IR) analysis showed that the FT-IR spectrum of SH fucoidan is similar to that of commercial fucoidan. In Conclusion, our results showed that SH fucoidan which is likely a fucoidan has anti-inflammatory activities and is a potential candidate for the formulation of a functional food ingredient or/and drug to treat inflammatory diseases.

**Key words:** Sargassum horneri; anti-inflammation; sulfated polysaccharide; RAW 264.7 cells; zebrafish

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## 1. Background

*Sargassum horneri* china strain is a Sargassaceae (Fig. 1A) edible brown algae and its habitat reported from the shores of Korea, China and Japan [1-3]. Generally, individual *S. horneri* can grow more than 7m height with the fresh weight of 3 Kg. Other than as a traditional food source and vital component of coastal water ecology recently *S. horneri* has been recognized as a promising source of novel bioactive compounds [2]. Recently, extreme amount of *S. horneri* was moved into Jeju island East coast of China sea. Due to high amount of seaweed it make adverse effect on the shores of Jeju by effecting to its beauty, economic losses to the fisheries industries as well as it creates imbalance in marine habitats. Moreover, compounds isolated from *S. horneri* China *Strain* (fucoidans, phlorotannins, fucoxanthin) have been demonstrated promising bioactivities as an anticancer, anti-microbial, anti-viral and antioxidant in different studies [3-5].

Fucoidan is a complex sulfated polysaccharide found in brown seaweeds (Fig. 1B) with interesting bioactive properties such as anti-oxidant and anti-cancer, anti-coagulant, immune modulatory and anti-inflammatory [6, 7]. Moreover, number of studies reveal that fucose-rich sulfated polysaccharide and/or fucoidan have promising anti-inflammatory properties in both *in vitro* and *in vivo* conditions [8-10]. In addition, studies reveals that the bioactive polysaccharide isolated from *S. horneri* contains considerable amounts of fucose [6]. However anti-inflammatory potentials and their underline biological mechanisms of polysaccharide isolated from *S. horneri* are not yet discovered properly. Therefore, in the present study emphasis was given to isolate fucoidan from *S. horneri* evaluate its anti-inflammatory effects on LPS-stimulated RAW 264.7 cells and zebrafish model.

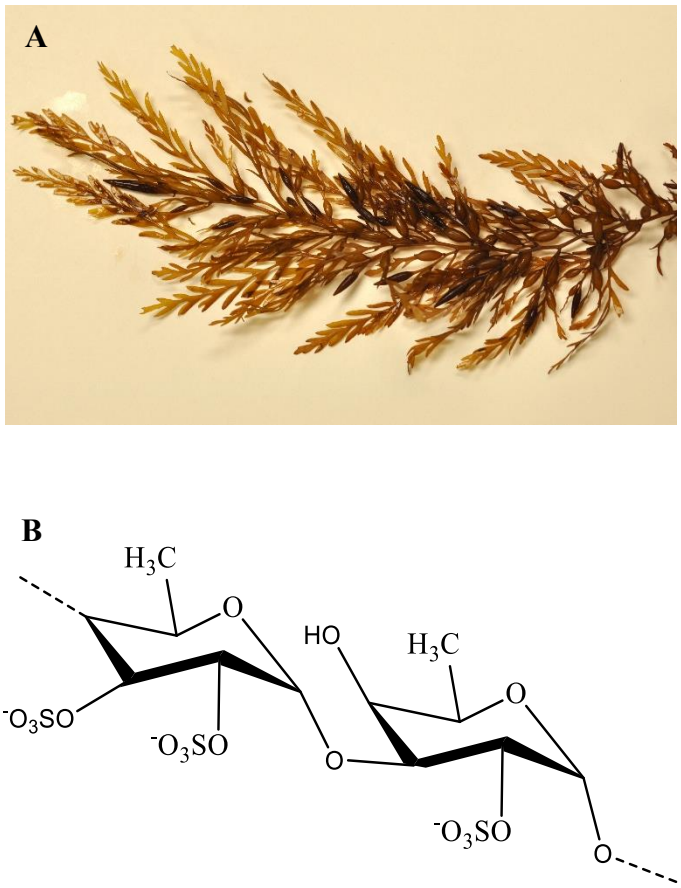


**Figure 1**. (A) Image of brown seaweed *Sargassum horneri*, (B) Proposed structure of brown algal fucoidans.

Inflammation is a normal biological response to infection or tissue injury. In general, tissue injury and pathological infections are major causes to trigger inflammatory responses in biological systems. Moreover, it is believed that the controlled inflammatory responses are beneficial for the host to protects themselves from pathological agents [11]. However, deregulated inflammatory responses are giving catastrophic outcome to their host cells or tissues as prolong inflammatory responses can lead pathogenesis of autoimmune disease, atherosclerosis, diabetic, cardiovascular diseases and cancer [11-13]. Figure 2. illustrate the causes and outcomes of inflammation.

Inducible nitric oxide synthase (iNOS) and Cyclooxygenase-2 (COX-2) are important enzymes that mediate inflammatory processes [14]. Moreover, expression of iNOS led to producing tiny, reactive radical gas nitric oxide (NO) and expression COX-2 led to producing Prostaglandin E2 (PGE2) in LPS-stimulated RAW cells [15-18]. Recent studies have demonstrated that the activation of nuclear factor-kappa B (NF-κB) and The mitogen-activated protein kinase (MAPK) signaling cascades are involved in regulation of iNOS and COX-2 expression [8, 14, 19]. In addition, accumulating evidence suggesting that the MAPK signaling cascade has important role in activation of NF-κB signaling cascade and meditate pro-inflammatory cytokine expression in LPS-stimulated macrophage cells [14, 20, 21]. Moreover, studies have demonstrated that the Phosphorylation of p38 kinase and ERK1/2 are compulsory for activate pro-inflammatory cytokines including interleukin 1β (IL-1β), IL-6 and tumor necrosis factor alpha (TNF-α) [20, 22-24]. Thus, regulation or inhibitory actions of MAPK and NF-κB signaling cascades are found to be the basic principle of anti-inflammatory drugs. Figure 3. illustrate the mechanisms underlying the inhibitory action of F2 on LPS-stimulated RAW 264.7 cells.

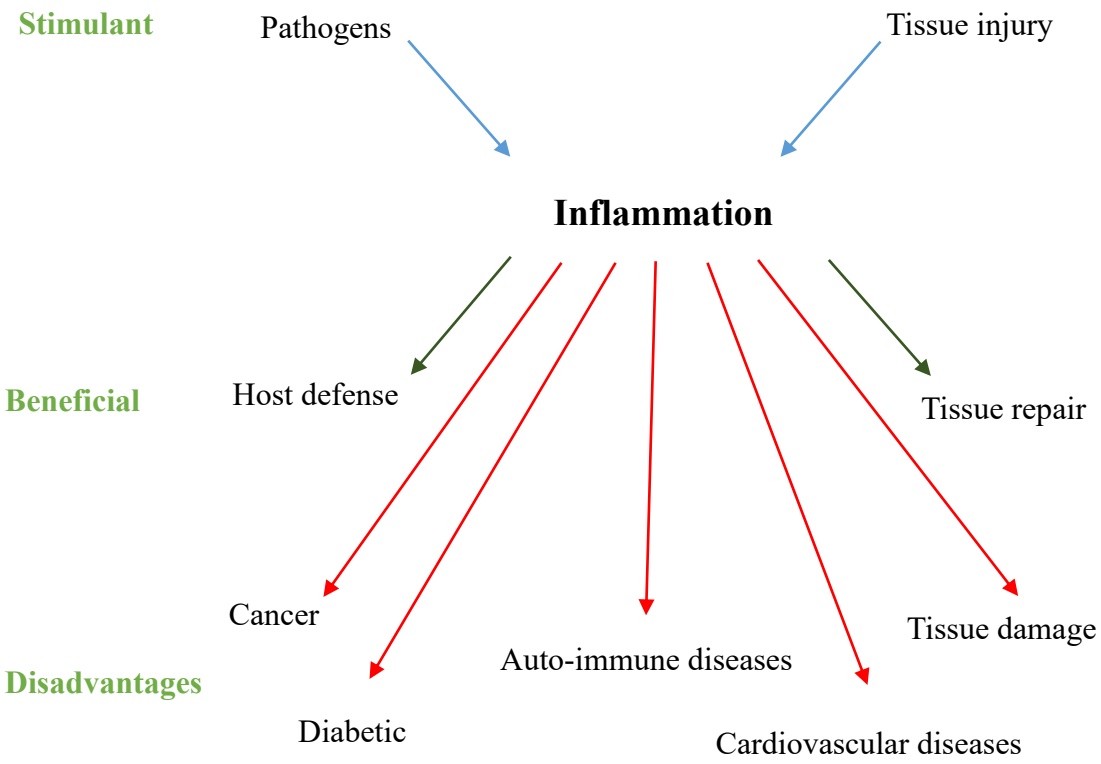


**Figure 2**. Causes and outcomes of inflammatory responses

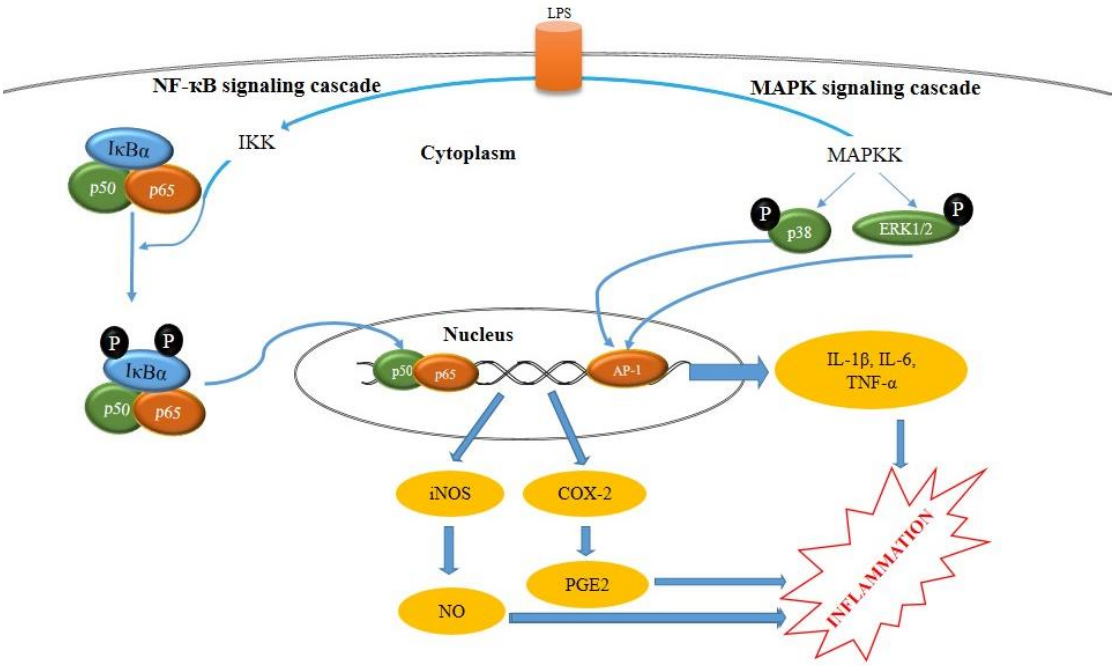


**Figure 3.** Schematic diagram illustrate the mechanisms underlying the inhibitory action of F2 on LPS-stimulated RAW 264.7 cells.

Direct use of *in vitro* results to develop isolated compounds as a functional foods or as a pharmaceutical is doubtable due to the physiological and economic relevance in *in vivo* situations. On the other hand use of animal model or human models are also have limitations due to limited amounts of pure compounds, high cost, laborious routine work, ethical law enforcements for animal and human models [25].

Recently, zebrafish (*Danio rerio*) has been recognized as a promising *in vivo* model to use in research areas such as cancer, stem cell research and immunology and infectious diseases research due to its morphological and physiological similarity to the mammals, transparency, easy to handle, less maintenance cost and only the innate immune system is present until several weeks post-fertilization. Moreover, due to these specific morphological and physiological features zebrafish has a great potential to provide opportunities to accelerate the process of drug discovery including target identification, disease modelling, lead discovery and toxicology [26-28]. Thus, we used LPS-exposed zebrafish as our *in vivo* model to evaluate cytotoxic and anti-inflammatory properties of F2.

In this work authors explored the anti-inflammatory effect of fucose rich sulfated polysaccharide isolated from *Sargassum horneri* in *in vitro* LPS-stimulated RAW 264.7 cells and in *in vivo* zebrafish model.

## 2. Materials and Methods

### 2.1 Chemicals and reagents

Murine macrophage cell line (RAW 264.7) was used to measure *in vitro* NO production and African green monkey kidney (Vero) cell line was used to evaluate cell viability of samples. RAW 264.7 cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Gibco/ BRL (Burlington, ON, Canada). 3-(4, 5-Dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). The enzyme linked immunosorbent assay kit (ELISA) for TNF–α and protaglndin $E_2$ ($PGE_2$) were purchased from R & D System Inc. (Minneapolis, MN, USA). Three carbohydrate degrading enzymes (AMG, Celluclast, Viscozyme) and two proteases (Alcalase and Protamex) were donated by Novozymes (China) Biotechnology Co., Ltd. (No. 150, Nanhai road, TEDA, Tianjin, Chin). All other chemicals and reagents used in these experiments were of analytical grade.

### 2.2 Sample collection and preparation

*Sargassum horneri* samples were collected during the winter season in 2015 along the sea shore of Jeju Island South Korea. Collected seaweed samples were washed with running tap water to remove salt, epiphytes and sand on seaweeds. The washed *S. horneri* biomass were frozen at -80°C and preserved by freeze dry.

## 2.3 Preparation of enzymatic extracts from seaweeds

The freeze dried *S. horneri* sample was homogenized with a grinder to obtain a fine powder. Two grams of *S. horneri* powder was homogenized with 100 mL of distilled water and then mixed with 20 µl (or 20 mg) of enzymes. The enzymatic hydrolytic reactions were performed under optimum pH and temperatures (Table 1.) for 24 hours to achieve the optimum degree of the hydrolysis. After 24 hours digested samples were boiled at 100 ˚C for 10 minutes to deactivate enzymes and then extracts were adjusted to pH into 7.0 and solution mixtures were centrifuged at 3500 RPM for ten minutes to remove unhydrolyzed residues. Supernatants were vacuum filtered and resulted solutions were stored in -20˚C for further experiments and designated as enzymatic digests (ED).

**Table 1.** Optimum hydrolyzation conditions for commercial food grade enzymes

| Enzyme | Optimum condition | | Enzyme composition | Reference |
|---|---|---|---|---|
| | pH | Temperature | | |
| **Carbohydrases** | | | | |
| AMG | 4.5 | 60˚C | Amyloglucosidase | [29] |
| Celluclast | 4.5 | 50˚C | β-glucanases | [29] |
| Viscozyme | 4.5 | 50˚C | Arabanase, cellulase, β-glucanase, hemi-cellulase and xylanase | [29] |
| **Proteases** | | | | |
| Alcalase | 8.0 | 50˚C | A endo protease | [29] |
| Protamex | 6.0 | 40˚C | Endo-proteases | [29] |

## 2.4 Crude polysaccharide separation

The enzymatic digests (ED) were mix 95% ethanol solution until the ethanol concentration reached to 2/3 of ED solution. Then the mixture was allowed to settle 24 hours at 4 ºC cold room, and then crude polysaccharide fractions were collected by centrifugation at 12000 rpm for 10 minutes at 4 ºC. Precipitates were named used as crude polysaccharide (CP). CP were concentrated using rotary evaporator at less than 40 °C and removed all ethanol, and then samples were dissolved in PBS for further experiments.

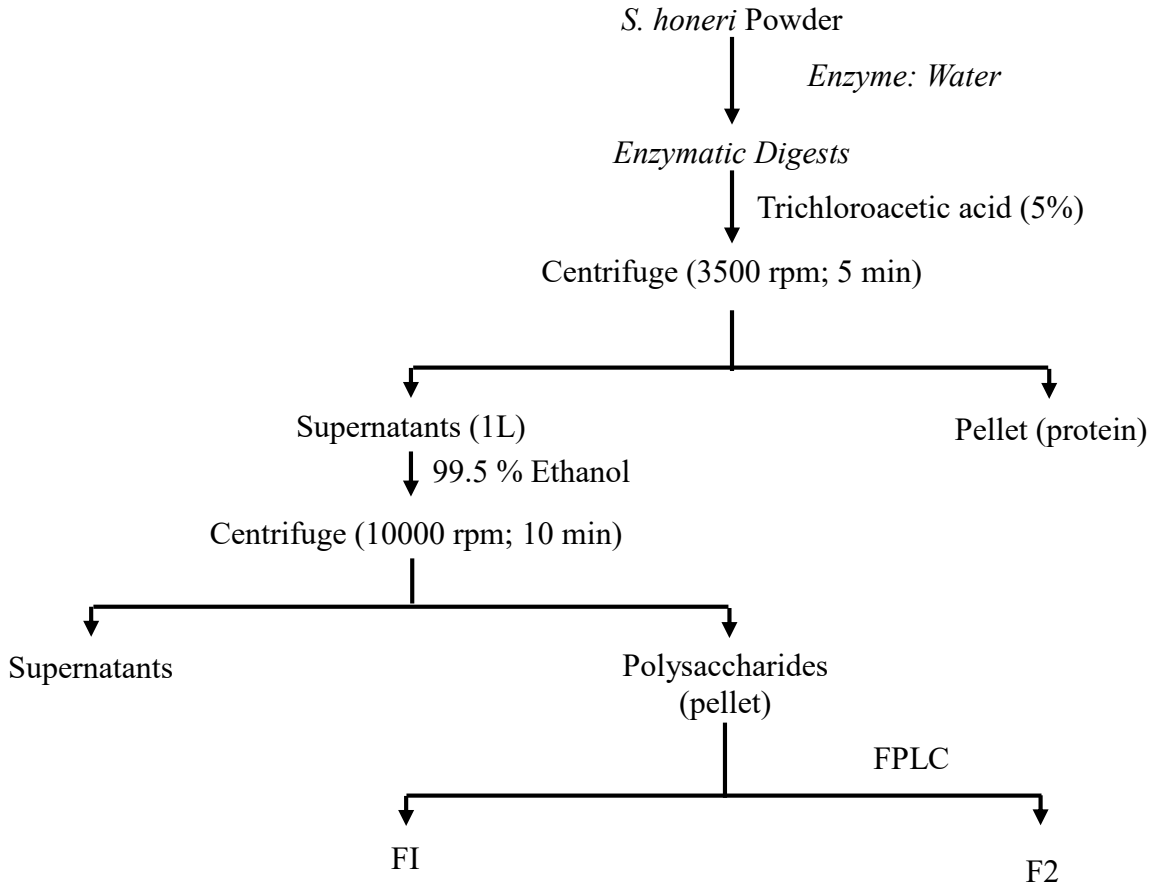


**Figure 4.** Flow chart of polysaccharide separation from *S. honeri*

## 2.6 Anion exchange chromatography

The crude polysaccharide sample (200 mg) was purified by an anion exchange chromatography using DEAE-cellulose column as documented by Pereira, Mulloy, and Mourao (1999) with slight modifications[30]. The crude polysaccharide sample was applied to a DEAE-cellulose column (HiPrep™ DEAE FF 16/10) linked to FPLC system (GE Health care, Uppsala, Sweden). The DEAE-cellulose column was washed with 50 mL of the sodium acetate buffer (pH 5.0) containing 2 M NaCl to remove unbound proteins and others on the column. Then the column was pre-equilibrated same sodium acetate buffer (pH 5.0). The column was eluted by a linear gradient prepared by mixing 400 mL of 50 mM sodium acetate (pH 5.0) containing 2 M NaCl with 400 mL of 2 M NaCl in the same buffer with the flow rate of 5 mL/Min. Then, 15 mL fractions were collected and polysaccharide contents were measured by using the phenol-sulfuric acid method as described by DuBois et al, (1956) [31].

## 2.7 Analytical procedures

The chemical compositions of *S. horneri* were identified by measuring the contents of moisture, proteins, and/or carbohydrates according to the Association of Official Analytical Chemists (AOAC) method.

Total polysaccharide contents of polysaccharides were measured by using the phenol-sulfuric acid method as described by DuBois et al, (1956) [31]. Phenolic contents were determined by using a protocol similar to Chandler and Dodds (1983) with some modifications. Briefly, 1 mL of seaweed extract, 5 mL of distilled water, 1 mL of 95% Ethanol and 50% Folin-Ciooocalteu regent were mixed together and were allowed to react five minutes. Then 1 mL of 5% sodium carbonate was mixed and then vertex the solution and placed in the dark room for one hour. Then absorbance was

measured at 725 nm and gallic acid standard curve was obtained for the calibration of phenolic content. [32]. Protein percentages of ED and CP were quantified using Pierce™ BCA Protein Assay Kit and bovine serum albumin used as the standard. Finally sulfate content of CP were quantified using $BaCl_2$ gelatin method as described by Saito et al., (1968) with slight modifications [33].

### 2.8 Quantification of neutral sugar composition on purified polysaccharides

To determine monosaccharide contents of the F2 fraction, first F2 was hydrolyzed in a sealed glass tube with 2 M trifluoroacetic acid for 4 h at 100°C and digested with 6 N of HCl for 4 h. Then, the F2 was applied separately to CarboPacc PA1 (4.5 × 250 mm, Dionex, Sunnyvale, CA, USA) with a CarboPac PA1 cartridge (4.5 × 50 mm) column to analyze neutral and amino sugars, respectively. The columns were eluted by using 16 mM of NaOH at 1.0 mL/min of flow rate. Monosaccharide contents of the samples (fucose, galactose, and xylose) were detected by using an ED50 Dionex electrochemical detector (Dionex), and the results were analyzed with Peack Net software (Dionex).

### 2.9 Fourier Transform Infrared (FT-IR) Spectrophotometer analysis

IR spectrums of the fractionated and separated polysaccharides were recorded using a FT-IR spectrometer (Nicolet 6700 FT-IR spectrometer). Samples were homogenized with KBr powder and then pressed into pellets for FT-IR measurement in the frequency range of 500-4000 $cm^{-1}$.

## 2.10 *In vitro* experiments

### *2.10.1 Cell culture*

RAW 264.7 cells were cultured in a Dulbecco's minimal essential (DMEM) medium. RPMI and DMEM were supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% streptomycin (100 µg/mL), and penicillin (100 unit/mL). The cells were maintained and incubated in modified atmospheric condition which contained 5% $CO_2$ and at 37˚C (ambient temperature). Cells were sub-cultured within two days intervals and exponential phase cells were used for experiments.

### *2.10.2 Determination of Cytotoxicity of extracts and purified polysaccharides on RAW 264.7 cells*

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay, is a test of metabolic competence predicated on the assessment of mitochondrial performance. Briefly, mitochondria located in living cells produce succinate dehydrogenase during metabolism. Production of succinate dehydrogenase enzyme can measure with the yellow color tetrazolium salt MTT as MTT reduce into it its blue-black formazan crystals after reacting with mitochondrial succinate dehydrogenase produced in viable cells during metabolism [34]. Cell cytotoxicity of all cell lines were estimated via a colorimetric MTT assay as described by Mosmann in 1983 [35], with slight modifications. Briefly, 190 µL of cells were seeded in 96 well plates with 1 X $10^5$ cells per milliliter concentration and plates were incubated for 24 hours, and then treated with 10 µL from measured concentration of tested samples and again incubated for 24 hours. Ten microliter of MTT reagent (2 mg $mL^{-1}$ in PBS) was added to each well and aspirate the medium after 3 hours of incubation and formazan crystals were dissolved in DMSO, and then the amount of blue-black formazan was measured using ELISA

reader at 540 nm.

### *2.10.3 Determination of Nitric oxide production*

Nitric oxide (NO) production was assayed using the method described by Leiro et al, (2002) with slight modifications [36]. Nitrate production in Raw 264.7 culture supernatants, measured with Griess solution. Briefly, 450 µL of cell suspensions (1 X $10^5$ cells per milliliter) were cultured in twenty-four well plates and incubated 24 hours. Then the incubated 24 well plates were treated with 25 µL of measured concentrations of samples to each well. Again twenty-four well plates were incubated for 1 hour and treated with 25 µL of LPS. Then sample treated well plates were again incubated for another 24 hours. Finally, 100 µL cell suspensions were mixed with equal volume of Griess reagent. After a 10 minute incubation, the absorbance was read with an ELISA reader at 540 nm.

### *2.10.4 Western blot analysis*

To determine the effects of F2 on the protein expression of inflammatory mediators, including, iNOS and COX2, in LPS-stimulated RAW 264.7 cells, a western blot analysis was performed. Briefly, cells ($1 \times 10^5$ cells/ml) were seeded in 6-well plates and incubated for 24 h. Then, the cells were treated with F2 (12.5 µg/ml, 25 µg/ml, and 50 µg/ml) for 1 h, and then cells were stimulated with LPS (1 µg/ml) for 24 h. RAW 264.7 macrophage cells were incubated with or without F2 (12.5 µg/ml, 25 µg/ml, and 50 µg/ml) for 1 h and then stimulated with LPS (1 µg/ml) for 25 min. cytosolic and nucleus proteins were extracted with the PROPREP protein extraction kit (iNtRON Biotechnology, Sungnam, Korea). After separation on a 10% SDS–polyacrylamide gel under denaturing conditions, the cytoplasmic proteins (40 µg) were electro-transferred

onto a nitrocellulose membrane. Then blocked with 5% nonfat milk for 1 h, the blots were separately incubated with the following primary antibodies: rabbit polyclonal anti-rabbit iNOS, p44/42 (ERK), phosphorylated p44/42 (PERK), p38, phosphorylated p38, NFκB p65, NFκB p50, C-23, COX2 and mouse monoclonal anti-mouse β-actin (Santa Cruz Biotechnology, CA) for 1.5 h. The blots were washed twice with Tween 20/Tris-buffered saline (TTBS) and then incubated with HRP-conjugated anti-mouse or anti-rabbit IgG for 1 h. Antibody binding was visualized by using enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL, USA).

### *2.10.5 Determination of Pro-inflammatory cytokine TNF–α production*

The Inhibitory effect of *S. horneri* polysaccharide on the pro-inflammatory cytokines TNF–α production of LPS-stimulated RAW-264.7 cells were determined method similar to Cho et al, (2000) with slight modifications [37]. Supernatants were used to measure pro-inflammatory cytokines assayed using mouse ELISA kit according to the manufacture's instruction.

### *2.10.6 Determination of $PGE_2$ Production*

Prostaglandins are play and important role in regulating inflammatory responses [38]. Therefore, to determine PGE2 production in LPS-stimulated macrophage cells, cultured RAW-264.7 cells (1 $x10^5$ cells per mL) were treated with measured concentrations of polysaccharides and after one-hour incubation cells were stimulated with LPS (1µg/ mL). After 24 hours incubation $PGE_2$ concentration in the supernatants were quantified by using a competitive enzyme immuno assay kit according to the manufacture's instruction.

## 2.11 *In vivo* Zebrafish experiments

### *2.11.1 Origin and maintenance of parental zebrafish*

Adult zebrafish were purchased from a commercial dealer (Seoul aquarium, Seoul, Korea) and were kept in a 3 L acrylic tank with modified environmental conditions as follows; 28.5 °C temperature, 14:10 h light: dark cycle and the zebrafish were fed twice a day (Tetra GmgH D-49304 Melle, Germany). Embryos were obtained by interbreeding two males and one female. In the morning (8 a.m), embryos were obtained from natural spawning and collection of the embryos were completed within 30 min in petri Dishes (containing embryo media).

### *2.11.2 Measurement of the toxicity of F2 on zebrafish embryo*

Toxicity of polysaccharides on zebrafish embryos was determined by means of survival rate and the heartbeat rate of the zebrafish embryos method described by Wijesinghe et al, (2014) with slight modifications [39] . Briefly, the zebrafish embryos ($n$ = 15) were transferred to 12-well plates containing 950 µL of embryo media from approximately 3 to 4 hours post-fertilization (hpf). Then 50 µL of different concentration of fucoidan mixed with the embryo medium. The survival rate of zebrafish embryos exposed to fucoidan was determined until 5-day post-fertilization (5dpf).

### *2.11.3 Measurement of heart-beating rate of zebrafish*

The heartbeat rate of both atrium and ventricle of the sample or LPS-stimulated zebrafish embryo was recorded at 2 dpf for 1 min under the microscope. The results were presented as the percentage of the heart-beating rate per min.

### *2.11.4 Measurement of the toxicity of the fucoidan on zebrafish embryo by means of the cell death*

Acridine orange staining used to detect cell death of fucoidan treated zebrafish embryo. At 3 dpf, a zebrafish were transferred to 24 well plate and incubated 30 minutes with acridine orange solution (7 μg/mL) in the dark room at 28.5 ± 1 °C. After 30 min acridine orange removed from 24 well-plate and washed twice with embryo medium. Then zebrafish larvae were anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) and observed under the microscope equipped with CoolSNAP-Pro color digital camera (Olympus, Japan). Individual zebrafish larvae fluorescence intensity was quantified using an image J program.

### *2.11.5 Estimation of oxidative stress-induced ROS generation and image analysis*

At the 3 dpf, zebrafish larvae were transferred to 24-well plate, treated with DCF-DA solution (20 μg/mL) and incubated for 1h in the dark room at 28.5 ± 1 °C. Then the zebrafish larvae were washed twice with fresh embryo media. Finally, zebrafish larvae were anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) and observed under the microscope equipped with CoolSNAP-Pro color digital camera (Olympus, Japan). Individual zebrafish larvae fluorescence intensity was quantified using an image J program.

### *2.11.6 Measurement of in vivo NO production*

NO production of LPS-stimulated zebrafish model was analyzed using a fluorescent probe dye, diaminofluorophore 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). At the 3 dpf, zebrafish larvae were transferred to 24-well plates and incubated with DAF-DM-DA solution (5 μM) for 1.5 h in the dark room at

28.5 ± 1 °C. After, incubation, the zebrafish larvae were rinsed in fresh embryo medium and anaesthetized by 2-phenoxy ethanol (1/500 dilution sigma) and observed under the microscope equipped with CoolSNAP-Pro color digital camera (Olympus, Japan). Individual zebrafish larvae fluorescence intensity was quantified using an image J program.

## 2.12 Statistical analysis

All the data were expressed as the mean ± standard deviation of three determinations. Analysis of variance was carried out for the collected data using the SPSS V20 statistical analytical package. Mean values of each experiment were compared using one-way analysis of variance. The Duncan's multiple range test (DMRT) was used for the mean separation. *P*-values less than 0.05 was considered significant.

# 3. Result and Discussion

## 3.1 Proximate chemical composition of *S. horneri*

Seaweeds are one of the potential renewable source found in the marine environment, which are carrying thousands of bio-active compounds could be developed as functional ingredients in industrial products dealing with human health [40]. *S. horneri* is an edible brown seaweed found along the shores of Jeju Island South Korea. Traditionally *S. horneri* has been used as a traditional medicine and as a food in Korea, China and Japan [5, 41]. The results of proximate chemical composition (Table 2) of *S. horneri* gives a better understanding of its medicinal and high nutritional value. According to the proximate chemical composition results *S. horneri* contained 57.94 % carbohydrate and 18.4% crude protein. Moreover, Ermakova et al, (2011) reported that generally brown seaweeds contain a higher percentage of polysaccharides which may vary from 40% to 80%. According to the results we all so discovered that *S. horneri* contained high percentage of polysaccharides compared to other nutrients (protein & lipid).

**Table 2.** Proximate chemical composition of *Sargassum horneri*

| Compound | Content (% wt) |
|---|---|
| Moisture | 9.3 |
| Ash | 14.16 |
| Crude carbohydrate | 57.94 |
| Crude proteins | 18.4 |
| Crude lipids | 0.2 |

### 3.2 Yield and general components of the *S. horneri* extracts.

Extraction yield of enzymatic digests were calculated to compare extraction efficiency of enzyme assisted extraction against distilled water extraction and to compare extraction efficiency of different enzymes used in this experiment. The extraction yields of all the carbohydrases and proteases evaluated in this study were significantly higher than that of the DW extraction (Table 3). The Celluclast enzymatic extraction (CCP) yielded the highest polysaccharide content (65.47 ± 0.66%), Protamex extraction yielded the highest protein content (24.64 ± 2.63%), and the AMG enzymatic extraction yielded the highest polyphenol content (5.57 ± 0.32%).

**Table 3.** Yield and general components of seaweed extracts

| Enzymatic digests | Yields (%) | Polysaccharides (%) | Proteins (%) | Polyphenols (%) |
|---|---|---|---|---|
| None | 09.50 ± 0.87[a] | 45.78 ± 0.42[a] | 7.66 ± 0.13[a] | 1.44 ± 0.06[a] |
| AMG | 16.00 ± 0.50[b] | 60.26 ± 0.98[e] | 8.70 ± 1.54[a] | 5.57 ± 0.32[c] |
| Celluclast | 20.17 ± 0.76[c] | 65.01 ± 1.22[e] | 14.13 ± 1.54[a] | 3.88 ± 0.16[b] |
| Viscozyme | 21.0 ± 1.00[c,d] | 49.55 ± 1.20[b] | 10.87 ± 1.54[a,b] | 4.10 ± 0.16[b] |
| Alcalase | 22.17 ± 0.76[d] | 56.69 ± 0.24[d] | 23.91 ± 1.54[c] | 3.65 ± 0.16[b] |
| Protamex | 15.50 ± 0.87[b] | 53.32 ± 2.61[c] | 24.64 ± 2.63[d] | 2.00 ± 0.09[a] |

Means in each column having common letters are not significantly different at 0.05 sigma levels.

### 3.3 *In vitro* cytotoxic effect and NO inhibitory properties of enzymatic digests separated from *S. horneri* on LPS-Stimulated RAW 264.7 cells

The yellow color of the MTT reagent changes to blue-black formazan after reacting with mitochondrial succinate dehydrogenase, which is produced in viable cells, but not in dead cells. Therefore, the MTT assay is used to measure the viability of cells after treatment [34, 42]. The cytotoxicity of the five EDs and a DW extract in RAW 264.7 cells was first evaluated at different concentration to determine the most suitable concentrations for further study. The results showed that the EDs had no cytotoxic effects on RAW 264.7 cells at all tested concentrations (50 μg/ml). Furthermore, all the EDs inhibited NO production in LPS-stimulated RAW 264.7 cells compared to the levels in control cells. Interestingly, the EDs had higher NO inhibitory activities than the DW extract. Based on these results, the enzyme extraction technique improved the extraction yields and the polysaccharide contents as well as the inhibitory effects on NO production.

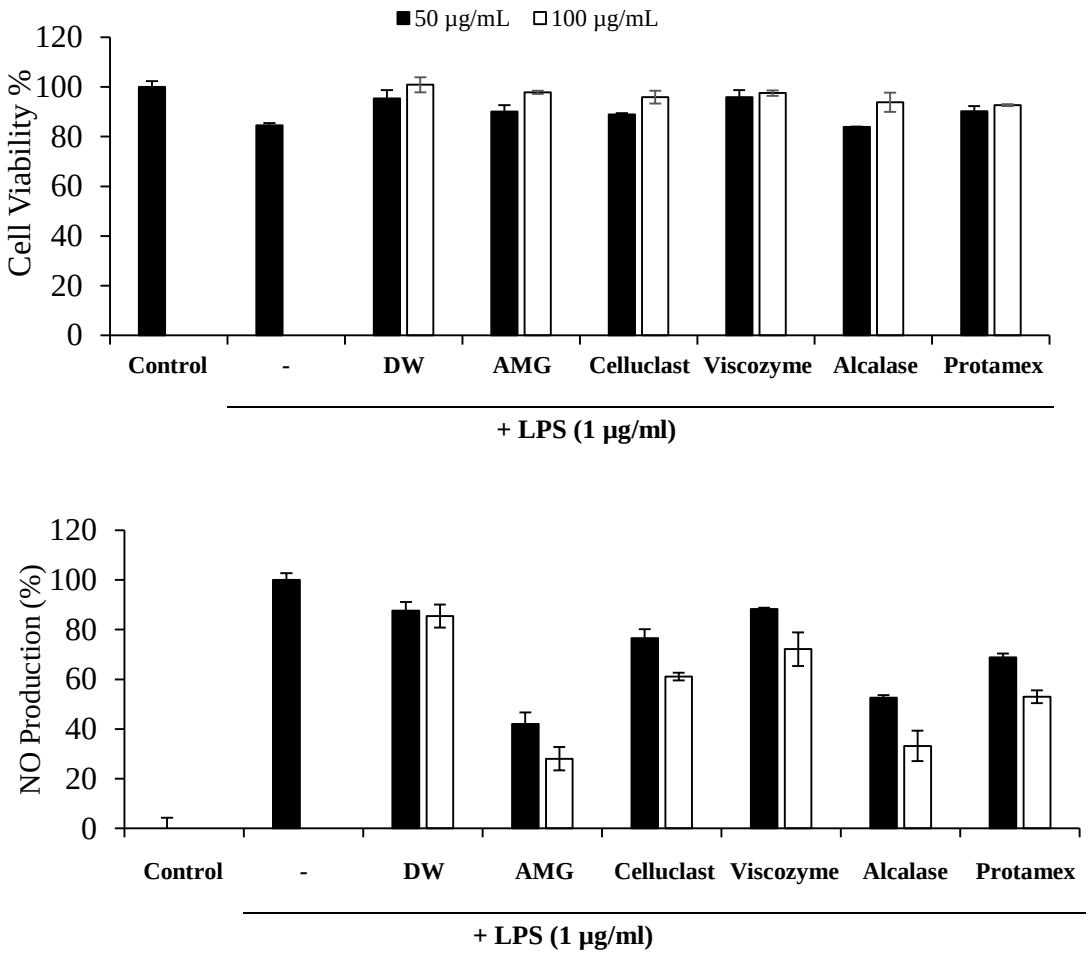


**Figure 5.** (A) Cytotoxicity of EDs on RAW 264.7 cells in the presence of LPS. (B) Dose-dependent inhibition of NO production by EDs in LPS-stimulated RAW264.7 macrophages. *P < 0.05.

### 3.4 *In vitro* cytotoxic effect and NO inhibitory properties of crude polysaccharides separated from *S. horneri* on LPS-Stimulated RAW 264.7 cells

Polysaccharides separated form AMG, Celluclast, Viscozyme and Alcalase were nominated as AMGCP, CCP, VCP and AlCP. Table 4 illustrates the composition of the CPs separated from the four enzymatic digests. CCP showed the highest polysaccharide and sulfate content ($88.7 \pm 2.44^{b}$ and $12.01 \pm 0.98^{b,}$ respectively).

It has been reported that the brown seaweeds are a potential source of sulfated polysaccharides that could potentially be exploited as functional ingredients for human health. Furthermore, sulfated polysaccharides (fucoidan) separated from brown seaweeds carrying numerous biological activities including promising anti-inflammatory activities [43]. In this point of view we separated the CP's from the ED's by ethanol precipitation for subsequent experiments and checked its effects on NO production. Cell viability experiments showed no cytotoxicity in RAW 264.7 cells for any of the CPs, with more than 90% viability (Fig. 6A). In addition, at 100 μg/ml, AMGCP, CCP, VCP, and AlCP inhibited NO production in RAW 264.7 cells induced by LPS stimulation (Fig. 6B). CCP had the highest inhibitory effect on NO production in LPS-stimulated RAW 264.7 cells, with an $IC_{50}$ value of 95.7 μg/ml, and the effect was dose-dependent. Our results indicated that CCP has an inhibitory effect on NO production in LPS-stimulated RAW 264.7 cells, without cytotoxicity. Therefore, CCP was used for all subsequent experiments.

**Table 4.** General components of CPs.

| Samples | Polysaccharide (%) | Sulfate content (%) | Protein (%) | Polyphenol (%) |
|---|---|---|---|---|
| AMGCP | $71.63 \pm 2.17^{a}$ | $11.51 \pm 0.07^{b}$ | $8.80 \pm 0.82^{b}$ | $4.63 \pm 0.30^{c}$ |
| CCP | $88.7 \pm 2.44^{b}$ | $12.01 \pm 0.98^{b}$ | $4.01 \pm 1.06^{a}$ | $3.90 \pm 0.15^{b}$ |
| VCP | $84.68 \pm 1.63^{b}$ | $11.34 \pm 0.18^{b}$ | $11.45 \pm 0.00^{c}$ | $3.68 \pm 0.15^{a,b}$ |
| AlCP | $81.25 \pm 2.44^{b}$ | $2.20 \pm 0.07^{a}$ | $15.77 \pm 0.57^{d}$ | $3.37 \pm 0.00^{a}$ |

Means with same letters are not significantly different at 0.05 sigma levels.

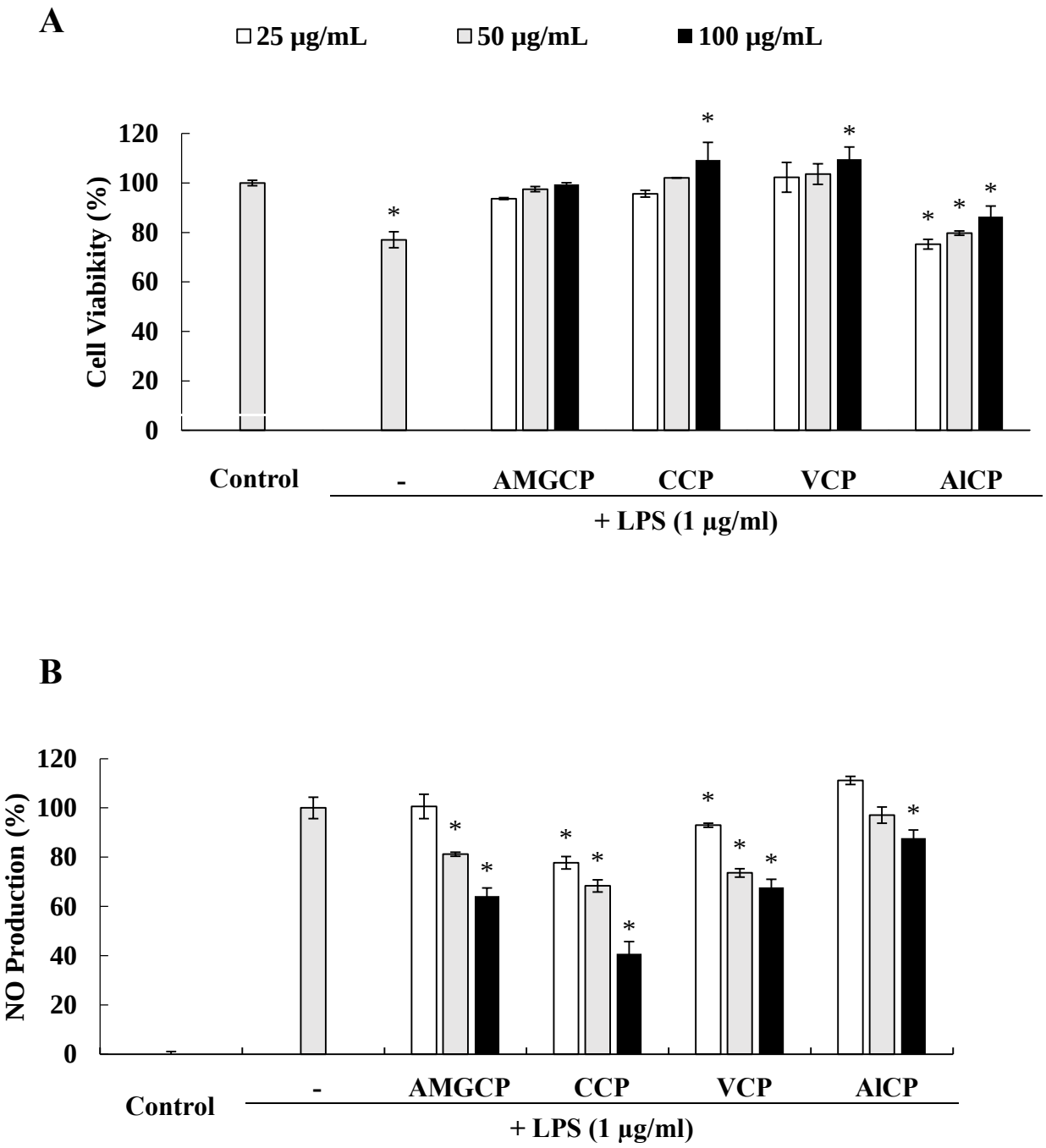


**Figure 6.** (A) Cytotoxicity of CPs on RAW 264.7 cells in the presence of LPS. (B) Dose-dependent inhibition of NO production by CPs in LPS-stimulated RAW264.7 macrophages. *$P < 0.05$.

### 3. 5 Isolation and Characterization of anti-inflammatory polysaccharides from *S. horneri*

According to the cytotoxic and NO inhibitory data of the four polysaccharide fractions, study was only continued with CCP. To purify the responsible anti-inflammatory polysaccharide mixed in CCP, 2 ml of 100 mg/ml CCP was applied to the DEAE-cellulose column (HiPrep™ DEAE FF 16/10) linked to FPLC system. Elution was separated into two fractions (F1, F2) according to their polysaccharide composition evaluated via phenol sulfuric acid method. The first peak was eluted at the beginning of the salt gradient and second minor peak was eluted at the higher NaCl gradient. However, *S. horneri* F2 fraction was small compared to the F1 fraction. The profile of DEAE cellulose chromatography is shown in Fig. 7. The fractions were pooled and lyophilized.

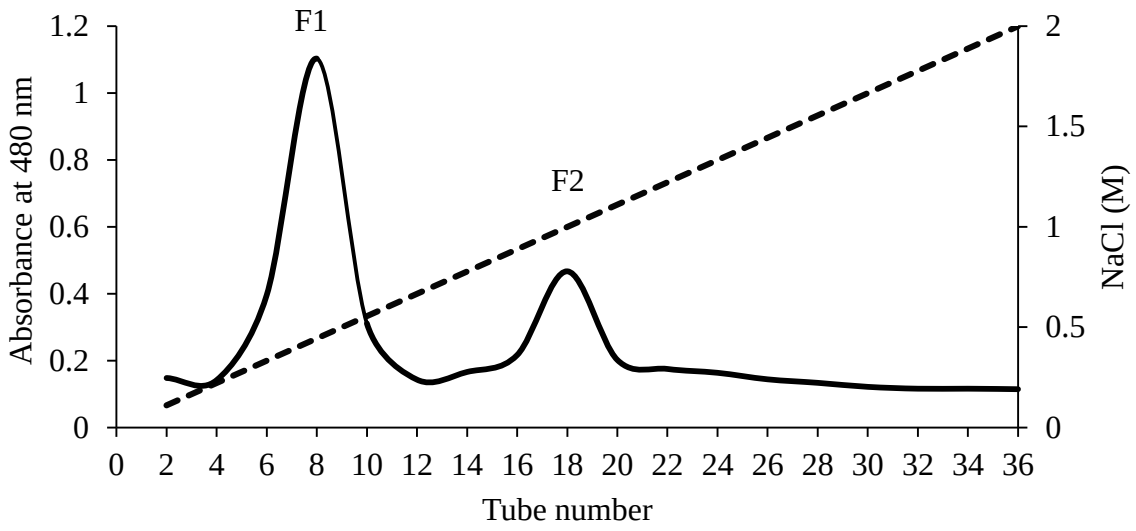


**Figure 7.** Purification of the sulfated polysaccharides (obtained from celluclast enzymatic extraction followed by ethanol precipitation) from the brown alga, *Sargassum horneri* by DEAE-cellulose column HiPrep™ DEAE FF 16/10) linked to FPLC system. Fractions of 15 ml were collected and checked by phenol–$H_2SO_4$ acid method.

### 3.6 Effect of F1 and F2 on cell viability and NO production in LPS-stimulated RAW 2647 cells.

According to the cytotoxic and NO inhibitory data of the four polysaccharide fractions, study was only continued with CCP. Two polysaccharide fractions isolated from CCP via DEAE column chromatography were used to evaluate *in vitro* anti-inflammatory activities of LPS-stimulated RAW 264.7 cells. Cell viability experiment confirmed that the both F1 & F2 have no cytotoxic effect on RAW 264.7 cells at tested concentrations (12.5 ~ 50.0 μg/ml) (Fig. 8A). Furthermore, F2 dose dependently inhibited the NO production in LPS-stimulated RAW 264.7 cells ($IC_{50}$ = 48.03 μg/ml), and F2 not exhibited considerable NO inhibitory activity under same experiment conditions (Fig. 8B). Therefore, F2 was selected to further study.

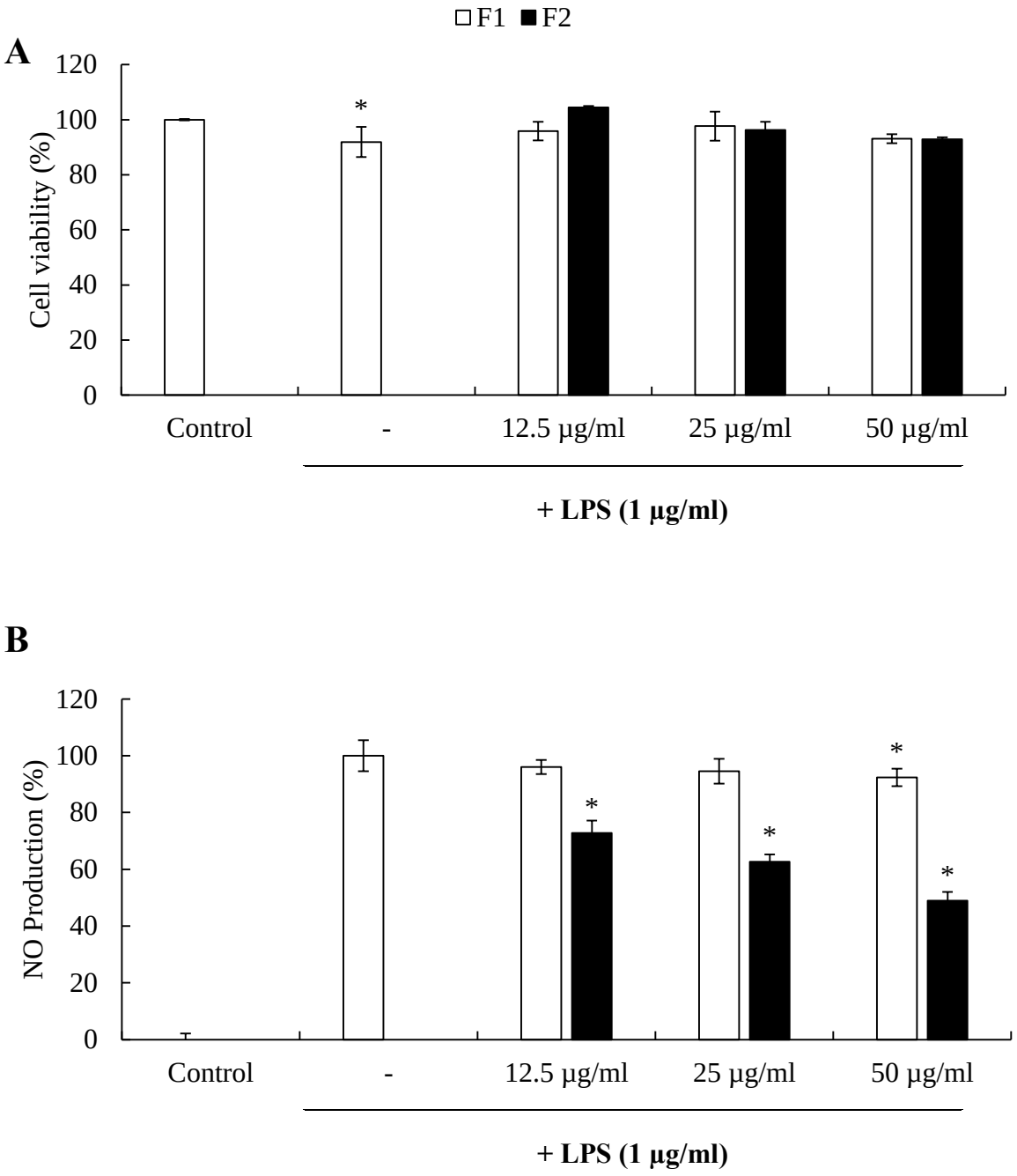


**Figure 8**. (A) Cytotoxicity of F1 & F2 on RAW 264.7 cells in the presence of LPS. (B) Dose-dependent inhibition of NO production by F1 in LPS-stimulated RAW264.7 macrophages. *$P < 0.05$.

### 3.7 Effects of F2 on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells.

LPS is a bacterial lipopolysaccharide which has an ability to induce inflammatory responses in macrophage cells [44]. Exposure of macrophage cells into the LPS stimulate the production of inflammatory genes from exposed cells including iNOS and COX-2 [45]. Furthermore, expression of iNOS activates the NO production and expression of COX-2 triggers the production of pro-inflammatory $PGE_2$ [46]. In addition, It is well-established fact that the sulfated polysaccharides located in brown seaweed cell walls are possesses promising bioactive properties including anti-inflammatory activities [47]. Kang et al (2011), isolate sulfated polysaccharide containing fucose from *Eckonia cava* to test anti-inflammatory activities *in vitro* on LPS-stimulated RAW 264.7 [48]. According to their observations algal fucoidan inhibited NO production in LPS-stimulated RAW 264.7 at dose dependent manner by down-regulating iNOS and COX-2 protein expression as well as inhibiting production levels of pro-inflammatory cytokines ($PGE_2$). Therefore, compounds having a suppressive effect on inflammatory protein expression might have a potential to develop as therapeutic drugs for inflammatory diseases [45]. According to the western blot analysis it was clearly observed that the LPS stimulation (1 μg/ml) of RAW 264.7 cells dramatically induce the iNOS and COX-2 protein expression levels compared to the control (Fig.9). However, pre-incubation of RAW 264.7 cells with F2 significantly down-regulated the elevated levels of iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells.

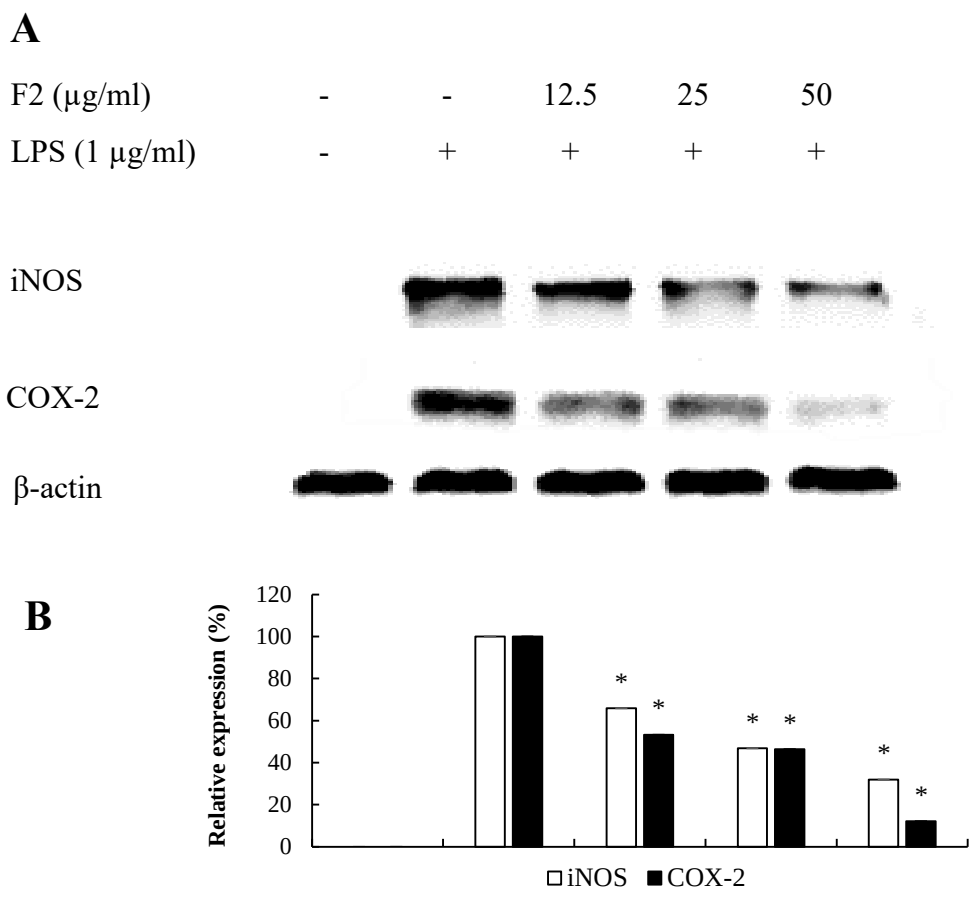


**Figure 9.** (A) Effects of F2 on LPS-stimulated iNOS and COX-2 protein expression in RAW 264.7 cells. Cells ($1 \times 10^5$ cells/ml) were treated with indicated concentrations of F2 (12.5, 25, or 50 μg/ml) for 1 h before LPS (1 μg/ml) treatment for 24 h. Cell lysates (40 μg) were resolved by 10% SDS–PAGE, transferred to nitrocellulose membranes, and probed with antibodies against iNOS and COX-2. The proteins were then visualized by ECL. The intensity of the bands was measured by ImageJ software. Relative amounts of iNOS and COX-2 (B) compared to β-actin. The gel shown is a representative of the results from three separate experiments. *$P < 0.05$.

### 3.8 Inhibitory effect of F2 on $PGE_2$ secretion in LPS-stimulated RAW 264.7 cells

During inflammatory responses, the expression of iNOS and COX2 in macrophages leads to the production of $PGE_2$ [49, 50]. $PGE_2$ produced during inflammation cytotoxic to the host cells, as well as induce pro-inflammatory activities [50]. Furthermore, studies have proved that the prolonged production of $PGE_2$ from inflammated cells as an inflammatory response can cause pathogenesis of inflammatory diseases as well as cancers [50-52]. In the present experiment, authors observed incubation of F2 with LPS-stimulated RAW 264.7 cells dose-dependently reduced the $PGE_2$ secretion (Fig. 10).

### 3.9 Effect of F2 on secretion of pro-inflammatory cytokines from LPS-stimulated RAW 264.7 cells.1

It is a well-established fact that the over expression of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, have an important role in the pathogenesis of various inflammatory diseases [53-55]. Recently, LI & Ye (2015) reported that the pre-treatment of fucoidan has potential to down-regulate expression of pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, CXCL-10, VCAM-1, and ICAM-1 in reperfusion injury-induced inflammation on Sprague–Dawley rat model in a dose-dependent manner [56]. To evaluate anti-inflammatory effects of F2 in LPS-stimulated RAW 264.7 cells, the levels of inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, in culture supernatants were measured by ELISA. The results clearly demonstrated that the secretion of pro-inflammatory cytokines were sufficiently suppressed by F2 pretreatment compared to the LPS-stimulated RAW 264.7 cells in a dose-dependent manner (Fig. 10).

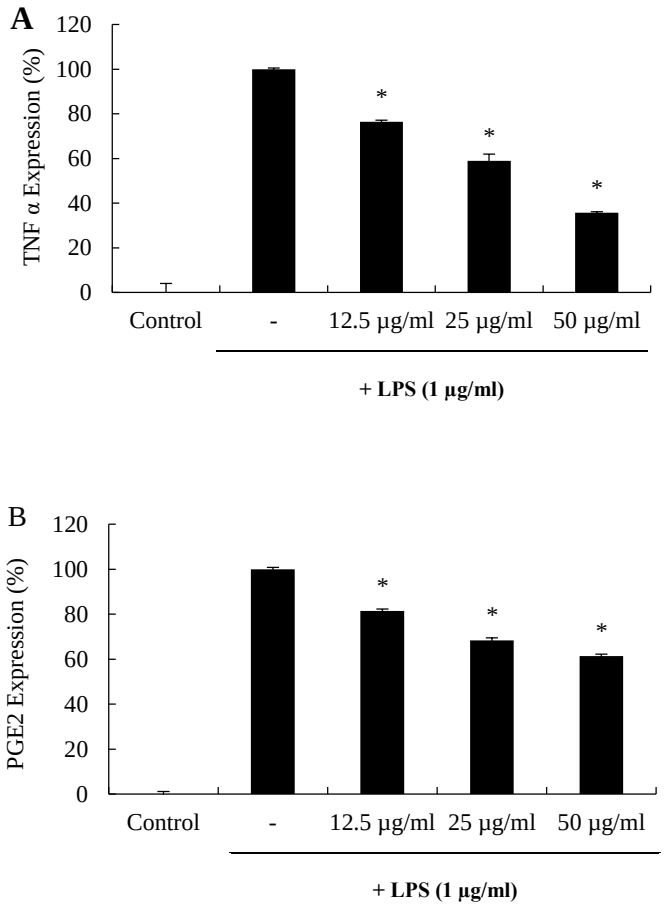


**Figure 10.** The inhibitory effect of F2 on the production of pro-inflammatory cytokines and $PGE_2$ in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells ($1 \times 10^5$) were stimulated with LPS (1 μg/ml) for 24 h with or without CCP. Supernatants were collected, and TNF-α (A levels in the culture supernatant were determined by ELISA according to the manufacturer's instructions. Data points and bars represent the arithmetic means ± SD (n = 3). Means with same letters are not significantly different ($P > 0.05$).

### 3.10 Effects of F2 on the NFκB and MAPK signaling pathway in LPS-stimulated RAW 264.7 cells

When macrophage cells exposed to the inflammatory stimulus such as LPS, macrophage cells start inflammatory responses via activating the NFκB and MAPK signaling cascades [8, 19]. Generally, when cells exposed to the inflammatory stimulus the cytosolic NFκB proteins are moved into the nucleus and start transcription of pro-inflammatory genes, including iNOS and COX-2, as well as the genes encoding pro-inflammatory cytokines [8, 57]. In addition, activation of MAPK signaling cascade also regulates the inflammatory responses in LPS-stimulated cells by regulating the induction of iNOS gene expression, inducing PGE2 production, as well as the transcription of pro-inflammatory cytokines [58],[59],[60]. Therefore, inhibition of NFκB and MAPK signaling pathways are considered as a useful approach to treat inflammation associated diseases [57]. In addition, a number of studies have suggested fucose-rich sulfated polysaccharides and/or fucoidan isolated from brown seaweeds exert promising anti-inflammatory properties by down-regulating NFκB and MAPK signaling cascades [58],[8],[61]. Taken together, to determine the effect of F2 on NFκB and MAPK signaling cascades, authors evaluate the activation levels of NFκB and MAPK related protein expression by means of western blot analysis.

The effects of F2 on the activation of p50 and p65 were examined to confirm inhibition of the expression of pro-inflammatory genes and cytokines. The results demonstrated that the pre-treatment of F2 (12.5 μg/ml, 25 μg/ml, 50 μg/ml) has great potential to inhibit LPS-induced activation of p50 and p65 (Fig. 11). According to the results, it is clear that the incubation of F2 down regulate the phosphorylation levels of p50 and p65 in the cytosol, as well as F2 inhibit translocation of p50 and p65 to the nucleus. Therefore, we can conclude that F2 has a potential to regulate inflammatory

responses via blocking NFκB signaling pathway.

To determine whether F2 has an effect on the MAPK signaling pathway, we examined the activation levels of MAPK pathway components after 30 minute LPS stimulation of RAW 264.7 cells. According to the results, LPS stimulation led to activation of p38 and ERK, significantly compared to the control. However, up-regulated levels of p38 and ERK were sufficiently down-regulated F2 treatment (Fig. 11). Therefore, we suggest that F2, inhibits inflammation, including the production of NO and PGE2, by decreasing the expression levels of inflammatory mediators and pro-inflammatory cytokines via inhibition of the NFκB and MAPK signaling pathway.

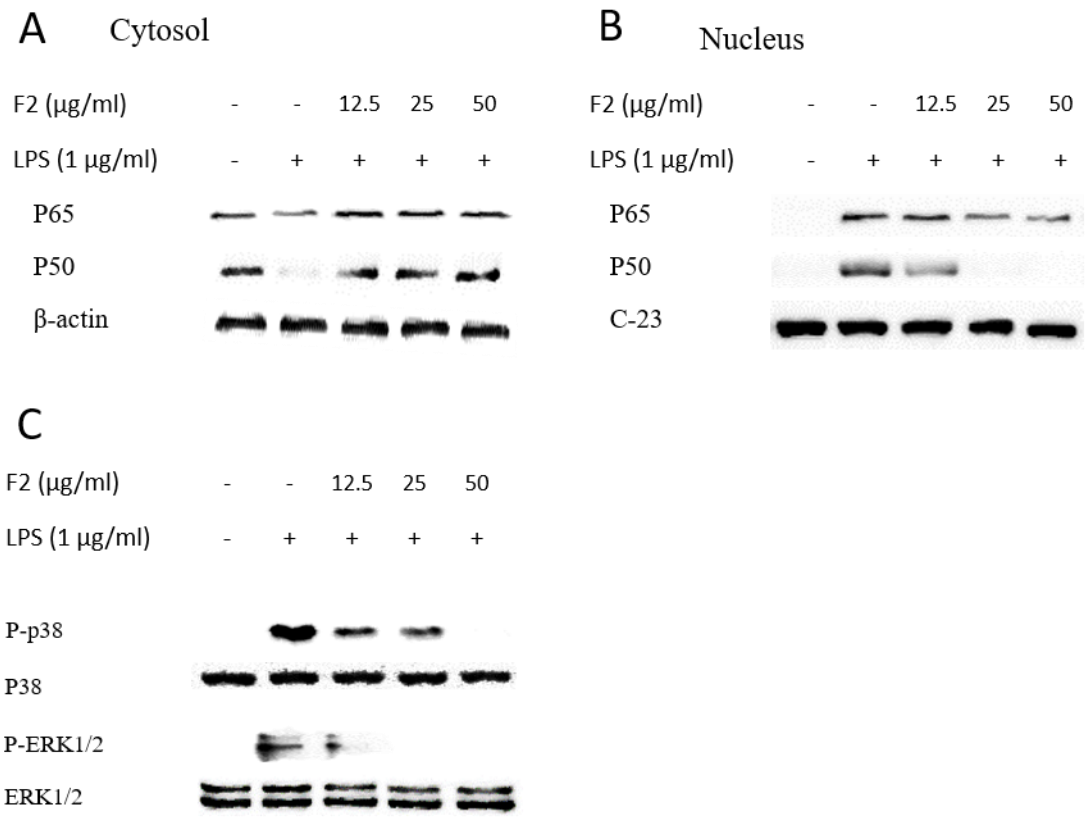


**Figure 11.** Inhibitory effects of F2 on NFκB and MAPK activation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/ml) or F2 (12.5 μg/ml, 25 μg/ml, 50 μg/ml) for 30 min. Cell lysates (A) and nucleus (B) lysates(40 μg) were resolved by 10% SDS–PAGE, transferred to nitrocellulose membranes, and probed with antibodies against NFκB p50 and p65. The proteins were then visualized by ECL (C). Effects of F2 on MAPK activation induced by LPS in RAW 264.7 cells. RAW 264.7 cells were treated with LPS (1 μg/ml) or F2 (12.5 μg/ml, 25 μg/ml, 50 μg/ml) for 30 min. Total protein (40 μg) was separated by 10% SDS–PAGE, transferred to nitrocellulose membranes, and probed with antibodies against ERK and p38. The proteins were then visualized by ECL. The results shown are representative of those obtained from three independent experiments.

### 3.11 *In Vivo* toxicity of LPS and F2 in zebrafish model

Direct use of *in vitro* results has several drawbacks due to their physiological and economic relevance in *in vivo* situations [25]. However, use of animal model or human models are also have limitations due to limited amounts of pure compounds, high cost, laborious routine work, ethical law enforcements for animal models [25]. Within recent decades the zebrafish has become a popular *in vivo* model due to its similar organ systems and gene sequences to humans [62],[28]. Taken together in the present study we used zebrafish as *in vivo* animal model to validate *in vitro* anti-inflammatory results obtained from F2. To determine the toxicity of F2 on zebrafish embryo authors evaluate the survival rates of zebrafish embryos. The zebrafish incubated with F2 (12.5 ~ 50 μg/ml) were not expressed any toxic effect on zebrafish embryo (Data not shown). Therefore, as next part of study the survival rate of embryo after treatment with 10 μg/mL LPS and various concentrations of F2 was measured for 5dpf. The survival rate of LPS alone incubation was dramatically decreased the survival rate up to ~ 80% compared to the control. However, co-treatment of LPS with F2 (12.5 ~ 50 μg/ml) significantly increased the survival rate of zebrafish embryos (Fig. 12A).

### 3.12 Effects of F2 on LPS-exposed heart-beating rate of zebrafish model

In the zebrafish model increased levels of heart beating rate, tail bending and small head size are the some parameters to evaluate toxic effect of testing compounds [25]. LPS alone treatment significantly affected the heart beating rate of zebrafish larvae compared to the control group (Fig. 12B). However, the treated concentration of F2 (12.5 ~ 50 μg/ml) in the LPS treated zebrafish significantly decreased the elevated heart beating rates. Previously, Lee et al, (2013) documented that the treatment of fucoidan (100 μg/ml) separated from *Ecklonia cava* has potential to reduce elevated heart beating rates in LPS exposed zebrafish embryo [10]. In addition, Kim at al, (2014) also reported that the fucoidan (100 ~ 200 μg/ml) isolated brown alga *Ecklonia cava* has potential to increase survival rates and reduce elevated heart beating rates in zebrafish model induced by AAPH [63]. Our results also demonstrated that the survival rates and heart beating rates of F2 treated zebrafish were similar to that of non-treated group even the embryos exposed to the LPS. Thus, these finding suggests that the F2 has a potential to protect zebrafish embryo against the toxic effect induced by LPS.

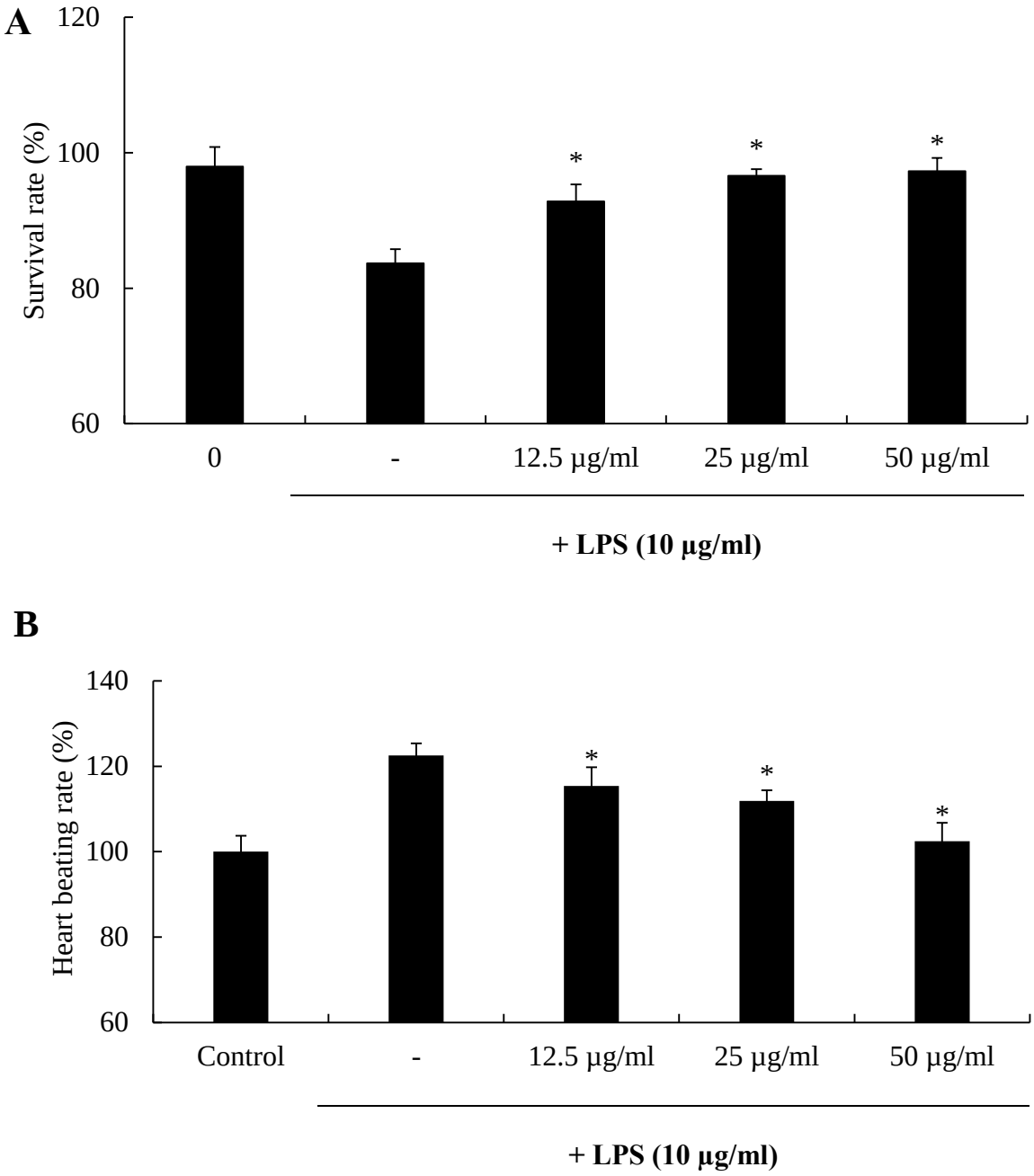


**Figure 12.** Measurement of LPS toxicity and LPS-co treated with F2 on survival and heart beating rates. (A) Survival rate and (B) heart beating rate. Experiments were performed in triplicate, and the data are expressed as mean ± SD, *$p < 0.01$.

### 3.13 Protective effect of F2 against LPS-induced cell death in *in vivo* zebrafish model.

Acridine orange is a nucleic acid selective methachromatic dye which can stains cells with disturbed plasma membrane permeability. Therefore, this dye can be used to identify necrotic or apoptotic cells [64]. In addition number of studies have reported that the exposure of zebrafish embryo to LPS has potential to increase cell death in zebrafish embryo due to the excessive production of pro-inflammatory cytokines and other molecules in LPS exposed embryos [27, 64]. Therefore, in the present study authors attempted to investigate the protective effect of F2 on LPS-exposed cell death in zebrafish larvae. In contrast, LPS-treatment (10 μg/ml) significantly increased the acridine orange intensity (Fig. 13A). However, present study demonstrated that the pre-incubation of F2 has a potential to reduce toxicity on zebrafish larvae induced by the LPS exposure in dose-dependent manner.

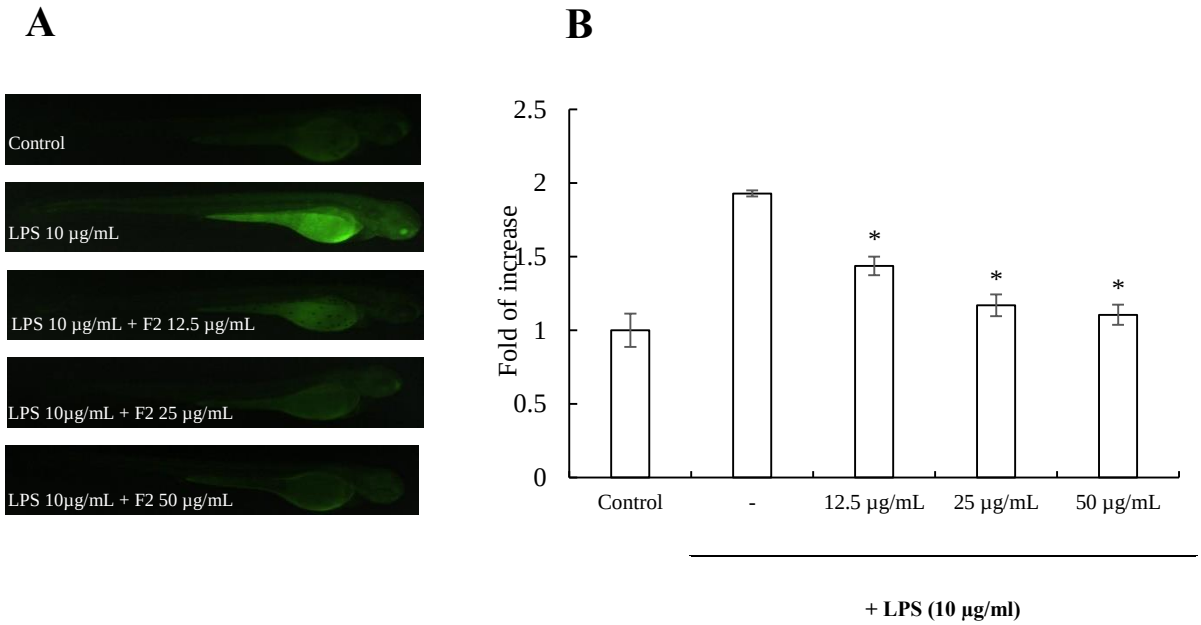


**Figure 13**. Effect of F2 on cell death in zebrafish embryo. (A) The cell death levels were measured after acridine orange staining by image analysis and fluorescence microscope. (B) The cell death embryos were quantified using an image J program. Experiments were performed in triplicate, and the data are expressed as mean ± SD, *$p < 0.01$.

### 3.14 protective effect of F2 on LPS –induced ROS production in *in vivo* zebrafish model.

DCF-DA is an oxidant-sensitive fluorescent probe which can use to measure levels of ROS in its surrounding environment. In general, the reaction of DCF-DA with intercellular ROS lead to convert DCF-DA into its oxidized form known as DCF. Furthermore, DCF emits fluorescent which can visualize under dark conditions [65]. Figure 14A shows the *in vivo* ROS production in LPS exposed zebrafish embryo and inhibitory effect of F2 against ROS generation in LPS-exposed zebrafish embryo. According to the exposure of LPS to the zebrafish embryo has significantly increased the levels of ROS production in zebrafish embryo. However, in this experiment authors observed pre-treatment of F2 before LPS exposure has potential to reduce elevated ROS levels in dose-dependent manner.

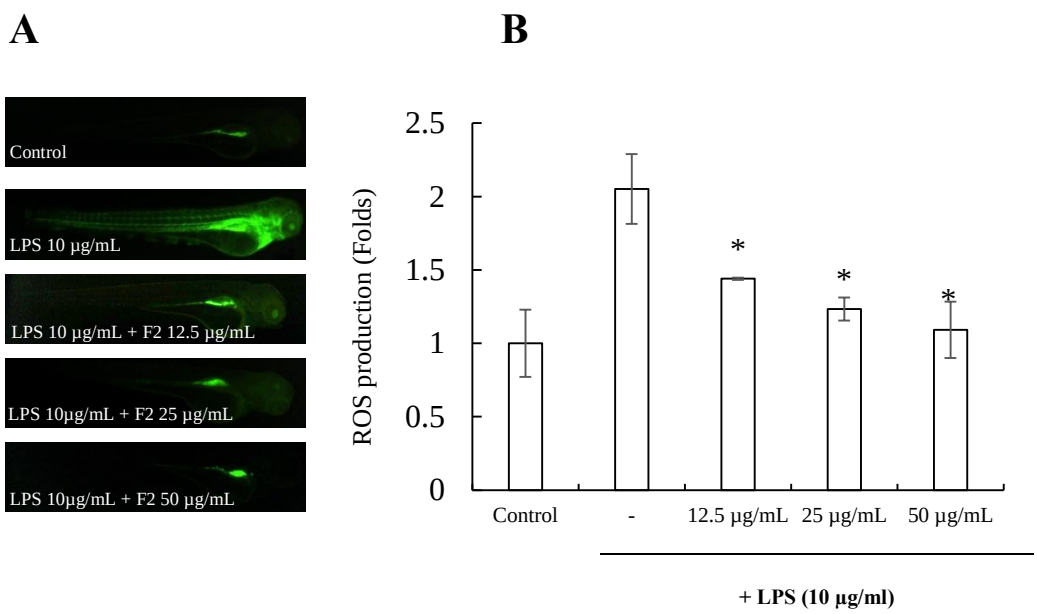


**Figure 14.** Protective effect of F2 against LPS-induced ROS generation in zebrafish embryo. (A) The cell death levels were measured after acridine orange staining by image analysis and fluorescence microscope. (B) The cell death was quantified using an image J program. Experiments were performed in triplicate, and the data are expressed as mean ± SD, *$p < 0.01$.

### 3.15 protective effect of F2 on LPS –induced NO production in *in vivo* zebrafish model.

The DAF-FM DA is a membrane-permeating dye which transformed by intracellular esterases into the highly water-soluble dye DAF-FM. furthermore, DAF-FM traps NO produced by NO synthase (NOS) to yield a highly fluorescent triazole compound in cells. Therefore, monitoring with a fluorescence microscope allows identifying intracellular NO production and location of NO[66]. The inhibitory effect of F2 on NO production in the LPS exposed zebrafish larvae are indicated in Fig. 15A. According to the image analysis level of NO production in LPS exposed cells have been dramatically increased, but the pre-treatment of F2 dose-dependently (12.5 ~ 50 μg/ml) decreased the elevated levels of intercellular NO in zebrafish Larvae.

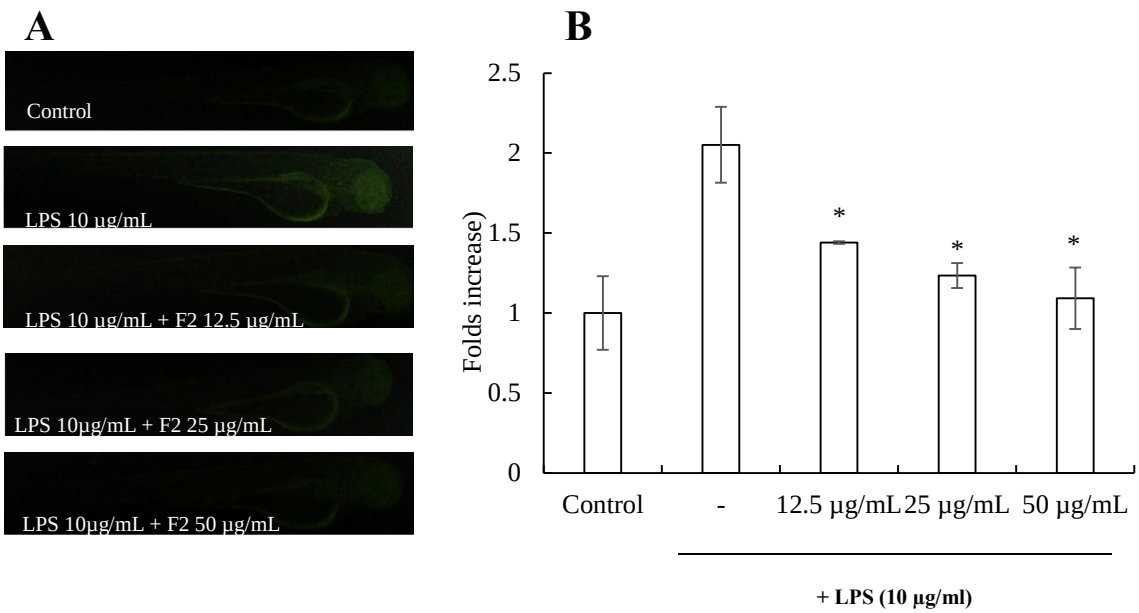


**Figure 15.** Inhibitory effect of F2 against LPS-induced NO production in zebrafish larvae. ) The NO levels were measured by image analysis and fluorescence microscope. (B) Individual zebrafish fluorescence intensity was quantified using an image J program. Experiments were performed in triplicate, and the data are expressed as mean ± SD, *$p < 0.01$.

### 3.16 FT-IR spectrum of F2 fraction and its monosaccharide composition

Contemporary, Fourier-transform spectroscopy technique uses to investigate the vibrations of molecules and polar bonds between the atoms. Moreover, FT-IR spectrum is a useful tool analyze structures of the polysaccharides, including functional groups, glucosidic bonds and monosaccharaides [67]. In general, bands around 970 – 1200 $cm^{-1}$ are developed in FT-IR spectrum due to the activities of polysaccharides. Furthermore, bands in this region represent the C−C and C−O stretching vibrations in the pyranoid ring and C−O−C stretching of the glycosidic bonds [68]. The strong absorption band at the region around 1240 – 1255 $cm^{-1}$ represent the S=O starching of sulfated ester polysaccharides. The shoulder absorbance at 840 $cm^{-1}$ together with sharp absorbance at 820 $cm^{-1}$ responsible for the C−S−O substitution, primarily at C-4 position (axial C-4 substitution of α-linked L-fucopyranose) [69]. Specifically characteristic bands in 1240 – 1255 $cm^{-1}$ and 820-840 $cm^{-1}$ are unique feature of natural fucoidans can use to identify natural fucoidan [70]. In addition, major absorbance at 3000 to 3500 $cm^{-1}$ develop due to O−H stretching and the absorbance at 1641 $cm^{-1}$ represent the carboxyl groups of Uronic acid [71]. When consider about FT-IR spectrum of F2 fraction (Fig. 6) we also observed band patterns at 820, 840, 1165, 1255, 1641 and 3422 $cm^{-1}$ which are represent the specific features of fucoidan.

Composition of F2 fraction is summarized in table 5. Without proteins detection of sulfate is indicative of the sulfated polysaccharide [72]. Moreover, neutral sugar analysis confirmed that fucose (49%) and galactose (31%) are the major neutral sugar in F2 fraction. Previous studies reported that natural fucoidan contains around 45-60% fucose and around 30-40 % galactose [9, 10, 72]. Our neutral sugar composition of F2 fraction also similar to the previously separated brown algal fucoidan. Collectively neutral sugar analysis and FT-IR spectrums of F2 fractions are similar to the previously reported fucoidans. Thus, active compound in F2 fraction might be fucoidan

**Table 5.** Neutral sugars composition of the F2 fraction and sulfate content

| Neutral sugars (mol% of all neutral sugars) | | | | | |
|---|---|---|---|---|---|
| Fucose | Galctose | Mannose | Xylose | Rhamanose | Sulfate |
| 48.69 | 31.13 | 8.72 | 7.27 | 4.19 | 16.85 |

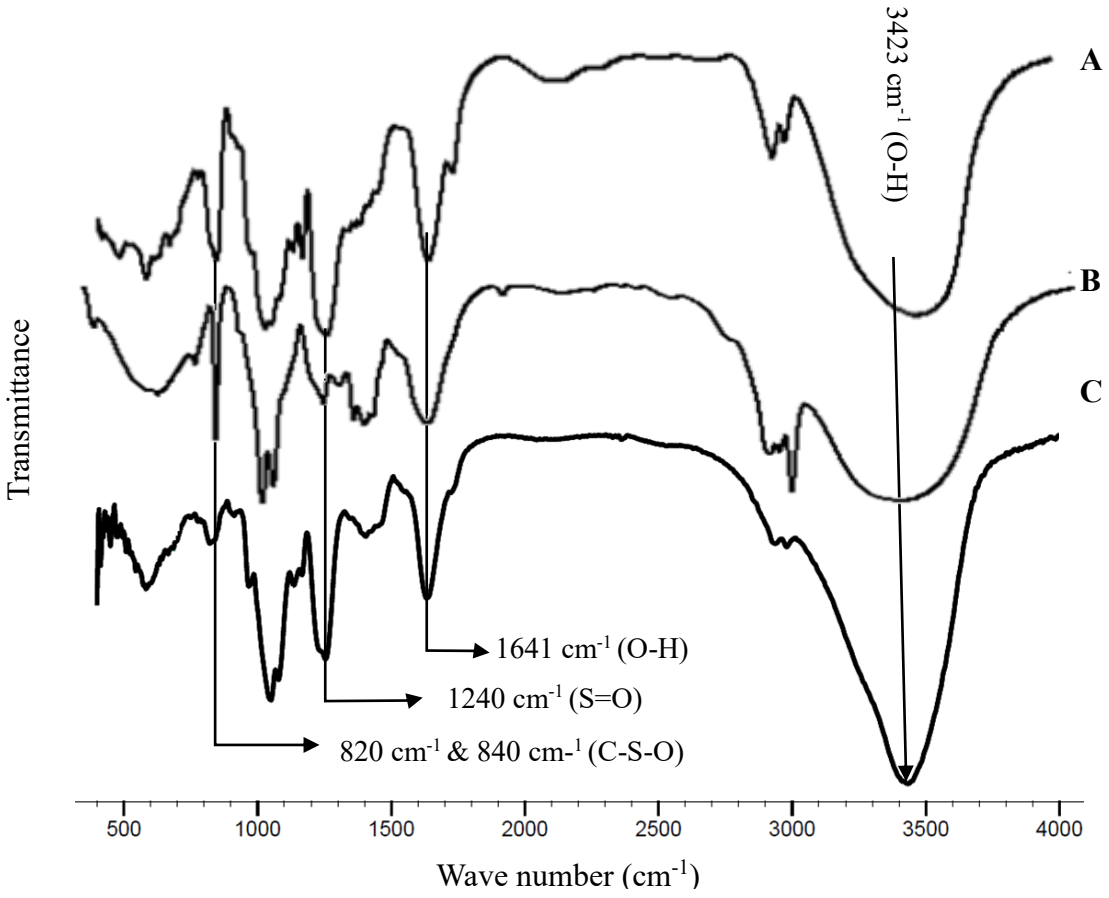


**Figure 16. (A)** Infrared analysis spectroscopy (FT-IR) of commercial fucoidan. (B) Infrared analysis spectroscopy (FT-IR) of CCP. (C) Infrared analysis spectroscopy (FT-IR) of F2 fraction obtained from *Sargassum horneri* via celluclast enzymatic extraction followed by ethanol precipitation and FPLC.

# 4. Conclusion

In conclusion, we have demonstrated fucoidan isolated from *S. horneri* via enzyme assistance extraction followed by FPLC chromatography has a great deal of potential to inhibit production of pro-inflammatory cytokines, NO and PGE2 by suppressing NF-κB and MAPK signaling cascades in *in vitro* on LPS-stimulated RAW 264.7 cells. Moreover, we have demonstrated *S. horneri* fucoidan has potential to reduce NO and ROS production dose-dependently in LPS-exposed zebrafish embryo without expressing any cytotoxic effect. Taken together, fucoidan isolate from *S.horneri* might have a potential to develop and/or incorporate as a function food, nutraceuticals or as a cosmeceutical due to its promising anti-inflammatory effects.

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