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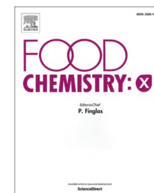

Determining the potent immunostimulation potential arising from the heteropolysaccharide structure of a novel fucoidan, derived from *Sargassum Zhangii*

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A R T I C L E I N F O

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A B S T R A C T

A preliminary study was conducted of the chemical, structural properties and immunomodulatory activities of fucoidan isolated from *Sargassum Zhangii* (SZ). *Sargassum Zhangii* fucoidan (SZF) was determined to have a sulfate content of 19.74 ± 0.01% (w/w) and an average molecular weight of 111.28 kDa. SZF possessed a backbone structure of (1,4)-α-D-linked-galactose, (3,4)-α-L-fucose, (1,3)-α-D-linked-xylose, β-D-linked-mannose and a terminal (1,4)-α-D-linked-glucose. The main monosaccharide composition was determined as (w/w) 36.10% galactose, 20.13% fucose, 8.86% xylose, 7.36% glucose, 5.62% mannose, and 18.07% uronic acids, respectively. An immunostimulatory assay showed that SZF, compared to commercial fucoidans (*Undaria pitnnaifida* and *Fucus vesiculosus* sources), significantly elevated nitric oxide production *via* up-regulation of cyclooxygenase-2 and inducible nitric oxide synthase at both gene and protein levels. These results suggest that SZ has the potential to be a source of fucoidan with enhanced properties that may act as a useful ingredient for functional foods, nutritional supplements, and immune enhancers.

Introduction

Marine algae (or, as they are more colloquially known, seaweeds) are found in oceans all over the world. There are approximately 30,000 identified types of algae in the ocean globally, which are usually divided into red, green, and brown seaweeds (Gomez-Zavaglia, Prieto Lage, Jimenez-Lopez, Mejuto, & Simal-Gandara, 2019). Seaweeds have the potential to be an outstanding target for blue farming, as they are low in fat, and contain abundant functional phytochemicals, such as polysaccharides, proteins, polyphenols, vitamins, and minerals (Yao, Qiu, Cheong, & Zhong, 2022). Marine algae are enriched with a wide range of complex polysaccharides, with a content ranging from 4% to 76% of dry weight (Tian et al., 2020).

A number of these polysaccharides have been identified as being of interest as high-value products. Of these, a notable type is those found in brown seaweeds, known as fucoidans. These are a class of highly sulfated polysaccharides residing chiefly in the intercellular spaces and fibrillar cell walls of brown algae (Cheong, Yu, Chen, & Zhong, 2022).

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Fucoidans are promising targets for compounds of value in the functional food and nutraceutical industries. In fact, fucoidans have been approved to be produced as food in China in 2019. Biologically, they have been found to have many beneficial effects such as antiviral, anti-inflammatory, anticancer, antithrombosis (Wang et al., 2017), and immunomodulatory activities with low side effects (Zha et al., 2018).

Increasing the activity of the immune system is a significant biological function of fucoidans through either improved release of pro-inflammatory mediators or decreased anti-inflammatory response (Bahramzadeh, Tabarsa, You, Li, & Bita, 2019). The multifunctionality of fucoidan is highly variable, and greatly dependent upon its complex molecular structure. Therefore, it is commonly believed that the magnitude of immunomodulatory activities of fucoidans is dominated by compositional characteristics such as monosaccharide composition, molecular weight, and sulfate content (Borazjani, Tabarsa, You, & Rezaei, 2018). Structurally, fucoidans are highly branched hetero-polysaccharides, and these structures vary within species, season, harvest location and maturity of the plant (Fletcher, Biller, Ross, & Adams, 2017). Therefore, considering the complexity of fucoidan structures and their multiple functional abilities, significant potential exists to explore new seaweed sources in order to acquire natural polysaccharides with improved health benefits and biological activities.

Sargassum species are present throughout subtropical and tropical areas globally and have been shown to produce many structural classes of metabolites such as sulfated polysaccharides, glycerides, polyphenols, and terpenoids, *etc.*, which possess many therapeutic activities (Yende, Harle, & Chaugule, 2014). Therefore, *Sargassum* species have great potential in the neutralceutical field, and have been identified as a key medicinal food of the twenty-first century. *Sargassum Zhangii* fucoidan (SZF) is an relatively unexplored compound; although it has been reported to have cholesterol-lowering activity (Lin, Chen, & Zhong, 2022), little research has been conducted into its immunomodulatory activities.

Motivated by this, the objective in this study was to determine the immune cell stimulating activities of SZF, and characterize its physiochemical and structural properties through a number of approaches including ion chromatograph, high performance gel-permeation chromatography, Fourier transformation infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR), *etc.* The immunoregulatory activities of the fucoidans on RAW 264.7 macrophage cells were determined by the production of nitric oxide (NO), a key component of the immunomodulatory pathway and tissue regeneration. Furthermore, the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) immune cytokines, were also determined based on protein abundance and gene expression. The results show that SZF exhibit previously unreported immune stimulating activities. These results, taken together, identify SZF as an outstanding candidate for further development in the functional or nutraceutical food fields.

Materials and methods

Chemical suppliers

Fucoidan Fuc1 (*Undaria pinnatifida* source, batch# 572001015) was obtained from Qingdao bright moon seaweed group co., LTD. (Qingdao, China). Samples Fuc2 (*Undaria pinnatifida* source, Lot# SLCK7680) and Fuc3 (*Fucus Vesiculosis* source, Lot# SLCJ3576) were purchased from Sigma-Aldrich (MO, USA). Murine RAW 264.7 cells were obtained from Biofavor Biotech (Wuhan, China). High glucose Dulbecco's modified Eagle's medium (DMEM) (with 4500 mg/L D-glucose, 3700 mg/L sodium bicarbonate, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate and 15 mg/L phenol red), Fetal bovine serum (FBS), streptomycin and penicillin Trizol reagent were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Cytoplasmic and nuclear protein extraction kit, and nitric oxide (NO) detecting kit were obtained from Beyotime (Haimen, China). cDNA Synthesis SuperMix TransScript® and One-Step gDNA Removal were purchased from Transgen Biotechnology Co. (Beijing, China). Fluorescence real-time quantitative PCR premix (SYBR) and total RNA isolation kit were purchased from Tiangen Biotechnology Co. (Beijing, China). GAPDH, iNOS, COX-2 antibodies and HRP-linked anti-rabbit IgG (secondary antibody) were purchased from Abcam (Shanghai, China). The monosaccharide standards were obtained from BoRui Saccharide Biotech Co. Ltd (Yangzhou, China). Other chemical reagents were of analytical grade.

Collection of seaweeds

Five batches of SZ, with each 30 kg, were collected from the coastal area of Leizhou, Zhanjiang, Guangdong, China in October 2020, and were in the breeding period identified immediately after picking by Professor En-Yi Xie of Guangdong Ocean University. The collected SZ was washed with sea water and fresh water, and then was sunshine dried to a constant weight. The dried samples were ground using a food grinder and sieved using a 100-mesh sieve, then put into a sealed bag and deposited at −20 °C at the College of Food Science and Technology, Guangdong Ocean University, Zhanjiang, China.

Extraction of SZF

The SZF powder was extracted *via* the method described in our previous work (Li et al., 2022). The SZ powder (20.0 g) was suspended in 600 mL distilled water, and the pH of the solution was adjusted to 6.0. The crude polysaccharide was extracted under magnetic stirring at 650 r/min, 80 °C for 3.5 h, then extracted using ultrasonication at room temperature for 50 min at a working power of 350 W, and the crude polysaccharide solution was obtained.

Purification of SZF

The SZF solution obtained in Section 2.3 was centrifuged for 10 min at 4000 r/min, and the precipitation was disposed. A rotary evaporator (EYELA, 100 N-1300 V-WB, Tokyo, Japan) was used to concentrate the supernatant to 33% of its original volume. 30% (v/v) of anhydrous ethanol based on the volume of the concentrated supernatant was added, then centrifuged for 10 min at 4000 r/min. 80% (v/v) of anhydrous ethanol based on the volume of the supernatant was added to the supernatant. Anhydrous ethanol and acetone were used to wash the precipitation, respectively, then the precipitation was dissolved in 30 mL distilled water. Next, the protein was removed by the addition of savage reagent in the ratio of 6:1 (v/v), and oscillated for 20 min using a vortex oscillator. A dialysis bag (15000 kDa) was used to dialyze the samples for 24 h at 4 °C, and distilled water was replaced 3 times during this process. Then SZF was obtained after freeze-drying of the extracted sample. The yield rate and extraction rate were calculated using the following equations, respectively.

$$\text{Yield rate}(\%) = \frac{\text{Weight of SZF}}{\text{Weight of seaweed powder}} \times 100\% \quad (1)$$

$$\text{Extraction rate}(\%) = \frac{\text{Weight of SZF}}{\text{Weight of total sugar content of seaweed powder}} \times 100\% \quad (2)$$

Chemical analysis

The total sugar content was determined using the phenol–sulfuric acid method at 490 nm, and D-fucose was used as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The content of sulfate was tested using the barium chloride gelatin method at 360 nm, and potassium sulfate was used as the standard (Dodgson & Price, 1962). The protein content was analyzed using the Coomassie bright blue at 595 nm, with bovine serum albumin as the standard (Kawai, Seno, & Anno, 1969). The total phenolic content of the sample was estimated by the Folin-Ciocalteu reagent method at 760 nm, with gallic acid as the standard, and the results are expressed in milligrams of gallic acid equivalent (mg GAE) per gram of SZF (Singleton, Orthofer, & Lamuela-Raventós, 1999).

Monosaccharide analysis

Monosaccharide components were tested using ion chromatograph (ThermoFisher, America) according to the approach we used previously (Li et al., 2022). Samples were hydrolyzed thoroughly using trifluoroacetic acid (TFA, 3 mol/L) at 120 °C for 3 h, and then dried by nitrogen blowing. The residue obtained was fully dissolved in 5 mL of pure water through vortexing, and diluted to 20 times the volume of the sample, then centrifuged at 12000 rpm for 5 min. Next, the supernatant was washed out at 30 °C with a Dionex Carbopac TMPA20 column (3.0 mm × 150 mm) at an elution speed of 0.3 mL/min. A volume of 5 μL of the sample was injected, and the eluent was composed of A, H_2O; B, 15 mM NaOH; C, 15 mM NaOH and 100 mM NaOAC. The samples were detected using an electrochemical detector. Standard monosaccharide mixture containing fucose; galactose hydrochloride, rhamnose, arabinose, glucosamine hydrochloride galactose; glucose, *N*-acetyl-D-glucosamine, xylose, mannose, fructose, ribose, galactose acid, guluronic acid, glucuronic acid, and mannuronic acid. The amount of each monosaccharide was calculated based on peak aera of each standard.

Molecular property determination

Molecular property of SZF was analyzed by high-performance gel-permeation chromatography (Shimadzu, Australia). A BRT105-104-102 tan-dem gel column (7.8 mm × 300 mm i.d.) (Borui Saccharide, Biotech. Co. Ltd.) equipped with a refractive index detector (RI-10A) was used to determine the molecular property of SZF. Briefly, the samples and standards were prepared at 5 mg/mL concentration, and centrifuged for 10 min at 12,000 rpm. The supernatant was filtered using a 0.22 μm Millipore membrane, then 20 μL of sample was transferred to a 1.8 mL vial. Dextran with different relative Mw (5000, 11,600, 23,800, 48,600, 80,900, 148,000, 273,000, 409,800, 667,800 kDa) was used as standards.

FTIR analysis

The featured groups of SZF were analyzed using an FTIR spectrophotometer (BRUKER TENSOR-2, BRUKER, Germany). The sample was ground with pure potassium bromide (KBr, 1:100) and pressed into the disc under vacuum. The scanning range was from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} with 32 scans, and the wavenumber ranges used for analysis in this study was 4000–500 cm^{-1}.

NMR analysis

^1H and ^{13}C NMR analysis was conducted using an advance spectrometer (600 MHz Bruker model). 10 mg of the fucoidan was dissolved in 0.25 mL of D_2O (Deuterium oxide), and 0.5 mL was transferred to a ^1H and ^{13}C NMR tube, respectively. NMR spectra was recorded at 25 °C, and the chemical shift was expressed in parts per million (ppm).

Table 1
Yields and chemical analysis of *Sargassum Zhangii* fucoidan (SZF).

	Composition	Content
Yields	Yield rate	2.85 ± 0.35 (%, w/w)
	Extraction rate	10.06 ± 0.56 (%, w/w)
Chemical composition	Total sugar	82.77 ± 0.40 (%, w/w)
	Sulfate	29.74 ± 0.01 (%, w/w)
	Total protein	1.92 ± 0.38 (%, w/w)
	Total polyphenol	1.40 ± 0.12 (mgGAE/g SZF)

Cell culture and determination of nitric oxide production

RAW 264.7 macrophages were cultivated in DMEM complemented with 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% FBS, and kept in a 37 °C humidified incubator with 5% CO_2. The experiment was performed using Griess assay. 100 μL of RAW 264.7 cells (1×10^5 cells/well) were inoculated into 96-well plates, then treated with 100 μL of fucoidan samples at 25, 50, and 100 μg/mL. The supernatants were collected to detect NO contents after 24 h incubation.

RT-qPCR analysis

RAW 264.7 macrophages (1×10^6 cells/mL) were cultured with SZF at concentrations of 25, 50 and 100 μg/mL, respectively. After 24 h incubation, a Trizol assay kit was applied on the conditioned cells to extract total RNA. Briefly, 1 μg of RNA was determined using qRT-PCR, and PCR amplification was carried out *via* incorporation of SYBR green (Roche). The amplification primer sequences GAPDH, iNOS, and COX-2 involved in this experiment were purchased from Sangon Biotech (Shanghai), GAPDH: Forward-GGTGAAGGTCGGTGTGAACG, Reverse-CTCG-CTCCTGGAAGATGGTG; iNOS: Forward-CCTCCTCGTTCAGCTCA CCT, Reverse-CAATCCACAACTCGCTCCAA; COX-2: Forward- TGAGTA CCGCAAA-CGCTTCT, Reverse- ACGAGGTTTTTCCACCAGCA. The expre ssion of genes was analyzed using the $2^{-\triangle\triangle Ct}$ method.

Immunofluorescence staining

The RAW 264.7 macrophages (1×10^5 cells/mL) were cultured with samples at the concentration of 25, 50, and 100 μg/mL for 24 h. Cells were then fixed with 4% polyformaldehyde for 10 min and then blocked with 10% BSA for 1 h. Next, RAW 264.7 cells were cultured with the primary COX-2 and iNOS antibodies at 4 °C overnight, respectively, then washed three times using TBST, followed by incubation with a fluorescence-conjugated secondary antibody for 1 h at room temperature. Finally, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1:1000 for 10 min. Between each procedure, cells were washed three times with PBS. A Cytation 5 cell-imaging multimode reader (Olympus SpinSR10 spinning disk confocal super resolution microscope, Tokyo, Japan) was used for imaging.

Western blot analysis

Cells were treated with fucoidan at a concentration of 25, 50, and 100 μg/mL for 2 h, respectively, then cells were extracted in lysis buffer. Aliquots of protein (30 μg) were electrophoresed on a 4–12% Bis-Tris SDS–PAGE gel and then transferred to nitrocellulose membranes (ThermoFisher, Waltham, MA, USA), and the membranes were incubated in 1 × TBST containing 4% milk for 1 h at room temperature, then incubated with primary antibodies (GAPDH, iNOX, and COX-2 solutions) at 4 °C overnight, and next incubated with secondary antibody for 1 h at room temperature. Bio-Rad image analysis system (Bio-Rad Laboratories, Hercules, CA) was used to image protein bands. Protein expressions were quantified using ImageJ 1.53q (Wayne Rasband and contributors, National Institutes of Health, Bethesda, Maryland, USA).

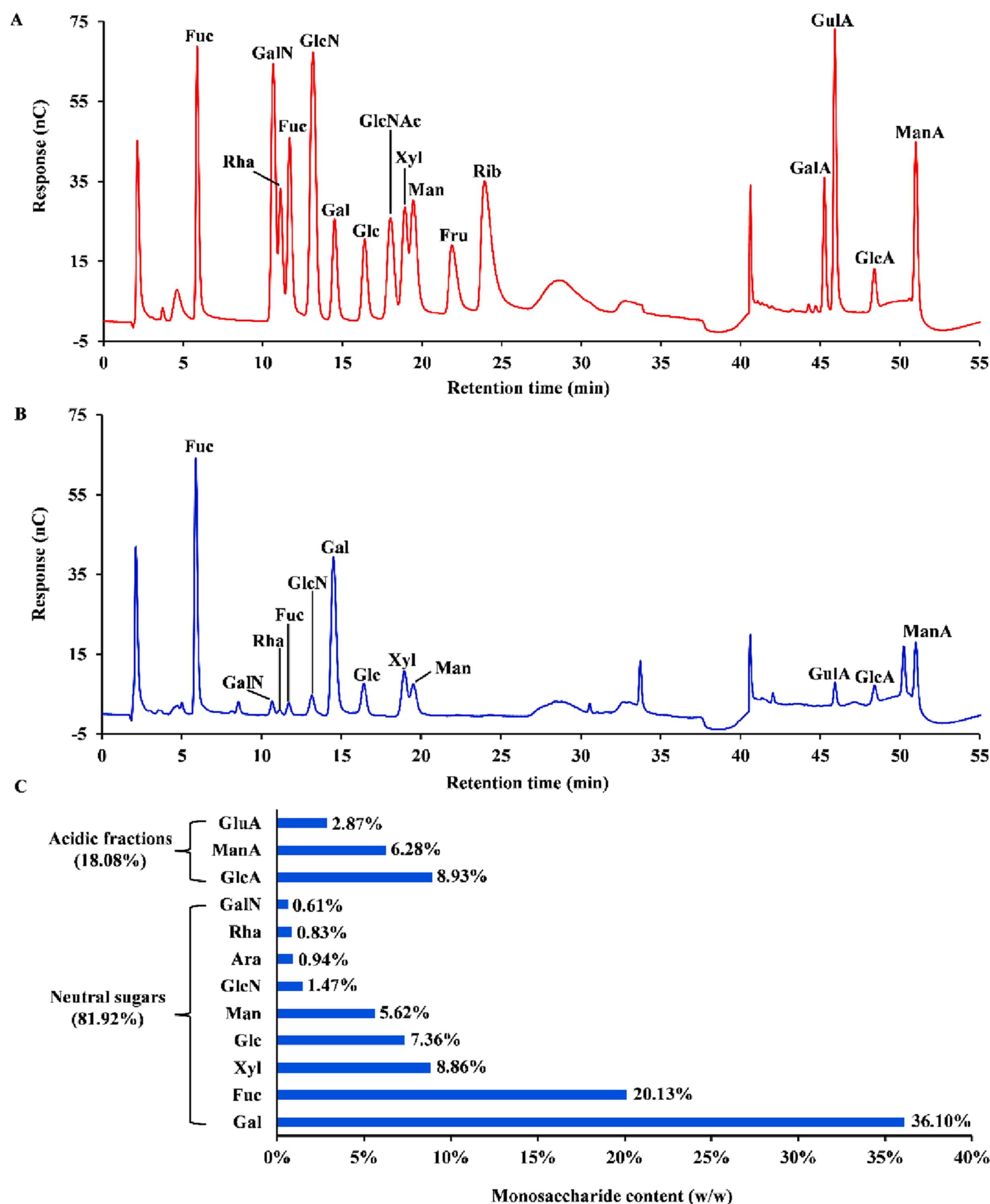


Fig. 1. The monosaccharide composition chromatogram of *Sargassum Zhangii* fucoidan (SZF). (A) Standards of monosaccharide and (B) monosaccharide composition of SZF. (1. Fuc, fucose; 2, GalN, galactose hydrochloride; 3. Rha, rhamnose; 4. Ara, arabinose; 5. GlcN, glucosamine hydrochloride; 6. Gal, galactose; 7. Glc, glucose; 8. GlcNAc, *N*-acetyl-D-glucosamine; 9. Xyl, xylose; 10. Man, mannose; 11. Fru, fructose; 12. Rib, ribose; 13. GalA, galactose acid; 14. GulA, guluronic acid; 15. GlcA, glucuronic acid; 16. ManA, mannuronic acid.)

Statistical analysis

Results were expressed as the means ± standard deviation (SD) of triplicate tests. One-way analysis of variance (ANOVA) and Tukey's multiple comparisons tests were used to analyze the differences between samples using GraphPad Prism 8 (GraphPad Software LLC., San Diego, California, USA).

Results and discussion

Yield rate and extraction rate

The total sugar content of SZ powder was 27.88 ± 0.36%. As shown in Table 1, the yield rate and extraction rate of SZF were 2.85 ± 0.35% (based on the weight of raw material) and 10.06 ± 0.56% (based on the

Table 2
Molecular properties of *Sargassum Zhangii* fucoidan (SZF).

Relative molecular weight (kDa)				Relative Percentage of peak area (%)
Mw	Mn	Mp	Polydispersity (PDI, Mw/Mn)	
7121.79	3380.83	4231.04	2.11	0.71
739.71	411.33	504.15	1.80	9.01
111.28	70.63	85.07	1.58	90.27

weight of the total sugar content of raw material), respectively. However, the yield rate was lower than that of other polysaccharide-rich species such as *Nizamuddinia zanardinii* (*6.5%*) and *Sargassum angustifolium* (6.35%) (Tabarsa et al., 2020). Previous research has shown that both extraction method and seaweed species had a significant effect on extraction yield rate (Rodrigues et al., 2015). Some research has found that there were no statistical differences in extraction by ultrasound procedures in comparison to hot water extraction (Rodrigues et al., 2015), or even lower (Alboofetileh et al., 2019; Wang et al., 2021), this could be due to the loss of low molecular weight polysaccharides during dialysis, because ultrasonication assisted extraction can degrade polysaccharides based on the extraction time and ultrasonic power (Wang et al., 2021). Furthermore, the diversity, amount, and complexity of polysaccharides in the seaweeds' cell wall could reduce the yield rate especially with traditional extraction methods (Wijesinghe & Jeon, 2012). The low yield rate of this research may be related to the amount of the polysaccharide distributed in the cell wall of the seaweed, and also may be due to the loss of extracted polysaccharide during the dialysis

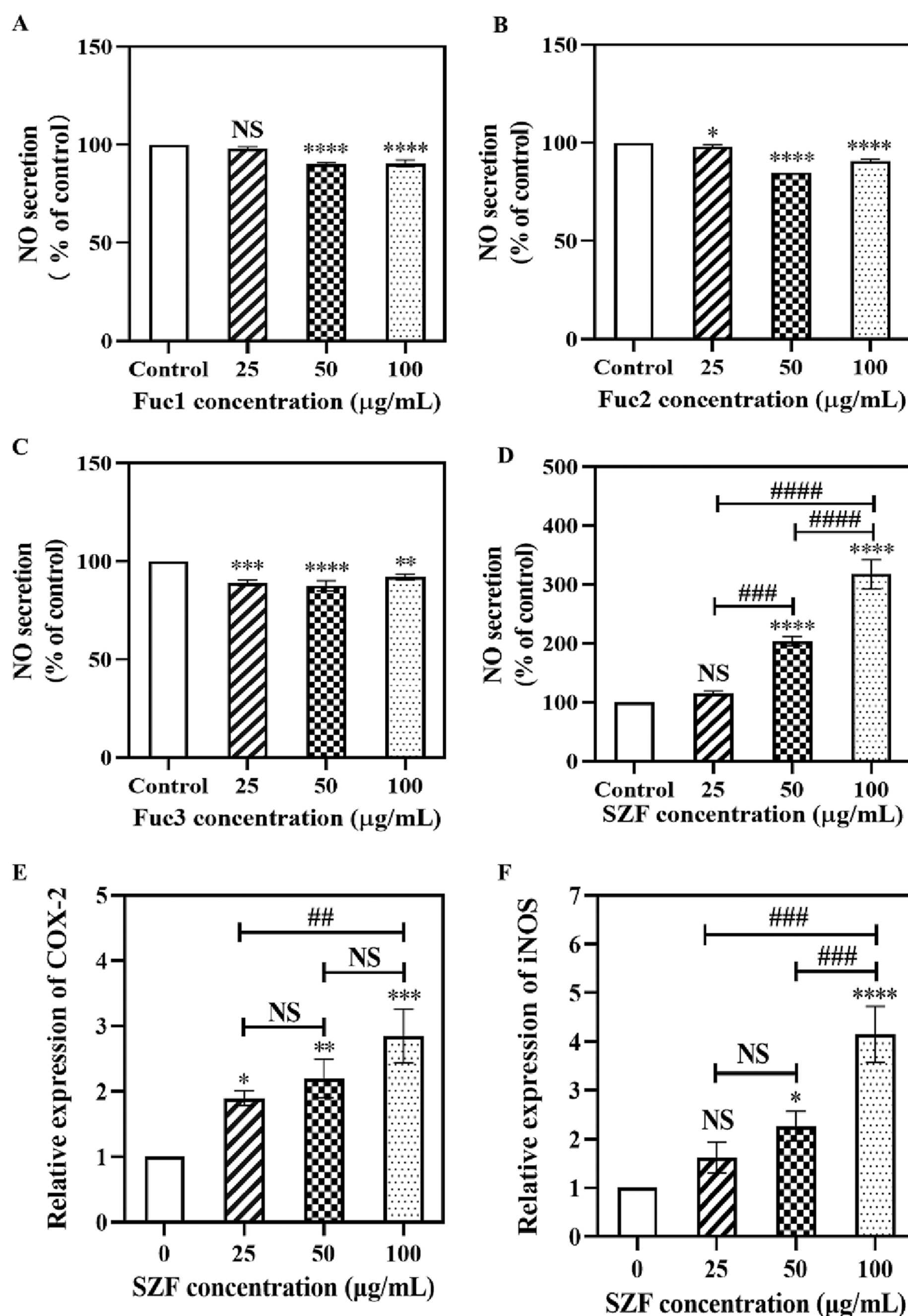


Fig. 2. (A) Fourier transformation infrared spectroscopy (FTIR) spectra of *Sargassum Zhangii* fucoidan (SZF); (B) ^{1}H NMR analysis of SZF. (C) ^{13}C NMR analysis of SZF.

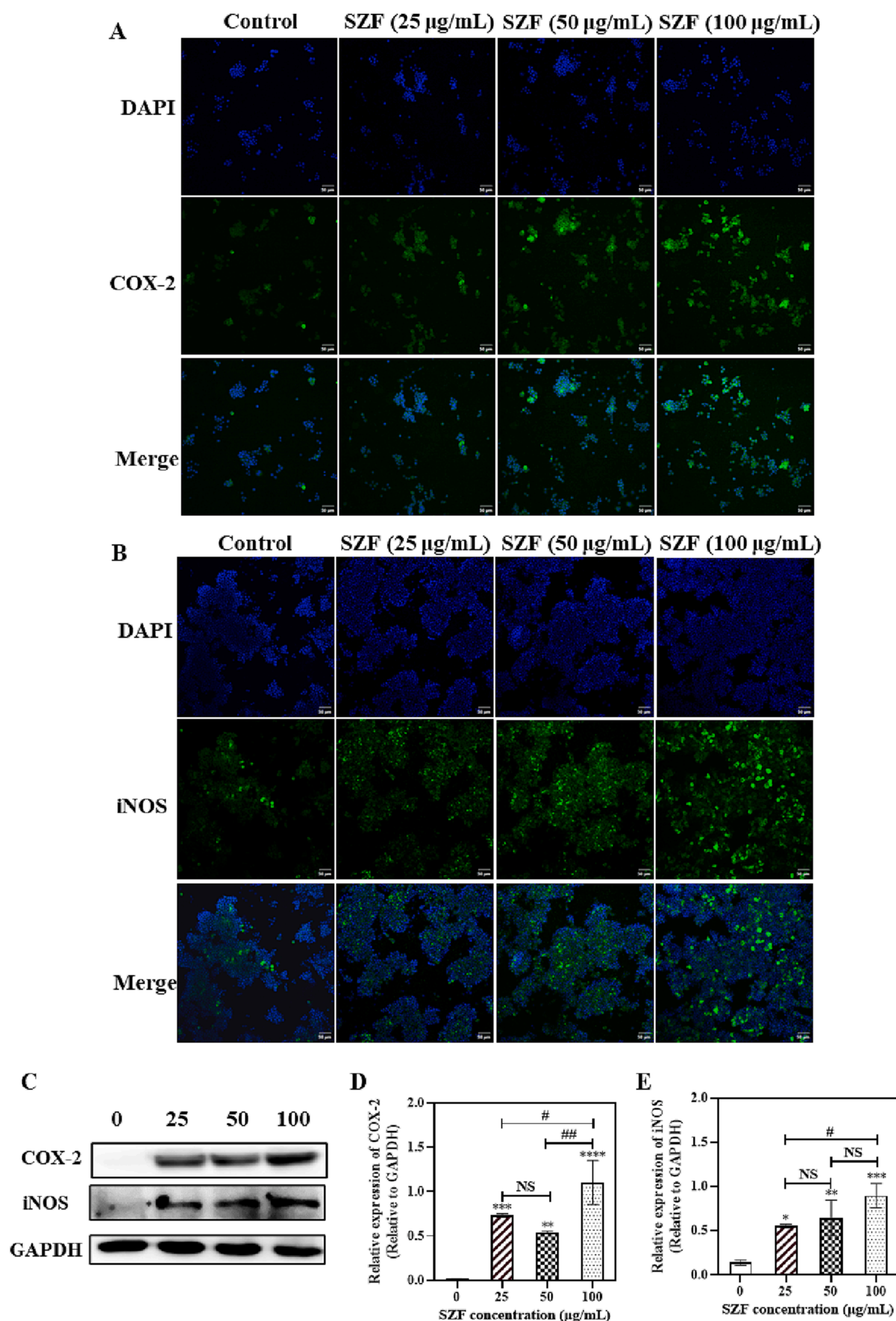


Fig. 3. Effects of commercial fucoidan samples and *Sargassum Zhangii* fucoidan (SZF) on nitric oxide (NO) release of RAW 264.7 cells. (A) Fucoidan 1 (*Undaria pitnnaifida* source) of Qingdao bright moon seaweed group co., LTD., Mw 23.91 kDa; (B) Fucoidan 2 (*Undaria pitnnaifida* source) of Sigma, Mw 19.52 kDa; (C) Fucoidan 3 (*Fucus vesiculosus* source) of Sigma, Mw 11.61 kDa; (D) SZF, Mw 111.28 kDa. (E) Effect of SZF on the mRNA expression level of cyclooxygenase-2 (COX-2), and (F) inducible nitric oxide synthase (iNOS). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$) compared to the control, respectively. # ($p < 0.05$), ## ($p < 0.01$), ### ($p < 0.001$), and #### ($p < 0.0001$), compared with each other of sample groups.

process. However, other research has shown that the extraction yield of *Sargassum wightii* fucoidan was much higher of ultrasonication assisted method than that of the hot water extraction method (Hanjabam et al., 2019).

Chemical analysis of SZF

The total sugar content of SZF was 82.77 ± 0.40% (Table 1). The sulfate content was 29.74 ± 0.01%, which was lower than that of the commercial fucoidan samples Fuc1 (42.30 ± 0.51%), Fuc2 (57.54 ± 5.25%) and Fuc3 (47.44 ± 3.24%) used in this study (Tai et al., 2022). Other similar research has reported that fucoidans extracted from the brown algae *Fucus distichus* subsp. *evanescens* and *Saccharina latissimi* had a sulfate content ranging from 15.5% to 38.0% (Nguyen et al., 2020). Finally, small amounts of protein (1.92 ± 0.38%) and polyphenol (1.40 ± 0.12 mg GAE/g SZF) were also detected.

Fig. 1(A-B) gives a representative monosaccharide chromatogram of SZF. In Fig. 1C, it is shown that neutral sugars (81.92%, w/w) constituted most of the structures of SZF, with some acidic fractions (18.08%, w/w). Specifically, it was observed that the neutral sugars of SZF were mainly composed of relative amounts of galactose (36.10%), fucose (20.13%), xylose (8.86%), glucose (7.36%), mannose (5.62%) (w/w), etc., and three uronic acids including glucuronic acid (8.93%), mannuronic acid (6.28%), and guluronic acid (2.87%) (w/w). In other similar studies, galactose, fucose, mannose and xylose were characterized as the majority of fucoidan but in different ratios, in which galactose was the majority of a fucoidan fraction extracted from Nizamuddinia zanardinii (Tabarsa et al., 2020). Our recent research showed that the main monosaccharides of Sargassum Hemiphyllum fucoidan consisted of glucose (32.68%), galactose (24.81%), fucose (20.75%) (w/w), and a low level of uronic acid content (8.91%) compared to that of SZF (Li et al., 2022).

Molecular properties

The molecular properties of SZF are shown in Table 2. The PDI shows the ratio of Mw and Mn, indicating a wide distribution of polymer molecular weights distribution, as a larger PDI denotes a more inhomogeneous molecular weight distribution. Table 2 shows that the molecular weight of SZF was 111.28 kDa (90.27%, w/w). Rioux *et al.* showed that the molecular weight of fucoidan was dependent on the sources and species of brown algae ranging from 1.4 kDa (Yuan & Macquarrie, 2015) to 2000 kDa (Fitton, Dell'Acqua, Gardiner, Karpiniec, Stringer, & Davis, 2015), which agrees well with our findings. Fucoidans can be classified as low-molecular-weight (<10 kDa), medium-molecular-weight (10–10000 kDa), and high-molecular-weight fucoidans (>10,000 kDa) (Van Weelden et al., 2019). From Table 2 we can see that over 90% of SZF's molecules possessed an Mw of 111.28 kDa, which places them amongst the medium molecular weight fucoidans. The Mn and Mp of SZF were 70.63 kDa and 85.07 kDa, respectively.

Most genetically encoded biopolymers (such as proteins) are usually monodispersed with a PDI of about 1. However, polysaccharides are produced in a less tightly regulated fashion, and as such are polydisperse with PDIs higher than 1. The PDI of SZF was 1.58 (over 90% of its molecules), and it fell within the reported fucoidan PDI value range of 1–6.2 (Alboofetileh et al., 2019; Ammar, Hafsa, Le Cerf, Bouraoui, & Majdoub, 2016). The PDI value also shows that SZF was heterogeneous and had a relative broad molecular weight distribution.

Structural characterization of SZF

FT-IR analysis

Fig. 2(A) shows that the broad peak at 3468 cm^{-1} corresponded to the O—H stretching vibration of SZF. The band at 2945 and 1611 cm^{-1} was corresponding to C-6 group of fucose, galactose or C—H stretching pyranoid ring and O—H vibrations, respectively. Furthermore, the band at 1611 cm^{-1} also indicated C═O vibration of uronic acid. Another peak at 1256 cm^{-1} was due to the sulfate esters (S═O), and a peak at 829 cm^{-1} indicated the presence of the sulfate group (C—O—S) and α-type glycosidic bond (Alboofetileh et al., 2019). The band at 1423 cm^{-1} indicated a variety of C—H vibration of polysaccharides composed of fucose, D-glucose, D-mannose, D-xylose, and galacturonat acid (Palanisamy, Vinosha, Marudhupandi, Rajasekar, & Prabhu, 2017). Meanwhile, the absorption at 1124 cm^{-1} was due to the stretching of the glycosidic C—O group of SZF, a strong peak near 1038 cm^{-1} was responsible for hemiacetal stretching, and the weak signals near 970 cm^{-1} were assigned to the asymmetrical stretching vibration of C—O—S bond (Lim et al., 2014). The above results were similar to the bands observed for fucoidan of *Sargassum polycystum* and other brown algae (Marudhupandi, Ajith Kumar, Lakshmanasenthil, Suja, & Vinothkumar, 2015; Palanisamy et al., 2017).

^{1}H and ^{13}C NMR

The chemical structure of SZF was further determined using NMR spectra of ^{1}H (Fig. 2B) and ^{13}C (Fig. 2C). With the ^{1}H NMR chemical shifts of SZF at δH 5.23 (H-1), 3.59 (H-2), 3.77 (H-3), 3.90 (H-4), 4.04 (H-5), and δH 3.77 (H-6), and the 4.80 ppm signal was attributed to D_2O (Fig. 2B), and ^{13}C NMR spectrometry chemical shifts at δC 100.02 (C-1), 69.93 (C-2), 71.36 (C-3), 77.91 (C-4), 75.79 (C-5), and 61.57 (C-6) (Fig. 2C), these data supported the signal corresponding to a (1,4)-α-D-linked-galactose (Zhang et al., 2021), and the absorption at δC 75.79 (C-4) correlated with absorption at δH 3.59 (H-4) was corresponding to a terminal (1,4)-α-D-linked-glucose (Chen et al., 2019). Similarly, as shown in the NMR spectra, the absorption at δC 102.75 (C-1) correlated with absorption at δH 5.06 (H-1) represented to (3,4)-α-L-fucose (Schilling, Klau, Aachmann, Rühmann, Schmid, & Sieber, 2022), and the signal at δ5.06/97.89 and δ4.66/102.75 were assigned to H1/C1 of β-D-linked-mannose and (1,3)-α-D-linked-xylose respectively (Luo, Wang, Li, & Yu, 2018; Zhang et al., 2022), which confirmed the major residues of the SZF.

Pro-inflammatory assessment via activation of Macrophages

Analysis of cellular nitric oxide (NO) production

Macrophages play important roles on the immunomodulatory system by keeping homoeostasis and providing defense against pathogen invasion. It is commonly recognized that pro-inflammatory mediator NO produced by activated macrophages is directly involved in the immunomodulatory activities, and the detection of NO content is one of the most reliable experiments to assess classical macrophage activation (Green, Mellouk, Hoffman, Meltzer, & Nacy, 1990). Therefore, NO secretion from a macrophage cell line, RAW 264.7, was investigated as an indicator of the immunomodulatory activity of SZF. In this study, RAW 264.7 murine macrophages in attachment-based tissue culture plates were incubated with solution of fucoidans at concentrations of 25, 50, and 100 μg/mL, and three commercial fucoidans Fuc1 (*Undaria pitnnaifida*), Fuc2 (*Undaria pitnnaifida*), and Fuc3 (*Fucus vesiculosus*) were used as positive controls. Fig. 3(A-D) shows that the solutions of the three commercial fucoidans had a small yet significant reduction in NO production compared to the control ($^*p < 0.05$) that was independent of concentration, whereas NO release in SZF-induced RAW 264.7 cells was increased significantly at concentrations of 50 and 100 μg/mL in a dose-dependent manner ($^*p < 0.001$). At a concentration 25 μg/mL, SZF exhibited only a weak effect on NO release in macrophages ($^*p > 0.05$), suggesting a minimum concentration required for effect. Compared to our recent study, the production of NO was higher than that of the *Sargassum Hemiphyllum* fucoidan (SHF) treated RAW 264.7 cells at the same concentration (Li et al., 2022), indicating that SZF was a more effective agent to stimulate proliferation of macrophages. The different effects over the commercial fucoidans, SHF and SZF on NO secretion may be due to differences in their chemical structure, *e.g.*

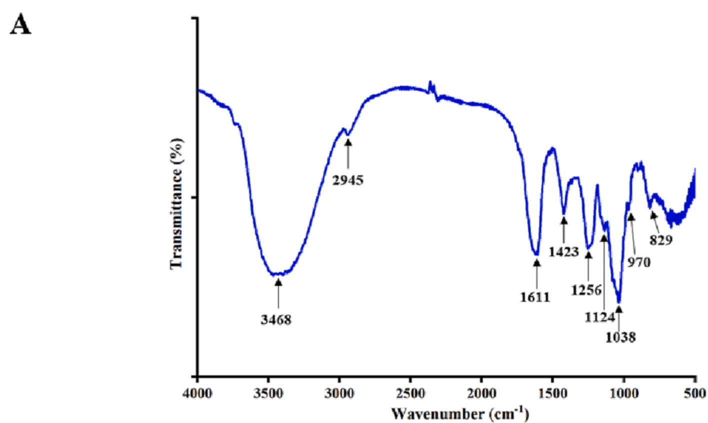
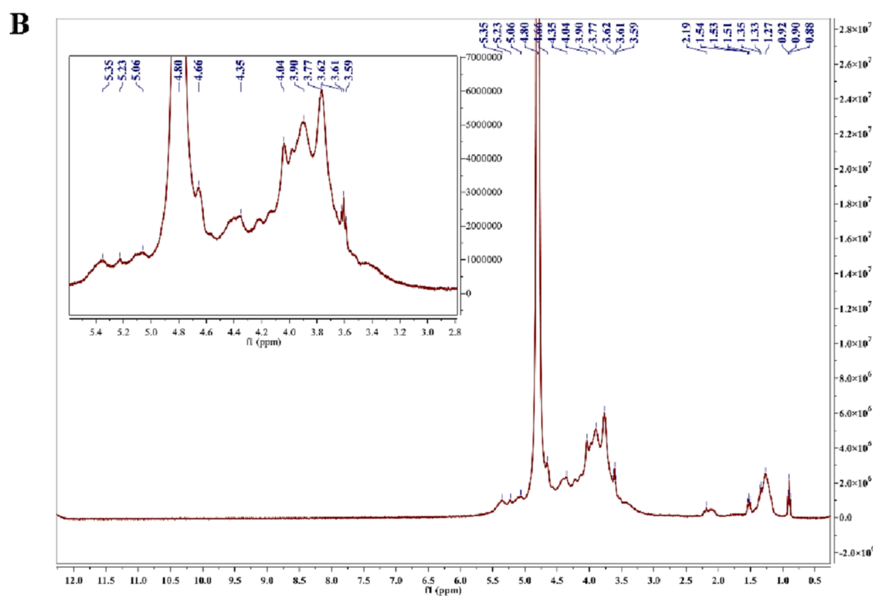
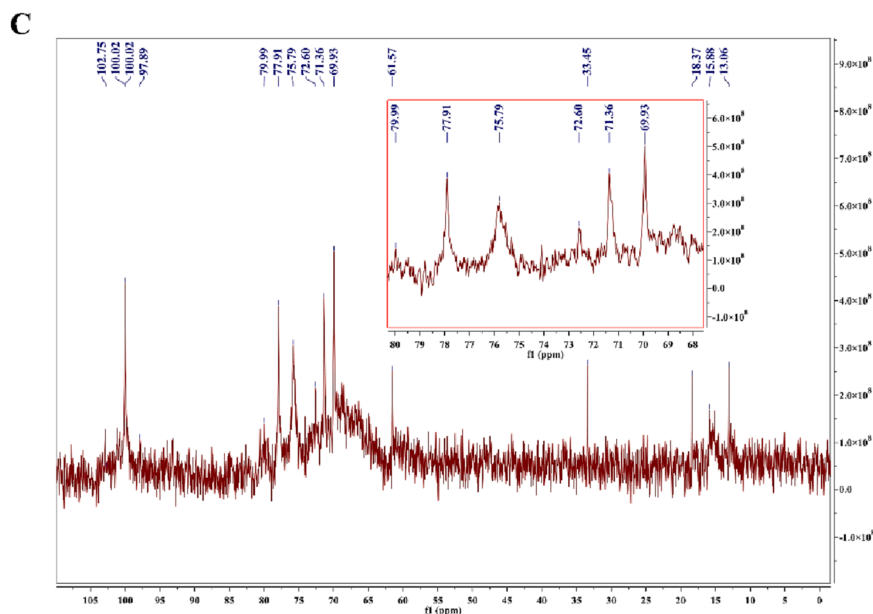


Fig. 4. *Sargassum Zhangii* fucoidan (SZF) up-regulated expression of cyclooxygenase-2 (COX-2) (A) and inducible nitric oxide synthase (iNOS) (B) detected by immunofluorescence staining. COX-2 and iNOS were shown by green fluorescence, and DAPI-stained nuclei were shown by blue fluorescence. Scale bar = 50 μm. Western blot images of effects of SZF (25, 50, 100 μg/mL) on COX-2 and iNOS promotion (C), and quantitative analysis of COX-2 (D) and iNOS (E). * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$) compared to the control, respectively. # ($p < 0.05$), ## ($p < 0.01$), ### ($p < 0.001$), and #### ($p < 0.0001$), compared with each other of sample groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)





molecular weight, sulfate content, and monosaccharide composition (Hao et al., 2019). Some studies have suggested that polysaccharides with lower molecular weights had better stimulating effects on the immune system (Liao et al., 2015), which confirms the trend we have observed in SZF and SHF.

COX-2 and iNOS mRNA expression in macrophages activated by SZF

Since both COX-2 and iNOS are isoenzymes that are responsible for the production of NO (Raso et al., 2002), the influence of SZF on the ability of RAW 264.7 cells to secrete NO was analysed at the molecular level. Here, the mRNA associated with the expression of COX-2 and iNOS were investigated using quantitative RT-PCR.

As shown in Fig. 3E, the mRNA expression of COX-2 was significantly up-regulated by SZF in a concentration-dependent manner ($*p < 0.05$), and the expression of COX-2 mRNA of cells treated by 100 μg/mL SZF was dramatically higher than that of cells treated by 25 μg/mL SZF ($^{\#}p < 0.01$), although there was no significant difference between the adjacent sample groups ($^{\#}p > 0.05$). Similarly, the mRNA expression of iNOS was elevated dose-dependently with SZF stimulation at concentrations of 50 and 100 μg/mL ($*p < 0.05$), and significant difference was observed between each group ($^{\#}p < 0.001$) (Fig. 3F). These results show an increasing trend as demonstrated by the NO secretion, which further supported the result that macrophages could be activated by SZF. This result also indicates that the NO release of macrophages induced by SZF stimulation matches the up-regulation of COX-2 and iNOS mRNA.

COX-2 and iNOS protein expression activated by SZF

To further confirm the stimulation of RAW 264.7 macrophages, the protein expression of COX-2 and iNOS were assessed by immunofluorescence and Western blotting. In the SZF-triggered groups, the fluorescence intensity of COX-2 (Fig. 4A) and iNOS (Fig. 4B) increased with the increase of SZF concentration compared with the control group, suggesting that the SZF treatments resulted in an up-regulated protein expression of COX-2 and iNOS in the RAW 264.7 cells. This result again was consistent with the observed trends in NO production of the RAW 264.7 cells.

As shown in Fig. 4C, the appearance of strong and distinctive bands of COX-2 and iNOS indicated the promotion of protein expression in activated cells. After quantifying the Western blot images using ImageJ, we could see that the protein content of COX-2 and iNOS in the SZF treated cells were significantly higher than those of the control ($*p < 0.05$), and both proteins showed the consistent trend in concentration-dependent induction by SZF at concentrations of 50 and 100 μg/mL. The protein expression closely matched the trends observed in, and further confirmed the results of, the NO secretion, COX-2 and iNOS mRNA upregulation. Therefore, the activation assays of RAW 264.7 cells all indicate that SZF is a potent potential candidate to act as functional compound for a range of applications such as food ingredient, immune enhancer or immunodulator, to name a few.

Conclusions

In this study, the unreported potential of the sulfated heteropolysaccharide, SZF were identified. SZF possessed a main chain of (1,4)-α-D-linked-galactose, (3,4)-α-L-fucose, (1,3)-α-D-linked-xylose, β-D-linked-mannose and a terminal (1,4)-α-D-linked-glucose. SZF had immune-stimulating activities, resulting in promoting NO production in RAW 264.7 cells *via* the up-regulation of both the mRNA and protein expression of iNOS and COX-2 in a dose-dependent manner, while the commercial fucoidans (*Undaria pitnnaifida* source and *Fucus vesiculosus* source) decreased NO production in macrophages. The preliminary results of SZF indicate it has great potential to act as functional food and/or nutritional food ingredients, immune enhancer or immunodulators due to its excellent immune-boosting activities. Furthermore, SZF is also a promising raw material for the fabrication of novel delivery systems such as nanoemulsions, nanoparticles, *etc.* by interacting with other natural biopolymers such as proteins due to its polyanion property, thereby providing synergistic immunomodulatory effects during the delivery of the packaged functional components. The promising data highlighted in this paper warrants further research, exploring and expanding the applications of SZF and its responding immune-stimulating activities *in vivo.* Future study should focus on the further purification and the precise chemical structure characterization of SZF; the *in vivo* experiments about the immune-boosting activities of SZF also need further research.

CRediT authorship contribution statement

Rui Li: Conceptualization, Formal analysis, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Qing-Ling Zhou:** Methodology, Formal analysis, Validation, Software. **Rui-Yu Yang:** Methodology, Formal analysis, Validation, Software. **Shu-Tong Chen:** Methodology, Formal analysis, Validation, Software. **Rui Ding:** Methodology, Formal analysis. **Xiao-Fei Liu:** Methodology, Software. **Lian-Xiang Luo:** Methodology. **Qiu-Yu Xia:** Writing – review & editing, Supervision. **Sai-Yi Zhong:** Project administration, Funding acquisition, Validation. **Yi Qi:** Writing – review & editing, Supervision. **Richard J. Williams:** Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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